HUMAN MONOCYTE SUBSETS

IN CORONARY ARTERY DISEASE

AND MYOCARDIAL INFARCTION

by

Luke David Tapp

A thesis submitted to the University of Birmingham

For the degree of

DOCTOR OF MEDICINE

Department of Cardiovascular Medicine

School of Clinical & Experimental Medicine

University of Birmingham

2014

ABSTRACT

Coronary artery disease (CAD) is a disease of inflammatory aetiology, and remains the commonest cause of death globally despite therapeutic advances. Monocytes are implicated in the pathogenesis of CAD, but also in reparative mechanisms after myocardial infarction (MI) due to subset heterogeneity. The aim of this thesis was to provide a detailed phenotypic comparison of differences between the three human monocyte subsets in CAD and after MI, with particular emphasis on CD16+ monocytes which have previously been analysed as a single population rather than two distinct subsets. Longitudinal changes were analysed following MI and relationships explored with plasma cytokines. Multiple significant novel changes in monocyte phenotype attributable to specific subsets were identified, particularly related to the CD14++CD16+CCR2+ 'Mon2'/'Intermediate' subset which increased in number on day 1 after MI and appeared highly functionally active. There were significant changes in expression of a range of receptors associated with inflammation, migration and reparative processes. Significant relations to plasma cytokines and the degree of myocardial damage were observed. Most monocyte parameters predictive of left ventricular ejection fraction six weeks after MI were related to the Mon2 subset. This suggests an important role for this subset in the acute phase of MI.

I dedicate this thesis

to my late parents John & Madeline

and to Anna & Boris, for their unfailing love and support.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor Professor Gregory Lip for giving me the opportunity to undertake research in his department and for his advice, encouragement and guidance along the way. This has been a very useful and productive experience in my career development and I am most grateful.

Special thanks are extended to my co-supervisor Dr Eduard Shantsila, for his support, friendship and unbelievable hard work in ensuring this project was so successful. His unlimited time, energy and devotion to this research have been truly inspirational and I will be forever grateful. I am particulalry appreciaitive of both my supervisors for their support and understanding during the particularly tough period around the time of my Mother's illness and death.

I sincerely thank all the patients and healthy volunteers who took part, without whom this research would not have been possible.

Ronnie Haynes and all the members of the ASCOT team have been very helpful in the daily running of this project. In particular I would like to thank Tony Jaipersaid, Burak Pamukcu, Silvia Montoro Garcia and Balu Balakrishnan for all their efforts. Thanks also to Dr Andrew Blann for his input into development and approval of the standard operating procedures used in this research and for his tuition on statistics, along with Dr Deirdre Lane.

Ben Wrigley deserves special mention as a fellow rider of the research rollercoaster, co-conspirator and partner in crime during this unique experience. His help and support during this project, together with his friendship through good times and bad times has been invaluable.

Dr Richard Watkin and Dr Ken Lee were very supportive during patient recruitment from Heart of England NHS Trust.

I thank my late parents John and Madeline Tapp for all their love, support and encouragement through the years. Sadly my Father died just after I qualified as a doctor and did not see my career in cardiology begin or develop, and my Mother died during the write-up phase of this thesis. I would not have come this far without them and I know they would have been proud of my achievements.

The final thanks go to those who kept me going when things got tough and gave me the strength to carry on. My beloved cat, Boris, was a constant source of joy and happiness, love and affection and we will never forget him. To my wife, Anna, I give my thanks for her endless love, support, patience and understanding. None of this would have been possible without her.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ACC: American College of Cardiology

ACE: angiotensin converting enzyme

ACS: acute coronary syndrome

ADAM: A disintegrin and metalloproteinase

ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs

ADP: adensoine diphosphate

ANOVA: analysis of variance

ApoE: apoliporotein E

BD: Becton Dicinson

BMI: body mass index

BP: blood pressure

CA: coronary artery

CABG: Coronary artery bypass grafting

CAD: coronary artery disease

CCB: calcium channel blocker

CCU: coronary care unit

COPD: chronic obstructive pulmonary disease

CRP: C-reactive protein

CT: computerised tomography

CV: coefficient of variability

DAPT: dual antiplatelt therapy

DC: dendritic cell

DC: density centrifugation

DTB: door to balloon

ECG: electro cardiogram

EDTA: ethylene diamine tetra-acetic acid

EF: ejection fraction

ELISA: enzyme-linked immunosorbant assay

EPC: endothelial progenitor cell

ESC: European Society of Cardiology

FGF: fibroblast growth factor

FSC: forward scatter

GM-CSF: granulocyte-macrophage colony-stimulating factor

GP: glycoprotein

GRACE: global registry of acute coronary events

HC: healthy controls

HF: heart failure

HSP: heat shock protein

hsCRP: high sensitivity C-reactive protein

IC: isotype control

ICAM: intracellular adhesion molecule

IFN: interferon

IKK: inhibitory κB kinases

IL: interleukin

IL6r: interleukin 6 receptor

IM: immunomagnetic

IQR: interquartile range

KDR: kinase domain receptor, VEGF receptor 2

LBBB: left bundle branch block

LDL: low density lipoprotein

LMWH: low molecular weight heparin

LPS: lipopolysaccharide

LV: left ventricle

MACE: major adverse cardiovascular event

MCP-1: monocyte chemoatractant protein 1

M-CSF: macrophage colony stimulating factor

MFI: median fluorescent intensity

MI: myocardial infarction

MMP: marix metalloproteinase

Mon1: CD14++CD16-CCR2+ (‘classical’) monocytes

Mon2: CD14++CD16+CCR2+ (‘intemediate’) monocytes

Mon3: CD14+CD16++CCR2- (‘non-classical’) monocytes

MPA: monocyte plateley aggregates

MRI: magnetic resonance imaging

mRNA: messanger ribonucleic acid

MRP: myeloid related protein

NA: not available

NHS: national health service

NKκB: nuclear factor kappa B

NO: nitric oxide

NST-ACS: non ST-elevation acute coronary syndrome

NSTEMI: non ST-elevation myocardial infarction

OCT: optical coherence tomography

OMT: optomised medical therapy

PAI-I: plasminogen activator inhibitor type 1

PB: peripheral blood

PBS: phosphate buffered saline

PCI: percutaneous coronary intervention

PET: positron emission tomography

PPCI: primary percutaneous coronary intervention

RNAi: ribonucleic acid interference

ROS: reactive oxygen species

RU: relative units

SD: standard deviation

SDF: stromal derived factor

SOP: standard operating procedure

SR-AI: scavenger receptor class A type 1

SSC: side scatter

STE-ACS: ST-elevation acute coronary syndrome

STEMI: ST-elevation myocardial infarction

SWBH: Sandwell and West Birmingham Hospitals

TF: tissue factor

TIMI: thrombolysis in myocardial infarction

TIMP: tissue inhibitor of metalloproteinase

TLR: toll-like receptor

TNF: tissue necrosis factor

t-PA: tissue-type plasminogen activator

UFH: unfractionated heparin

uPA: urokinase-type plasminogen activator

VCAM: vascular cell adhesion molecule

VEGF: vascular endothelial growth factor

VEGFR1: vascular endothelial growth factor receptor 1

CHAPTER 1

MONOCYTE SUBSETS AND THEIR ROLE IN PLAQUE DESTABILISATION AND MYOCARDIAL INFARCTION

1.1 ATHEROSCLEROSIS, CORONARY ARTERY DISEASE AND MYOCARDIAL INFARCTION

1.1.1 Epidemiology of coronary artery disease and myocardial infarction

Cardiovascular disease is already the commonest cause of death in industrialised countries, but it is also projected to become so in developing countries by 2020 (Murray & Lopez, 1997). More specifically, coronary artery disease (CAD) is the most common cause of death globally with over 7 million deaths annually, accounting for 12.8% of all mortality (WHO, 2011). The prevalence of angina increases with age and is estimated to be 2-4000/100,000, with an annual mortality rate of up to 1.4% (Fox et al, 2006). The annual incidence of ST-elevation myocardial infarction (STEMI) is approximately 66/100,000 (Widimsky et al, 2010). The average incidence of non ST-elevation myocardial infarction (NSTEMI) is approximately 300/100,000 (Fox et al, 2010). The incidence of STEMI appears to be declining, in contrast to the incidence of NSTEMI which is rising (Yeh et al, 2010; Roger et al, 2012). Within Europe, in-hospital mortality following STEMI varies between countries, ranging from 6% to 14%, with an average of approximately 12% within 6 months (Mandelzweig et al, 2006). Survivors of myocardial infarction (MI) have a high risk of recurrent re-infarction, measured at 17.4% at 1 year in a population study (Milonas et al, 2010). These secondary events may occur because MI itself accelerates underlying atherosclerosis, rather than occurring as a simple manifestation of pre-existing advanced disease (Dutta et al, 2012). Although there have been major advances in the management of MI over the last 60 years, there clearly remains a significant morbidity and mortality associated with this condition. The figures speak for themselves; atherosclerosis remains a

major healthcare problem and even optimally treated patients may experience recurrent ischaemic and thrombotic events. This drives the need for ongoing research into the pathophysiological determinants of plaque development, progression, instability, rupture and MI.

1.1.2 Coronary artery disease and angina

The term angina pectoris was coined by William Heberden in 1772 (Heberden, 1772), and the underlying mechanism proposed by Parry in 1799 (Parry, 1799). This refers to the typical symptoms of chest discomfort, precipitated by exercise and relieved by rest, caused by atheromatous plaques in coronary arteries of sufficient proportion (typically >50% luminal diameter reduction) to reduce coronary blood flow beyond that which can be maintained by reduction of coronary vascular bed resistance. This results in an imbalance between myocyte oxygen supply and demand which causes myocardial ischaemia and pain. Angina is diagnosed clinically on the basis of typical symptoms, complemented by invasive or non-invasive tests of coronary ischaemia (Fox et al, 2006). Of note, all subjects recruited for the stable CAD control group in my study (see 3.1.2.1) had symptoms typical of angina and angiographic evidence of obstructive coronary artery narrowings, and received standard treatment according to contemporary guidelines (Fox et al, 2006). This maximised homogeneity amongst this cohort.

1.1.3 Definition of myocardial infarction

Due to variation in the definition of MI, a joint consensus statement from the European Society of Cardiology (ESC) and American College of Cardiology (ACC) 'First Global MI Task Force' was published in 2000 (ESC/ACC, 2000). Subsequently, there have been major changes in the availability and sensitivity of biomarkers used for the diagnosis of MI, meaning that hitherto undetectable small amounts of myocardial damage can now be identified. However, the fundamental principle remains that the diagnosis of MI can be made when there is evidence of myocardial necrosis in a clinical context consistent with myocardial ischaemia. The criteria to make the diagnosis of MI were summarised in the 2007 'Universal Definition of MI' consensus document, summarised in table 1.1 (Thygesen et al, 2007). This was the contemporary guidance during the conception and conduct of my study.

Table 1.1 Universal definition of myocardial infarction

UNIVERSAL DEFINITION OF MYOCARDIAL INFARCTION
Detection of rise and/or fall of cardiac biomarker values (preferably troponin) with at least one value above the 99th percentile of the upper reference limit and with at least one of the following: • Symptoms of ischaemia • New or presumably new significant ST-T changes or new left bundle branch block • Development of pathological Q waves on the electrocardiogram • Imaging evidence of new loss of viable myocardium, or new regional wall motion abnormality • Identification of an intracoronary thrombus by angiography or autopsy.

The universal definition of MI was updated in 2012 to include the diagnosis of MI under special circumstances (related to angioplasty, cardiac surgery or coronary stent thrombosis),

and a classification of subtypes of MI based on the underlying pathology (Thygesen et al, 2012). This update does not affect the inclusion criteria for my study (see 3.1.1.1).

In my study, the diagnosis of STEMI was made acutely at the time of hospital admission on the basis of symptoms and electrocardiogram (ECG) changes of ST elevation (see 3.1.1), rather than the development of Q waves, regional wall motion abnormalities or identification of intracoronary thrombus which are retrospective diagnostic criteria. When the diagnosis of STEMI was made, all patients proceeded immediately to primary percutaneous coronary intervention (PPCI). The detection of a longitudinal rise and fall of biomarkers (troponin) is confirmatory and is made in the days after STEMI. Similarly, NSTEMI in my study was diagnosed on the basis of characteristic symptoms of myocardial infarction and compatible ECG changes (ST segment depression and/or T wave changes, but no ST elevation). All NSTEMI patients had a rise in biomarkers indicative of myocardial damage (see 3.1.1). This distinguishes this cohort as a group with definite myocardial infarction, compared to groups with 'troponin-negative acute coronary syndromes' or 'unstable angina' where symptoms may be due to ischaemia alone rather than additional infarction per se. This ensured that the NSTEMI cohort was as homogeneous as possible.

It should be noted that the ESC guidelines for diagnosis and management of both STEMI and NSTEMI were updated after the conception and initiation of this study. The contemporary STEMI guidelines during the conduct of this study were those published in 2008 (Van de Werf et al, 2008), subsequently updated in 2012 (Steg et al, 2012). The contemporary NSTEMI guidelines during the conduct of this study were those published in 2007 (Bassand

et al, 2007), subsequently updated in 2011 (Hamm et al, 2011). There are no major changes in these updates relevant to the management of patients in my study. There is a subtle change in terminology in the new guidelines, whereby the umbrella term of 'acute coronary syndromes' (ACS) is now used to encompass patients with a range of clinical phenotypes related to similar underlying pathophysiological substrate (see 1.1.4). The terms 'ST-elevation acute coronary syndrome' (STE-ACS) and 'non ST-elevation acute coronary syndrome' (NSTE-ACS) have been introduced to define the acute presentation with ischaemic chest pain and ECG changes before the cardiac biomarkers (usually troponin) are measured. Most STE-ACS will progress to STEMI. Patients with NSTE-ACS are subsequently discriminated based on the presence (NSTEMI) or absence ('unstable angina') of biomarker elevation.

1.1.4 Underlying pathology

Atherosclerosis is a complex disease of inflammatory aetiology, characterised by the formation of atherosclerotic plaques in the wall of medium and large diameter arteries. These plaques are composed of lipids, lipid-laden monocyte-derived macrophages (foam cells), inflamed smooth muscle cells, endothelial cells and immune cells (Gerrity, 1981). Inflammation as identified by elevated levels of high-sensitivity C-reactive protein (hsCRP) in the blood was found to be a strong predictor of future major adverse cardiovascular events in the prospective JUPITER clinical trial (Ridker et al, 2008).

The initial events of plaque development include impairment of endothelial function promoting vasoconstriction, enhanced coagulation and leucocyte adhesion. This endothelial

activation may result from numerous triggers including disturbed arterial flow, modified low-density lipoprotein (LDL), bacterial antigens and endogenous inflammatory mediators. Endothelial activation promotes leucocyte invasion into the intima and proliferation of smooth muscle cells, leading to the formation of a potentially reversible 'fatty streak', which may be present even during intrauterine life in humans (Napoli et al, 1997). Fatty streaks are the precursors of mature atherosclerotic plaques and are characterised by accumulation of foam cells in the vascular intima (Jonasson et al, 1986). These atherogenic macrophages are predominantly derived from the proinflammatory Ly6C^{hi} subset of monocytes in mice (discussed in section 1.2) (Swirski et al, 2007). Over time, the plaque core becomes surrounded by a fibrous cap composed of smooth muscle cells and collagen-rich matrix which confers biomechanical strength. The biological role of this process is thought to be elimination of excessive amounts of toxic oxidised LDL from the vascular wall via a number of monocyte scavenger receptors (Teupser et al, 1999; Febbraio et al, 2000; Silverstein & Febbraio, 2000). Although this may initially be a physiologically beneficial process, *excessive* lipid accumulation by foam cells leads to their death and the resultant self-perpetuating process of plaque progression. This constitutes progressive core enlargement, deposition of collagen, fibrosis, calcification, extracellular matrix formation and migration of smooth muscle cells into the intima, ultimately resulting in plaque expansion into the arterial lumen and obstruction of coronary blood flow (Gerrity, 1981).

STEMI and NSTEMI represent an acute and potentially life-threatening manifestation of atherosclerosis. The underlying CAD is often previously clinically quiescent, as the responsible plaque alone is often not sufficiently large to cause a significant reduction in luminal cross-sectional area to obstruct coronary flow during rest or exercise and may not

therefore cause stable exertional angina (Stone et al, 2011). The hitherto stable CAD is complicated by atherosclerotic plaque erosion and rupture. Activation of inflammatory cells within the atherosclerotic plaque is a key feature of this destabilisation process (Libby, 1995). The presence of a single 'inflamed' plaque as the substrate for MI has more recently been extended to the concept of a more generalised inflammatory process with multiple vulnerable plaques (Buffon et al, 2002). Plaque rupture is associated with varying degrees of platelet aggregation, thrombosis and vasoconstriction, causing acute obstruction and reduction in coronary artery blood flow. Myocardial infarction due to ischaemia-induced death of myocardial cells in the territory of the heart supplied by the culprit coronary artery is the end result of this process (Stone et al, 2011). Although this may lead to sudden death, the most frequent presentation is with characteristic chest pain associated with a number of accompanying features including dyspnoea, nausea and diaphoresis. Rarely, the cause of STEMI or NSTEMI is not due to atherosclerotic plaque rupture. Alternative causes include an imbalance between myocardial oxygen supply and demand (e.g. profound sustained hypotension), spontaneous coronary artery dissection, trauma, intense vasospasm (spontaneous 'Prinzmetal's angina', or related to cocaine use) and coronary arteritis. It should be noted that the underlying pathological mechanism in all STEMI and NSTEMI patients in my study was coronary atherosclerosis (3.1.1). This ensured pathophysiological uniformity amongst these cohorts.

1.1.5 Contemporary management of ST-elevation myocardial infarction

Since the 1960s there has been an exponential rise in research into MI. The precise management of MI inevitably varies, within countries, regions, hospitals and between doctors.

Efforts have been made to collate available evidence into consensus guidelines to aid physicians in delivering optimal care at institutional level and to individual patients.

The aims of treating MI in the acute phase are to relieve the symptoms associated with coronary ischaemia and to promptly restore coronary artery blood flow to improve both the short and long-term prognosis. The management of MI has undergone major changes over the last few decades which have been paralleled by an impressive reduction in morbidity and mortality. Initially, the treatment for MI was based on managing the symptoms, primarily relief of pain, and the complications rather than treating the underlying condition. In 1961, Desmond Julian described the treatment of cardiac arrest as a complication of MI in 5 patients with cardiac massage and electrical cardioversion (3 by direct cardiac massage via thoracotomy and 2 by external chest compressions) (Julian, 1961). Although only 1 of the 5 patients ultimately survived, the experiences gained highlighted the need for rapid, co-ordinated treatment of patients with MI by experienced staff and lead to the establishment of coronary care units (CCU) and resuscitation protocols for cardiac arrest. Management of STEMI patients on CCU alone lead to a reduction in the rate of death for MI patients by approximately one-third (Julian, 1968).

1.1.5.1 Reperfusion strategies

The central management strategy in treating STEMI is prompt and successful restoration of flow within the 'culprit' coronary artery, so called 'reperfusion' therapy. This reperfusion may occur spontaneously, but the 2 mainstays of active therapy are (i) pharmacological with drugs to break down the thrombus with 'clot-busting drugs', known as 'thrombolysis' or (ii) mechanical by PPCI.

1.1.5.1.1 Thrombolysis

The landmark UK co-ordinated ISIS-2 study (International study of infarct survival) published in 1988 was a randomised trial comparing intravenous streptokinase, aspirin, neither or both in 17, 187 patients with acute STEMI. The combination of aspirin with streptokinase was significantly better than either agent alone, with a marked improvement in vascular deaths and all cause mortality. The vascular mortality rate in the placebo arm was 13.2% at 35 days, compared to 8.0% in the aspirin + streptokinase arm ($p<0.00001$), a 42% reduction by the combination (ISIS-2 collaborative group, 1988). The striking reduction in mortality associated with early thrombolysis highlighted the crucial requirement to deliver proven treatments as soon as possible after the onset of MI. Although the attendant risks associated with both aspirin and especially streptokinase (principally haemorrhage) were noted, the overwhelming balance was clearly in favour of these drugs, and they subsequently became a routine treatment for STEMI. Subsequently, the tissue plasminogen activator (tPA) drugs (alteplase, retaplase and tenectaplase) were shown to be more potent thrombolytic agents compared with streptokinase, at the cost of an increased risk of haemorrhagic complications (GUSTO investigators, 1993). Emphasis was placed on giving thrombolytic drugs expeditiously, thus minimising the 'door to needle time', by re-organisation and streamlining of care for patients with STEMI, for example by direct admission to CCU.

1.1.5.1.2 Primary percutaneous coronary intervention

PPCI is defined as 'emergent percutaneous catheter intervention in the setting of STEMI, without previous fibrinolytic treatment' (Steg et al, 2012). This involves a percutaneous 'key hole' approach, where the culprit coronary artery occlusion is visualised by angiography and

treated mechanically to restore flow. This may involve removal of thrombus, dilating the occluded area with a balloon catheter and deployment of a metal stent to maintain patency. A landmark meta-analysis by Keeley et al in 2003 assessed 23 randomised trials comparing thrombolysis versus PPCI for STEMI. The results showed a significant benefit in reduction in major adverse cardiac events (MACE) including death in both the short and long term (Keeley et al, 2003). The net benefit appeared to be due to a combination of improved restoration of coronary blood flow with a concomitant avoidance of the complications of thrombolysis, principally haemorrhage. This data prompted a sea-change in the way STEMI care has been delivered. PPCI has emerged as the reperfusion modality of choice, as long as this can be delivered without unacceptable delays in transfer to PPCI-capable centres. Major organisational and logistical changes were required to deliver the 24 hours a day, 7 days a week (24/7) primary PCI service that exists for the majority of the UK population. Recent data from the Freeman Hospital in Newcastle-upon-Tyne showed that this is achievable with no differences seen in short or long-term mortality between STEMI patients presenting to the PPCI service during working-hours compared to outside working-hours (Noman et al, 2012). Peri-procedural adjuncts during PPCI recommended to further increase the net benefit of PPCI include radial rather than femoral arterial access (Jolly et al, 2011), thrombus aspiration (Svilaas et al, 2008) and the use of drug-eluting stents (Kastrati et al, 2007).

1.1.5.2 Pharmacological therapy

All patients undergoing PPCI are recommended to receive two oral anti-platelet drugs, so called ‘dual antiplatelet therapy’ (DAPT), with aspirin and an adenosine diphosphate (ADP) receptor antagonist (Steg et al, 2012). Aspirin is prescribed for life (ISIS-2 collaborative

group, 1988). The second drug is prescribed for 12 months, until the coronary stent has re-endothelialised. The aim of antiplatelet drugs is to maintain patency of the culprit coronary artery occlusion by prevention of recurrent thrombosis. Until recently, clopidogrel (Chen et al, 2005) was the most widely used ADP antagonist, but this has been superceded by prasugrel (Wiviott et al, 2007); the third recommended agent is ticagrelor (Wallentin et al, 2009; Steg et al, 2012) but use of this drug is not yet widespread in the UK, and no patients in my study received it.

An intravenous anticoagulant is recommended in all patients undergoing PPCI (Steg et al, 2012), which may be unfractionated heparin (UFH), low-molecular weight heparin (LMWH) (Montalescot et al, 2011) or bivalirudin (a direct thrombin inhibitor) (Stone et al, 2008). The use of platelet glycoprotein IIbIIIa receptor inhibitors may also be considered in selected cases at the operator's discretion, usually where the thrombus burden is judged to be high. Options include abciximab (De Luca et al, 2005), tirofiban (Valgimigli et al, 2010) and eptifibatide (Zeymer et al, 2010).

1.1.5.3 Long-term management

There are several evidence-based drugs which are routinely recommended for long-term therapy post-MI to improve prognosis. These included HMG co-enzyme A reductase inhibitors ('statins') (Cannon et al, 2004; Ray et al, 2005) and angiotensin converting enzyme (ACE) inhibitors (Fox, 2003). The aldosterone antagonist eplerenone may be used in patients with evidence of a left ventricular ejection fraction $\leq 40\%$ or symptoms of heart failure (Pitt et al, 2003).

Emphasis is placed on optimal control of recognised modifiable CAD risk factors including dyslipidaemia, hypertension and diabetes, which may involve lifestyle and pharmacological treatment. Lifestyle advice includes smoking cessation, regular exercise, healthy diet and maintaining normal BMI. Other therapies with proven mortality benefit after MI include cardiac rehabilitation (Lawler et al, 2010) and induced hypothermia after cardiac arrest acutely after STEMI (Bernard et al, 2002; Holzer et al, 2002).

1.1.6 Contemporary management of non ST elevation myocardial infarction

Many of the broad management principles described above for STEMI patients in terms of long-term secondary prevention strategies with pharmacological agents (DAPT, ACE inhibitors and statins) and lifestyle measures to improve prognosis also apply to NSTEMI patients (Hamm et al, 2011). Additionally, beta-blocker drugs are routinely used to reduce symptoms of ischaemia but there is no evidence that they alter prognosis. The main difference between STEMI and NSTEMI is that the underlying pathology in NSTEMI is usually not complete occlusion of the coronary artery. Although both conditions are associated with significant acute complications, NSTEMI does not require an urgent interventional procedure in the form of PPCI. A range of 'risk-stratification' tools can be employed to help identify those NSTEMI patients who are likely to benefit from an 'invasive' management strategy to reduce the risk of recurrent ischaemia and resultant complications (Fox et al, 2010). This invasive approach involves timely assessment of the coronary arteries by angiography with a view to 'revascularisation' by either PCI or coronary artery bypass grafting (CABG), or continuation of drug-treatment alone – 'optimised medical therapy' (OMT). The most commonly used risk stratification systems for NSTEMI are the TIMI

(Antman et al, 2000) and GRACE (Fox et al, 2006) scores. The decision between revascularisation by PCI or CABG depends upon factors including co-morbidities and the angiographic pattern of coronary artery narrowing(s) (Hamm et al, 2011).

As for STEMI patients, all NSTEMI patients receive initial DAPT with aspirin and clopidogrel. This DAPT is continued for 12 months after PCI, followed by lifelong aspirin. Additionally, anticoagulation with either fondaparinux (factor Xa inhibitor) or LMWH is recommended for the first few days (Hamm et al, 2011). Fondaparinux is not in widespread use in the UK currently; all NSTEMI patients in my study received LMWH (see 3.1.4). Additional anticoagulant therapy with platelet glycoprotein receptor IIbIIIa inhibitors or bivalirudin may be used 'upstream' before PCI, or as a peri-procedural adjunct during PCI. Use of these drugs is at the discretion of the individual physician and consequently varies widely.

1.2 MONOCYTES AND MONOCYTE SUBSETS

1.2.1 Monocytes

Monocytes were first recognised to be derived from haematopoietic precursor cells in the bone marrow. They develop from a common myeloid progenitor cell shared with neutrophils. Entry into the blood is regulated by CCR2. They circulate for three to five days, then reside in reservoirs including the spleen or migrate into tissues and develop into macrophages and dendritic cells (Ebert & Florey, 1939; Volkman & Gowans, 1965; van Furth & Cohn, 1968; Swirski et al, 2009; van der Laan et al, 2013). More recently, the development of precursors which give rise to monocytes outside the bone marrow ('extra medullary monocytopoiesis') has been described (Robbins et al, 2012; Leuschner et al, 2012). Monocytes in circulation exist in a constant but reversible interaction with platelets ('monocyte platelet aggregates', MPAs) which is calcium dependent (Shantsila et al, 2014). Monocytes exhibit high developmental plasticity and intrinsic heterogeneity (van de Veerdonk & Netea, 2010). Moreover, the monocyte pool may include a population of cells with pluripotent progenitor potential (Zhao et al, 2003).

The primary function of circulating monocytes is in host defence against foreign pathogens as part of the innate immune system, primarily by phagocytosis. However, accumulating evidence indicates that monocytes and macrophages also play key roles at all stages of atherosclerotic cardiovascular disease (reviewed by Pamukcu et al, 2010). These roles include processes taking place within the plaque itself but also whilst the monocyte is in the circulation. This indicates that monocyte-associated plaque generation, progression and destabilisation is both a local and systemic process.

A role for monocytes and macrophages in atherogenesis was first hypothesised when they were detected in atherosclerotic plaques of animals (Watanabe et al, 1985) and humans (Gown et al, 1986). Monocyte depletion from circulation was found to significantly reduce plaque formation in rabbits (Ylitalo et al, 1994), although this depended upon the stage of atherosclerosis. Indeed, depletion of monocytes at the early stages of atherosclerosis decreases the number and activity of macrophages within plaques, inhibits lesion development, and alters plaque composition by reducing collagen content and necrotic core formation. However, monocyte depletion at more advanced stages of atherosclerosis does not yield such effects (Stoneman et al, 2007). This highlights the principle that monocytes play fundamental roles from the very earliest stages of lesion formation, at least in experimental studies.

In addition to these pathological processes, monocytes may also exert beneficial effects such as a contribution to angiogenesis and tissue repair. Indeed, monocytes express a wide range of angiogenic factors and appear to be critical for orchestrating arrangement of different cell types in neovessels (Shantsila et al, 2008).

1.2.2 Monocyte subsets and heterogeneity

The numerous and very distinct roles attributed to monocytes in homeostasis, inflammation and repair lead to the concept of monocyte heterogeneity and the hypothesis that monocytes may commit to specific functions whilst in circulation (reviewed by Woollard & Geissmann, 2010). This prompted the identification of phenotypic subsets in humans and mice (Ziegler-Heitbrock et al, 1988; Passlick et al, 1989). However, the majority of research into monocyte subsets in atherogenesis has been performed in mice, and as humans and animals do not

express directly comparable monocyte surface markers, comparisons between species and therefore extrapolation of data are difficult and require caution.

1.2.2.1 Mouse monocytes

Mouse monocyte subsets were first identified according to their expression of CCR2, CD62L (L-selectin) and fractalkine chemokine receptor 1 (CX_3CR1). Subsequently Ly6C (part of the epitope of GR1) was identified as an additional marker of $CCR2^+$ monocytes, and two mouse monocyte subsets were described; $CCR2^+CD62L^+CX_3CR1^{lo}Ly\text{-}6C^{hi}$ and $CCR2^-CD62L^-CX_3CR1^{hi}$ $Ly\text{-}6C^{lo}$ (or more simply $Ly\text{-}6C^{high}$ and $Ly\text{-}6C^{low}$, respectively) (Gordon & Taylor, 2005). Mouse monocyte subsets do not arise from distinct progenitors but convert from the $Ly\text{-}6C^{high}$ to the $Ly\text{-}6C^{low}$ subset (Yona et al, 2013). Subsequently, evidence emerged identifying subset-specific involvement in the pathophysiology of atherosclerosis (Swirski et al, 2007; Tacke et al, 2007).

$Ly\text{-}6C^{high}$ monocytes (see 1.2.2.1) produced by extramedullary haematopoiesis infiltrate atherosclerotic plaques (Robbins et al, 2012). They are derived from splenic stores where release is mediated by angiotensin II and bone marrow pools where release is mediated by CCR2 (Swirski et al, 2009). Release from bone marrow is triggered by the sympathetic nervous system and may be inhibited with β3 adrenoceptor blockers (Dutta et al, 2012). $Ly\text{-}6C^{high}$ monocytes actively migrate into the myocardium during the early phase of MI (Nahrendorf et al, 2007). This has recently been imaged in real time, in-vivo, using intravital microscopy (Lee et al, 2012; Jung et al, 2013). In mice, monocytes within infarcted myocardium turnover very quickly; after an average of 20 hours they become apoptotic and are replaced by newly recruited cells (Leuschner et al, 2012).

Ly-6C^{high} monocytes have prominent proinflammatory, proteolytic and phagocytic properties within plaques. Through protease activity they digest dead cells and extra cellular matrix and clear the debris by phagocytosis to allow for the subsequent healing processes of elaboration of granulation tissue and scar formation. They are implicated in plaque destabilisation via production of cytokines, myeloperoxidase and matrix metalloproteinases (MMPs) (Swirski et al, 2007; Libby et al, 2008). Prolongation of Ly-6C^{high} monocytosis seen in atherosclerotic mice disturbs resolution of inflammation, impairs infarct healing and is associated with adverse left ventricular (LV) remodelling following experimental MI (Panizzi et al, 2010). Blockade of their recruitment improves LV healing after MI (Majmudar et al, 2013).

In contrast, Ly-6C^{low} monocytes play a role in the immune response, including replenishing the population of tissue-macrophages and dendritic cells (Auffray et al, 2009). They differentiate from the Ly-6C^{high} subset and have a lower content of inflammatory molecules and proteases. They feature more in the recovery phase following MI, where they promote healing and repair of the myocardium via myofibroblast accumulation, deposition of collagen and stimulation of angiogenesis via expression of vascular endothelial growth factor (VEGF) and transforming growth factor-β (Nahrendorf et al, 2007). Recent data suggests that Ly-6C^{low} monocytes are enriched within capillaries and function as intravascular scavenging 'housekeepers' that recruit neutrophils to mediate focal necrosis of endothelial cells and phagocytosis of cellular debris (Carlin et al, 2013). These two phases of monocyte recruitment after MI should not be viewed in isolation, as there is a close relationship between the acute inflammation during the early days after MI and the ensuing chronic inflammation that promotes remote recruitment of inflammatory cells which fuels progression of plaque growth and destabilisation (Dutta et al, 2012; Swirski et al, 2013).

1.2.2.2 Human monocytes

Human monocytes were first identified by their abundant expression of CD14 (part of the lipopolysaccharide (LPS) receptor) (Passlick et al, 1989). Differential expression of CD16 (the low affinity receptor for IgG) allowed the discrimination of two subsets; $CD14^{++}CD16^{-}$ (~85% total monocytes) and $CD14^{+}CD16^{+}$ (~15% total monocytes) by two-colour flow cytometry (Ziegler-Heitbrock et al, 1988; Passlick et al, 1989).

$CD14^{+}CD16^{-}$ monocytes are large cells with inflammatory roles, high phagocytic and proteolytic activity. They respond to LPS stimulation in vitro by releasing interleukin (IL)-10 (Skrzeczyńska-Moncznik et al, 2008). The remaining monocytes in humans have usually been considered as one subset, $CD14^{+}CD16^{+}$. In contrast with the $CD14^{+}CD16^{-}$ subset, $CD14^{+}CD16^{+}$ monocytes are small and possess low phagocytic activity. They 'patrol' the endothelium and rapidly extravasate in response to inflammatory stimuli (Auffray et al, 2007) in a similar manner to the mouse Ly-$6C^{low}$ subset (Cros et al, 2010).

CD16+ monocyte numbers are significantly increased in subjects with CAD (Schlitt et al, 2004; Hristov et al 2010). High CD16+ monocyte count was associated with subclinical atherosclerosis assessed by an association with increased carotid artery intima medial thickness (Rogacev et al, 2010). In one study of patients with acute MI treated by PPCI, $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocytes were sequentially mobilised with peaks in venous blood on day 3 and day 5 respectively. The $CD14^{+}CD16^{-}$ monocyte count was inversely correlated with LV recovery after MI (Tsujioka et al, 2009). Subsequently, the peak $CD14^{+}CD16^{-}$ monocyte count following MI was significantly higher in-patients with evidence of ongoing microvascular obstruction detected by gadolinium enhanced

cardiovascular magnetic resonance imaging (MRI), which is associated with LV impairment and a poor prognosis (Tsujioka et al, 2010). CD16+ monocytes were positively associated with the presence of vulnerable coronary plaques (Kashiwagi et al, 2010) and with fibrous cap thickness (Imanishi et al, 2010). Recent data from post-mortem tissue from humans describes the spatio-temporal distribution of monocytes in the myocardium following MI. In the 'early' stage after MI (3 - 12 hours), there were no differences in myocardial monocyte number or distribution compared to controls. In the acute 'inflammatory' phase after MI (12 hours - 5 days), monocytes accumulated in the infarct border zone surrounding the necrotic core, adjacent and adherent to viable cardiomyocytes, of which approximately 85% were CD14+CD16- monocytes versus 15% CD14+CD16+ monocytes. In the 'proliferative' phase after MI (5 - 14 days), there was a massive accumulation of monocytes in the infarct core consisting of granulation tissue, with very few in the border zone. At this stage 60% were CD14+CD16-, and 40% CD14+CD16+. After acute MI, the number of monocytes (CD14+ cells) in the bone marrow was 39% lower than controls, and in the spleen was 58% lower than controls (van der Laan et al, 2013), suggesting that the spleen may be an important reservoir for monocytes in humans. This temporal pattern described mirrors the previous observations in peripheral blood by Tsujioka and colleagues where CD14+CD16+ monocyte number peaked on day 5 after MI. Unfortunately there was no discrimination between the CD16+ monocytes in these studies (Tsujioka et al, 2009; van der Laan et al, 2013), so it is unclear which of the CD16+ subsets is implicated in these observations.

The majority of studies, including even the most recently published (Arslan et al, 2013; van der Laan et al, 2013), consider CD16+ monocytes as a single population, whereas it is increasingly apparent there are two distinct subsets within this group (Zawada et al, 2011;

Wong et al, 2011, Ziegler-Heitbrock & Hofer, 2013). This is a major limitation of many studies. Initial attempts at categorisation divided CD16+ monocytes into $CD14^{low}CD16^{+}$ and $CD14^{+}CD16^{+}$ subsets (Table 1.2). However, this is not an ideal nomenclature as expression of CD14 represents a continuum, and division into $CD14^{low}$ and $CD14^{+}$ is inherently artificial and therefore inexact. More recently efforts have been made to definitively discriminate CD16+ cells phenotypically as two distinct subpopulations (Kim et al, 2010; Tallone et al, 2011; Shantsila et al, 2011; Hristov et al, 2012; Ziegler-Heitbrock & Hofer, 2013). Our research group described that the three human monocyte subsets can be unequivocally defined by addition of CCR2 to the panel of the markers which allows accurate discrimination between CD16+ cells. We define the three subsets as: 'Mon1' (CD14++CD16-CCR2+), 'Mon2' (CD14++CD16+CCR2+), and 'Mon3' (CD14+CD16++CCR2-) (Shantsila et al, 2011) (Table 1.3). These definitions of monocyte subsets are used in my study. Our findings are in keeping with the work of Zawada and colleagues who demonstrated a 2-fold higher expression of TIE2 protein by the 'intermediate' subset (Zawada et al, 2011).

The definition of human monocytes as three subsets is a recent change, and there have consequently been few studies specifically investigating their individual functions. However, emerging data has been very interesting, with the potential to focus in and refine our understanding of their individual roles in cardiovascular disease. Of particular interest, amongst CD16+ monocytes the CD14++CD16+ subset appears to be especially pro-atherogenic. CD14++CD16+ monocytes selectively express the chemokine receptor CCR5 (Ancuta et al, 2003; Zawada et al, 2011; Rogacev et al, 2011) which has been associated with atherosclerosis in experimental and epidemiological studies (Gonzalez et al, 2001; Pai et al, 2006; Muntinghe et al, 2009). They are also major producers of reactive oxygen species

which propagate atherosclerosis (Zawada et al, 2011). Furthermore, the pro-angiogenic features of CD14++CD16+ monocytes (Venneri et al, 2007; Zawada et al, 2011) may link them to the process of plaque neovascularization seen in advanced stages of atherosclerosis which is associated with instability and the potential for rupture (Michel et al, 2011). In a recent observational cohort study, CD14++CD16+ monocytes were the only independent predictor of adverse cardiovascular outcomes after adjustment for cardiovascular risk factors (Rogacev et al, 2012). This association with atherosclerosis is in keeping with evidence of the proinflammatory capacity of CD16+ monocytes (Zawada et al, 2011; Belge et al, 2002) along with their preferential affinity and adherence to activated endothelial cells (Steppich et al, 2000; Ancuta et al, 2003). Additionally, CD16+ monocytes secrete IL-6, matrix metalloproteinases (MMPs), and chemokines which attract further monocytes and lymphocytes (Ancuta et al, 2006) propagating atherosclerosis. These findings raise the possibility that CD14++CD16+ monocytes may represent a future therapeutic target in the treatment of cardiovascular disease, and highlight the requirement for more research in this area.

The association of CD14++CD16+ monocytes with cardiovascular risk is in keeping with mouse data suggesting they are the human counterparts of pro-atherogenic Ly-6C^{high} monocytes (Cros et al, 2010). However, drawing parallels between mouse and human monocyte subset data has been difficult, as most mouse studies of monocyte heterogeneity considered only two subsets, Ly6C^{high} and Ly6C^{low}. Moreover, many mouse studies have been performed in apolipoprotein knockout mice fed an atherogenic diet. This induces marked elevations in lipid levels which may cause massive rises in Ly6C^{high} monocyte numbers but no effect on Ly6C^{low} numbers. Such changes were not seen in wild-type mice

fed this diet (Swirski et al, 2007). Cros and colleagues recently showed that both human CD14++CD16- and CD14++CD16+ monocytes cluster together with mouse Ly6C^{high} monocytes, whereas CD14+CD16++ monocytes cluster together with Ly6C^{low} monocytes (Cros et al, 2010) which has helped reconcile the differences (see table 1.3).

Differences in terminology and definitions of monocyte subsets between studies have been confusing and have lead to difficulties in comparing data. To address this issue, the International Union of Immunological Sciences and the World Health Organisation issued a statement in 2010, proposing a consensus on monocyte subset nomenclature. This document recommends describing human monocytes as three subsets, namely 'Classical' (CD14+CD16-), 'Intermediate' (CD14++CD16+) and 'Nonclassical' (CD14+CD16++) (Ziegler-Heitbrock et al, 2010), where '+' denotes an expression level 10-fold above the isotype control and '++' denotes expression level 100-fold above the isotype control. The consensus statement suggests that they correspond to three equivalent populations in mice, as summarised in table 1.2.

Table 1.2 Definition of monocyte subsets (adapted from Ziegler-Heitbrock et al, 2010)

INTERNATIONAL UNION OF IMMUNOLOGICAL SCIENCES & WORLD HEALTH ORGANISATION DEFINITION OF MONOCYTE SUBSETS		
	Human	**Mouse**
Classical	CD14++CD16-	Ly6C++CD43+
Intermediate	CD14++CD16+	Ly6C++CD43++
Nonclassical	CD14+CD16++	Ly6C+CD43++

This consensus nomenclature proposal is a welcome attempt to introduce consistency in describing subsets. The majority of current data published was conceived and executed before this nomenclature was proposed, including that in this thesis. The definitions of monocyte subsets in this thesis are those our group first described in healthy subjects in 2011 (Shantsila et al, 2011). Characteristics of these subsets and their correspondence to the 2010 nomenclature are described in table 1.3.

Table 1.3 Characteristics of human monocyte subsets

Human monocyte subsets	'Classical' $CD14^{+}CD16^{-}CCR2^{+}$ 'Mon1'	'Intermediate' $CD14^{+}CD16^{+}CCR2^{+}$ 'Mon2'	'Non-classical' $CD14^{low}CD16^{+}$ $CCR2^{-}$ 'Mon3'	Ziegler-Heitbrock et al, 2010 Shantsila et al, 2011
Corresponding mouse subset	Ly-$6C^{high}$	Not clear	Ly-$6C^{low}$	Grage-Griebenow et al, 2001 Geissmann et al, 2003
	Ly6C++ CD43+	Ly6C++ CD43++	Ly6C+ CD43++	Ziegler-Heitbrock et al, 2010
Approximate proportion of overall monocytes	85%	5%	10%	Ziegler-Heitbrock et al, 1988 Passlick et al, 1989 Shantsila et al, 2011
Size	++	+++	+	Shantsila et al, 2011
Granularity	++	++	+	Shantsila et al, 2011
Number in plaques	Frequent, associated with plaque destabilisation		Infrequent	Swirski et al, 2007
Primary roles	Rapid recruitment to sites of inflammation Phagocytosis Scavenging necrotic debris Release of MMPs Cytokine production Give rise to macrophages in atheromata	Angiogenesis Anti-inflammatory cytokine production (IL-10)	Anti-inflammatory Collagen deposition Healing 'Patrol' endothelium	Sunderkötter et al, 2004 Swirski et al, 2007 Libby et al, 2008 Skrzeczyńska-Moncznik et al, 2008 Auffray et al, 2007 Cros et al, 2010

Phagocytic activity	High	High	Low	Cros et al, 2010 Shantsila et al, 2011
NFκB activity	High	Moderate	Moderate	Shantsila et al, 2011
Chemokine receptors	Mobilisation via CCR2 receptor in response to MCP-1	Mobilisation via CX3CR1 in response to fractalkine	Data not available	Weber et al, 2000 Aukrust et al, 1998 Ancuta et al, 2004
LPS stimulated cytokine production	Potent producers of IL6, IL-1β, MCP-1	Maximal production of IL10	Unresponsive to LPS	Shantsila et al, 2011 Frankenberger et al, 1996 Mizuno et al, 2005
Surface expression of angiogenic receptors (VEGFR1, VEGFR2, CXCR4, Tie 2)	Low	High	Low	Shantsila et al, 2011 Zawada et al, 2011
Surface expression of adhesion molecules (ICAM, VCAM)	Moderate expression of ICAM-1 and VCAM-1	High expression of ICAM-1 receptor. High expression of adhesion molecules and increased monocyte endothelial adherence	High expression of VCAM-1 receptor	Shantsila et al, 2011 Ancuta et al, 2004
Surface expression of scavenger receptors CD163 CD204	 Medium Low	 High Medium	 Low High	 Shantsila et al, 2011 Buechler et al, 2000

Change in stroke	No effect	Increased, and predictive of subsequent infections. High level associated with better survival.	Decreased	Urra et al, 2009
Change in severe asthma	Not studied	Increased Decrease on allergen challenge	No effect	Moniuszko et al, 2009 Kowal et al, 2012
Change in rheumatoid arthritis	Decreased	Increased High level predicts reduced response to therapy		Cooper et al, 2012
Changes in colorectal cancer		Increased, especially in localised rather than metastatic cancer		Schauer et al, 2012
Changes in survivors of ALL		Increased		Sulicka et al, 2013
Association with adverse CV events in CKD dialysis patients		Positive association with adverse outcomes		Heine et al, 2008 Rogacev et al, 2011
Association with adverse CV events in patients undergoing elective coronary angiogram		Positive association with adverse outcomes		Rogacev et al, 2012

Changes in heart failure	Increased in acute heart failure	Increased in acute and chronic heart failure. Increased expression of markers of activation (CD14) and chemotaxis (CCR2). Associated with adverse prognosis	No difference	Wrigley et al, 2013

ALL, Acute Lymphoblastic Leukaemia; MMP, Matrix Metalloproteinase; ECM, Extra-Cellular Matrix; ROS, Reactive Oxygen Species; IL-10, Interleukin 10; NFκB, Nuclear Factor Kappa B; LPS, Lipopolysaccharide; IL-6, Interleukin 6; IL-1β, Interleukin 1β; MCP-1, Monocyte Chemoattractant Protein-1; VEGFR1, Vascular Endothelial Growth Factor Receptor 1; VEGFR2, Vascular Endothelial Growth Factor Receptor 2, CXCR4, C-X-C chemokine receptor type 4; ICAM-1, Intercellular Adhesion Molecule-1; VCAM-1, Vascular Cell Adhesion Molecule-1; CCR2, Chemokine (C-C motif) Receptor 2; CX3CR1, Chemokine (C-X3-C motif) receptor; CV, cardiovascular; CKD, chronic kidney disease; CAD, coronary artery disease

1.3 THE ROLE OF MONOCYTES IN ATHEROSCLEROTIC PLAQUE DESTABILISATION

1.3.1 Plaque development

Although the primary function of circulating monocytes is in host defence against foreign pathogens as a part of the innate immune system, there is a growing body of data which has linked specific monocytes subsets to plaque formation and destabilisation in animals and human studies. Plaque development as the first step in the development of atherosclerosis was discussed in section 1.1.4.

1.3.2 Plaque destabilisation

Plaque destabilisation is an extremely complex multifaceted process, triggered by inflammatory environment within the plaque (van der Wal et al, 1994). Mechanisms implicated include an unbalanced generation of inflammatory cytokines and angiogenic growth factors which promote pathological plaque neovascularisation (Maseri & Fuster, 2003). However, not all atherosclerotic plaques become unstable and rupture. The risk of plaque rupture depends on plaque composition rather than size per se, of which monocyte and macrophages are key determinants. Macrophages (Moreno et al, 1994) and T-lymphocytes (Neri Serneri et al, 1997) are the principal inflammatory cells in human coronary unstable plaques where they are present in increased density compared to stable plaques (Richardson et al, 1989; Lendon et al, 1991; Davies et al, 1993). Characteristic histological features of vulnerable plaques include a thin fibrous cap, high ratio of macrophages to vascular smooth-muscle cells (Moreno et al, 1994), reduced collagen content and high lipid content.

Beside the cellular interactions taking place inside the plaque, plaque destabilisation is also facilitated by circulating monocytes which are able to generate and secrete mediators of all the major factors involved in plaque destabilisation, including inflammation, matrix degradation, and thrombogenesis (Shantsila & Lip, 2009). This indicates that plaque destabilisation is both a local and systemic process. Intriguingly, recent data suggests that after MI in experimental mice, pre-existing plaques become larger with a more advanced 'vulnerable' morphology. This acceleration of disease progression was maintained over time and was associated with a significant and persistent increased recruitment of monocytes. These monocytes are liberated from the bone marrow and spleen via sympathetic nervous system signalling after MI (Dutta et al, 2012).

Hitherto stable atherosclerotic plaques may progress to become acutely unstable which is the substrate for MI. The fibrous cap which separates the plaque's content from contact with blood becomes thinned and may rupture. This exposes the plaque's procoagulant contents to circulating clotting factors and discharges plaque debris and tissue factor. Activation of the coagulation cascade results in thrombus formation, coronary occlusion, tissue ischaemia and necrosis. Early identification of coronary plaques prone to rupture is challenging as they are often clinically silent, are non-obstructive and appear similar to stable plaques on a coronary angiogram.

However, there is emerging evidence linking plaque vulnerability with changes in circulating levels of specific monocyte subsets. For example, an increased count of CX_3CR1-expressing monocytes was associated with coronary plaque rupture in patients with unstable angina (Ikejima et al, 2010). Imanishi and colleagues examined fibrous cap thickness of non-culprit

lipid-rich plaques by optical coherence tomography (OCT) in patients with unstable angina who underwent PCI. Monocytes were measured and OCT performed at baseline (day of PCI) and 9 months post PCI. Monocyte populations were defined as $CD14^{+}CD16^{+}CX3CR1^{+}$ and $CD14^{+}CD16^{-}CCR2^{+}$. At 9 months, fibrous cap thickness increased by 100% in subjects taking statins and 73% in those not on statins. The percentage change in fibrous cap thickness was significantly correlated with the percentage change in $CD14^{+}CD16^{+}CX3CR1^{+}$ monocytes, but not $CD14^{+}CD16^{-}CCR2^{+}$ monocytes. The percentage change in $CD14^{+}CD16^{+}CX3CR1^{+}$ monocytes was significantly decreased in the statin group compared to the non-statin group (Imanishi et al, 2010). This study therefore demonstrates a negative correlation between $CD14^{+}CD16^{+}CX3CR1^{+}$ monocytes and fibrous cap thickness, suggesting a relationship between this monocyte subset and coronary plaque vulnerability, which statins may be able to modulate. As statins have the ability to increase fibrous cap thickness (Takarada et al, 2009), the finding of a correlation between statin use and a decreased percentage in $CD14^{+}CD16^{+}CX3CR1^{+}$ monocytes suggests that statins may have the potential to increase integrity and thickness of the fibrous cap by reducing number of $CD14^{+}CD16^{+}CX3CR1^{+}$ monocytes. A limitation of this study is the consideration of $CD14^{low}CD16^{+}$ and $CD14^{+}CD16^{+}$ monocytes as a single population (defined as $CD14^{+}CD16^{+}CX3CR1^{+}$), so it is not possible to ascertain which of the $CD16^{+}$ monocytes may be implicated in this regard.

An elevated $CD14^{+}CD16^{+}$ monocyte count has also been associated with characteristics of plaque vulnerability assessed by multidetector computed tomography (CT) in patients with stable CAD without raised hs-CRP (Kashiwagi et al, 2010). Whether this monocyte subset has a causal role in plaque vulnerability or is a marker of an inflammatory process in the

coronary artery is unclear. Again, this study did not discriminate subsets of $CD16^+$ monocytes which should be considered a potential study limitation.

1.3.3 The monocyte journey towards plaque destabilisation

The pathogenesis of atherosclerosis and plaque destabilisation begins with the activation of monocytes and mobilisation from the bone marrow into the circulation, and thence recruitment to the vascular wall. This process occurs in response to the inflammatory milieu of chemokines produced locally by damaged tissues or released in the context of systemic inflammation. This is reflected by high circulating levels of pro-inflammatory cytokines and adhesion molecules in atherosclerotic disease (Ross, 1986).

1.3.3.1 Monocyte activation

Plaque destabilisation is closely associated with monocyte activation and change in phenotype. Activated T cells within vulnerable plaques produce interferon (IFN) γ which subsequently activates plaque monocytes and macrophages (Serneri et al, 1992). Monocytes are activated via a number of pattern-recognition receptors, such as the endotoxin receptor CD14 and toll-like receptors (TLRs) (Medzhitov, 2007). Bone marrow-derived monocytes expressing TLR2 and TLR4 are pivotal in arteriogenesis and collateral artery growth in mice (de Groot et al, 2011). TLR1, TLR2, and TLR4 are upregulated in the endothelium and expressed by monocytes within atherosclerotic plaques (Vink et al, 2002). TLRs may be stimulated by a variety of molecules expressed during cardiovascular injury (Ohashi et al, 2000; Okamura et al, 2001; Smiley et al, 2001; Johnson et al, 2002; Termeer et al, 2002; Scheibner et al, 2006) with the net result of activation of monocytes via the nuclear factor κ B

(NFκB pathway). TLR4, functionally linked to its co-receptor CD14 is overexpressed on the surface of monocytes at sites of atherosclerotic plaques (Ishikawa et al, 2008). The number of circulating $TLR4^{+}CD14^{+}$ monocytes is twice as high in ACS patients compared to subjects with stable CAD (Ishikawa et al, 2008). The density of CD14 on the monocyte surface is much higher in patients with MI (Methe et al, 2005).

Overexpression of TLR4 by monocytes in circulation and on ruptured plaques following acute MI has been associated with high levels of inflammatory cytokines, IL-6 and tumour necrosis factor (TNF) α (Ishikawa et al, 2008). Prolonged expression of these markers which amplify the inflammatory response after MI has been associated with the development of heart failure (Satoh et al, 2006). The intracellular NFκB signalling pathway is the principal mechanism for the monocyte inflammatory response at every stage of atherosclerosis, including plaque destabilisation (Ghosh & Karin, 2002). The NFκB pathway regulates expression of lipoxygenase (Zhao & Funk, 2004) and cyclo-oxygenase (Burleigh et al, 2002) involved in the atherogenic modification of LDL. The NFκB pathway also regulates the expression of adhesion molecules, including P-selectin, E-selectin, intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (Collins et al, 2000; Cybulsky et al, 2001). Furthermore the NFκB pathway regulates transcription of the extracellular matrix-degrading enzymes, MMP-9 and myeloperoxidase (Sugiyama et al, 2001; Tavora et al, 2009) in monocytes found in fibrous caps. Indeed, overexpression of these cytokines is closely linked to plaque destabilisation and atherothrombosis.

1.3.3.2 Monocyte mobilisation

Monocyte chemoattractant protein (MCP-1) is the most important chemokine implicated in the regulation of mobilisation, migration and infiltration of monocytes and macrophages. There is much evidence highlighting its contributions to vascular inflammation, thrombosis, plaque formation, progression and destabilisation. MCP-1 accelerates atherosclerosis in apolipoprotein E (ApoE)-deficient mice (Aiello et al, 1999). Production in vitro is upregulated by inflammatory cytokines (Morimoto et al, 2006) and by the interaction between monocytes and endothelium (Lukacs et al, 1995) where it is tethered to proteoglycans on the luminal surface of endothelial cells. MCP-1 levels were found to be significantly elevated in samples taken distal to the culprit coronary artery lesion immediately after PPCI in STEMI patients compared to samples taken in patients with stable angina (Benson et al, 2013). Localised overexpression of MCP-1 by the vascular wall promotes macrophage infiltration and development of atherosclerosis (Namiki et al, 2002), neovascularization (Salcedo et al, 2000) and expression of cytokines and chemokines (Morimoto et al, 2006; Park et al, 2005). Additionally, MCP-1 promotes smooth muscle proliferation and plaque neovascularisation, leading to rapid lesion progression and instability (Doyle & Caplice, 2007). Also, MCP-1 may enhance MMP expression and release by plaque monocytes leading to the plaque rupture (Robinson et al, 2002).

Although the underlying mechanism is unknown, an elevated level of MCP-1 and associated monocytosis are linked with an adverse prognosis following MI (de Lemos et al, 2003; de Lemos et al, 2007). This may reflect a greater extent of atherosclerotic disease, a prothrombotic state related to monocytosis which facilitates recurrent occlusive coronary events, or a more intense and persistent proinflammatory state with ongoing ‘vulnerable

plaques'. Indeed, MCP-1 gene polymorphisms were associated with up to 2-fold increased prevalence of MI in-patients in the Framingham Heart Study (McDermott et al, 2005). Excess MCP-1 may cause abnormal accelerated monocyte recruitment into the arterial wall and myocardium, and an imbalance in the pathological and reparative roles of monocytes. Importantly, MCP-1 level varies with sampling time after MI (Korybalska et al, 2010), which may be related to the sequential recruitment of different monocyte subsets following MI (Nahrendorf et al, 2007; Tsujioka et al, 2009) as described in section 1.2.

Interestingly, in contrast to the post-MI setting, plasma MCP-1 levels appear to have little prognostic importance in patients with stable CAD (Haim et al, 2005). This may be due to a disparity between local and systemic MCP-1 levels. High local expression of MCP-1 on the luminal surface of endothelial cells in the vascular wall at the site of a plaque, which promotes localised monocyte migration, may not be associated with elevated MCP-1 level in circulation.

Genetic deletion of MCP-1 (Gu et al, 1998) or its receptor (CCR2) (Boring et al, 1998) reduces development of atherosclerotic lesions in mice. The technique of Ribonucleic acid interference (RNAi) directed towards silencing monocyte CCR2 improves healing and attenuates LV remodelling after MI in apoE-/- mice with a 41% reduction in $LyC6^{high}$ monocyte recruitment to infarcted myocardium (Majmudar et al, 2013). Blockade of the MCP-1 pathway using gene therapy (Ni et al, 2001) or an inhibitory antibody (Lutgens et al, 2005) attenuates initiation and progression of the atheroma (Inoue et al, 2002) in ApoE knockout hypercholesterolaemic mice. Moreover, plaques develop a more stable phenotype,

with reduced macrophage content and more smooth muscle cells and collagen. These beneficial effects are likely due to a reduction in recruitment and activation of monocytes.

HMG Co-enzyme A reductase inhibitor ('Statin') drugs decrease MCP-1 expression and neointimal inflammation in a rabbit model of atherosclerosis (Bustos et al, 1998) and reduce MCP-1 levels in circulating monocytes from hypercholesterolaemic humans (Rezaie-Majd et al, 2002; Lewandowski et al, 2008). Via suppression of NFκB activity, the lipid-modifying drug ezetimibe reduces plaque MCP-1 expression, monocyte migration and hence plaque macrophage and lipid content in a rabbit model of atherosclerosis (Gómez-Garre et al, 2009). These mechanisms may promote plaque stabilisation, which could contribute to the beneficial effects of these drugs in human atherosclerotic disease.

1.3.3.3 Monocyte adhesion and migration

In addition to their systemic actions in the circulation, monocytes migrate into the vascular wall where they exert local effects contributing to plaque destabilisation. The migration of different monocyte subsets to the endothelium depends on specific chemokines (Huo et al, 2001; von Hundelshausen et al 2005) causing differential receptor upregulation and interaction with specific ligands. In mice, $Ly6C^{high}$ monocytes are dependent upon CCR2, CX3CR1 and CCR5 for effective recruitment, whereas recruitment of $Ly6C^{low}$ monocytes is CCR5-dependent and not CCR2-dependent (Tacke et al 2007; Combadière et al, 2008). The hypothesis that CCR2 specifically mediates the recruitment of 'harmful' monocytes but not 'reparative' monocytes suggests that this may be a potential therapeutic target. In humans, the $CD14^{+}CD16^{-}$ subset expresses CCR2 and migrates in response to MCP-1, whereas $CD14^{+}CD16^{+}$ monocytes express CX3CR1 and respond to the chemokine CX3CL1

(fractalkine) (Weber et al, 2000; Ancuta et al, 2003; Nahrendorf et al, 2007). This illustrates the ability of the endothelium to selectively recruit monocyte subsets dependent upon specific local chemokines expression. Of note, polymorphisms of the CX3CR1 gene in humans are reported to be associated with a genetic predisposition to CAD. Heterozygosity for CX3CR1-V2491/T280M is associated with a marked reduction in risk of thrombotic coronary events (Moatti et al, 2001), possibly due to impaired ability of CX3CR1-expressing monocytes to invade the vascular wall and contribute to plaque development and destabilisation.

Healthy arterial endothelium resists adhesion and recruitment of monocytes. However, under the proinflammatory shift driven by cardiovascular risk factors including hypertension, dyslipidaemia, hyperglycaemia and cigarette smoking, the endothelium becomes activated (Falk, 2006) and upregulates expression of a variety of adhesion and chemoattractant molecules (Nelken et al, 1991; Couffinhal et al, 1993; Hansson et al, 2002). The key molecules involved in monocyte adhesion include P-selectin, E-selectin, VCAM-1 and ICAM-1. P-selectin and E-selectin mediate the tethering and rolling of circulating monocytes upon the endothelium (van der Wal et al, 1992; Weyrich et al, 1995). The selectins stimulate monocyte expression of integrins including ICAM-1 and VCAM-1 (Weyrich et al, 1995) which are over-expressed locally at sites of atherosclerosis (Gerszten et al, 1998) and can be seen in high concentration in the circulation from the early stages of an ACS (Meisel et al, 1998). Aggregation with platelets and formation of MPAs leads to upregulation of monocyte receptors to adhesion molecules (Simon et al, 1993) which may facilitate monocyte migration to sites of inflammation and atherogenesis. The interaction between monocytes and the endothelium activates monocytes, which results in an inflammatory cytokine cascade. This is

enhanced by high levels of CRP or heat shock proteins (HSP) seen in ACS (Satoh et al, 2006).

1.3.3.4 Monocyte proliferation

The ongoing survival, proliferation and differentiation of monocytes in atherosclerotic plaques is dependent upon macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and MCP-1 (Irvine et al, 2009). Stimulation of macrophages by M-CSF increases expression of scavenger receptors (including scavenger receptors A and B, CD36, CD68 and CXCL16) which promote phagocytosis of modified lipoproteins and apoptotic cells. The reduction in atherosclerosis in M-CSF deficient mice results from markedly decreased macrophage accumulation within plaques (Smith et al, 1995).

1.3.4 Proteolytic enzyme pathways

Systemic and local inflammation adversely influences collagen metabolism, the major component of the extracellular matrix that determines strength and stability within the fibrous cap. Inflammation results in degradation and proteolytic destruction of the extracellular connective tissue matrix and inhibition of production of new collagen by smooth muscle cells. This degradation of collagen leads to reduced tensile strength, thinning and weakening of the fibrous cap, especially in the 'shoulder' regions which are subject to intense shear stress. This ultimately leads to plaque destabilisation and rupture (Galis et al, 1994; Schwartz et al, 2007).

Monocyte-derived macrophages were first implicated in plaque destabilisation by demonstration that their incubation with human aortic plaques was associated with an increase

in collagen breakdown (Shah et al, 1995). Macrophages were subsequently acknowledged as the predominant source of a variety of synergistically-acting extracellular proteases responsible for plaque rupture. One of the additional beneficial mechanisms of statin drugs may be the inhibition of macrophage activation and subsequent protease production (Bocan, 2002).

1.3.4.1 Matrix metalloproteinases

The major enzymes responsible for active breakdown of the collagen-rich connective tissue matrix are the MMPs (Newby, 2008). In humans they comprise a family of 23 genetically-related proteins classified according to their structure and substrate. MMPs are produced as pro-enzymes requiring activation. Monocytes within atherosclerotic plaques and in the circulation are a major source of MMPs as well as molecules regulating their activation and inhibition, emphasising the key role of monocytes as mediators of plaque integrity (Table 1.4). Tissue homeostasis is maintained via a precarious balance between beneficial and deleterious effects of MMPs. Abnormal activity of MMPs in inflammation overwhelms the inhibitory effects of tissue inhibitors of metalloproteinases (TIMPs), which tips the balance towards plaque instability. Hence, TIMP gene transfer or TIMP knock-out may alternately promote plaque stability or instability (Johnson et al, 2006). Importantly, both the absolute level of MMP content within a plaque and its activation status are crucial in determining whether the net outcome is beneficial or destructive. For example, physiological levels of MMP-9 may promote plaque stability by promoting smooth muscle migration and fibrous cap formation (Johnson et al, 2005). Conversely, higher levels of activated MMP-9 may promote plaque inflammation, matrix destruction and hence instability and rupture (Luttun et al, 2004). Of note, circulating monocytes in humans with MI significantly overexpress MMP-2 and

MMP-9 genes even prior to their migration to the tissues. MMP-9 overexpression by macrophages induces plaque instability and rupture in Apo-E deficient mice (Gough et al, 2006). Conversely, MMP-9 knockout mice are less likely to suffer myocardial rupture following induced MI (Heymans et al, 1999), suggesting the presence of attenuated myocardial damage.

MMP synthesis by monocytes occurs in response to a complex interwoven network of stimuli. Catecholamines were shown to potentiate LPS-induced synthesis of MMP-1 and MMP-9 in circulating monocytes and macrophages (Speidl et al, 2004). Oxidised LDL also stimulates monocyte expression of the urokinase receptor and consequent urokinase-mediated MMP-9 generation (Ganné et al, 2000). Increased monocyte expression of extracellular MMP inducer (EMMPI) and cyclooxygenase-2 which enhances the production of MMP-1 and MMP-9 has also been demonstrated in acute MI (Schmidt et al, 2006). High expression of TNFα and reactive oxygen species (ROS) by monocytes further promotes MMP production by other cell lines within the vascular wall (Poitevin et al, 2008). Moreover, human monocytes stimulated with TNF-α release angiotensin II, which additionally mediates an increase in MMP-1 synthesis (Kim et al, 2005).

As MMPs produced by monocytes appear to be central to plaque destabilisation, this pathway has been investigated as a therapeutic target to reduce the risk of plaque rupture. For example, reduced plaque expression of MMP-9 and MMP-13 by a gene transfer technique preserves interstitial collagen (Herman et al, 2001) which is considered to be a critical determinant of plaque stability. Inhibition of MMP-2 with drugs or gene deletion protects

against cardiac rupture in mice by reducing matrix degradation and impairing the removal of necrotic myocardium by macrophages (Matsumura et al, 2005).

Table 1.4 Effects of manipulation of Tissue Inhibitors of Metalloproteinases or Matrix Metalloproteinases in atherosclerosis

TIMP or MMP	Setting	Mechanism	Observation	Reference
TIMP-1	ApoE null mice	overexpression by gene therapy	Reduces atherosclerotic lesions	Rouis et al, 1999
TIMP-2	ApoE knock-out mouse	Inhibition by gene therapy	Reduces plaque progression Reduces plaque instability	Johnson et al, 2006
	Humans with ACS	Gene expression evaluation	Monocytes in ACS overexpress TIMP-2	Kułach et al, 2010
MMP-2	Humans with ACS	Gene expression evaluation	Monocytes in ACS overexpress MMP-2 Standard ACS treatment down-regulates MMP-2 gene	Kułach et al, 2010
MMP-3	ApoE/MMP knock-out mouse	Reduced expression	Limits plaque growth Promotes stable plaque phenotype	Johnson et al, 2005
MMP-7	ApoE/MMP knock-out mouse	Reduced expression	No effect on plaque growth or stability	Johnson et al, 2005
MMP-9	ApoE null mice[113]	Overexpression by gene therapy	Promotes plaque instability	Gough et al, 2006
	Humans with ACS	Gene expression evaluation	Monocytes in ACS overexpress MMP-9	Kułach et al, 2010
	ApoE/MMP knock-out mouse	Reduced expression	Limits plaque growth Promotes stable plaque phenotype	Johnson et al, 2005
	ApoE/MMP knock-out	MMP-9 deficiency	Reduced atherosclerotic burden	Luttun et al, 2004

	mouse		Reduced macrophage infiltration Reduced collagen deposition	
MMP-12	ApoE/MMP knock-out mouse	Reduced expression	Promotes lesion expansion Promotes plaque destabilisation	Johnson et al, 2005
	ApoE/MMP knock-out mouse	MMP-12 deficiency	No effect on lesion growth	Luttun et al, 2004

TIMP: Tissue Inhibitor of Metalloproteinase; MMP: Matrix Metalloproteinase; ApoE: Apolipoprotein E; ACS: Acute Coronary Syndrome

1.3.4.2 Cathepsins

The cathepsin enzymes of the cysteine protease family have emerged as another key component involved in the activation of the innate and adaptive immune systems. Additionally, through their involvement in breakdown of the extracellular matrix and overexpression in unstable plaques, they have been shown to contribute to atherogenesis and its complications. Macrophages in human plaques produce cathepsins F, K, L, S and V, which have proteolytic activity, degrade collagen and elastin (Sukhova et al, 1998; Yasuda et al, 2004; Li et al, 2009; Barascuket al, 2010) and modify LDL (Oörni et al, 2004). These processes are linked with macrophage apoptosis, necrosis of the plaque core and cap rupture supporting their role in plaque destabilisation.

Under normal physiological conditions, human arteries express cystatin C, an endogenous inhibitor of cathepsins, whereas atherosclerotic plaques express reduced levels of the enzyme (Liu et al, 2004). Interestingly, the genes encoding several of the cathepsins are expressed in adipose tissue and their deregulation is increased with elevated body mass index (Lafarge et al, 2010). This may represent one of the mechanisms linking obesity with cardiovascular risk.

The effects of reduced levels of cathepsins, either through deficiency or blockade further demonstrate their importance in atherosclerotic plaque progression and stability. Cathepsin deficiency in a mouse model of atherosclerosis reduces collagen and elastin degradation, macrophage accumulation and overall plaque burden (Sukhova et al, 2003; Lutgens et al, 2006, Kitamoto et al; 2007). An inhibitor of cathepsin S inhibits atherosclerotic plaque burden in Apo-E knockout mice (Samokhin et al, 2010). This data is summarised in table 1.5.

Table 1.5 Effects of manipulation of Cathepsins in atherosclerosis

Cathepsin	Setting	Mechanism	Observation	Reference
S	LDL-R deficient mouse	Cathepsin S deficiency	Smaller plaques, less intimal thickening, reduced adverse plaque content	Sukhova et al, 2003
L	LDL-R knock-out mouse	Cathepsin L deficiency	Reduced atherosclerotic burden Reduced plaque lipid content Reduced matrix breakdown	Kitamoto et al, 2007
S	ApoE deficient mouse	Renin-angiotensin-aldosterone pathway blockade with Olmesartan	Reduced plaque macrophage content Halted plaque progression	Sasaki et al, 2010
S	ApoE knock-out mouse	Drug inhibitor of cathepsin S	Reduced plaque burden	Samokhin et al, 2010
K	ApoE knock-out mice	Disrupted cathepsin K gene	Limits plaque progression 41.8% reduced plaque area Plaque fibrosis Reduced macrophage foam cell formation	Lutgens et al, 2006

LDL-R: Low Density Lipoprotein Receptor; ApoE: Apolipoprotein

1.3.4.3 ADAM and ADAMTS

More recently, the 'a disintegrin and metalloprotease' (ADAM) and 'ADAM with thrombospondin motifs' (ADAMTS) family of proteases have been identified as important enzymes in extracellular matrix protein turnover (Salter et al, 2010). They have much greater substrate specificity than MMPs. Their altered regulation has been implicated in a variety of disorders including cancer, arthritis and atherosclerosis (Jönsson-Rylander et al, 2005). Although data regarding their role in plaque pathophysiology is currently limited, they have been found to be released by plaque macrophages, and promote matrix and fibrous cap remodelling which predisposes to plaque rupture (Wågsäter et al, 2008).

1.3.4.4 CD40 ligand pathway

The MCP-1/CCR2 and CD40/CD40 ligand (CD40L) systems appear to be interlinked in atherogenesis. The immune response-mediating factor CD40L and its receptor CD40 have been implicated in the initiation of atheroma formation and plaque progression and destabilisation (Schönbeck & Libby, 2001). Stimulation of the CD40 induces production of inflammatory cytokines, chemokines, MMPs and tissue factor by macrophages which impair the collagen structure within the plaque and promote its destabilisation and rupture (Mach et al, 1998). Blockade of MCP-1 was shown to reduce immunohistochemically-detectable CD40 and CD40L expression within plaques of hypercholesterolaemic ApoE-knockout mice (Inoue, 2002).

1.3.4.5 Neopterin

Neopterin is a pteridine derivative produced via the guanosine triphosphate pathway. It is produced by activated macrophages upon stimulation by IFNγ released by T-lymphocytes,

and may have a role as a pro-oxidant. Neopterin is a marker of activated monocytes/macrophages, and serum levels are raised in ACS (Sugioka et al, 2010). More recently, immunohistochemical techniques demonstrated significantly higher content of neopterin-rich macrophages in coronary atherectomy samples of unstable plaques in humans following ACS. This suggests that neopterin content correlates with plaque instability (Adachi et al, 2007).

1.3.5 Monocytes and angiogenesis

Monocytes play pivotal roles in the processes of angiogenesis and intimal and plaque neovascularisation, which are further important mechanisms related to plaque progression and destabilisation (Ribatti et al, 2008). In vitro models of angiogenesis and gene expression studies in humans (Zawada et al, 2011) and the ability of TIE-2 expressing monocytes in mouse to induce a capillary network (De Palma et al, 2005) suggest a prominent role for the Mon2/intermediate subset in this process. As a plaque develops, the central core becomes hypoxic due to impaired diffusion of oxygen from the arterial lumen through the thickened intima. Upregulated monocyte expression of pro-angiogenic factors including VEGF and fibroblast growth factor (FGF)-2 (Murdoch et al, 2007) stimulates proliferation of endothelial cells, as well as mobilisation and homing of bone marrow-derived endothelial progenitors (Lambert et al, 2008). This promotes neovascularisation of the plaque from the adjacent adventitia. Hence, the angiogenic process sets up a vicious cycle whereby new vessels produced under the influence of monocytes concurrently provide an additional route for their enhanced entry into plaques.

Thus, although the initial effect of monocytes is beneficial, aiming to improve tissue perfusion, this therapeutic effect may become pathological. Progressive hypoxia and enhanced plaque vascularity promotes ongoing accumulation of inflammatory cells including monocytes via the neo-vessels, and the fragile network of neovessels predisposes to intra-plaque haemorrhage (Virmani et al, 2005). These processes contribute to atheroma progression and ultimately plaque destabilisation.

As vulnerable plaques contain increased number of vasa-vasorum, inhibition of this process of neovascularisation may represent a potential therapeutic target aiming to reduce the risk of plaque rupture. For example, angiostatin (an anti-angiogenic molecule) inhibits intraplaque angiogenesis and thus reduces accumulation of intraplaque monocytes and macrophages, which appears to promote plaque stability (Moulton et al, 2003). However more data is needed to explore this concept.

1.3.6 Monocytes and apoptosis

The LPS receptor CD14 mediates recognition and phagocytosis of apoptotic cells by monocytes (Devitt et al, 1998). This is inherently a biologically protective mechanism, which may attenuate atherosclerosis in early plaques. However, it also perpetuates atherogenesis via the inflammatory reactions it causes. Incomplete apoptotic cell clearance in mature plaques may lead to an enhanced proinflammatory response and further apoptotic signals for endothelial cells, smooth muscle cells and leukocytes within atherosclerotic plaques (Tabas, 2005), leading to plaque destabilisation and rupture.

1.3.7 Conclusions

Cells of the monocyte-macrophage lineage are key contributors to the complex process of initiation and propagation of atherosclerotic plaques. They are clearly implicated in plaque destabilisation via a number of complex inter-linked mechanisms. Importantly, monocytes may be involved in the pathogenesis of atherosclerosis, including plaque destabilisation locally at the site of atheroma, and also whilst in circulation.

Plaque destabilisation and rupture is clearly an active process rather than a passive phenomenon. Therefore, modulation of monocytes and the pathways via which they are activated and exert their effects may be viable therapeutic targets in the treatment of atherosclerosis and the prevention of plaque progression and destabilisation. Heterogeneity of human monocytes may allow for specific targeting of pathological pathways whilst sparing beneficial roles, thus avoiding the adverse effects associated with indiscriminate monocyte inhibition. Further functional characterisation of the specific human monocyte subsets in atherosclerosis and their contributions to disease progression and repair is of fundamental importance in this regard.

CHAPTER 2

AIMS AND HYPOTHESES

The overall aim of this thesis was to provide a detailed phenotypic comparison of differences between the three human monocyte subsets in the setting of CAD and MI, which has not been done before. Particular emphasis was placed on the 'Mon2'/'Intermediate' and 'Mon3'/'Nonclassical' subsets which have hitherto been grouped together as a single population, and hence little is known about their individual characteristics in these settings. Based on our previous observations demonstrating marked differences between subsets in healthy subjects, I hypothesised that the Mon2 subset exhibits distinct changes compared to Mon1 and Mon3.

Emphasis was placed on examining expression of monocyte surface markers associated with monocyte activation, inflammation, repair and angiogenesis, and also aggregates with platelets. I also sought to explore associations between monocyte subset parameters and cytokines, markers of fibrinolysis, MCP-1 and residual LV ejection fraction (EF) in the recovery phase following STEMI which is the most important determinant of long-term prognosis.

In chapter 4, the focus was placed on investigating differences in expression of surface receptors implicated in inflammatory and reparative responses in CAD by comparing with healthy subjects. Additionally, the functional status of monocyte subsets was investigated via measurement of intracellular inhibitory κB kinase (IKK) (a marker of activation of the NFκB pathway). Monocyte interactions with platelets were examined, and associations between monocyte characteristics and plasma markers of inflammation and fibrinolysis were explored. As monocyte subsets have been proposed to play different roles in the pathogenesis of CAD, I

hypothesised that there are significant differences in these subset characteristics between healthy subjects and those with CAD.

In chapter 5, my aim was to investigate the dynamic changes of the three human monocyte subsets and their associations with MPAs in STEMI, from the acute to the recovery phase. I hypothesised that the Mon2 subset exhibits distinct dynamic changes following STEMI compared to Mon1 and Mon3, and that these changes differ from healthy subjects or those with stable CAD. I also hypothesised that these changes are related to the degree of myocardial damage and to changes in inflammatory cytokines and MCP-1. Finally I hypothesised that the dynamic changes in monocyte subsets acutely after STEMI are related to LVEF in the recovery phase.

My objective in chapter 6 was to assess numbers of TLR4 expressing monocytes in each of the three human subsets in STEMI and NSTEMI, their expression of TLR4, and relations to markers of monocyte pro-inflammatory activation, myocardial damage, inflammatory cytokines and LVEF in the recovery phase. I hypothesized that there are distinct differences in TLR4 parameters between patients with MI compared with CAD controls, and that there are dynamic changes between the acute phase of MI and the recovery phase.

In chapter 7, my aim was to focus on the associations of monocyte subsets with surface markers implicated in reparative processes following STEMI. The numbers of CXCR4+, CD34+ and KDR+ cells attributed to the three human monocyte subsets were assessed, along with monocyte expression of CD204 and CD163. Their associations with biological and physiological parameters related to myocardial damage and recovery following MI were

assessed. I hypothesised that the number of CXCR4+ monocytes, CD34+/KDR+ cells and expression of monocyte scavenger receptors is changed in patients following STEMI. Additionally, I hypothesised that these changes are attributable to specific monocyte subsets and are associated with biomarkers of inflammation and LVEF in the recovery phase after STEMI.

My aim in chapter 8 was to examine expression of ICAM-1r, VCAM-1r and IL-6 receptor (IL6r) on individual subsets in patients with MI and to assess dynamics of monocyte expression of these receptors during the acute and recovery phases after STEMI which has not been described. I also explored possible associations between these receptors and markers of systemic inflammation, myocardial damage and cardiac functional recovery following STEMI. Having observed significant differences in expression of these receptors between the three monocyte subsets in healthy humans, I hypothesised that there are marked differences in their expression in the setting of MI.

CHAPTER 3

METHODS AND MATERIALS

3.1 SUBJECT SELECTION

3.1.1 ST elevation and non-ST elevation myocardial infarction patients

Patients with STEMI admitted to Sandwell and West Birmingham Hospitals (SWBH) NHS Trust meeting the study criteria (see 3.1.1.1 and 3.1.3) were recruited between November 2009 and November 2010. Patients were recruited from both the City Hospital and Sandwell General Hospital sites within the Trust. The cardiology Consultants within the Trust work on both sites, including undertaking PPCI for STEMI. All operators adhere to the Trust policy on management of patients with STEMI, and hence there is little variation in practice and management of patients with STEMI.

Patients with NSTEMI meeting the study criteria (see 3.1.1.1 and 3.1.3) were recruited from SWBH and Heart of England NHS Foundation Trust between November 2009 and May 2011 (Birmingham Heartlands Hospital, Solihull Hospital and Good Hope Hospital).

3.1.1.1 Inclusion criteria

STEMI was diagnosed according to the ESC definition; patients presenting with symptoms of cardiac ischaemia with persistent ST-segment elevation or presumed new left bundle branch block (LBBB) on ECG (Van de Verf et al, 2008).

NSTEMI was diagnosed according to the ESC definition; patients presenting with symptoms of cardiac ischaemia, new ECG changes compatible with cardiac ischaemia other than

persistent ST-segment elevation or presumed new LBBB, and dynamic changes in biomarkers of myocyte necrosis (troponins) (Bassand et al, 2007).

3.1.1.2 Time points

The counts of the 3 monocyte subsets and MPAs were measured at 4 time points following STEMI: day 1 (during the first 24 hours after PPCI), day 3, day 7 and day 30. A proportion of patients did not complete follow-up due to withdrawal of consent or death.

3.1.2 Control groups

Two age- and sex-matched control groups were studied:

3.1.2.1 Stable coronary artery disease

'Disease controls' were recruited, with angiographically proven CAD and no episodes of hospital admissions or instability for ≥3 months. Additionally, none of the CAD patients had a history of prior STEMI, LV systolic impairment or heart failure. Patients with stable CAD were identified from out-patient clinics and cardiac rehabilitation services at SWBH. The 'disease control' group was chosen in order to allow comparison with the STEMI group and examine changes in study parameters related to the onset of and recovery from MI on a background of CAD.

3.1.2.2 Healthy controls

Healthy subjects were identified as people with no evidence of cardiovascular disease on history taking and routine clinical examination, with no disqualifying exclusion criteria (see 3.1.3). The healthy control group was chosen in order to allow comparison with the 'disease control' group and examine differences in study parameters related to the presence of stable CAD and its associated risk factors.

3.1.3 Exclusion criteria

Exclusion criteria comprised factors that could affect monocyte count (infectious disease, inflammatory disorders and their treatment (including steroids and non-steroidal anti-inflammatory drugs), cancer, haemodynamically significant valvular heart disease, atrial fibrillation, renal failure and hormone replacement therapy. Additionally, no STEMI patients had a history of previous STEMI, LV systolic impairment or heart failure. All subjects were asked to refrain from extreme physical exertion for at least 1 hour prior to blood sampling (Shantsila et al, 2012) (3.3.2).

3.1.4 Patient management

This study was purely observational and there were no alterations in patient management. All study patients received standard treatment according to contemporary guidelines (Van de Verf et al, 2008; Bassand et al 2007). All patients with STEMI were treated with PPCI. The SWBH NHS Trust is very experienced in the management of patients with STEMI and has been undertaking PPCI for STEMI since 2005. The Trust's median 'door-to-balloon' (DTB) time which indicates the time between admission to the hospital accident and emergency

department and restoration of coronary artery blood flow was 68 minutes (IQR 50-91 minutes) at the time of this study (Kong et al, 2009). The national standard median DTB time is ≤90 minutes.

3.2 FLOW CYTOMETRY

3.2.1 Equipment & software

Flow cytometry was undertaken using the BD FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK [BD]). VenturiOne, Version 3.1 software (Applied Cytometry, Sheffield, UK) was used for data analysis.

3.2.2 Absolute count of monocytes, monocyte subsets and monocyte platelet aggregates

We have published a paper utilising the flow cytometry protocols and methodology used in this thesis to further characterize and describe the three monocyte subsets in healthy humans (Shantsila et al, 2011) identified using contemporary nomenclature (Ziegler-Heitbrock et al, 2010.

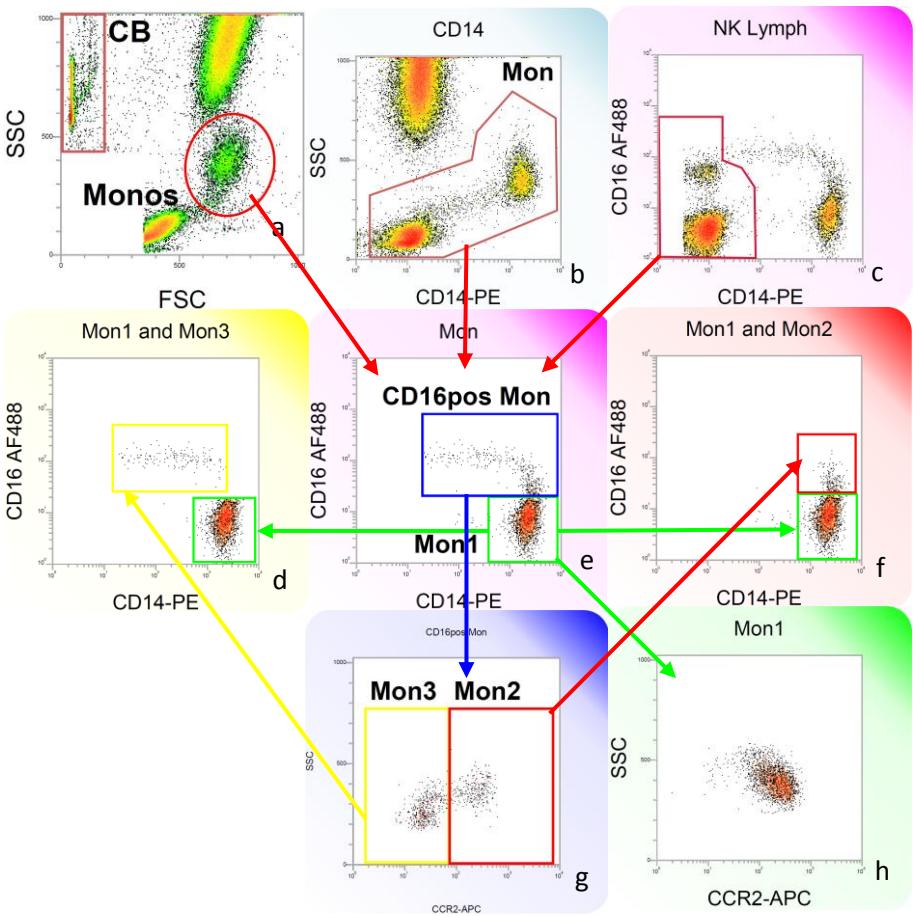
3.2.2.1 Monocytes and monocyte subsets

Mouse anti-human monoclonal fluorochrome-conjugated antibodies anti-CD16-Alexa Fluor 488 (clone DJ130c, AbD Serotec, Oxford, UK), anti-CD14-PE (clone MϕP9, BD), anti-CD42a-PerCP (clone Beb1, BD) and anti-CCR2-APC (clone 48607, R&D) were mixed with 50µl of ethylene diamine tetra-acetic acid (EDTA)-anticoagulated whole blood in TruCount tubes (BD) which contain a precise number of fluorescent count beads. Following incubation for 15 minutes, red blood cells were lysed using 450µl of lysing solution® (BD) for 15 minutes. Subsequently the sample was diluted in 1.5 ml of phosphate buffered saline (PBS) and immediately analysed by flow cytometry.

Definitions of CD14, CD16 and CCR2 positivity were made using appropriate isotype controls (Ziegler-Heitbrock et al, 2010). Monocyte subsets were defined as CD14++CD16-CCR2+ (‘Mon1’), CD14++CD16+CCR2+ (‘Mon2’) and CD14+CD16++CCR2- (‘Mon3’) (Shantsila et al, 2011).

The gating strategies employed to discriminate monocytes into the three subsets are illustrated in figure 3.1. Monocytes were firstly selected by a gating strategies based on forward scatter versus side scatter (figure 3.1, panel a). Granulocytes were excluded by CD14 expression versus side scatter (figure 3.1, panel b). CD16+CD14- natural killer lymphocytes were excluded from CD16+ monocytes based on ungated CD14 versus CD16 expression (figure 3.1, panel, c). Mon1 are identified as CD14++CD16- cells (figure 3.1, panel e). The CD16+ cells (figure 3.1, panel e) are separated into Mon 2 and Mon 3 based on their expression of CCR2 (figure 3.1, panel g).

Figure 3.1 Gating strategies and definition of the three monocyte subsets



The monocyte region is selected based on the forward scatter (FSC) versus side scatter (SSC) plot (a). Exclusion of granulocytes by CD14 expression versus SSC (b). Exclusion of CD16+CD14- natural killer lymphocytes from CD16+ monocytes (c). Monocytes (e) are defined as cells that correspond to the gate 'Monos' in panel (a) and 'Mon' in panel (b) and do not correspond to natural killer lymphocytes in panel (c). In panel (e) Mon 1 are identified as CD14++CD16- cells (green box). CD14+CD16+ cells (e, blue box) are discriminated based on their expression of CCR2 (g) into Mon 2 (CCR2+, red box) and Mon 3 (CCR2-, yellow box). Panel (h) demonstrates CCR2 expression by the Mon1 subset. Panels (d) and (f) demonstrate the location of Mon3 (d, yellow box) and Mon2 (f, red box) respectively on the CD14 versus CD16 plot.

The absolute count of monocytes (cells/µl) was obtained by calculating the total number of monocytes proportional to the number of count beads in the TruCount tube according to the manufacturer's recommendations. The count of each subset and their relative proportions of the absolute monocyte count were defined according to the gating strategy described above in 3.2.2.1.

3.2.2.2 Monocyte platelet aggregates

MPAs were defined as events positive to both the monocyte markers described in 3.2.2.1 and the platelet marker CD42a (glycoprotein IX). The total MPA count and MPA count associated with each monocyte subset was calculated based on the number of count beads as described in 3.2.2.1. Thus, MPAs associated with Mon1 are CD14++CD16-CCR2+CD42a+, MPAs associated with Mon 2 are CD14++CD16+CCR2+CD42a+ and MPAs associated with Mon 3 are CD14+CD16++CCR2-CD42a+.

3.2.3 Expression of surface antigens on monocyte subsets

For the analysis of expression of surface antigens (summarised in table 3.1) by monocytes and the three subsets, 100µl of whole blood was incubated with mouse anti-human monoclonal fluorochrome-conjugated antibodies for 15 minutes in the dark. Subsequently red blood cells were lysed with 2ml of BD lysing solution® for 10 min, followed by washing in PBS and immediate analysis by flow cytometry. The three monocyte subsets were defined as CD14++CD16– monocytes ('Mon1'), CD14++CD16+ monocytes ('Mon2') and CD14+CD16++ monocytes ('Mon3') in accordance with contemporary guidelines (Ziegler-Heitbrock et al, 2010) using anti-CD16-Alexa Fluor 488 (clone DJ130c, AbDSerotec, Oxford, UK) and anti-CD14-PerCP-Cy5.5 (clone M5E2, BD) antibodies.

PE-conjugated antibodies were used against TLR4 (clone 285219, R&D), ICAM-1 receptor (integrin β2/CD18) (clone 212701, R&D Systems Europe Ltd, Abingdon, UK [R&D]), CXCR4 (SDF-1 receptor) (clone 12G5, R&D), CD34 (clone 8G12, BD) and VEGF receptor 1 (clone 49560, R&D). APC-conjugated antibodies were used against IL6r (clone 17506, R&D), VCAM-1 receptor (integrin α4/CD49d) (clone 7.2R, R&D), CD163 (clone 215927, R&D), KDR (VEGF receptor 2) (clone 89106, R&D) and CD204 (clone 351520, R&D). Monoclonal antibodies for CD204 were conjugated to APC using the LLAPC-XL conjugation kit [Innova Biosciences, UK].

Table 3.1 Summary of monocyte markers, fluorochromes and antibodies

Surface antigen	Fluorochrome	Antibody
TLR4	PE	clone 285219, R&D
ICAM-1 receptor (integrin β2/CD18)	PE	clone 212701, R&D
CXCR4 (SDF-1 receptor)	PE	clone 12G5, R&D
CD34	PE	clone 8G12, BD
VEGF receptor 1	PE	clone 49560, R&D
IL6 receptor	APC	clone 17506, R&D
VCAM-1 receptor (integrin α4/CD49d)	APC	clone 7.2R, R&D
CD163	APC	clone 215927, R&D
KDR (VEGF receptor 2	APC	clone 89106, R&D
CD204	APC	clone 351520, R&D conjugated to APC using LLAPC-XL conjugation kit [Innova Biosciences, UK]

3.2.4 Assessment of activation of the nuclear factor κ B pathway

Stimulation of monocyte surface receptors (including TLRs and CD14) by a variety of stimuli leads to activation of the NFκB pathway. Specific inhibitory κB kinases promote

phosphorylation and disintegration of IκBs. Subsequently cytoplasmic NFκB is translocated into the nucleus where it binds to the promoter region of various genes which activates their transcription. These genes include cytokines (including TNF-α and IL-1β), cyclooxygenase-2, inducible nitric oxide (NO)-synthase and MMPs (Yamamoto et al, 2004; Hayden et al 2004).

The IKK complex consists of two catalytic subunits, IKKα and IKKβ. IKKβ is 20-fold more active than IKKα, and plays an essential role in activation of the NFκB pathway activation mediated by lipopolysaccharides, TNFα, or IL-1 (Hu et al, 1999; Mercurio et al, 1997). As the activated form of NFκB (translocated into nuclei) is identical to the resting cytoplasmic form, measurement of total NFκB level within a cell is not informative. The assessment of NFκB in isolated nuclei is problematic as it necessitates multistep procedures including cell isolation and preparation which are thought to contribute to artificial activation (Ritchie, 1998). In this study, direct measurement of intracellular levels of IKKβ was used as a surrogate cytoplasmic marker of activation of the NFκB pathway, thereby aiming to assess activation of monocytes which has been associated with changes in phenotype and function.

Intracellular level of IKKβ in monocytes was measured from whole blood. 100µl of blood was incubated with monoclonal mouse anti-human antibodies against CD16-Alexa Fluor 488 (clone DJ130c, AbD Serotec, Oxford, UK) and CD14-PerCP-Cy5.5 (clone M5E2, BD) for 15 minutes. Red blood cells were then lysed with 2ml of BD PharmLyse™ for 10 minutes followed by washing in staining buffer. The resulting pellet was resuspended in Fixation/Permeabilization solution (BD) for 20 minutes. Following centrifugation the pellet was resuspended in 2 ml of BD Perm/Wash™ buffer for 10 minutes. After further

centrifugation, the pellet was incubated for 30 minutes with monoclonal mouse anti-human APC-conjugated antibodies (LL-APC-XL conjugation kit [Innova Biosciences, UK]) against IKKβ (clone 10A9B6, Abcam, Cambridge, UK), washed and resuspended in 200µl of 2% PBS/2% paraformaldehyde solution (PharmFix, BD) and immediately analysed by flow cytometry.

3.2.5 Plasma markers

Plasma levels of IL-1β, IL-6, IL-10 and MCP-1 were measured by cytometric bead array technology. The BD FACSCalibur flow cytometer was used for data acquisition, with FCAP Array v2.0.2 software (Burnsville, Minnesota, USA) for data analysis. Commercially available reagent sets, Human IL-1β Flex Set, Human IL-6 Flex Set, Human IL-10 Flex Set and Human MCP-1 Flex Set (all from BD) were used according to the manufacturer's recommendations.

3.2.6 Reproducibility of assay results

Intra-assay variability is a measure of the ability of an assay to give the same result when the same sample is tested multiple times. Intra-assay reproducibility was assessed during development of the Standard Operating Procedure (SOP) for studying monocytes in the preparatory stages for this project. An SOP is a fundamental requirement for all laboratory investigations in the Atherosclerosis Thrombosis and Vascular Biology Unit of the University Of Birmingham Department Of Medicine at City Hospital, Birmingham where this project was conducted. All SOPs must be evaluated and 'signed off' by the department's Consultant Clinical Scientist, Dr Andrew Blann, before they may be used in research projects. The SOP for this project is SOP 201 "Monocyte subsets, monocyte platelet aggregates by flow

cytometry" (see appendix 3). This SOP was developed by Dr Eduard Shantsila with Dr Luke Tapp and Dr Ben Wrigley.

The intra-assay reproducibility of the methods was assessed on six samples of blood; one set of three from a healthy male (subject A) and second set of three from a woman with a history of renal and ovarian cancer (subject B) (table 3.2).

Table 3.2 Mean intra-assay coefficients of variation (%) for monocyte subset and monocyte platelet aggregate.

	Subject A	**Subject B**	**Mean**
Total Mon	0.6	0.4	0.5
Mon1	0.9	1.4	1.15
Mon2	10.6	9.9	10.25
Mon3	3.9	4.7	4.3
Total MPA	3.2	4.5	3.85
MPA1	3.4	5.2	4.3
MPA2	14.1	9.7	11.9
MPA3	8.2	6.8	7.5
Median intra assay coefficient of variation 4.6%			

Total Mon, total monocyte count; Mon1, CD14++CD16-CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2- monocytes; MPA, monocyte platelet aggregates; Total MPA, total MPA count; MPA1, MPAs associated with Mon1; MPA2, MPAs associated with Mon2; MPA3, MPAS associated with Mon 3; CV, coefficient of variability.

Similarly, assessments of intra-assay reproducibility were made for an example monocyte surface marker CCR2 (median CV 4.1%) and IKKβ (median CV 5.3%). The reproducibility of each surface marker used in the study was not assessed as the core protocol is identical other than different antibodies being used in each tube.

The CVs for the assays used in this study are low. This suggests that the assays are sound and that differences measured subsequently in my study are genuine and due to real changes in the parameters measured in the disease settings evaluated.

The acknowledgement that reproducibility is limited by measurement error along with extrinsic variables including diurnal variation and exercise status should be considered a limitation of this study.

My ability to perform the assays required for this study was assessed and approved by Dr Andrew Blann prior to commencing subject recruitment. The other operators involved in processing some laboratory samples in this study (Dr Ben Wrigley and Dr Burack Pamukcu) were assessed and approved in a similar manner.

3.3 BLOOD SAMPLING AND STORAGE

As part of the preparatory work for this thesis, I undertook experiments to determine the optimal conditions under which blood samples should be collected, stored and processed. I hypothesized that total monocyte count, monocyte subset count and MPAs would be affected by physical exercise and that there would be diurnal variation in their numbers. I also hypothesized that monocyte and MPA parameters would be affected by the time delay between taking the sample and processing.

3.3.1 Effect of exercise

The effects of a Bruce protocol exercise test was assessed on 12 healthy volunteers (age 35.3 ± 7.6 years, 8 male) with no evidence of cardiovascular disease. Venous blood samples were taken pre- and post exercise. Total monocyte count and Mon1 increased significantly 15 minutes after ceasing exercise, followed by a significant reduction by 1 hour. Mon2 and Mon3 followed similar dynamic changes, but only the reduction in Mon3 count at 1 hour post exercise reached statistical significance. The proportions of the overall monocyte population contributed to by each subset were not affected by exercise. The total MPA count and MPAs associated with Mon1 decreased significantly 15 minutes post exercise, with a further significant reduction at 1 hour compared to pre-exercise. The number of MPAs associated with Mon2 also decreased significantly by 1 hour. No changes in MPAs associated with Mon3 were apparent. Additionally, Mon1 expression of CD14 decreased significantly 1 hour post exercise. No such changes were observed for the Mon2 or Mon3 subset. CD16 and CCR2 expression was not affected by exercise (Shantsila et al, 2012). Having observed these effects on monocyte subsets and MPAs, participants in my study were asked to refrain from

strenuous exertion for at least 1 hour prior to sample collection in order to minimise the effect of exercise on monocyte parameters.

3.3.2 Effect of diurnal variation

Diurnal variation in monocyte parameters was assessed in 16 healthy volunteers (age 36.0 ± 8.7 years, 7 male) with no evidence of cardiovascular disease on examination or history-taking. Samples were taken five times at six hourly intervals over a 24 hour period (06.00, 12.00, 18.00, 00.00 and 06.00). The number and proportion of Mon2 was observed to undergo significant diurnal variation. These values peaked at 18.00 and reached their nadir at 06.00. There were no significant variations in total monocyte count, or count of Mon1 or Mon 3. Expression of CCR2 by Mon2 exhibited the same pattern. There was no variation in expression of CD14 or CD16 (Shantsila et al, 2012). Given these observations on the effects of exercise on monocyte parameters, the increase noted during the daytime may be attributable to a generally higher level of background physical activity. It was logistically impossible to plan to undertake sample collection at the same time each day. However, the majority of samples were collected from all subject groups between 09.00 and 12.00. The remaining influence of diurnal variation on monocyte parameters should be considered as a limitation of my study.

3.3.3 Effect of delay in sample preparation

The effect of time delay on monocyte and MPA parameters between sample collection and processing was assessed in 12 healthy volunteers with no evidence of cardiovascular disease. Following venesection, the sample was processed immediately and then repeated at 1, 2 and 4 hours after collection whilst being stored at ambient room temperature. The count and

proportions of monocyte subsets was not affected by a delay of up to 2 hours before sample processing. By 4 hours, significant changes had become apparent; the proportion of Mon2 increased, the proportion of Mon1 decreased and Mon 3 counts increased. The expression of CD14 and C16 by Mon1 increased significantly by 2 hours and significantly further by 4 hours. Expression of CCR2 was not affected by a delay in sample preparation. MPAs appeared more sensitive to a delay in sample processing. MPA count increased significantly at 2 hours followed by a further significant increase at 4 hours. This trend was apparent for all three monocyte subsets (Shantsila et al, 2012). In order to minimise the effect of time delay between sample collection and processing on monocyte parameters in my study, all samples were analysed within 1 hour of venesection.

3.3.4 Plasma storage

Plasma was separated by centrifugation and stored at -70^{o}C for batched analysis at the end of my study.

3.4 ENZYME-LINKED IMMUNOSORBENT ASSAYS

Enzyme-linked immunosorbent assay (ELISA) of citrated plasma was used to quantify plasma levels and activity of fibrinolytic parameters. All measurements were performed using recognized high quality ZYMUTEST kits (Hyphen Bio-Med, Neuville-sur-Oise, France distributed by Quadratech Ltd, Epsom, UK); tissue type plasminogen activator (tPA) antigen (Cat. No. RK011A), plasminogen activator inhibitor type 1 (PAI-1) antigen (Cat. No. RK012A), PAI-1 Activity (Cat. No. RK019A) and urokinase type plasminogen activator (uPA) antigen (Cat. No. RK013A). All measurements were performed according to the manufacturer's recommendations.

3.5 LABOROATORY MEASUREMENTS

Routine haematology (full blood count) and biochemistry (urea and electrolytes, troponin) tests were conducted by the clinical pathology laboratories at SWBH NHS Trust.

3.6 ASSESSMENT OF LEFT VENTRICULAR FUNCTION

All patients underwent echocardiographic assessment of LVEF by Simpson's method 6 weeks after STEMI as part of their routine care (Van de Werf et al, 2008). The scans were undertaken by British Society of Echocardiography accredited echocardiographers blinded to the study results (Lang et al, 2005).

3.7 POWER CALCULATION

Based on our previous work (Shantsila et al, 2011), the calculated minimum number of participants required to achieve 80% power to detect a difference of 0.5 standard deviations in mean monocyte count between the study groups was n=35 for the cross-sectional study and n=25 for the longitudinal study in STEMI patients. For additional confidence, and in anticipation of the potential for subjects to drop-out of the longitudinal study, I aimed to recruit 50 STEMI patients and 40 subjects in each control group.

3.8 STATISTICAL ANALYSIS

Following a test of statistical normality, normally distributed continuous data are expressed as mean [standard deviation, SD]. Non-normally distributed continuous data are expressed as median [interquartile range]. Cross-sectional data were analysed by one way analysis of variance (ANOVA) and follow-up data were analysed by repeated measures ANOVA. The

Kruskal-Wallis or Friedman test was used for non-normally distributed data. A post-hoc Tukey test was performed to assess inter-group differences, where appropriate. Arithmetic (log or inverted) transformation of non-normally distributed variables was performed prior to post-hoc analysis of ANOVA. Statistical significance of differences between monocyte subsets was determined by independent sample T-test for normally distributed variables, and by Mann-Whitney test for non-normally distributed variables. Correlations between study parameters were assessed using Pearson's method (for normally distributed parameters) or Spearman's method (for non-normally distributed parameters). Univariate and multivariate linear regression analyses were used to establish predictive value of the study parameters for LVEF at 6 weeks in STEMI patients. A p-value of <0.05 was considered statistically significant. In order to minimise the possibility of finding a significant association by chance due to the multiple comparison effect, the Bonferroni adjustment of p value was used using the formula: p[significant] = 0.05/number of comparisons per a receptor in an analysis where appropriate. SPSS18 (SPSS, Inc, Chicago, Illinois, USA) software was used to perform statistical analyses.

Formal training in statistical methodology was undertaken as part of the curriculum for MD students of the University of Birmingham working in the University of Birmingham Department of Medicine at City Hospital, Birmingham. This was taught by Dr Andrew Blann who is a Trust Statistician for SWBH NHS Trust and Dr Deirdre Lane.

3.9 ETHICAL CONSIDERATIONS

This study was performed in accordance with the Helsinki declaration. Ethical approval was granted by the Coventry Research Ethics Committee (reference number 09/H1210/11) and approval was obtained from the Research & Development departments at SWBH NHS Trust and Heart of England NHS Foundation Trust. All participants provided written informed consent.

CHAPTER 4

MONOCYTE SUBSETS IN CORONARY ARTERY DISEASE

Contributory publication: Monocyte subsets in coronary artery disease and their associations with markers of inflammation and fibrinolysis. Shantsila E, Tapp LD, Wrigley BJ, Pamukcu B, Apostolakis S, Montoro-Garcia S, Lip GY. Atherosclerosis 2014; 234(1): 4-10.

Abstract

The diverse roles of monocytes in atherogenesis are attributed to the existence of distinct monocyte sub-populations. Scarce data are available regarding changes in phenotype and functional status of human monocyte subsets in patients with CAD, especially when evaluated as three subsets.

Monocyte subset surface expression of receptors implicated in inflammation and repair and their activation status (intracellular IKKβ) was assessed by flow cytometry in 53 patients with CAD and compared to 50 age- and sex-matched healthy subjects. Monocyte subsets were defined as CD14++CD16–CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2), and CD14+CD16++CCR2– (Mon3). Plasma levels of inflammatory cytokines (by FACSArray) and fibrinolytic factors (by ELISA) were measured in CAD.

Compared to HC, CAD was associated with lower expression of CD14 on Mon1 ($p=0.02$) and Mon3 ($p=0.036$), lower expression of CD16 on Mon1 ($p=0.002$) and Mon2 ($p=0.001$), higher expression of IL6 receptor on Mon1 ($p=0.025$) and Mon2 ($p=0.015$), higher expression of CXCR4 on Mon3 ($p=0.003$), and higher expression of CD34 on all subsets (all $p<0.007$). There were no significant differences in levels of intracellular IKKβ. Monocyte CD163 expression correlated negatively with IL6 levels ($p<0.01$ for all subsets). IL1β levels correlated positively with CD42a expression on Mon1 aggregated with platelets ($r=0.36$, $p=0.009$). Expression of VEGFR1 correlated positively with uPA levels on Mon1 ($r=0.51$, $p=0.003$) and Mon2 ($r=0.44$, $p=0.012$), and VEGFR1 expression on Mon2 correlated positively with PAI-1 antigen levels ($r=0.47$, $p=0.006$).

The marked differences observed in subset phenotype in CAD supports the presence of unique roles for the three human monocyte subsets in CAD pathogenesis.

4.1 INTRODUCTION

4.1.1 Monocytes in coronary artery disease

Monocyte-derived 'foam' cells have been established as a hallmark and key pathogenic feature of atherosclerosis, a disease of inflammatory aetiology (Gerrity, 1981). High total monocyte count is a strong predictor of increased risk of CAD or MI (Hristov & Weber, 2004; Woollard & Geissmann, 2010; Shantsila & Lip, 2009; Maekawa et al, 2002). However the roles of monocytes in atherosclerosis are diverse, including involvement in inflammatory responses, the regulation of thrombogenic status (eg via tissue factor expression and the modulation of fibrinolysis) and also physiologically beneficial processes related to scavenging of redundant or pathological substances, angiogenesis and repair (Panizzi et al, 2010; Dresske et al, 2006; Nahrendorf et al 2007; Shantsila et al, 2012).

4.1.2 Monocyte subsets in coronary artery disease

This diversity of monocyte functions has been partly attributed to the existence of different monocyte subsets with specific phenotypic and functional properties (Geissmann et al, 2003; Auffray et al, 2009; Shantsila & Lip, 2009; Cros et al, 2010; Zawada et al, 2011). Specific monocyte subsets have been shown to be differentially involved in the pathogenesis and outcomes of a range of diseases including heart failure (Wrigley et al, 2013) and stroke (Urra et al, 2009) (Table 1.3).

Evidence supporting functional diversity of monocyte subsets in atherogenesis has primarily been derived from animal experiments (Swirski et al, 2007; Combadiere et al, 2008; Tacke et al, 2007). Experimental work in mice suggests that in the pathogenesis of MI, 'classical' Ly-

$6C^{high}$ monocytes contribute to inflammation, phagocytic and proteolytic processes, whilst 'non-classical' Ly-$6C^{low}$ monocytes are involved in myocardial healing, recovery and angiogenesis (Nahrendorf et al, 2007). However, there is limited published data describing the phenotypic and functional characteristics of human monocyte subsets in CAD. Marked differences in monocyte subset phenotype and the pathogenesis of atherosclerosis between rodents and humans preclude direct translation of data between species. Also, variation in the definition of subpopulations and methodological differences between published papers renders comparison between studies problematic.

In humans, an increased number of $CD14^{dim}CD16^{+}$ monocytes was observed in hypercholesterolaemic patients, and an increased number of CD14+CD16+ monocytes was found in patients with CAD (Rothe et al, 1996; Schlitt et al, 2004). In stable CAD a negative correlation was found between $CD14^{low}CD16^{+}$ monocyte count and cumulative risk factor score, cigarette smoking, family history of CAD and history of hypertension (Hristov et al, 2010). However, the majority of available data describes human monocytes as two subsets (CD14+CD16- and CD14+CD16+) where CD16+ monocytes are considered as a single subset, pre-dating the recently published consensus nomenclature which describes three subsets (Ziegler-Heitbrock et al, 2010) (section 1.2.1). The hitherto poorly characterised CD14++CD16+ subset has been the focus of much recent interest. An association was noted between this subset and adverse cardiovascular outcomes in patients with chronic kidney disease (Rogacev et al, 2011) and a cohort of high cardiovascular risk patients referred for coronary angiography (Rogacev et al, 2012). However, there is very little data regarding the three monocyte subsets in the setting of stable CAD.

4.2 AIMS AND HYPOTHESES

The aim of this study was to provide a detailed phenotypic comparison of differences between the three human monocyte subsets in patients with CAD and healthy subjects defined according to contemporary nomenclature (Ziegler-Heitbrock et al, 2010; Shantsila et al, 2011). The focus was placed on investigating differences in expression of surface receptors implicated in inflammatory and reparative responses between groups. Additionally, the functional status of monocyte subsets was investigated via measurement of intracellular IKKβ, a marker of activation of the NFκB pathway. Monocyte interactions with platelets were examined, and associations between monocyte characteristics and plasma markers of inflammation and fibrinolysis were explored.

As monocyte subsets have been proposed to play different roles in the pathogenesis of CAD, I hypothesised that there would be significant differences in subset characteristics between healthy subjects and those with CAD.

4.3 METHODS AND MATERIALS

4.3.1 Coronary artery disease subjects

53 patients were recruited with angiographically proven stable CAD and normal LVEF from SWBH NHS Trust as described in section 3.1.2.1.

4.3.2 Control group

50 age and sex-matched healthy controls (HC) were recruited as described in section 3.1.2.2. Exclusion criteria comprised factors which may affect monocyte numbers (section 3.1.3).

4.3.3 Sample collection

All subjects were asked to refrain from extreme physical exertion for at least 1 hour prior to sample collection (section 3.3.1). Peripheral venous blood samples were collected from all participants and processed by flow cytometry within 60 minutes (section 3.2). Plasma was separated by centrifugation and stored at –70^{o}C for batched ELISA analysis (section 3.4).

Routine haematology and biochemistry investigations were undertaken by the hospital laboratory (section 3.5).

4.3.4 Flow cytometry

Flow cytometric analysis was performed as described in section 3.2. Briefly, the absolute count of monocytes and counts of the three monocyte subsets and associated MPAs were performed, as described in section 3.2.2. The three monocyte subsets were defined as CD14++CD16-CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2) and CD14+CD16++CCR2-

(Mon3) in keeping with contemporary nomenclature (Shantsila et al, 2011; Ziegler-Heitbrock et al, 2010). Expression of surface antigens on monocyte subsets was measure as described in section 3.2.3.

Intracellular level of IKKβ was measured as described in section 3.2.4.

Plasma levels of IL-1β, IL-6, IL-10 and MCP-1 were measured by cytometric bead array technology as described in section 3.2.5.

4.3.5 Enzyme-linked immunosorbent assays

ELISA of citrated plasma was used to quantify levels and activity of fibrinolytic parameters as described in section 3.4.

4.3.6 Statistical analysis

Statistical analyses were performed as described in chapter 3.8. Statistical significance of differences between monocyte subsets was determined by independent sample T-test for normally distributed variables, and by Mann-Whitney test for non-normally distributed variables. Correlations between monocyte parameters and inflammatory and fibrinolytic markers in CAD were assessed using Pearson's method (for normally distributed variables) and Spearman's method (for non-normally distributed variables).

4.4 RESULTS

4.4.1 Subject demographics

The study groups were well matched for age, sex, body mass index, blood pressure and renal function (Table 4.1). Healthy subjects included significantly less cigarette smokers. Among patients with CAD, 28 (53%) had a history of hypertension, 9 (17%) had diabetes mellitus, and 24 (46%) had a history of previous MI. The CAD patients received the following background pharmacological treatment: aspirin 46 (87%), clopidogrel 23 (43%), prasugrel 2 (4%), statins 47 (89%), angiotensin converting enzyme inhibitors/angiotensin receptor antagonist 41 (77%), beta-blockers 38 (72%), calcium channel blockers 15 (28%).

Table 4.1 Demographic and clinical characteristics, leukocyte counts and plasma inflammatory cytokines in the study groups

	CAD (n=53)	Healthy controls (n=50)	p value
Age, years	64 [12]	61 [14]	0.22
Male, n (%)	38 [72]	33 [66]	0.53
Systolic BP, mm Hg	134 [16]	128 [17]	0.35
Diastolic BP, mm Hg	72 [10]	75 [4]	0.38
Body mass index, kg/m^2	29 [5]	27 [4]	0.26
Creatinine, μmol/l	88 [20]	77 [14]	0.17
Smoking, n (%)	19 [36]	2 [4]	<0.001
Leukocytes, $x10^3$ per μl	6.4 [1.9]	5.9 [1.3]	0.23
Neutrophils, $x10^3$ per μl	3.9 [1.4]	3.5 [1.4]	0.25
Lymphocytes, $x10^3$ per μl	1.8 [0.6]	1.8 [0.6]	0.94
Platelets, $x10^3$ per μl	235 [71]	263 [67]	0.089
Monocytes, per μl	567 [171]	508 [182]	0.095
MPA, per μl	74 [53-104]	62 [37-90]	0.061
Interleukin 1β, pg/ml	0.64 [0.11-0.75]	0.52 [0.3-0.75]	0.45
Interleukin 6, pg/ml	2.1 [1.2-3.1]	1.7 [0.8-2.7]	0.098
Interleukin 10, pg/ml	0.13 [0.07-1.26]	0.83 [0.13-1.63]	0.009
MCP-1, pg/ml	52 [28-107]	107 [63-170]	<0.001

BP: blood pressure, CAD: coronary artery disease, MCP-1: monocyte-chemoattractant protein-1, MPA: monocyte-platelet aggregates. Data are presented as mean [SD] or median [IQR] for normally and non-normally distributed variables.

4.4.2 Leucocytes and inflammatory cytokines

There was no significant difference in leukocyte levels between the groups, although there was a trend towards higher monocyte count and lower platelet count in CAD (Table 4.1). Subjects with CAD had significantly lower plasma IL10 and MCP-1 levels than controls ($p=0.009$, and $p<0.001$, respectively), with a trend towards higher IL6 concentrations. There was no difference in IL1β levels between the groups.

4.4.3 Monocyte subset characteristics

There were similar numbers of the Mon1 and Mon2 subsets and their aggregates with platelets in the groups. MPA number associated with the Mon3 subset was higher in CAD ($p=0.03$), with a non-significant trend observed in Mon3 count (Table 4.2).

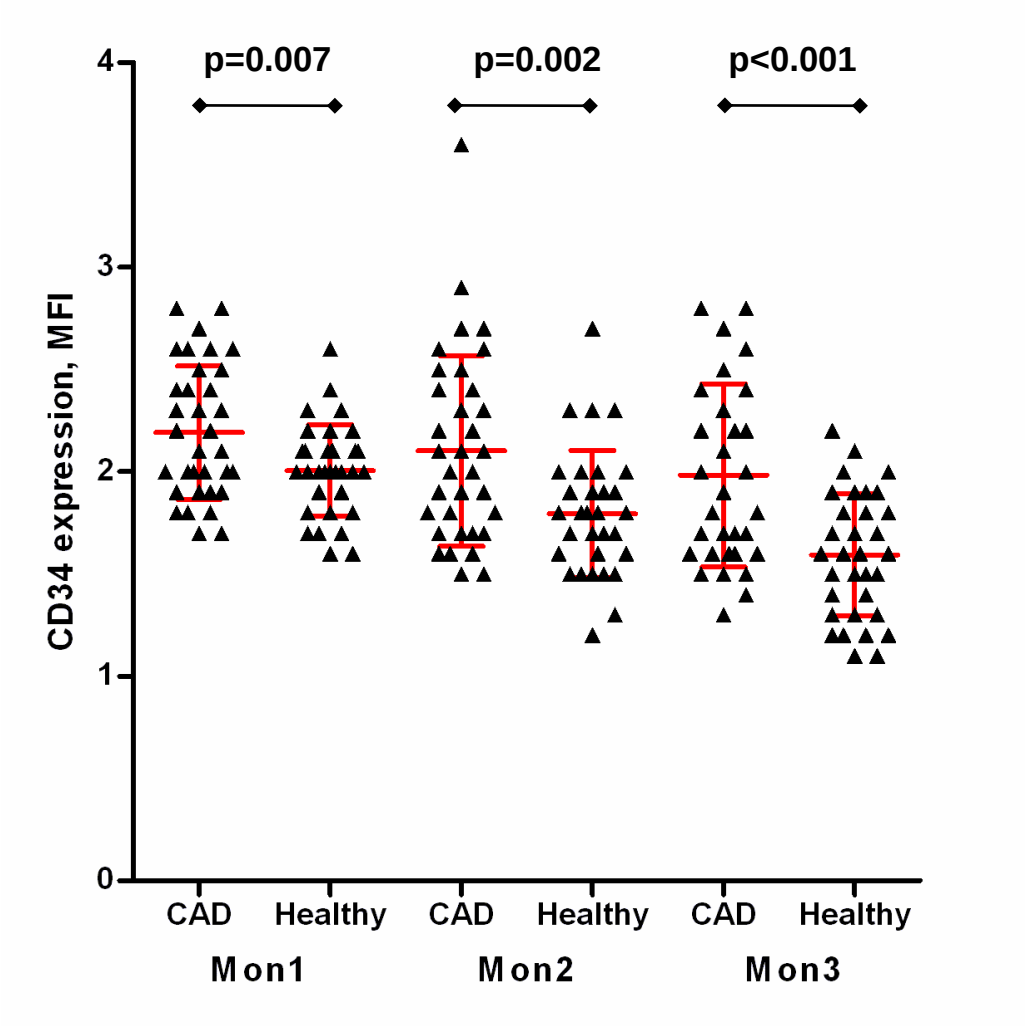
CAD was associated with lower expression of CD14 on Mon1 and Mon3 ($p=0.02$, and $p=0.036$, respectively), and lower expression of CD16 on Mon1 and Mon2 ($p=0.002$, and $p=0.001$, respectively). Patients with CAD had significantly higher expression of IL6 receptor on Mon1 ($p=0.025$) and Mon2 ($p=0.015$), CXCR4 on Mon3 ($p=0.003$), and CD34 on all three subsets as compared to HC ($p=0.007$ for Mon1, $p=0.002$ for Mon2, and $p<0.001$ for Mon3) (Figure 4.1). There were no significant differences in other monocyte parameters including levels of intracellular IKKβ (Table 4.2).

Table 4.2 Characteristics of monocyte subsets in the study subjects

	Mon1			Mon2			Mon3		
	CAD	Healthy	p value	CAD	Healthy	p value	CAD	Healthy	p value
Count, per μl	469 [149]	423 [160]	0.15	30 [18-48]	29 [10-51]	0.52	54 [39-79]	49 [35-62]	0.094
MPA, per μl	60 [41-81]	48 [28-74]	0.11	6 [3-10]	5 [3-8]	0.22	8 [5-11]	7 [4-9]	0.030
CCR2, MFI	148 [43]	147 [51]	0.94	115 [33]	106 [33]	0.16	15 [3]	15 [2]	0.37
CD14, MFI	1268 [342]	1423 [312]	0.02	1287 [391]	1281 [495]	0.95	141 [44]	158 [36]	0.036
CD16, MFI	9 [3]	10 [3]	0.002	49 [14]	59 [16]	0.001	142 [60]	143 [61]	0.89
TLR4, MFI	5.8 [1.7]	5.7 [2.0]	0.70	9.2 [7-13]	10 [6-16]	0.47	3.9 [1.2]	3.9 [1.5]	0.88
IL6R, MFI	70 [16]	62 [20]	0.025	64 [14]	55 [20]	0.015	35 [23]	32 [12]	0.40
Integrin β2, MFI	33 [15]	38 [18]	0.23	60 [24]	66 [30]	0.28	29 [15]	28 [12]	0.78
Integrin α4, MFI	11 [5]	11 [4]	0.80	23 [9]	24 [11]	0.55	36 [14]	36 [12]	0.83
CXCR4, MFI	17 [13]	14 [3.2]	0.13	26 [17]	21 [8]	0.069	9 [6]	6 [2]	0.003
CD34, MFI	2.2 [0.3]	2.0 [0.2]	0.007	2.1 [0.5]	1.8 [0.3]	0.002	2.0 [0.4]	1.6 [0.3]	<0.001
VEGFR1, MFI	6.3 [1.6]	6.8 [1.8]	0.28	13.3 [4.7]	15.2 [6.4]	0.13	4.2 [1.5]	4.0 [1.8]	0.55
VEGFR2, MFI	3.3 [1.0]	3.4 [1.4]	0.78	4.4 [1.9]	4.4 [2.3]	0.95	3.5 [0.9]	3.9 [0.8]	0.57
CD163, MFI	142 [60]	136 [50]	0.57	236 [90]	222 [76]	0.42	16 [12-22]	14 [9-24]	0.55
CD204, MFI	12 [8-23]	9 [7-13]	0.13	30 [14-61]	30 [19-100]	0.43	32 [19-73]	38 [23-71]	0.46
IKKβ, MFI	68 [17]	67 [13]	0.78	75 [20]	74 [16]	0.95	64 [15]	64 [10]	0.95

Mon1, CD14++CD16-CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2– monocytes; CAD, coronary artery disease; IL6R, interleukin 6 receptor; MFI, median fluorescent intensity; MPA, monocyte-platelet aggregates (associated with individual monocyte subsets); TLR4, toll-like receptor 4; VEGFR, vascular endothelium growth factor receptor. Data are presented as mean [SD] or median [IQR] for normally and non-normally distributed variables respectively.

Figure 4.1 CD34 expression by monocyte subsets in subjects with coronary artery disease and healthy controls



Mon1, CD14++CD16-CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2– monocytes; CAD, coronary artery disease; MFI, median fluorescent intensity. Red bars represent mean and standard deviation. Horizontal lines indicate significant differences between groups.

4.4.4 Correlations between expression of monocyte receptors and intracellular IKKβ levels

In this analysis a p value <0.0167 was considered significant, based on the Bonferroni adjustment for multiple testing. Significant correlations were found between intracellular IKKβ levels and monocyte parameters as follows: for Mon1 with expression of CXCR4 (r=0.54, p=0.003), CD34 (r=0.54, p=0.003), VEGFR1 (r=-0.51, p=0.003) and CD42a expression on aggregates with platelets (r=0.43, p=0.007); for Mon2 with expression of VEGFR1 (r=-0.51, p=0.006) (Table 4.3).

4.4.5 Correlations between expression of monocyte surface receptors, plasma cytokines and monocyte chemoattractant protein-1

In this analysis a p value <0.0125 was considered significant based on the Bonferroni adjustment for multiple testing. CD163 expression by all monocyte subsets correlated negatively with IL6 levels (r=-0.50, p<0.001 for Mon1, r=-0.41, p=0.004 for Mon2, r=-0.41, p=0.004 for Mon3) (Table 4.3). MCP-1 levels correlated positively with expression of TLR4 on Mon1 (r= 0.47, p=0.001) and Mon2 (r=0.52, p<0.001) and VEGFR1 on Mon2 (r=0.44, p=0.004) and negatively with CD204 expression on Mon1 (r=-0.64, p<0.001) and Mon2 (r=-0.57, p<0.001). Also, IL1β levels correlated positively with CD42a expression on Mon1 aggregated with platelets (r=0.36, p=0.009) (Table 4.3).

4.4.6 Correlations between expression of monocyte receptors and plasma fibrinolytic factors

In this analysis a p value <0.0125 was considered significant based on the Bonferroni adjustment for multiple testing. Plasma uPA levels correlated positively with VEGFR1

expression on Mon1 (r=0.51, p=0.003) and Mon2 (r=0.44, p=0.012). VEGFR1 expression on Mon2 correlated with positively with PAI-1 antigen levels (r=0.47, p=0.006). IL6R expression on Mon2 correlated negatively with PAI-1 antigen (r=-0.45, p=0.005) and PAI-1 activity (r=-0.47, p=0.004) (Table 4.3).

Table 4.3 Correlations between expression of monocyte receptors, intracellular IKKβ levels, inflammatory and fibrinolytic markers

	r	p value
Correlations with intracellular IKKβ levels		
CXCR4 (Mon1)	0.54	0.003
CD34 (Mon1)	0.54	0.003
VEGF receptor 1 (Mon1)	-0.51	0.003
VEGF receptor 1 (Mon2)	-0.51	0.006
CD42a (Mon1)	0.43	0.007
Correlations with inflammatory markers		
Plasma interleukin-6 levels		
CD163 (Mon1)	-0.50	<0.001
CD163 (Mon2)	-0.41	0.004
CD163 (Mon3)	-0.41	0.004
Plasma MCP-1 levels		
TLR4 (Mon1)	0.47	0.001
TLR4 (Mon2)	0.52	<0.001
CD204 (Mon1)	-0.64	<0.001
CD204 (Mon2)	-0.57	<0.001
VEGF receptor 1 (Mon2)	0.44	0.004
Plasma interleukin-1β levels		
CD42a (Mon1)	0.36	0.009
Correlations with fibrinolytic factors		
Plasma uPA levels		
VEGF receptor 1 (Mon1)	0.51	0.003
VEGF receptor 1 (Mon2)	0.44	0.012
Plasma PAI-1 antigen levels		
VEGF receptor 1 (Mon2)	0.47	0.006
Interleukin-6 receptor (Mon2)	-0.45	0.005
Plasma PAI-1 activity		
Interleukin-6 receptor (Mon2)	-0.47	0.004

VEGFR, vascular endothelium growth factor; MPA, monocyte-platelet aggregates; TLR4, toll-like receptor 4; PAI-1, plasmin activator inhibitor-1; uPA, urokinase type plasminogen activator

4.5 DISCUSSION

This study observed multiple significant novel changes in human monocyte phenotype in patients with CAD compared to healthy subjects, which are attributable to specific monocyte subsets. A significant up-regulation of IL6R expression on Mon1 and Mon2 subsets was found. There was significant up-regulation of CXCR4 expression on Mon3, with a similar trend for Mon2. A significant increase in CD34 expression was observed on all monocyte subsets.

In a recently published meta-analysis, Sarwar et al provided compelling evidence of a causal relationship between the pro-inflammatory IL6 pathway and risk of coronary artery disease. A genotype linked to accelerated shedding of the IL6r into the circulation (Asp358A1a) was associated with potent atheroprotective properties, independent of traditional cardiovascular risk factors (Sarwar et al, 2012). This was associated with reduced levels of downstream mediators of the IL6 pathway and disruption of IL6-driven inflammatory responses. Their findings imply that it is not IL6r shed into the circulation, but that left on effector cells which is of major importance, but data on cells that up-regulate expression of IL6r in CAD were not provided. Thus the observations in this study support the data of Sarwar and colleagues, that patients with CAD express higher IL6r levels, and suggest that Mon1 and Mon2 may be those effector cells responsible for IL6r-mediated atherogenesis (Sarwar et al, 2012). This is also in accordance with the recent finding of an independent association between CD14++CD16+ monocyte count and future risk of cardiovascular events in a large prospective cohort study of patients at high cardiovascular risk (Rogacev et al, 2012). The observations in my study suggest what one of the mechanisms underlying this association may be. Although limited

methodologically by the assessment of CD16+ monocytes as a single population, the findings that statin drugs lower the proportion of the CD16+ subset (population with higher expression of IL6r in my study) may explain the potential therapeutic pathway of this class of drug in lowering cardiovascular risk (Imanishi et al, 2010).

Although the CXCL12 (Stromal cell-derived factor-1)/CXCR4 axis is thought to play a critical role in vascular repair and remodelling, its role in atherogenesis and post-infarct recovery is controversial, with conflicting data (Zernecke et al, 2008). Mobilisation of CXCR4+ cells has been shown to mediate the benefit of treatment with macrophage-colony stimulating factor (M-CSF) in reduction of LV dysfunction and accelerated neointima formation following vascular injury in mice (Morimoto et al, 2007; Shiba et al 2007). Additionally, inhibition of the CXCL12/CXCR4 axis in mice exacerbated atherogenesis (Zernecke et al, 2008). However, CAD risk alleles at rs1746048 and rs501120 have been associated with higher plasma levels of CXCL12 (Mehta et al, 2011; Liehn et al, 2011). This discrepancy may be partly due to the fact that both inflammatory and progenitor cells are CXCR4+ and are both recruited by this pathway. My finding of a significantly higher expression of CXCR4 by Mon3 in CAD compared to HC suggests that these 'non-classical' monocytes may be a subset with enhanced CXCR4-dependent mobilisation to tissues in CAD, in keeping with their 'patrolling' behaviour (Cros et al, 2010).

This study found a significant increase in CD34 expression by all three monocyte subsets in CAD. This may reflect a compensatory mechanism attempting to enhance angiogenic processes in response to likely low-grade ischaemia in stable CAD. Indeed, potent angiogenic potential related to CD34+ cells has been observed in animal models (Shantsila et al, 2007;

Harraz et al, 2001). Of interest, in a cohort of subjects of South Asian origin (a high risk population for CAD and it's complications) no difference in CD34+ monocyte counts was found between patients with CAD and healthy subjects. Ethnicity was independently predictive of CD34+ monocyte levels in heart failure subjects, with lower values seen in South Asian subjects that in Caucasians (Shantsila et al, 2012).

In this study I observed significant reductions in CD14 expression on Mon1 and Mon3, and CD16 expression on Mon1 and Mon2 in patients with CAD compared to HC. Although a reduction in CD16 expression on Mon1 (described as CD16- cells) may appear paradoxical, it may reflect that some of these cells do in fact express very low levels of the CD16 (~14-fold lower expression that Mon3, and ~5-6 fold lower expression than Mon2 in this study). This low-level expression may not be reliably discriminated using isotype-control samples, but may be detectable by measuring MFI. Lower density of these receptors, which have been implicated in inflammatory reactions, may reflect their enhanced release into circulation or the partial suppression of inflammatory signalling previously observed in monocytes from patients with stable CAD (Schirmer et al, 2009).

No significant differences were seen in monocyte expression of the other receptors tested between CAD and HC in this study. This may be related to the comprehensive contemporary medical therapy prescribed to patents with CAD and their subsequent stable status. This is supported by the lack of differences in IKKβ expression between study groups, in contrast to the significant up-regulation (especially the Mon2 subset) seen in patients with STEMI (discussed in chapter 5).

Whilst the role of the NFκB pathway in induction of synthesis of inflammatory cytokines, MMPs and other biological molecules in monocytes is well established (Pamukcu et al, 2011), this study shows for the first time an association between monocyte activation and increased surface expression of receptors associated with angiogenesis, repair and remodelling (CXCR4, CD34 and VEGFR1) on monocyte subsets in stable CAD. This suggests a more complex role of the NFκB system in monocyte responses to tissue injury. The positive correlation observed between IKKβ levels in the Mon 1 subset and CD42 expression on their aggregates with platelets (a measure of platelet load on monocytes) provides further support to the growing evidence on the role that interaction with platelets plays in regulation of monocyte activity (Simon et al, 1993; van Gils et al, 2008; Kuckleburg et al, 2011).

This study also reinforces the anti-inflammatory properties of the haptoglobin scavenger receptor CD163, as expression on all monocyte subsets was found to correlate negatively with plasma IL6 levels. Additionally, the positive correlations between VEGFR1 expression on Mon1 and Mon2 with uPA levels and the negative correlation between IL6r expression on Mon2 and plasma PAI-1 level may reflect a role of fibrinolytic enzymes in the regulation of expression and cleavage of these receptors, in accordance with previous data reporting links between monocytes and the fibrinolytic system (Shantsila et al, 2012).

4.6 LIMITATIONS

This study is limited by its cross-sectional design, and any clinical relevance of the findings should be assessed in prospective and ideally interventional studies. As with all contemporary human studies, I was limited to analysing monocytes in peripheral venous blood, and direct extrapolation of these findings into physiological processes within atherosclerotic plaques and the myocardium should be made with caution.

Robust mechanistic implications cannot be inferred from this purely observational data. These mechanisms should be explored in future experiments. The differences and similarities observed between study groups may be partly attributable to the medication taken by CAD patients rather than the presence of CAD per se. Thus my findings may only be relevant to patients receiving such treatment. An examination of the effects individual medications exert on the observations made in this study would be of interest.

Although the majority of samples were collected between 09.00 and 12.00 hours, it was logistically impossible to collect all samples at the same time each day. The remaining influence of diurnal variation on monocyte parameters should be considered as a limitation (Shantsila et al, 2012).

4.7 CONCLUSIONS

There are multiple significant differences in monocyte phenotype in patients with CAD, which are differentially attributable to individual monocyte subsets. There appears to be a complex interplay between monocyte activity, phenotype, plasma cytokines and fibrinolytic factors. The findings of this study support accumulating data describing the presence of diverse roles of human monocyte subsets in the pathogenesis of CAD, with specific emphasis on the CD14++CD16+ subset. This data suggests potential mechanisms which may underlie the recently reported associations between this 'intermediate' subset and prognosis in CAD. Further research into the heterogeneity of human monocyte populations in CAD may ultimately allow for cell-specific therapeutic strategies for modulation of atherosclerosis. CD14++CD16+ 'intermediate' monocytes and their relations with the IL6r may be a promising target.

CHAPTER 5

MONOCYTE SUBSETS AND MONOCYTE PLATELET AGGREGATES IN ST-ELEVATION MYOCARDIAL INFARCTION

Contributory publication: The CD14++CD16+ monocyte subset and monocyte-platelet interactions in patients with ST-elevation myocardial infarction. Tapp LD, Shantsila E, Wrigley BJ, Pamukcu B, Lip GY. J Thromb Haemost 2012; 10(7): 1231-41.

Abstract

Monocytes contribute to both myocardial damage and repair by virtue of subset heterogeneity. The hitherto unknown dynamic changes and relations to LVEF of the three human monocyte subsets and their aggregates with platelets (MPAs) following STEMI were studied.

The three monocyte subsets defined as CD14++CD16-CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2) and CD14+CD16++CCR2- (Mon3) and their contributions to MPAs were analysed by flow cytometry in 50 patients with STEMI, 40 patients with stable CAD and 40 healthy subjects. Parameters were measured within 24 hours of PPCI (day1) and on days 3, 7 and 30. Monocyte activation status was assessed by measuring the NFκB pathway. LVEF was measured by echocardiography 6 weeks after STEMI. Correlations between monocyte subsets and MPAs with plasma cytokines (by FACSArray) and troponin were assessed.

On day 1, marked differences in subset dynamics were observed, with a prominent (2.5 fold) increase in Mon2 ($p < 0.0001$) but no changes in Mon3. Significant increases in Mon2 CD14 ($P = 0.002$) and CCR2 ($p < 0.0001$) expression, and a reduction in CD16 expression ($p = 0.001$) were seen. NFκB pathway activity increased most prominently in Mon2 ($p = 0.007$). Mon2 count correlated with peak troponin ($r = 0.31$, $p = 0.044$) and plasma IL6 ($r = 0.65$, $p < 0.0001$) and IL-10 ($r = 0.34$, $p = 0.017$). Mon1 correlated with IL-6 ($r = 0.55$, $p < 0.0001$). Reduced Mon2 expression of CD16 on day 1 was independently predictive of higher LVEF ($b = -0.37$, $p = 0.013$) at 30 days. The increase in MPA count following STEMI persisted at 1 month.

The Mon2 ‘intermediate’ subset has unique dynamic and phenotypic characteristics following STEMI and significant correlations with troponin, plasma cytokines and convalescent LV function were observed. The persistent increase in MPA count 30 days after STEMI may reflect monocyte subset functional activity.

5.1 INTRODUCTION

5.1.1 Monocytes in myocardial infarction

The underlying pathophysiological mechanisms contributing to MI include vascular inflammation, plaque rupture, coronary artery thrombosis and occlusion resulting in myocardial necrosis (chapter 1.1). Monocytes are an essential component of the innate and adaptive immune systems and have been implicated in many inflammatory diseases including CAD and atherosclerosis (Woollard et al, 2010; Hristov et al, 2010, Hristov et al, 2011). Following MI, monocytosis has been associated with impaired myocardial recovery and unfavourable prognosis (Maekawa et al, 2002; Panizzi, et al 2010). However, unique characteristics of monocytes also suggest an intimate role in cardiovascular repair processes (Dresske et al, 2006; Nahrendorf et al, 2007). This functional diversity is thought to result from heterogeneity of monocyte subpopulations (Geissmann et al, 2003; Auffray et al, 2009, Shantsila et al, 2009; Zawada et al, 2011).

5.1.2 Monocyte subsets in myocardial infarction

Evidence supporting the hypothesis of monocyte functional diversity in MI in vivo is supported by the findings of sequential mobilisation of monocyte subsets, firstly in mouse (Nahrendorf et al, 2007) and more recently in humans (Tsujioka et al, 2009). Sequential mobilisation of subsets is thought to be due to the orchestrated release of subset-specific chemokines. In the mouse model of MI, the Ly-6C^{high} monocyte subset predominated within the myocardium during phase I after MI (peak level on day 3) and was found to exhibit phagocytic, proteolytic and inflammatory activity. In contrast, the Ly-6C^{low} monocyte subset predominated in phase II after MI (peak on day 7) and was implicated in myocardial healing

and recovery processes, including anti-inflammatory properties, promotion of myofibroblast accumulation, angiogenesis and collagen deposition (Nahrendorf et al, 2007). In humans, the CD14+CD16- and CD14+CD16+ monocyte subsets peaked in the circulation on days 3 and 5 respectively after MI (Tsujioka et al, 2009). A negative correlation was found between the peak level of the CD14+CD16- monocyte subset following MI and convalescent LVEF at 6 months assessed by cardiovascular MRI (Tsujioka et al, 2009). These studies illustrate the diverse and contrasting functions of monocytes and their subsets in homeostasis and the pathophysiology of MI. Further evaluation of monocyte subset biology and a greater understanding of their specific roles in MI may allow the development of novel therapeutic interventions specifically targeted towards inhibiting detrimental processes whilst selectively sparing beneficial roles.

CD16+ positive monocytes remain the most poorly characterised as they have historically been evaluated as a single population, including the paper by Tsujioka and colleagues (Tsujioka et al, 2009). However, more recently they have been shown to comprise two distinct subsets of CD14++CD16+ and CD14+CD16++ cells and there is emerging phenotypic, functional and genetic evidence suggestive of distinct roles for each subset (Cros et al, 2010; Zawada et al, 2011; Shantsila et al, 2011; Auffray et al, 2007; Rogacev et al, 2010; Wong et al 2011) (chapter 1.2).

5.2 AIMS AND HYPOTHESES

Our research group described marked phenotypic differences between the three human monocyte subsets in healthy subjects (Shantsila et al, 2011), but their characteristics in MI have not been studied. My objective in this study was to investigate their dynamic changes and associations with MPAs in STEMI, from the acute to the recovery phase, with particular emphasis on the hitherto poorly characterised Mon2 subset. Based on the accumulating data suggesting its unique characteristics (Cros et al, 2010; Rogacev et al, 2010; Wong et al, 2011; Shantsila et al, 2011; Zawada et al, 2011) I hypothesised that the Mon2 subset would exhibit distinct dynamic changes following STEMI compared to Mon1 and Mon3, and that these changes would differ from monocyte numbers in healthy people and those with stable CAD. I hypothesised that these changes may be related to the degree of myocardial damage and to changes in inflammatory cytokines and MCP-1. Finally I hypothesised that the dynamic changes in monocyte subsets acutely after MI is related to convalescent LVEF, which is the most important determinant of long-term prognosis following STEMI.

5.3 METHODS AND MATERIALS

5.3.1 ST elevation myocardial infarction patients

50 consecutive patients diagnosed with STEMI fulfilling the study criteria were recruited from SWBH NHS Trust between November 2009 and November 2010 (sections 3.2.1.1 and 3.1.3). The diagnosis of STEMI was made according to the universal definition (Thygesen et al, 2007) (chapter 1.1.3). All patients were treated successfully with PPCI and managed according to the contemporary ESC guidelines (Van de Verf et al, 2008) (chapter 1.1.5). The three monocyte subsets and MPAs were evaluated at 4 time points following STEMI: day 1 (during the 24 hours after PPCI), day 3, day 7 and day 30.

5.3.2 Control groups

For a cross-sectional comparison, STEMI patients were compared to two age- and sex-matched control groups: (i) 40 'disease controls' with stable CAD (chapter 3.1.2.1) and (ii) 40 'healthy controls' (HC) (chapter 3.1.2.2). Exclusion criteria are summarised in chapter 3.1.3. No STEMI patients had a history of previous MI, LV dysfunction or heart failure.

5.3.3 Sample collection

All subjects were asked to refrain from extreme physical exertion for at least 1 hour prior to sample collection (chapter 3.3.1). Peripheral venous blood samples were collected from all participants and processed by flow cytometry within 60 minutes (chapter 3.2). Plasma was stored at -70^{o}C for batched ELISA analysis (chapter 3.4).

Routine haematology and biochemistry investigations were undertaken by the hospital laboratory (chapter 3.5).

5.3.4 Flow cytometry

Flow cytometric analysis was as described in chapter 3.2. Briefly, monocyte subsets were defined as CD14++CD16–CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2) and CD14+CD16++CCR2– (Mon3) in accordance with contemporary nomenclature (Ziegler-Heitbrock et al, 2010; Shantsila et al, 2011) (chapter 1.2).

Intracellular level of IKKβ was measured as described in chapter 3.2.4.

Plasma levels of IL-1β, IL-6, IL-10 and MCP-1 were measured by cytometric bead array technology as described in chapter 3.2.5.

5.3.5 Assessment of left ventricular function

Patients underwent echocardiographic assessment of convalescent LVEF by Simpson's method 6 weeks after STEMI (chapter 3.6).

5.3.6 Statistical analysis

Statistical analyses were performed as described in chapter 3.8. T-test (for normally distributed parameters) or Mann-Whitney test (for non-normally distributed parameters) was performed to compare study parameters between patients with stable CAD and 30 day values in STEMI patients. Correlations between monocyte parameters and levels of troponin and

plasma markers on day 1 after STEMI were assessed using Pearson's method (for normally distributed parameters) or Spearman's method (for non-normally distributed parameters). Univariate and multivariate linear regression analyses were performed to assess the predictive value of the study parameters for convalescent LVEF 6 weeks after STEMI. In the longitudinal analysis, only STEMI patients who completed follow-up were included.

5.4 RESULTS

5.4.1 Patient characteristics

50 patients with STEMI (57.5 [11.7] years, 86% male), 40 patients with stable CAD (60.4 [10.8] years, 83% male) and 40 healthy volunteers (59.5 [13.4] years, 80% male) were recruited (Table 5.1). Thirty three STEMI patients completed follow-up. Two patients died; 1 on day 1 due to a retroperitoneal bleed from the femoral artery puncture and the second on day 2 from respiratory failure having suffered a cardiac arrest due to ventricular fibrillation on day 1, complicated by aspiration pneumonia. One patient developed recurrent non ST-elevation MI attributed to a different culprit coronary artery lesion than that implicated in their index STEMI and underwent a second PCI. Fourteen withdrew consent. The principle reasons for withdraw of consent were logistical difficulties in attending follow-up appointments and unwillingness to continue with the blood tests required for this study in addition to the frequent blood tests needed for routine care post-MI. Only 2 patients dropped out due to feeling too unwell to attend follow-up.

Table 5.1 Demographic and clinical characteristics of study patients

	STEMI (n=50)	**Stable CAD (n=40)**	**HC (n=40)**	**p value**
Demographic and clinical characteristics				
Age, years	57.5 [11.7]	60.4 [10.8]	59.5 [13.4]	0.50
Male, n (%)	43 (86)	33 (83)	32 (80)	0.75
BP systolic, mmHg	132 [18]	133 [15]	128 [17]	0.74
BP diastolic, mmHg	78 [14]	75 [9]	75 [4]	0.74
Body mass index, kg/m^2	30.0 [6.1]	28.8 [4.7]	26.7 [3.8]	0.25
Smoking, n (%)	29 (58)	17 (43)	1 (3)	<0.0001
Stroke, n (%)	3 (6)	5 (13)	-	0.25
Hypertension, n (%)	24 (48)	20 (50)	-	0.67
Diabetes, n (%)	16 (32)	8 (20)	-	0.25
COPD, n (%)	3 (6)	3 (8)	-	0.73
Creatinine, µmol/l	91.7 [18.5]	89.5 [17.9]	78.8 [15.2]	0.26
Glomerular filtration rate, ml/min	75.1 [15]	73.0 [14]	83.5 [9]	0.26
Platelets, per µl	263 [98]	226 [69]	252 [67]	0.14
Medications at time of recruitment				
Aspirin, n (%)	50 (100)	36 (90)	-	0.10
Clopidogrel or prasugrel, n (%)	50 (100)	30 (75)	-	<0.0001
Statin, n (%)	48 (96)	34 (85)	-	0.23
ACE inhibitor or ARA, n (%)	45 (90)	29 (73)	-	0.082
Diuretic, n (%)	2 (4)	5 (13)	-	0.12
Beta-blocker, n (%)	35 (70)	27 (68)	-	0.91
Calcium channel blocker, n (%)	8 (16)	11 (28)	-	0.14

ACE, angiotensin converting enzyme; ARA, angiotensin receptor antagonist; BP, blood pressure; CAD, coronary artery disease; COPD, chronic obstructive pulmonary disease; HC, healthy control; STEMI, ST-elevation myocardial infarction.

5.4.2 Monocyte subsets

5.4.2.1 Comparison with control groups

Counts of the Mon1 and Mon2 subsets were significantly higher on day 1 after STEMI compared to both control groups ($p<0.0001$ for all). There was a particularly prominent (over 2.5-fold) increase in Mon2 count. No difference was seen in Mon3 count. However, in stable CAD subjects, the proportion of Mon3 was 56% higher than Mon2. In contrast, the reverse pattern was seen on day 1 following STEMI, where Mon 2 predominated; the Mon 2 proportion was 47% higher compared to Mon3 (Table 5.2, Figure 5.1).

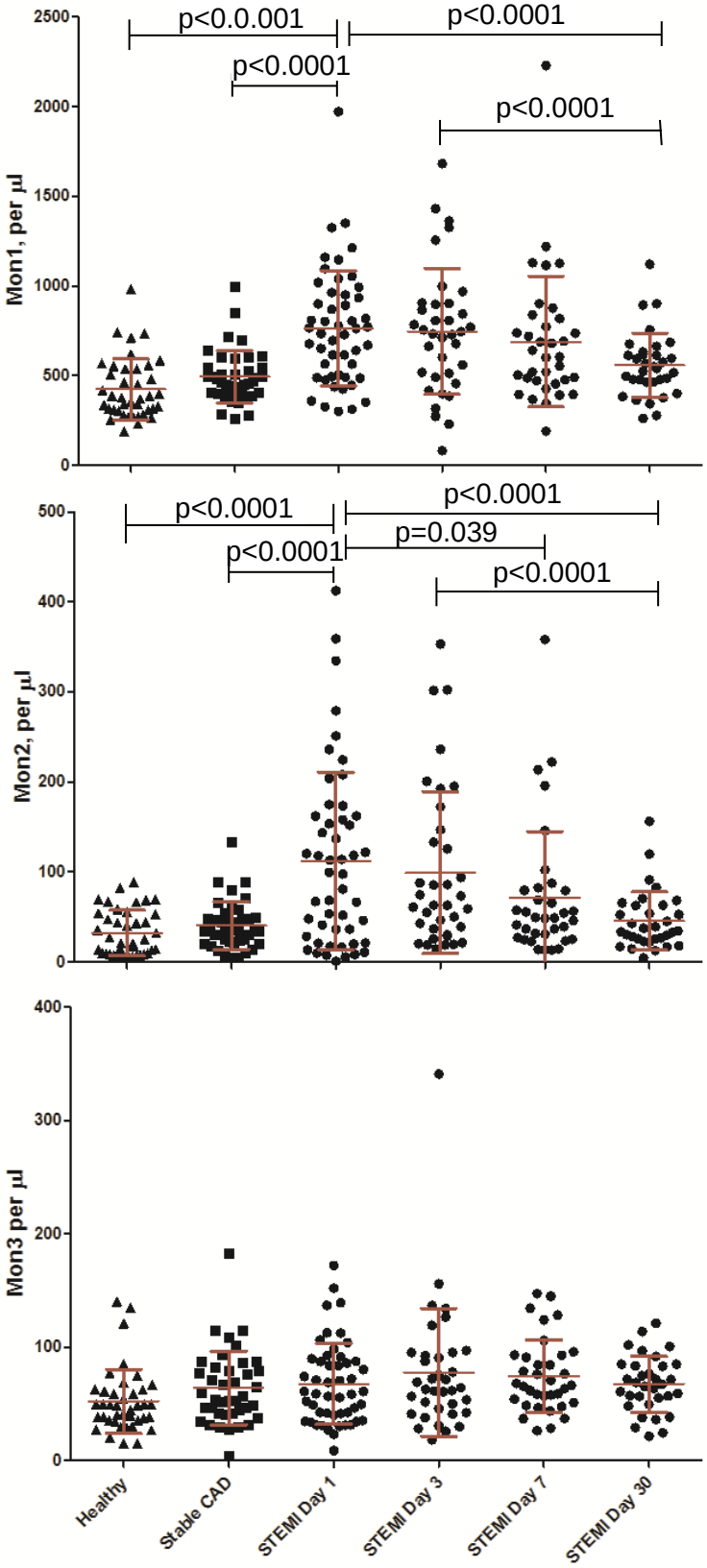
Table 5.2 Study parameters on day 1 after ST elevation myocardial infarction, compared with controls

	STEMI (n=50)	Stable CAD (n=40)	HC (n=40)	p value
Monocyte subsets				
Total monocytes, per µl	946 [378]*†	599 [165]	506 [192]	<0.0001
Mon1, per µl	765 [322]*†	494 [146]	422 [169]	<0.0001
Mon2, per µl	112 [98.9]*†	40.3 [26.3]	31.9 [25.3]	<0.0001
Mon3, per µl	67.5 [35.8]	63.9 [32.5]	52.0 [28.4]	0.074
Mon1, %	80.9 [8.9]	82.3 [5.8]	83.0 [7.7]	0.41
Mon2, %	11.3 [8.2]*†	6.92 [4.1]	6.41 [5.1]	<0.0001
Mon3, %	7.69 [4.4]*†	10.8 [3.9]	10.6 [4.5]	0.001
Monocyte platelet aggregates				
MPA, per µl	146 [78.4]*†	82.0 [28.6]	65.4 [36.5]	<0.0001
MPA (with Mon1), per µl	111 [61.2]*†	65.5 [24.7]	52.7 [31.8]	<0.0001
MPA (with Mon2), per µl	16.2 [7.2-26.4]*†	8.0 [3.7-9.8]	5.6 [2.5-8.6]	<0.0001
MPA (with Mon3), per µl	10.0 [5.8-13.1]†	7.4 [5.3-11.1]	6.6 [4.2-8.5]	<0.0001
MPA (with Mon1), %	13.5 [11.1-17.4]	12.6 [9.6 - 17.6]	11.4 [6.8 – 16.7]	0.088
MPA (with Mon2), %	19.4 [14.9-23.1]	20.4 [14.0-26.4]	21.2 [12.7-35.4]	0.65
MPA (with Mon3), %	14.5 [12.1-17.9]	12.7 [10.0-14.8]	12.0 [9.7-14.5]	0.011
MPA (with Mon1), MFI	40.1 [38.1-44.7]*	40.9 [37.7-45.1]	37.7 [36.3-42.8]	0.059
MPA (with Mon2), MFI	52.2 [44.9-71.3]†	73.8 [54.6-106]	89.5 [57.6-114]	<0.0001
MPA (with Mon3), MFI	55.3 [48.3-68.6]	54.6 [48.2-62.6]	54.5 [46.4-62.5]	0.47
Surface expression of monocyte markers				
CCR2 (Mon1), MFI	170 [51.6]	155 [45.0]	149 [47.5]	0.13
CCR2 (Mon2), MFI	159 [57.2]*†	121 [34.1]	106 [34.9]	<0.0001
CCR2 (Mon3), MFI	15.3 [2.4]	15.9 [2.6]	15.0 [2.1]	0.23
CD14 (Mon1), MFI	1315 [461]	1339 [268]	1472 [296]	0.11
CD14 (Mon2), MFI	1626 [527]*	1388 [329]	1286 [516]	0.003
CD14 (Mon3), MFI	195 [98.5]*	146 [37.4]	160 [38.4]	0.002
CD16 (Mon2), MFI	39.8 [36.7-48.6]†	55.6 [40.7-61.9]	61.0 [48.4-67.7]	<0.0001
CD16 (Mon3), MFI	130 [87.2-174]	143 [108-198]	149 [89.3-201]	0.22

Monocyte intracellular IKKβ				
IKKβ (Mon1), MFI	79.5 [18.1]*†	68.6 [16.3]	67.9 [12.6]	0.004
IKKβ (Mon2), MFI	90.9 [22.0]*†	76.2 [19.4]	76.6 [14.0]	0.002
IKKβ (Mon3), MFI	76.1 [14.]1*†	64.3 [14.2]	65.7 [10.4]	<0.0001
Inflammatory cytokines and MCP-1				
Interleukin 1β, pg/ml	0.5 [0.58]	0.6 [0.54]	0.4 [0.4]	0.41
Interleukin 6, pg/ml	10.8 [4.8-18.0]*	2.3 [1.4-3.4]	1.7 [0.7-2.7]	<0.0001
Interleukin 10, pg/ml	0.2 [0.1-1.2]	0.1 [0.1-1.4]	0.8 [0.1-1.7]	0.44
MCP-1, pg/ml	60.2 [39.2]*	73.3 [53.8]‡	122 [76.7]	<0.0001

CAD, coronary artery disease; HC, healthy control; MCP-1, monocyte chemoattractant protein 1; MPA, monocyte-platelet aggregates; MFI, median fluorescent intensity; STEMI, ST-elevation myocardial infarction; Mon1, CD14++CD16-CCR2+ ('classical') monocytes ; Mon2, CD14++CD16+CCR2+ ('intermediate') monocytes ; Mon3, CD14+CD16++CCR2- ('non-classical') monocytes. *p<0.05 STEMI vs. stable CAD (p=0.054 for CD14 (Mon2), MFI), †p<0.05 STEMI vs. healthy controls (p=0.055 for MPAs with Mon1, p=0.052 for CD14 (Mon3), MFI). ‡ p<0.05 stable CAD vs. healthy controls

Figure 5.1 Longitudinal changes in monocyte subset counts compared with control groups.



Numbers of Mon1 (upper), Mon2 (middle) and Mon3 (lower) per µl were measured in healthy controls and stable coronary artery disease (CAD) patients. This was compared with ST elevation myocardial infarction (STEMI) patients measured within 24 hours of primary PCI (day 1) and on days 3, 7 and 30. Red bars represent mean and standard deviation. Horizontal bars illustrate significant differences with individual p values. Mon1, CD14++CD16-CCR2+ ('classical') monocytes ; Mon2, CD14++CD16+CCR2+ ('intermediate') monocytes ; Mon3, CD14+CD16++CCR2- ('non-classical') monocytes.

5.4.2.2 Longitudinal changes in ST elevation myocardial infarction

The peak Mon1 and Mon2 counts were observed on day 1 after STEMI, with comparable values seen on day 3. The total monocyte count and Mon1 count decreased significantly by day 30 ($p<0.0001$ for both), where they reached levels not significantly different to those observed in stable CAD ($p=0.072$ and $p=0.086$, respectively). Mon2 count reduced earlier (by day 7), and on day 30 was similar to values seen in stable CAD ($p=0.35$). No significant dynamic changes were seen in Mon3 levels. The relative proportions of Mon2 and Mon3 had returned to levels similar to stable CAD by day 30 (Table 5.3).

Table 5.3 Dynamics of study monocyte parameters during 30 day follow-up after ST elevation myocardial infarction and relation to values in stable coronary artery disease

N=33 (27 for IKKβ)	**Day 1**	**Day 3**	**Day 7**	**Day 30**	**p value**	**CAD**	**p value‡**
Monocyte subsets							
Monocytes, per µl	994 [66.2]	967 [71.0]	861 [70.3]	670 [32.4]*†	<0.0001	599 [165]	0.072
Mon1, per µl	810 [57.7]	785 [58.5]	712 [62.9]	557 [31.3]*†	<0.0001	494 [146]	0.086
Mon2, per µl	108 [16.0]	105 [15.9]	73.6 [13.1]†	45.3 [5.6]*†	<0.0001	40.3 [26.3]	0.35
Mon3, per µl	72.2 [7.1]	77.1 [10.1]	75.1 [5.7]	67.3 [4.3]	0.55	63.9 [32.5]	0.65
Mon1, %	81.4 [1.6]	81.3 [1.6]	82.1 [1.6]	82.4 [1.3]	0.81	82.3 [5.8]	0.99
Mon2, %	10.9 [1.4]	10.5 [1.2]	8.58 [1.4]	7.36 [1.1]*†	0.002	6.92 [4.1]	0.63
Mon3, %	7.61 [0.8]	8.23 [0.8]	9.37 [0.7]	10.3 [0.6]*†	0.001	10.8 [3.9]	0.53
Monocyte-platelet aggregates							
MPA, per µl	157 [15.1]	148 [13.0]	136 [11.6]	107 [7.21]*†‡	<0.0001	82.0 [28.6]	0.003
MPA (with Mon1), per µl	123 [11.7]	113 [10.6]	109 [10.4]	87.4 [6.65]*†‡	0.001	65.5 [24.7]	0.004
MPA (with Mon2), per µl	18.0 [6.2-26.2]	14.1 [6.8-24.3]	10.6 [7.5-17.6]	8.70 [4.6-17.1]*†	0.006	7.95 [3.7-9.8]	0.069
MPA (with Mon3), per µl	10.3 [4.6-13.2]	9.50 [5.8-14.1]	11.3 [8.7-16.3]	8.50 [5.8-12.0]	0.10	7.40 [5.3-11.1]	0.50
MPA (with Mon1), %	14.1 [11.0-17.6]	15.1 [12.6-18.0]	15.1 [12.2-18.4]	14.9 [10.5-18.3]	0 38	12.6 [9.6 - 17.6]	0.16
MPA (with Mon2), %	19.7 [14.7-26.3]	20.3 [15.3-25.6]	22.0 [17.2-28.5]	22.2 [15.6-34.2]	0.11	20.4 [14.0-26.4]	0.15
MPA (with Mon3), %	13.8 [12.3-16.8]	15.2 [11.8-18.5]	15.6 [12.9-19.7]	12.8 [10.7-14.9]†	0.001	12.7 [10.0-14.8]	0.61
MPA (with Mon1), MFI	39.7 [37.9-43.7]	41.3 [37.6-44.5]	42.1 [38.4-45.8]	39.0 [36.9-41.4]	0.10	40.9 [37.7-45.1]	0.23
MPA (with Mon2), MFI	50.1 [44.7-71.8]	56.0 [45.8-74.7]	59.4 [48.9-111]	62.0 [49.0-77.4]	0.078	73.8 [54.6-106]	0.076
MPA (with Mon3), MFI	55.0 [48.1-65.0]	54.3 [48.6-58.6]	56.3 [51.0-63.6]	54.3 [49.6-60.3]	0.85	54.6 [48.2-62.6]	0.77
Surface expression of monocyte markers							
CCR2 (Mon1), MFI	173 [8.6]	177 [10.1]	166 [8.3]	144 [4.5]*†	0.003	155 [45.0]	0.27
CCR2 (Mon2), MFI	157 [10.5]	142 [8.4]	136 [8.5]	114 [4.2]*	<0.0001	121 [34.1]	0.36
CCR2 (Mon3), MFI	15.6 [0.4]	17.2 [0.4]*	14.9 [0.3]†	15.0 [0.3]†	<0.0001	15.9 [2.6]	0.082

CD14 (Mon1), MFI	1394 [79.7]	1338 [77.5]	1315 [49.8]	1330 [53.8]	0.76	1339 [268]	0.81
CD14 (Mon2), MFI	1687 [98.2]	1489 [96.3]	1381 [64.4]*	1382 [58.6]	0.001	1388[329]	0.93
CD14 (Mon3), MFI	192 [15.8]	223 [15.0]*	143 [8.1]*†	145 [7.1]*†	<0.0001	146 [37.4]	0.86
CD16 (Mon2), MFI	40.3 [37.3-55.7]	43.8 [38.5-58.5]	46.5 [37.3-60.7]	51.5 [37.1-63.1]	0.46	55.6 [40.7-61.9]	0.71
CD16 (Mon3), MFI	128 [83.4-177]	116 [88.1-191]	125 [95.4-173]	144 [98.3-194]	0.71	143 [108-198]	0.48
Monocyte intracellular IKKβ							
IKKβ (Mon1), MFI	80.4 [3.3]	72.6 [3.7]	69.4 [3.4]	61.8 [3.1]*	0.002	68.6 [16.3]	0.13
IKKβ (Mon2), MFI	92.8 [4.3]	80.4 [4.4]	76.0 [3.5]*	73.8 [4.2]*	0.003	76.2 [19.4]	0.66
IKKβ (Mon3), MFI	76.6 [2.5]	70.9 [2.8]	68.6 [2.8]	62.8 [2.8]*	0.003	64.3 [14.2]	0.71
Inflammatory cytokines and MCP-1							
Interleukin 1β, pg/ml	0.6 [0.6]	0.8 [0.6]*	0.8 [0.7]*	0.8 [0.7]	0.005	0.6 [0.5]	0.16
Interleukin 6, pg/ml	10.2 [4.8-18.1]	5.3 [2.9-10.6]	3.3 [2.2-5.9] *†	1.8 [1.1-2.6] *†	<0.0001	2.3 [1.4-3.4]	0.20
Interleukin 10, pg/ml	0.2 [0.1-1.2]	0.3 [0.1-1.3]	0.9 [0.2-1.3]*	0.5 [0.1-0.9]	0.023	0.1 [0.1-1.4]	0.52
MCP-1, pg/ml	64.1 [39.8]	55.4 [30.5]	41.3 [26.3]*†	58.3 [52.5]	0.024	73.3 [53.8]	0.27

CAD, coronary artery disease; MFI, median fluorescent intensity; MCP-1, monocyte chemoattractant protein; MPA, monocyte-platelet aggregates; Mon1, CD14++CD16-CCR2+ ('classical') monocytes; Mon2, CD14++CD16+CCR2+ ('intermediate') monocytes; Mon3, CD14+CD16++CCR2- ('non-classical') monocytes. *p<0.05 vs. day 1, †p<0.05 vs. day 3, ‡day 30 vs. stable CAD

5.4.3 Monocyte-platelet aggregates

5.4.3.1 Comparison with control groups

The total MPA count and MPAs associated with the Mon1 and Mon2 subsets were significantly higher on day 1 after STEMI compared with both control groups ($p<0.0001$ for all) (Table 5.2, Figure 5.3). MPAs associated with the Mon3 subset were increased in number on day 1 after STEMI compared to HC ($p=0.008$) (Table 5.3). There was a trend towards a higher proportion of Mon1 aggregated with platelets on day 1 after STEMI compared with HC ($p=0.055$). There was no observed difference in the proportion of the Mon2 subset aggregated with platelets ($p=0.65$). There was a trend towards a higher percentage of the Mon3 subset aggregated with platelets on day 1 after STEMI ($p=0.011$), but post-hoc analysis did not reach significance.

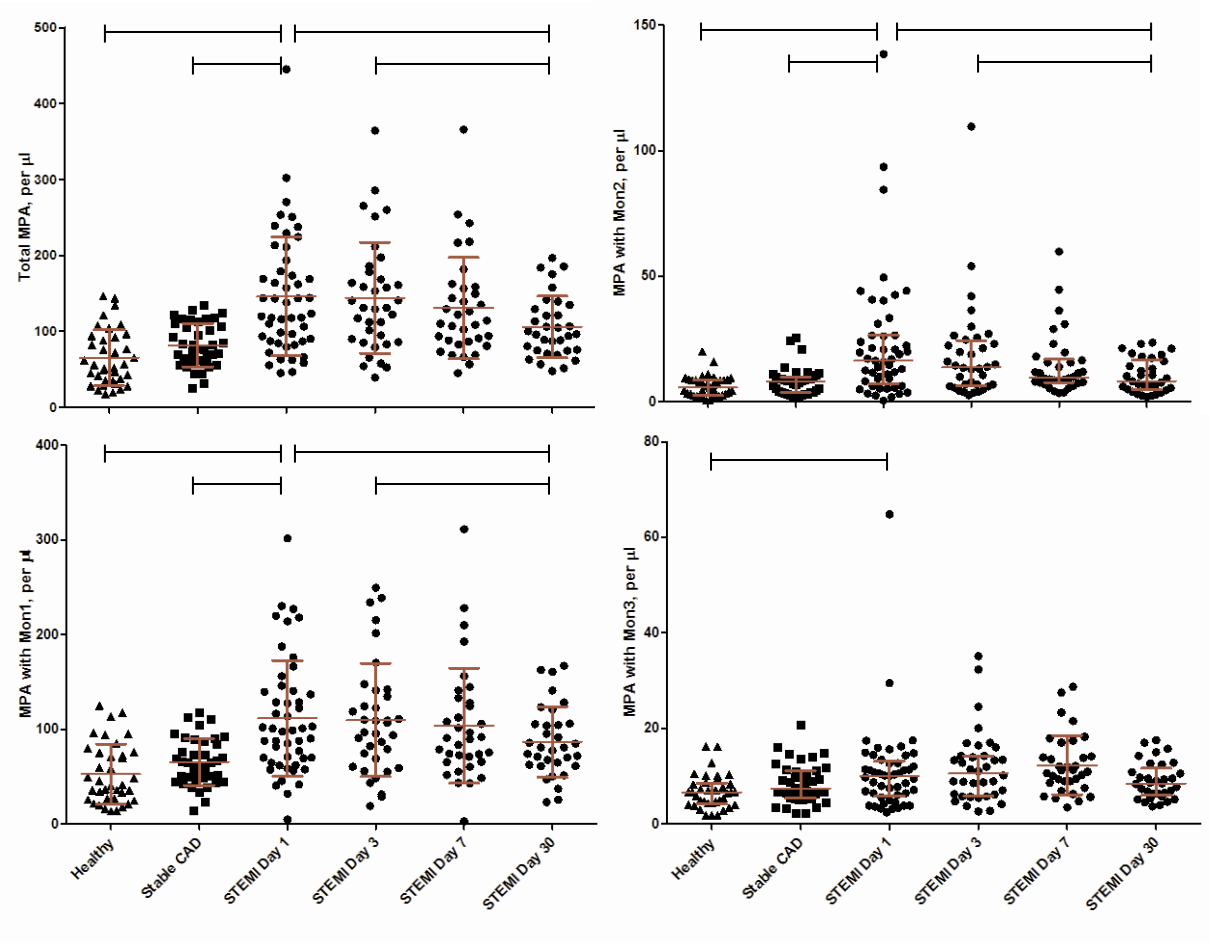
The number of platelets aggregated with the Mon1 subset (as indicated by MPA MFI) was increased on day 1 after STEMI compared with stable CAD ($p=0.041$). The number of platelets aggregated with the Mon2 subset was decreased in STEMI compared with HC ($p=0.001$) (Tables 5.2 and 5.3). The percentage of the Mon2 subset aggregated with platelets (19.4 [14.9-23.1]) on day 1 after STEMI was significantly higher than the percentage of aggregates with the Mon1 subset (13.5 [11.1-17.4]) ($p<0.001$).

5.4.3.2 Longitudinal changes in ST elevation myocardial infarction

After STEMI, the total number of MPAs and MPAs associated with Mon1 and Mon2 were reduced on day 30 ($p=0.005$ vs. day 1 and day 3 for total MPAs; $p=0.011$ and $p=0.055$ vs. day 1 and day 3 for Mon1; $p=0.001$ and $p=0.019$ vs. day 1 and day 3 for Mon2 respectively). At

day 30, total MPA count and MPAs associated with Mon1 remained significantly higher compared to stable CAD ($p=0.003$ and $p=0.004$, respectively). MPAs associated with Mon2 followed a similar but non-significant trend ($p=0.069$). The number of MPAs associated with Mon3 on day 1 after STEMI were increased compared to HC ($p=0.008$), but there was no significant longitudinal change during follow-up. The number of platelets aggregated with Mon1 (as indicated by MPA MFI) was increased in STEMI compared with stable CAD ($p=0.041$) and remained unchanged during follow-up (Table 5.3).

Figure 5.2 Longitudinal changes in monocyte-platelet aggregate number following ST elevation myocardial infarction compared with control groups



Total MPA count per µl (upper left) and MPAs associated with Mon1 (lower left), Mon2 (upper right) and Mon3 (lower right) were measured in healthy controls and stable coronary artery disease patients. Comparisons were made with STEMI patients measured within 24 hours of primary PCI (day 1) and on days 3, 7 and 30. Data are presented as median and interquartile range. Horizontal bars show significant differences between the groups at $p<0.05$ level. MPA, monocyte platelet aggregates; CAD, coronary artery disease; STEMI, ST elevation myocardial infarction; Mon1, CD14++CD16-CCR2+ ('classical') monocytes; Mon2, CD14++CD16+CCR2+ ('intermediate') monocytes; Mon3, CD14+CD16++CCR2- ('non-classical') monocytes; healthy, healthy control

5.4.4 Monocyte expression of CCR2, CD14 and CD16

5.4.4.1 Comparison with control groups

CCR2 expression by the Mon2 subset was significantly increased on day 1 after STEMI compared with both control groups ($p<0.0001$ for both) (Table 5.3).

In comparison with stable CAD, patients on day 1 after STEMI had increased expression of CD14 by the Mon2 ($p=0.002$) and Mon3 subsets ($p=0.02$).

Expression of CD16 by the Mon2 subset was reduced on day 1 after STEMI compared with healthy controls ($p=0.001$).

5.4.4.2 Longitudinal changes in ST elevation myocardial infarction

The significant elevation in CCR2 expression by Mon2 on day 1 after STEMI had reduced significantly by day 30 ($p<0.0001$) (Table 5.2). CCR2 expression by the Mon1 subset, although not significantly increased at admission, reduced by day 30 ($p=0.013$). Following STEMI, CCR2 expression by the Mon3 subset increased by day 3 ($p=0.007$), followed by reduced expression at subsequent time points (Table 5.3).

The elevation in CD14 expression by Mon 2 on day 1 after STEMI had reduced significantly by day 7 ($p=0.009$). The elevation in CD14 expression by Mon3 seen on day 1 after STEMI continued to increase on day 3 ($p=0.048$) followed by a reduction by day 7 ($p=0.008$ vs. day 1, and $p<0.0001$ vs. day 3).

No significant longitudinal changes in monocyte CD16 expression were seen (Tables 5.2 and 5.3).

5.4.5 Intracellular monocyte inhibitory κB kinases levels

5.4.5.1 Comparison with control groups

The intracellular level of IKKβ was significantly increased in all three monocyte subsets on day 1 after STEMI (most prominent increase in Mon2) compared with stable CAD and HC ($p=0.018$ and $p=0.015$ for Mon1, $p=0.007$ and $p=0.012$ for Mon2, $p<0.0001$ and $p<0.0001$ for Mon3, respectively) (Table 5.2). There was a significant negative correlation between the percentage of Mon1 with IKKβ levels ($r=-0.40$, $p=0.008$). A similar non-significant trend was observed for Mon2 ($r=-0.26$, $p=0.094$). There was no such association seen for Mon3 ($r= -0.20$, $p=0.20$).

5.4.5.2 Longitudinal changes in ST elevation myocardial infarction

The elevation in IKKβ level in all three monocyte subsets observed on day 1 after STEMI did not persist. IKKβ levels in Mon1 and Mon3 reduced significantly by day 30 ($p=0.003$ and $p=0.01$, respectively). A reduction in IKKβ levels in Mon2 was observed earlier, by day 7 ($p=0.02$). By day 30, IKKβ levels in all three monocyte subsets were similar to those in stable CAD (Table 5.3).

5.4.6 Inflammatory cytokines and monocyte chemoattractant protein-1

5.4.6.1 Comparison with control groups

Plasma IL-6 levels were significantly increased in patients on day 1 after STEMI compared to both stable CAD and healthy controls ($p<0.0001$ vs. both groups) (Table 5.2).

Patients on day 1 after STEMI and patients with stable CAD had lower plasma levels of MCP-1 than healthy controls ($p<0.0001$ and $p=0.001$, respectively).

No differences in plasma IL-1β and IL-10 levels were observed between STEMI patients on day 1 and control groups ($p=0.41$ and $p=0.44$, respectively).

5.4.6.2 Longitudinal changes in ST elevation myocardial infarction

During follow-up, the elevated plasma IL-6 level observed on day 1 after STEMI had reduced significantly by day 7 ($p=0.001$ for day 7 vs. day 3and $p<0.0001$ for day 30 vs. day 7) (Table 5.3).

In STEMI patients, the plasma MCP-1 level which was lower on day 1 compared to HC reduced further on day 7 ($p=0.002$ vs. day 1 and $p=0.031$ vs. day 3) and on day 30 had returned to levels not significantly different to stable CAD.

Having been unchanged on day 1 after STEMI, plasma IL-1β level was increased on day 3 ($p=0.035$ vs. day 1), and plasma IL-10 level had increased significantly by day 7 ($p=0.033$ vs. day 1).

On day 30, all plasma cytokine levels and MCP-1 values were similar to those observed in stable CAD.

5.4.6.3 Relations between monocyte subsets, plasma cytokines and monocyte chemoattractant protein 1

Plasma IL-6 levels in patients on day 1 after STEMI correlated positively with counts of Mon1 ($r=0.55$, $p<0.0001$), Mon2 ($r=0.65$, $p<0.0001$), total MPAs ($r=0.44$, $p=0.002$), MPAs associated with the Mon1 subset ($r=0.41$, $p=0.004$) and MPAs associated with the Mon2 subset ($r=0.55$, $p<0.0001$). Plasma IL-10 level correlated positively with the Mon2 subset count ($r=0.34$, $p=0.017$). No other significant correlations were observed for IL-10, and there were no significant correlations seen between plasma IL-1β or MCP-1 levels and monocyte or MPA count.

5.4.7 Relations of monocyte subsets and monocyte platelet aggregates to peak troponin level and left ventricular ejection fraction at 6 weeks after ST elevation myocardial infarction

The median peak troponin T value after STEMI was 2.50μg/l (interquartile range 1.19-5.50μg/l). Peak troponin level correlated negatively with LVEF measured 6 weeks after STEMI ($r= -0.51$, $p=0.002$), Mon2 count on day 1 ($r=0.31$, $p=0.044$), number of platelets on Mon2 on day 1 ($r=-0.41$, 0.01), CCR2 expression by Mon2 on day 1 ($r=0.30$, 0.056), and CD14 expression by Mon2 on day 1 ($r=0.34$, $p=0.032$). In contrast to Mon2, no correlations were observed between Mon1 count and peak troponin level ($r = 0.11$, $p=0.49$) or LVEF at 6 weeks ($r = -0.08$, $p=0.62$).

5.4.8 Monocyte subsets and monocyte platelet aggregates as predictors of left ventricular ejection fraction at 6 weeks after ST elevation myocardial infarction

Mean LVEF 6 weeks after STEMI was 54.1±14.6%. In a univariate regression analysis, significant predictors of LVEF at 6 weeks were age, peak troponin level, Mon2 count on day 1, the number of MPAs associated with Mon2 on day 1, Mon2 expression of CCR2 and CD14 on day 1, and Mon3 expression of CD14 on day 1. After adjustment for age, the number of MPAs associated with Mon2, and Mon3 expression of CD14 remained independent predictors of LVEF at 6 weeks. None of these parameters remained independent predictors of LVEF at 6 weeks after additional adjustment for peak troponin level. Lower expression of CD16 by the Mon2 subset, measured as MFI (which was not a significant predictor of LVEF in the univariate analysis) was an independent predictor of higher LVEF at 6 weeks after adjustment for age and peak troponin level (Table 5.4).

Table 5.4 Regression analysis of the predictive value of monocyte parameters for left ventricular ejection fraction

	B±SE	β	p value
Univariate analysis			
Age, years	-0.45±0.19	-0.36	0.023
Mon2, per μl	-0.47±0.02	-0.30	0.055
MPA with Mon2, per μl	-0.20±0.09	-0.34	0.03
MPA with Mon1, MFI	0.55±0.26	0.32	0.044
CCR2 (Mon2), MFI	-0.09±0.04	-0.32	0.044
CD14 (Mon2), MFI	-0.01±0.004	-0.31	0.053
CD14 (Mon3), MFI	-0.06±0.02	-0.38	0.015
Adjusted for age, troponin level			
CD16 (Mon2), MFI	-0.49±0.18	-0.37	0.013

B, regression coefficient; SE, standard error; β, adjusted regression coefficient; MFI, median fluorescent intensity; MPA, monocyte-platelet aggregates; Mon1, CD14++CD16-CCR2+ ('classical') monocytes ; Mon2, CD14++CD16+CCR2+ ('intermediate') monocytes ; Mon3, CD14+CD16++CCR2- ('non-classical') monocytes

5.5 DISCUSSION

The diverse functions attributed to monocytes is likely to stem from the presence of subsets with heterogeneous functions (Geissmann et al, 2003; Auffray et al, 2007; Auffray et al, 2009; Shantsila et al, 2009; Cros et al, 2010; Zawada et al, 2011; Rogacev et al, 2010; Wong et al, 2011). In this study I have described for the first time the differential dynamics of the three human monocyte subsets in venous blood after STEMI, defined according to the contemporary classification (Ziegler-Heitbrock et al, 2010). Particular emphasis was placed on the differences between the two types of CD16+ monocytes (Mon2 and Mon3), hitherto described as a single subset. Comparisons have been made between day 1 after STEMI and subjects with stable CAD and healthy subjects.

5.5.1 Monocyte subsets

The novel observation of a dramatically higher number of the Mon2 subset on day 1 compared to controls is particularly striking. This contrasts with a less prominent (54%) increase in number of the Mon1 subset and no change in Mon3 subset number. Proportionally, Mon2 predominates over Mon3 on day 1 after STEMI, with the reverse pattern seen on day 30 and in subjects with stable CAD. The observation of no change in number of Mon3 during 30 days after STEMI supports the recently proposed hypothesis that this subset has substantially different physiological roles compared to the more 'typical' functions of the Mon1 and Mon2 subsets, such as 'patrolling' the endothelium (Auffray et al 2007; Cros et al, 2010). Van der Laan and colleagues recently found that 40% of monocytes within the necrotic core during the proliferative phase after MI (5-14 days) are CD14+CD16+ cells) (van der Laan et al, 2013). This is clearly a much higher proportion than the total

proportion of Mon2 + Mon3 (ie total of CD14+CD16+ cells) seen in my study, which stayed remarkably similar at each time point after STEMI: 18.5% on day 1, 18.7% on day 3, 18.0% on day 7, 17.7% on day 30 (17.7% in stable CAD) (Table 5.3). This suggests that CD14+CD16+ cells may be selectively recruited into the myocardium after MI, although it should be noted that all subjects in the van der Laan study had died after MI suggesting a more severe and extensive burden of necrosis than in my cohort where the mean EF at 6 weeks was 54.1%. Moreover, CD16+ cells were considered as a single population which is a significant limitation (van der Laan et al, 2013). As I observed a marked increase then decrease in Mon2 subset number in venous blood after STEMI but no longitudinal change in Mon3 number, one may speculate that the CD14+CD16+ cells within the necrotic core may be of the Mon2 subset. Furthermore, as the number of monocytes in the spleen in the early phase after MI (3-12 hours) is significantly reduced (van der Laan et al, 2013), these Mon2 may have been liberated from a splenic reservoir. Alternatively, they may also be liberated from the bone marrow, as our previous work demonstrated that all three monocyte subsets are present in bone marrow, which contained relatively higher proportions of Mon2 than found in circulation (Shantsila et al, 2011). My observation of a significant elevation of Mon2 count on days 1 and 3 followed by a significant reduction on day 7 and return to levels comparable to controls at day 30 (compared to total monocyte and Mon1 counts which remained elevated on day 7) is in accordance with the findings of Nahrendorf and colleagues in mice, where levels of both the Ly-6C^{high} and Ly-6C^{low} subsets in circulation were higher during phase I than phase II. The significant reduction in Mon2 number on day 7 in my study is in keeping with the observed increased capacity for Ly-6C^{low} monocytes to migrate into the myocardium at this stage in mice (Nahrendorf et al, 2007).

Data supporting the hypothesis that the Mon2 subset has unique properties are supported by the findings of specific and highly significant changes in their phenotype after STEMI in this study, including increases in CD14 and CCR2 expression and reduction in CD16 expression. In contrast, the only significant phenotypic change observed in the other monocyte subsets after STEMI was an increase in CD14 expression by Mon3. Although the NFκB pathway appeared to be more active in all 3 monocyte subsets after STEMI when compared to controls (defined by an increased intracellular IKKβ level), the highest level of this key transcriptional factor was observed in the Mon2 subset, thus suggesting a prominent increase in their functional activity.

Our research group and others have shown that both the Mon1 and Mon2 subsets can produce high levels of pro-inflammatory cytokines in healthy subjects. However, a unique feature of the Mon2 subset is the ability to produce high levels of the *anti-inflammatory* cytokine IL-10 (Cros et al, 2010; Shantsila et al, 2011). In this study, I found that the Mon1 count on day 1 after STEMI correlates significantly with plasma levels of the pro-inflammatory cytokine IL-6, whilst Mon2 subset count correlates with levels of both IL-6 and IL-10.

As this study was purely observational in its approach, it is difficult to speculate on the exact biological roles of the changes in monocyte parameters seen. However, the vast majority of dynamic changes observed corresponded to the Mon 2 subset. For example, the only significant correlations between monocyte parameters and peak troponin level were with Mon2 number and characteristics. Additionally, univariate regression analysis showed that most monocyte-associated predictors of LVEF at 6 weeks were related to the Mon2 subset,

including the count of Mon2 and count of MPAs associated with Mon2. Furthermore, the only parameter in this study that independently predicted LVEF at 6 weeks following STEMI after adjustment for age and peak troponin level was CD16 expression by Mon2 (lower CD16 expression predicted better LVEF). These findings suggest a close relationship between numerous Mon2 subset characteristics and the degree of myocardial damage and recovery following STEMI. This is in accordance with recent data relating the Mon2 subset to cardiovascular prognosis (Rogacev et al, 2010; Rogacev et al, 2012), and suggests a more selective relationship between specific monocyte subsets and prognosis than absolute monocytosis per se. The observations made in this study lend support to the literature which suggests a specific role for the Mon2 subset in the pathophysiology of MI. There are several similarities between Mon2 and the potentially reparative and angiogenic monocytes described in experimental studies (De Palma et al, 2005; Morimoto et al, 2007; Shantsila et al, 2011). This suggests that further research into the reparative potential of the Mon2 subset may be of interest.

Several observations in this study contrast with those reported in humans by Tsujioka and colleagues (Tsujioka et al, 2009). Firstly, they observed no difference in CD14+CD16- monocyte count, but a significantly lower CD14+CD16+ count respectively after STEMI compared with stable angina subjects. They also described the sequential mobilisation of two monocyte subsets in circulation following STEMI, with peak CD14+CD16- monocyte count on day 2.6 and peak CD14+CD16+ monocyte count on day 4. However, the trends described did not reach statistical significance and should therefore be interpreted with caution. Additionally, in direct contrast to mouse data, Tsujioka and colleagues did not find a relationship between CD16+ monocyte count and myocardial recovery and outcome

following STEMI (Nahrendorf et al, 2007; Tsujioka et al, 2009). These discrepancies between our findings are likely to arise from methodological differences. These include variation in definitions of monocyte subsets, gating strategies, and counting the two CD16+ monocyte subsets (Mon2 and Mon3 in this study) together as a single subset (Tsujioka et al, 2009). These differences make comparisons between our findings difficult. As I discussed in section 5.2, my aim was to evaluate monocytes after STEMI using the contemporary definition of three subsets, which has not been previously undertaken (Ziegler-Heitbrock et al, 2010).

It remains unclear whether the differentiation of human monocytes into specific subsets is already complete in the bone marrow or whether this maturation occurs in the circulation and tissues as has been demonstrated in mice. The Mon1 subset has been reported to show some degree of CD16 expression after stimulation (Passacquale et al, 2011). The changes in phenotype observed may reflect their differentiation towards the Mon2 subset or maturation into CD16+ macrophages. Our previous work demonstrated that all three monocyte subsets are present in bone marrow, which contained relatively higher proportions of Mon2 than found in circulation (Shantsila et al, 2011). A rapid release of the Mon2 subset from bone marrow into the circulation may explain the dramatic increase in Mon2 count on day 1 after STEMI in this study. Further research is required to answer these questions about monocyte phenotypic differentiation.

5.5.2 Monocyte platelet aggregates

The finding of a significant (~80%) rise in MPA count on day 1 after STEMI is in accordance with previous findings (Furman et al, 2001). However, this study demonstrates for the first

time that the increase in MPA count persists at 30 days after STEMI, despite ongoing potent dual antiplatelet therapy, and after the monocyte count has returned to a level comparable to stable CAD. Although the pathophysiological implications of this observation are unclear, it is in accordance with the accumulating data describing the important regulatory role platelets have in modulating the functional activity of monocytes. Aggregation of monocytes with platelets up-regulates expression of surface receptors to adhesion molecules which promotes monocyte migration to tissues (Simon et al, 1993; van Gils et al, 2008; Kuckleburg et al, 2011).

Although the increase in MPA count observed in this study was partly attributable to an increase in total monocyte count, there was also a strong trend towards an increased proportion of the Mon1 subset aggregated with platelets after STEMI, which persisted throughout follow-up. Given the potent antiplatelet therapy used after STEMI, these results suggest that monocyte-platelet interactions may involve mechanisms beyond simple platelet aggregation. The finding that aggregation of the Mon1 subset with platelets was associated with a reduced IKKβ level provides indirect evidence of the regulatory role of platelet interactions on monocytes functional activity. The effect of platelets on monocytes is complex. Certain monocyte functions may be activated by interaction with platelets whereas others are inhibited. Moreover, the net effects on monocytes may also depend on the activation status of the platelets, which is dependent upon other factors.

These observations on monocyte-platelet interactions after STEMI may be related to the dynamic changes found in circulating CD16+ monocytes in this study and that of Tsujioka and colleagues, together with the circulating and myocardial levels of $Ly6C^{low}$ monocytes in

mice (Nahrendorf et al, 2007; Tsujioka et al, 2009). Although the absolute number of the Mon3 subset was unchanged after STEMI, the proportion of Mon3 aggregated with platelets was increased on day 1 and peaked on day 7. Similarly to healthy subjects, Mon2 following STEMI had the highest (over 20%) proportion of aggregation with platelets seen in the three monocyte subsets. The number of platelets per one Mon2 (as reflected by MFI) was significantly reduced on day 1, when the number of Mon2 peaked; however, the reverse trend was seen on day 7, when Mon2 count reduced significantly. Current evidence suggests that (i) CD16+ monocytes may promptly migrate to tissues following activation, (ii) formation of MPAs promotes monocyte migration and (iii) mouse 'equivalents' of human CD16+ monocytes predominate in the myocardium but not in circulation on day 7 after MI (Nahrendorf et al, 2007; Cros et al, 2010; Kuckleburg et al, 2011). Therefore, this raises the possibility that from day 7 onwards, levels of Mon2 and Mon3 in circulation may not directly reflect their level in the myocardium, due to platelet-mediated migration through the endothelium. However, my aim in this study was purely to characterise the three monocyte subsets in peripheral blood. Assessment of myocardial levels of the three human monocyte subsets following STEMI would be very informative and of great interest.

5.5.3 Comparison of healthy subjects and stable coronary artery disease

I did not observe any differences at all between monocyte subset count or proportion, or their association with MPAs between healthy subjects and patients with CAD. This suggests that the presence of CAD, the co-existing risk factors and co-morbidities associated with CAD and the medication used in its treatment do not affect these monocyte parameters, and the differences observed were related to the pathophysiological processes of STEMI.

5.6 LIMITATIONS

My study aimed to characterise the dynamic changes in the three human monocyte subsets in peripheral blood following STEMI in a real-world setting and explore relationships with convalescent LVEF. Mouse data suggests that monocyte counts and activity in circulation may not reflect their accumulation and functionality within the myocardium (Nahrendorf et al 2007). Hence, any extrapolation of my findings towards suggestions of how monocyte subsets may migrate into and accumulate in tissues is purely speculative and hypothesis generating. Mechanistic conclusions cannot be drawn from the observational approach of this study.

It is not possible to distinguish whether the changes observed in expression of surface markers following STEMI reflect activation of monocyte subsets or the process of their differentiation into other subsets. Further studies would be required to explore the significance of an increase in individual subset number, changes in expression of surface markers and whether these represent selective expansion or transformation between subsets.

Correlations were made between monocyte subset counts and certain cytokines. However, this does not confirm to what extent if any these cytokines were secreted by the respective monocyte subset.

Although statistically significant, the correlations observed between monocyte parameters, peak troponin level and convalescent LVEF are not strong and any pathological implications based upon them should be made with caution. However, strong correlations may perhaps not

be expected as the pathophysiological mechanisms underlying development of atherosclerosis, myocardial infarction and the ensuing recovery processes involve multiple cell types and are complex.

Although the number of subjects recruited exceeded the minimum number derived from the power calculation, the relatively small sample size and short follow-up in this study precludes evaluation of the impact of individual monocyte subsets and associated MPAs on clinical outcomes other than LVEF. The remarkable success of PPCI delivered expeditiously in restoring coronary blood flow is highlighted by the low 30 day mortality rate (n=2, 4%) and relatively minor impairment of LVEF recorded at 6 weeks after STEMI (54.1±14.6%). This suggests that the average 'magnitude' of myocardial infarction suffered by a patient in this study is relatively small. This is undoubtedly important for patients, as convalescent LV systolic function is the most important determinant of long-term prognosis. The observations made in this study may have been more striking, and perhaps trends detected may have become significant, if repeated in a cohort of patients with relatively larger degrees of myocardial damage.

There was a higher proportion of cigarette smokers in STEMI subjects compared to controls, and a higher proportion treated with clopidogrel or prasugrel. These factors may influence cross-sectional comparisons of MPAs.

Of the 50 patients with STEMI recruited, 33 completed follow up at 30 days. The principle reasons for withdraw of consent (n=14) were logistical difficulties in attending follow up appointments and unwillingness to continue with the blood tests required for this study in

addition to the frequent blood tests needed for routine care post-MI rather than being too unwell. Hence, the patients who withdrew consent are unlikely to differ pathophysiologically from those who completed follow-up.

It was logistically impossible to plan to undertake sample collection at the same time each day. However, the majority of samples were collected from all subject groups between 09.00 and 12.00. The remaining influence of diurnal variation on monocyte parameters is likely to be minimal but should be considered as a limitation (Shantsila et al, 2012).

5.7 CONCLUSIONS

This study describes for the first time the dynamic differences between the three human monocyte subsets, as defined according to contemporary nomenclature, following STEMI. Many significant dynamic and phenotypic differences were found, especially related to the CD14++CD16+ Mon2 subset. The count of the Mon2 subset increased dramatically after STEMI and correlated significantly with peak troponin level, plasma cytokines and convalescent LVEF. All monocyte subsets exhibited evidence of functional activation, but this was most prominent in Mon2 following STEMI. The increase in MPA count persisted 30 days after STEMI despite potent dual antiplatelet therapy. These findings are in accordance with data supporting distinct functional differences between the two CD16+ monocytes subsets. This confirms that these subsets should be analysed separately in future studies in order to fully understand their specific role.

CHAPTER 6

TOLL-LIKE RECEPTOR 4 EXPRESSION BY MONOCYTE SUBSETS IN MYOCARDIAL INFARCTION

Contributory publication: TLR4 expression on monocyte subsets in myocardial infarction. Tapp LD, Shantsila E, Wrigley BJ, Montoro-Garcia S, Lip GY. J Intern Med 2013; 273(3): 294-305.

Abstract

Monocyte TLR4 has been implicated in the pathogenesis of atherosclerosis, and increased levels observed following MI. This study aimed to assess the numbers of TLR4+ monocytes in the three human subsets following MI, their expression of TLR4 and their associations with markers associated with monocyte activation and inflammation, myocardial damage and post-MI cardiac contractility.

The three monocyte subsets were defined as CD14++CD16-CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2) and CD14+CD16++CCR2- (Mon3). Monocyte surface expression of TLR4 and the number of TLR4-expressing monocytes was assessed by flow cytometry of venous blood in 50 patients with STEMI, 48 with NSTEMI and 40 controls with stable CAD. Parameters were measured on days 1, 3, 7 and 30 post-MI in STEMI patients. Plasma inflammatory cytokine levels were assessed using cytometric bead array.

There were significant increases in counts of TLR4+ Mon1 and Mon2 in STEMI and TLR4+ Mon2 in NSTEMI compared with CAD. Monocyte TLR4 expression was similar between groups, and did not change during follow-up in STEMI. Plasma IL6 levels correlated positively with TLR4+ Mon2 count ($r = 0.54$, $P < 0.001$), but negatively with TLR4 expression by Mon2 ($r = -0.33$, $P = 0.021$).

Following acute MI, TLR4 expression by individual human monocyte subsets is unchanged. The increase in TLR4+ Mon1 and Mon2 count after STEMI and TLR+ Mon2 count after NSTEMI is due to an increase in monocyte subset number and not to changes in TLR4

expression. Monocyte count but not TLR4 expression correlates positively with plasma IL6 level. TLR4 expression may not be a reliable marker of monocyte activation after MI.

6.1 INTRODUCTION

6.1.1 Toll-like receptor 4

TLR4 is a member of the pattern recognition receptor family. It is involved in innate immunity via bacterial endotoxin-induced inflammatory responses, but it has also been implicated in the pathogenesis of atherosclerosis (Edfeldt et al, 2002). The role of TLR4 in cardiovascular disease may be attributed to responsiveness to host ligands, such as HSP released during myocardial damage, fibronectin, and reactive oxidative species (Vabulas et al, 2002; Asehnoune et al, 2004). Whilst the biological roles of TLR4-mediated pathways represent an anti-bacterial defense mechanism, its chronic stimulation or acute up-regulation in the setting of atherosclerotic CAD may be detrimental, by facilitating excessive inflammation, leukocyte accumulation and release of MMPs (Xu et al, 2001). TLR4 over-expression by monocytes in acute MI, both in the circulation and in ruptured plaques, has been associated with high levels of inflammatory cytokines, including IL-6 (Ishikawa et al, 2008). Conversely, inhibition of TLR4 in mouse models of MI attenuated the inflammatory process and was associated with smaller infarct size (Nakamura et al, 2007).

6.1.2 Toll-like receptor 4 and monocyte subsets

However, most published work on human monocyte TLR4 over-expression is in vitro, in isolated monocytes which may influence monocyte phenotype. Moreover, examination of the whole monocyte pool does not take into account the presence of distinct monocyte subsets (chapter 1.2). Even in the most recently published study considering monocyte subsets separately, 'classical' CD14++CD16– monocytes were compared with CD14+CD16+ monocytes; i.e. CD14++CD16+ ('Mon2' Intermediate') and CD14+CD16++ ('Mon3'

'Nonclassical') cells were grouped together as a single cell population (Kashiwagi et al, 2012). Hence, little is known about TLR4 parameters and their relationships in monocytes defined as three subsets according to contemporary nomenclature (Ziegler-Heitbrock et al, 2010; Shantsila et al 2011) in healthy subjects or in the setting of MI.

6.2 AIMS AND HYPOTHESES

In this study, my objective was to assess numbers of TLR4+ monocytes in each of the three human subsets in patients with STEMI and NSTEMI, their expression of TLR4, and relations to markers of monocyte pro-inflammatory activation, myocardial damage, inflammatory cytokines and convalescent post-MI cardiac contractility. Parameters of monocyte TLR4 expression in patients with MI were compared with matched subjects with stable CAD. As our research group has previously observed marked differences in monocyte subsets in healthy subjects (Shantsila et al, 2011), I hypothesized that there would be distinct differences in TLR4 parameters between patients with MI versus healthy subjects and that there would be dynamic changes between the acute phase of MI and the recovery phase.

6.3 METHODS AND MATERIALS

6.3.1 Study population

Fifty consecutive subjects with STEMI admitted to SWBH Hospitals NHS Trust and 48 with NSTEMI admitted to SWBH NHS Trust or Heart of England NHS Foundation Trust were recruited between November 2009 and May 2011 (chapter 3.1). MI was diagnosed according to the consensus universal definition (Thygesen et al, 2007) (chapter 1.1.3). All STEMI patients underwent PPCI (chapter 1.1.5.1.2). All study patients received standard treatment according to the contemporary ESC guidelines (Bassand et al, 2007; Van de Werf et al, 2008). STEMI patients and NSTEMI patients were compared to an age- and sex-matched group with stable CAD (n=40) (chapter 3.1.2.1). Exclusion criteria are described in chapter 3.1.3.

6.3.2 Blood sample collection

In STEMI patients, blood samples were collected at 4 time points: day 1 within 24 hours of MI (after PPCI), day 3, day 7 and day 30 (section 3.1.1.2). A proportion of STEMI patients did not complete follow-up due to withdrawal of consent, re-infarction or death (chapter 5.4.1).

In NSTEMI patients, blood samples were collected on day 1, before PCI. All patients had received loading doses of dual anti-platelet therapy (aspirin plus clopidogrel) and low-molecular weight heparin prior to venesection (chapter 1.1.6).

All subjects were asked to refrain from extreme physical exertion for at least 1 hour prior to sample collection (section 3.3.1). Peripheral venous blood samples were collected from all participants and processed by flow cytometry within 60 minutes (chapter 3.2). Plasma was separated by centrifugation and stored at –70^{o}C for batched ELISA analysis (chapter 3.4).

Routine haematology and biochemistry investigations were undertaken by the hospital laboratory (chapter 3.5).

6.3.3 Flow cytometry

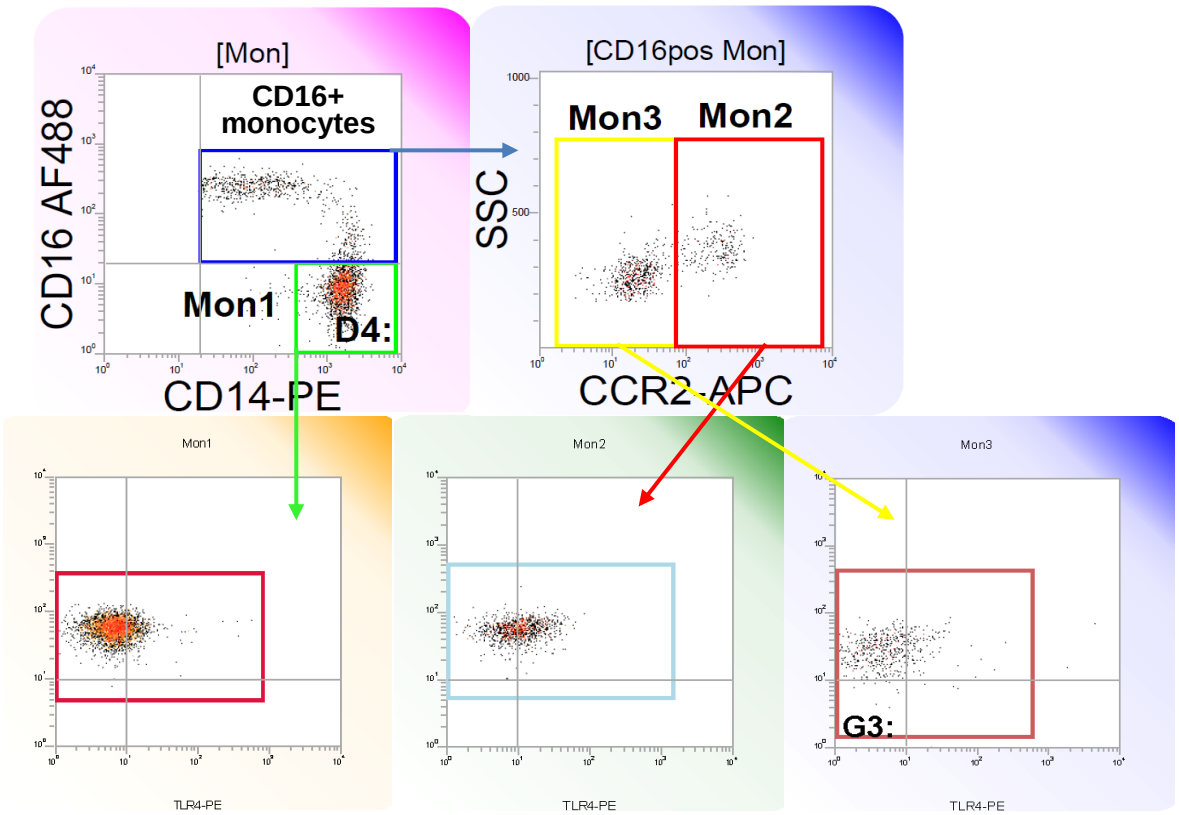
Flow cytometric analysis was performed as described in chapter 3.2. The three monocyte subsets were defined as CD14++CD16-CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2) and CD14+CD16++CCR2- (Mon3) in keeping with contemporary nomenclature (Shantsila et al, 2011; Ziegler-Heitbrock et al, 2010).

Monocyte surface TLR4 expression by each subset was analysed as described in chapter 3.2.3., quantified as MFI, using appropriate isotype controls to establish cut-off levels for positivity in keeping with current consensus guidance (Ziegler-Heitbrock et al, 2010) (figure 6.1).

Intracellular level of IKKβ was measured as described in chapter 3.2.4.

Plasma levels of IL-1β, IL-6, IL-10 and MCP-1 were measured by cytometric bead array technology as described in chapter 3.2.5.

Figure 6.1 Assessment of Toll-like receptor 4 expression by monocyte subsets.



Top figures, definition of monocyte subsets based on their CD14, CD16, and CCR2 expression; bottom figures, TLR4 expression is quantified for individual monocyte subsets. Mon1, CD14++CD16–CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2– monocytes; TLR4, toll-like receptor 4

6.3.4 Assessment of left ventricular function

Patients underwent echocardiographic assessment of convalescent LVEF by Simpson's method 6 weeks after STEMI (chapter 3.6)

6.3.5 Statistical analysis

Statistical analyses were performed as described in chapter 3.8. Univariate and multivariate linear regression analyses were used to establish predictive value of the study parameters for LVEF at 6 weeks in STEMI patients.

6.4 RESULTS

6.4.1 Subject characteristics

50 patients with STEMI (mean age [standard deviation] 58 [12] years, 86% male), 48 patients with NSTEMI (mean age 60 [12] years, 83% male) and 40 patients with stable CAD (mean age 60 [11] years, 83% male) were recruited. Demographic and clinical details are summarised in table 6.1. The study groups were well matched for most demographic and clinical parameters, and most medications. Glycoprotein IIbIIIa inhibitors were administered to 96% of STEMI patients but only 10% of NSTEMI subjects. Patients with MI had higher rates of clopidogrel/prasugrel (100%) usage that subjects with stable CAD (75%, $p<0.001$ for both STEMI and NSTEMI). Thirty three STEMI patients completed follow-up (chapter 5.4.1). Twenty seven STEMI patients had data on TLR4 expression at all four time-points; the remainder could not be analysed due to logistical difficulties in sourcing anti-TLR4 antibodies midway through the project. Despite this factor, the number of patients with data on TLR exceeded the minimum required from the power calculation.

Table 6.1 Subject demographics, clinical characteristics and medication

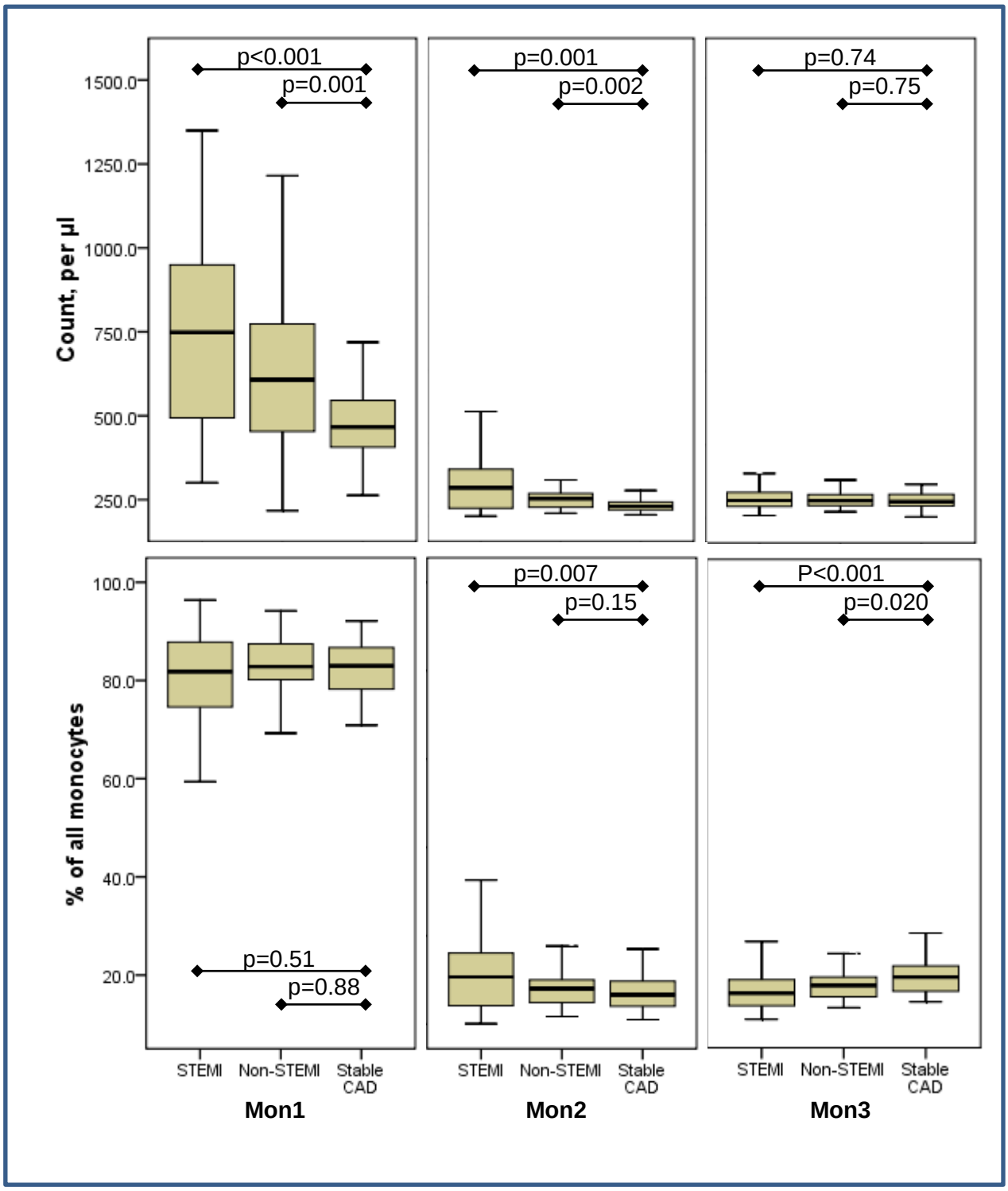
	STEMI (n=50)	**NSTEMI (n=48)**	**Stable CAD (n=40)**	**p value**
Demographic and clinical characteristics				
Age, years	57.5 [11.7]	60.6 [12.4]	60.4 [10.8]	0 .58
Male, n (%)	43 (86)	39 (83)	33 (83)	0.96
BP systolic, mmHg	132 [18]	137 [17]	133 [15]	0.56
BP diastolic, mmHg	78 [14]	82 [10]	75 [9]	0.15
Body mass index, kg/m^2	30 [6]	28 [6]	29 [5]	0.46
Smoking, n (%)	29 (58)	27 (57)	17 (43)	<0.001
Stroke, n (%)	3 (6)	2 (4)	5 (13)	0.27
Hypertension, n (%)	24 (48)	27 (57)	20 (50)	0.65
Diabetes, n (%)	16 (32)	14 (30)	8 (20)	0.50
Creatinine, μmol/l	91.7 [18.5]	92.3 [27.0]	89.5 [17.9]	0.51
Platelets, per μl	263 [98]	247 [58]	233 [56]	0.35
Medications at time of recruitment				
Aspirin, n (%)	50 (100)	47 (100)	36 (90)	0.075
Clopidogrel or prasugrel, n (%)	50 (100)	47 (100)	18 (45)	<0.001
Glycoprotein IIbIIIa inhibitor	48 (96)	5 (11)	-	<0.001
Statins, n (%)	50 (100)	47 (100)	34 (85)	0.063
ACE inhibitor or ARA, n (%)	45 (90)	39 (83)	29 (73)	0.22
Diuretics, n (%)	2 (4)	2 (4)	5 (13)	0.17
Beta-blockers, n (%)	35 (70)	34 (72)	27 (68)	0.97
Calcium channel blockers, n (%)	8 (16)	13 (28)	11 (28)	0.27

ACE, angiotensin converting enzyme; ARA, angiotensin receptor antagonist; BP, blood pressure; CAD, coronary artery disease; STEMI, ST-elevation myocardial infarction; NSTEMI, Non-ST-elevation myocardial infarction. [SD]. (%).

6.4.2 Cross-sectional comparison of monocytes, monocyte subsets and Toll-like receptor 4 parameters

Mirroring our previous observations following STEMI, there were significant but numerically less prominent increases in Mon1 and Mon2 subset count and no change in Mon3 subset count following NSTEMI (Figure 6.2) (Tapp et al, 2011). Also, patients with NSTEMI showed a trend similar (but not statistically significant) to that observed following STEMI, with a reduced relative proportion of Mon3 but increased relative proportion of Mon2, so that Mon2 predominated over Mon3 in STEMI and NSTEMI in contrast to stable CAD (Figure 6.2) (Tapp et al, 2011).

Figure 6.2 Counts and percentage of individual monocyte subsets in the study groups.



Mon1, CD14++CD16–CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2– monocytes; CAD, coronary artery disease; STEMI, ST-elevation myocardial infarction; NSTEMI, non ST-elevation myocardial infarction; p, p value. Boxes represent 25th, 50th and 75th centiles. Whiskers represent 5th and 95th centiles. Horizontal lines represent significant differences between groups.

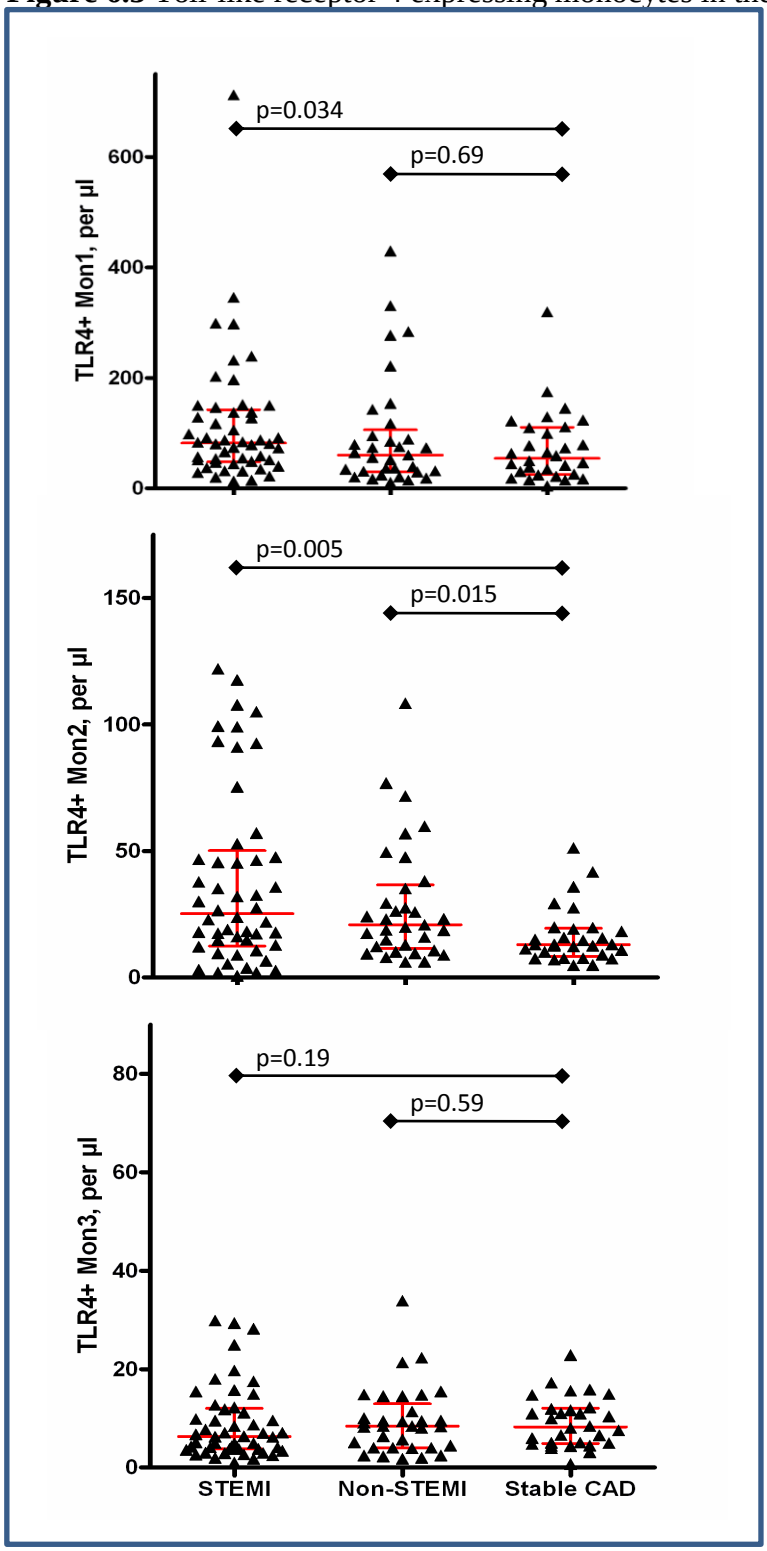
There was a significant increase in TLR4+ Mon1 and Mon2 subset count in STEMI and TLR4+ Mon2 count only in NSTEMI (Table 6.2, Figure 6.3). There was no difference in TLR4 expression on any monocyte subset (neither percentage, nor MFI) despite the study being powered to detect even small (20%) changes (Table 6.2).

Table 6.2 Toll-like receptor 4 expressing monocytes and monocyte subsets in the study groups

	STEMI (n=50)	**NSTEMI (n=48)**	**CAD (n=40)**	**p value STEMI vs. CAD**	**p value NSTEMI vs. CAD**
TLR4+ Monocytes, per µl	130 [68-190]	102 [52-162]	80 [39-145]	0.008	0.315
TLR4+ Mon1, per µl	82 [43-140]	60 [32-106]	54 [25-110]	0.034	0.690
TLR4+ Mon2, per µl	25 [13-49]	21 [11-36]	13 [8.7-19]	0.005	0.015
TLR4+ Mon3, per µl	6.3 [3.8-12]	8.4 [4.0-13]	8.3 [4.9-12]	0.193	0.591
TLR4+ Mon1, %	12 [7-18]	9.7 [5.3-17]	12 [6.8-20]	0.952	0.518
TLR4+ Mon2, %	39 [23-56]	47 [28-61]	40 [30-57]	0.554	0.640
TLR4+ Mon3, %	11 [7-18]	13 [6.9-22]	16 [9.5-20]	0.102	0.630
TLR4 (Mon1), MFI	6.4 [2.0]	5.9 [2.2]	6.0 [1.7]	0.36 [82%]*	0.93 [81%]*
TLR4 (Mon2), MFI	10.1 [4.8]	10.7 [5.1]	10.3 [4.8]	0.83 [52%]*	0.80 [50%]*
TLR4 (Mon3), MFI	4.0 [1.7]	4.1 [1.7]	3.9 [0.8]	0.85 [75%]*	0.71 [81%]*

CAD, coronary artery disease; MFI, median fluorescent intensity; Mon, monocytes; Mon1, CD14++CD16–CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2– monocytes; NSTEMI, non ST-elevation myocardial infarction; STEMI, ST-elevation myocardial infarction; TLR4, Toll-like receptor 4. Data expressed as median [interquartile range] or mean [standard deviation] *Statistical power (1-β) to detect a minimum 20% changes in TLR4 expression.

Figure 6.3 Toll-like receptor 4 expressing monocytes in the study groups



Mon1, CD14++CD16–CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2– monocytes; TLR4, toll-like receptor 4; CAD, coronary artery disease; STEMI, ST-elevation myocardial infarction; non-STEMI, non ST-elevation myocardial infarction; p, p value (horizontal lines). Red bars represent mean and standard deviation.

6.4.3 Longitudinal changes in Toll-like receptor 4 expression by monocyte subsets after STEMI

There was no change in TLR4 expression during follow up in STEMI (Table 6.3).

Table 6.3 Monocyte TLR4 expression during follow-up in patients with ST-elevation myocardial infarction

	Day 1	Day 3	Day 7	Day 30	p value
TLR4 (Mon1), MFI	5.9 [5.0-7.0]	5.5 [4.6-7.4]	5.7 [4.9-6.9]	5.1 [4.1-6.2]	0.17
TLR4 (Mon2), MFI	9.7 [7-13]	10 [7-16]	8.9 [7-15]	7.5 [5-15]	0.44
TLR4 (Mon3), MFI	3.6 [2.9-4.6]	4.1 [3.4-4.9]	4.5 [3.4-5.5]	3.8 [2.9-5.1]	0.29

MFI, median fluorescent intensity; Mon1, CD14++CD16–CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2– monocytes; TLR4, Toll-like receptor 4. Data expressed as median [interquartile range].

6.4.4 Correlations with monocyte Toll-like receptor 4 parameters

On day 1 following STEMI, troponin level correlated positively with TLR4+ Mon2 subset count ($r=0.31$, $p=0.044$) but not with TLR4 expression on Mon2 (nor any other subset). Plasma IL6 level correlated positively with TLR4+ Mon2 count ($r=0.54$, $p<0.001$), but negatively with TLR4 expression on Mon2 ($r=-0.33$, $p=0.021$). No significant association was found between TLR4–related monocyte parameters and convalescent LVEF 6 weeks after STEMI. No significant correlation was found between monocyte TLR4 expression and their IKKβ level.

6.5 DISCUSSION

This study shows for the first time that MI is associated with increased numbers of TLR4+ monocytes, but not with higher TLR4 expression by individual monocyte subsets. Moreover the increase in TLR4+ monocytes is only attributed to specific human monocyte subsets (Mon1 and Mon2 in STEMI and Mon2 only in NSTEMI). This suggests that TLR4 expression may not be a reliable marker of monocyte activation in MI.

Given that these findings contradict some previously published data on monocyte TLR4 expression in MI (as summarised in Table 6.3), the possible reasons for this merit discussion. Although all studies point towards increases in TLR4+ monocyte parameters, there is marked heterogeneity between these studies. For example the proportion of monocytes expressing TLR4 in comparable STEMI cohorts varies from 6% to about 80%; in the present study 12% of 'classical' monocytes (Mon1) expressed TLR4 in STEMI. Similar variations are also seen in control samples (Azeredo et al, 2010; Xie et al, 2010; Methe et al, 2005). These discrepancies are likely to reflect differences in methodological approaches used and populations tested. The lowest proportions of TLR+ expressing monocytes are seen in studies which analysed fresh whole blood samples when compared to protocols based on prolonged sample processing (including density centrifugation, adhesion to plastic or immune-magnetic separation, and often in vitro culture) (Table 6.3). As there is consistent evidence of monocyte activation in STEMI, and their high responsiveness to external stimuli results in prompt TLR4 up-regulation, it is likely that monocyte TLR4 expression may be increased by sample processing. Monocytes following STEMI may potentially be more sensitive in this regard due to their activated state (Satoh et al, 2006).

Another confounding factor is related to variations in the definition of TLR4-expressing monocytes by flow cytometry. Monocytes do not form a separate cluster of TLR4-expressing cells, but rather show a continuous spectrum of cells with varying TLR4 expression. With the exception of the Mon2 subset, this expression is rather low. In these circumstances definition of 'TLR4-positivity' critically depends on the cut-off value used. This is reflected in the lower proportion of TLR4+ cells in studies employing isotope controls (aiming to account for non-specific binding) compared to the studies using 'sham' controls (Xie et al, 2010). As TLR4 is functionally a co-receptor for CD14 which is a key part of the innate immune system, continuous low level expression of TLR4 would perhaps be expected rather than variable or inducible expression. Thus, TLR4 is likely to be expressed on all monocytes (at least Mon1 and Mon2), but in such low levels that its expression may be difficult to discriminate from isotype controls. Accordingly, the assessment of monocyte TLR4 expression may be preferable via MFI rather than percentage of TLR4+ cells. Lastly, several studies used healthy volunteers as controls, which makes drawing conclusions on whether differences observed are purely due to MI or other co-morbidities, cardiovascular risk factors and their treatments problematic.

Of interest, there appears to be a general trend towards lower monocyte TLR4 expression in more recent studies, which might indicate that more effective contemporary medical therapy underlies the relatively low TLR4 expression seen. This may partly explain the lack of significant differences in monocyte TLR4 expression between the groups in this study. Virtually all STEMI patients and a fraction of NSTEMI patients received platelet glycoprotein IIbIIIa inhibitors and all were on potent dual antiplatelet therapy (aspirin plus clopidogrel or prasugrel). Given that monocyte-platelet interactions play significant roles in regulation of

monocyte phenotype and activity, the use of such antiplatelet therapy may influence monocyte TLR4 expression (Shantsila & Lip, 2009).

Table 6.4 Published data on Toll-like receptor 4 expression in myocardial infarction

Study	Study patients [mean age]	Controls [mean age]	Source of sample, Sample processing	TLR4 measure	Findings	Mon counts	Other findings *Limitations*
Kashiwagi et al, 2012	22 MI (17 STEMI) [67]	27 CAD [65]	PB and CA blood, Fresh whole blood	TLR4+ Mon % for Mon1and all CD16+ Mon [Mon2 + Mon3]	↑in MI (~6% for Mon1 and ~15% for CD16+ Mon) vs. CAD (~2% for Mon1 and ~4% for CD16+ Mon). ↑in CA lesion site than in PB (CD16+ Mon only). TLR4 Mon% in MI ↓ by day 12.	NA, CD16+ Mon ↓ in STEMI vs. CAD	Plasma TNFα and CRP positively correlated with TLR4 expression on Mon (all subsets together). Mon2 and Mon3 analysed together.
Yonekawa et al, 2011	20 STEMI, 7 NSTEMI [53]	NA	PB and CA thrombi, DC, negative IM selection on CD14+ cells	Mon TLR4 MFI	MRP8/14 enhanced Mon TLR4 expression in thrombi, but not in PB	NA	
Sheu et al, 2008	43 STEMI [62]	20 HC [62]	PB DC, 2 h in culture, detached by scrapping, further 24h incubation with or without LPS	Mon TLR4 MFI	↑in MI with MACE (3.6 [0.9]) vs. MI without MACE (2.1 [0.7]) vs. HC (1.2 [0.3]).	NA	TLR4 increased after incubation with LPS in all groups. High TLR-4 expression was the most independent predictor of 30-day MACE. Very low MFI, no FC figure
Xie et al, 2010	70 STEMI [60]	32 CAD [57]	PB Fresh whole blood	TLR4+ Mon % (defined by sham controls, not IC)	↑ in MI (80 [11]) vs. CAD 60 [16].	NA	Positive correlation between TLR4 and TNFα/MMP-9. Not clear how monocytes were defined. All treatments were withdrawn 12 h prior to blood sampling.
Wyss et al, 2010	35 ACS (26 STEMI) [~57]	10 without CAD [61]	CA thrombi, aortic blood. 24 h in vitro incubation, lysed by osmotic shock	Mon TLR4 MFI	↑ in coronary artery thrombi (78 [64–85] vs. aortic blood (65 [57–70]). ↑ in ACS vs. controls (47 [40–49]).	NA	Proportion of related to total leukocyte count was 47% in coronary artery thrombi vs. 20% in aortic blood.
Ishikawa et al, 2008	62 STEMI [64]	20 CAD [65]	PB, CA blood, solid plaque samples	Mon TLR4 mRNA Mon TLR4 MFI	↑ in STEMI vs. CAD in PB ↑in coronary artery blood plaques vs.	NA	TLR4 expression responded to stimulation with HSP70.

			DC, adhesion to plastic dishes	Plaque Mon TLR4+ levels	PB		High Mon TLR4 expression predicted cardiac events at 6 months.
Satoh et al, 2006	52 STEMI [65]	20 HC [62]	PB DC, adhesion to plastic dishes, detached by incubation in cold PBS.	Mon TLR4 mRNA Mon TLR4 MFI	↑ in STEMI (2.64[0.17] RU) vs. HC (0.37 [0.06] RU). ↑ in STEMI (6.15[0.31] RU) vs. HC (1.08 [0.09]). Both measures ↓by day 14 (still ↑ vs. HC)	665 [40] in STEMI vs. NA in HC	Circulating HSP70 levels were positively correlated with Mon TLR4 MFI in MI
Satoh et al, 2006	65 STEMI [65]	20 HC [61]	As above	Intracellular Mon TLR4 MFI, Mon TLR4 mRNA	Both ↑ in STEMI - remained ↑ at 14 days STEMI onset. ↑ in those with HF vs. no HF	NA	After incubation with LPS TLR4 increased in MI. LPS-stimulated TLR4 correlated with IL6/TNFα.
Methe et al, 2005	28 STEMI [62]	20 CAD [60]	PB DC, positive IM selection of CD14+ cells	TLR4+ Mon %, Mon TLR4 mRNA, RU	↑in STEMI (78 [14]) vs. CAD (33 [5]) ↑in STEMI (1.82 [0.33]) vs. CAD (0.96 [0.19])	NA	A strong correlation between TLR4+ Mon % and B7-1 expression/IL-12 secretion across all patient groups
Shiraki et al, 2006	21 with MI or UA [NA]	79 CAD [NA]	PB Paraformaldehyde-fixed, lysed whole blood	Ratio of Mon TLR4 MFI to IC MFI	↑in ACS vs. CAD	NA	No correlation between hsCRP and TLR4

Mon, monocyte, Mon1; CD14++CD16–CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2– monocytes; CAD, coronary artery disease; CRP, C-reactive protein; hsCRP, high sensitivity CRP; HC, healthy controls; HSP, heat shock proteins; IC, isotype control; IL, interleukin; LPS, lipopolysaccharide; MFI, mean or median fluorescent intensity; MI, myocardial infarction; MRP, myeloid related protein; NA, not available; RU, relative units; STEMI, ST-elevation myocardial infarction; TLR4, Toll-like receptor 4; TNF α, tumour necrosis factor α; ↑, increased; ↓, decreased; PB, peripheral blood; CA, coronary artery; MACE, major adverse clinical events; ACS, acute coronary syndrome; mRNA, messenger ribonucleic acid; FC, flow cytometry; MMP-9, matrix metalloproteinase 9; HF, heart failure; DC, density centrifugation; UA, unstable angina; IM, immune-magnetic.

The diversity of monocyte subsets in terms of phenotype, function and pathophysiological implications in cardiovascular disease is increasingly recognised. Following MI in humans, only the Mon1 and Mon2 subset increase in number, but not Mon3, with the most prominent changes being seen in Mon2 (over 2.5-fold increase in STEMI) (chapter 5; Tapp et al, 2011). This study shows that in humans with MI managed according to contemporary guidelines and with monocytes defined as 3 distinct subsets, there is an increase in TLR4+ expressing Mon1 and Mon2 in STEMI and TLR4+ Mon2 in NSTEMI due to an increase in number of these monocyte subsets, but this is not due to a change in TLR4 expression per se.

The relative clinical importance of monocyte count rather than their individual level of TLR4 expression is suggested by the correlation observed between peak troponin level and TLR4+ Mon2 subset count (similarly to the previously reported correlation with total Mon2 subset count), but not with TLR4 expression on any subset (chapter 5; Tapp et al, 2011). Of interest, plasma IL6 level correlated positively with TLR4+ Mon2 number, but negatively with TLR4 expression on Mon2. This observation is in agreement with previous data describing the association of this subset with high IL6 levels. In contrast, the negative association with TLR4 expression by other monocytes may reflect partial loss of TLR4 from activated monocytes, indicating that TLR4 expression may not be a reliable marker of monocyte activation in STEMI. This possibility is also supported by the lack of any observed correlation between monocyte TLR4 expression and a marker of NFκB activation (intracellular IKKβ) as well as the lack of association between monocyte TLR4 expression and convalescent LVEF 6 weeks post STEMI.

Finally, as illustrated in Figure 6.3 and Table 6.2, in STEMI (and to a smaller degree in NSTEMI) there is an increase in the proportion of Mon2 with high TLR4 expression and a decrease in proportion of Mon3 with lower TLR4 expression. Accordingly an MI-related increase in TLR4 expression by CD16+ monocytes analysed together (i.e. Mon2 and Mon3 considered as a single population) may be partly due to an increase in proportion of Mon2 rather than a true increase in TLR4 expression per se (Kashiwagi et al, 2012).

6.6 LIMITATIONS

The study is descriptive in its nature, with monocyte functional activity assessed only by intracellular IKKβ levels in peripheral venous blood. Thus it is not possible to provide any mechanistic insight into TLR4 related monocyte pathways or into their roles within the myocardium.

Also, given that certain amounts of surface TLR4 could be shed into circulation, surface TLR4 expression may not be a comprehensive parameter of monocyte TLR4 production. Intracellular TLR4 mRNA measurement could be of additive value in this regard. In aiming to obtain a snapshot of monocyte TLR4 expression as close to the in vivo state as possible (by expeditious processing of fresh whole blood samples), the more prolonged sample processing time required for monocyte (subsets) isolation and RNA extraction was avoided.

It was logistically impossible to plan to undertake sample collection at the same time each day. However, the majority of samples were collected from all subject groups between 09.00 and 12.00. The remaining influence of diurnal variation on monocyte parameters should be considered as a limitation of my study (Shantsila et al, 2012).

6.7 CONCLUSIONS

Following acute MI treated according to contemporary guidelines, monocyte TLR4 expression by individual subsets is unchanged. The number of TLR4+ Mon1 and Mon2 subsets increases in STEMI as does the number of TLR+ Mon2 in NSTEMI. However, there is no change in their surface expression of TLR4. Monocyte count but not their TLR4 expression correlates positively with plasma IL6 levels. This suggests that TLR4 expression may not be a reliable marker of monocyte activation in MI.

CHAPTER 7

CXCR4 POSITIVE AND ANGIOGENIC MONOCYTES IN MYOCARDIAL INFARCTION

Contributory publication: CXCR4 positive and angiogenic monocytes in myocardial infarction. Shantsila E, Tapp LD, Wrigley BJ, Montoro-Garcia S, Lip GY. Thromb Haemost 2013; 109(2): 255-62.

Abstract

There are limited data on the role of human monocytes in cardiac repair. The aim of this study was to evaluate the dynamic alterations of monocytes with reparative and angiogenic potential, and expression of scavenger receptors by monocytes in patients following MI, with specific reference to the three human monocyte subsets defined as CD14++CD16-CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2) and CD14+CD16++CCR2- (Mon3).

CXCR4+ monocytes, CD34+ / KDR+ monocytes with angiogenic potential and monocyte expression of CD204 and CD163 were quantified by flow cytometry in 50 patients with STEMI and 40 controls with stable CAD. Parameters were measured on days 1, 3, 7 and 30 after STEMI. Plasma levels of inflammatory cytokines were assessed on day 1 by cytometric bead array.

The number of CXCR4+ and KDR+ monocytes were increased following STEMI, being most prominently associated with the Mon1 and Mon2 subsets (both $p=0.002$). The number of CXCR4+ Mon1 and Mon2 after STEMI reduced significantly by day 30 ($p=0.009$ and $p<0.001$ respectively). Expression of the pro-reparative scavenger receptor CD163 by Mon3 was reduced on day 1 after STEMI ($p=0.008$), but later on other subsets with lowest levels on day 3 post-STEMI ($p<0.001$ for Mon1, $p=0.02$ for Mon2).

IL-6 levels correlated positively with counts of Mon2-derived CXCR4+ ($r=0.63$, $p<0.001$) and KDR+ cells ($r=0.55$, $p<0.001$) and negatively with Mon2 expression of CD204 ($r=-0.29$, $p=0.044$). IL1β levels correlated positively with counts of KDR+ Mon1 ($r=0.34$, $p=0.016$)

and Mon2 (r=0.36, p=0.010). IL-10 concentration correlated positively with levels of CXCR4+ Mon2 (r=0.36, p=0.010) and negatively with KDR+ Mon3 (r=-0.29, p=0.039) and Mon2 expression of CD204 (r=-0.28, p=0.046).

No significant association was found between MCP-1 and any monocyte parameter. Peak troponin level correlated positively with counts of CXCR4+ Mon2 (r=0.35, p=0.022). Low number of CXCR4+ Mon2 and low CD163 expression by Mon2 were associated with higher LVEF six weeks after STEMI.

The Mon2 subset appears to have the most prominent changes in characteristics of the 'reparative' monocytes following STEMI. The association of reparative monocytes with both pro and anti-inflammatory cytokines indicates a complex interplay of these cells in the post-STEMI setting.

7.1 INTRODUCTION

7.1.1 The myocardium following infarction

Despite prompt coronary artery recanalisation and successful restoration of normal blood flow by PPCI in patients with MI, the injured myocardium undergoes complex healing and reparative processes. Myocardial salvage and preservation of cardiac contractility is rarely complete. This leads to scaring of the infarcted area, compensatory structural changes in non-infarcted segments and only partial recovery of the vascular bed. The process of angiogenesis after MI aims to improve myocardial perfusion via the formation of new capillaries, and is orchestrated by a number of growth factors (Lee et al, 2004).

The mechanisms underlying cardiac repair following MI are incompletely understood. These reparative processes are thought to involve several types of cells which respond to various tissue signals including chemokines that regulate cellular migration via interactions with their specific chemokine receptors. Most such chemokines are not expressed in normal myocardium but may be up-regulated and secreted by the heart following MI (Rijken & Lijnen, 2009).

7.1.2 The CXCL12/CXCR4 axis following myocardial infraction

CXCL12 (Stromal cell-derived factor-1) and its main receptor CXCR4 are thought to play a unique role in myocardial biology, with actions including a reparative potential. The CXCL12/CXCR4 axis may be rapidly activated following MI. This promotes several reparative mechanisms including angiogenesis and enhanced regenerative capacity of progenitor cells. However there is conflicting data as the CXCL12/CXCR4 axis has also been

associated with detrimental effects on the injured myocardium. Reduced post-MI scaring seen in CXCR4-deficient mice compared to wild type animals was associated with reduced myocardial infiltration of neutrophils and proinflammatory monocytes but earlier and more prevalent infiltration of 'reparative' monocytes. Interestingly, CXCR4 deficiency was not associated with worse post-MI contractility despite impaired neoangiogenesis (Liehn et al, 2011). These observations suggest that the net effect of the CXCL12/CXCR4 is finely balanced and may depend upon differential influences on individual cell types under different physiological conditions, both temporally and spatially (eg cardiovascular growth and development compared with post-MI). Thus, the CXCL12/CXCR4 axis represents a potential therapeutic target for enhancing cardiovascular recovery. As the majority of data in this area has been conducted in mice and rats, further research is required to explore the roles of CXCR4 expressing cells in humans.

7.1.3 Scavenger receptors following myocardial infarction

The role of monocytes in reparative processes may also be related to their activity mediated by scavenger receptors, such as CD204 (scavenger receptor class A type I) and CD163 (haptoglobin receptor). CD204 is involved in lipid accumulation (by macrophages) and atherogenesis. There are emerging data to implicate this receptor in reparative processes including clearance of apoptotic cells, attenuation of oxidative stress and inflammation, and myocardial remodelling (Kobayashi et al, 2007; Tsujita et al, 2007; Todt et al, 2008; Chen et al, 2010; Hu et al, 2011). CD163 is also implicated in a range of reparative processes in various tissues, and its expression has been linked with attenuated inflammatory properties of monocytes (Schaer et al, 2006). However little data are available on the dynamics and role of CD204 and CD163 expression on monocyte subsets following MI.

7.1.4 Relations to human monocytes, monocyte subsets and endothelial progenitor cells

Our research group has previously described phenotypic variation amongst the three human monocyte subsets in healthy subjects (Shantsila et al, 2011), defined according to contemporary nomenclature (Ziegler-Heitbrock et al, 2010). Whilst all three types of human monocyte subsets include a proportion of CXCR4-expressing cells, these proportions vary significantly between the subsets, being highest in CD14++CD16+CCR2+ (Mon2) 'intermediate' monocytes (Shantsila et al, 2011). However, the patterns of reparative CXCR4+ monocytes as well as CD34+ and KDR+ cells (i.e. cells with angiogenic potential) attributed to individual monocyte subsets post-MI have not been explored. The role of endothelial progenitor cells (EPCs) in angiogenesis after MI is generally acknowledged. Various blood cells have been shown to demonstrate angiogenic potential, with monocyte-derived cells being the largest pool of angiogenic cells (Shantsila et al, 2007).

7.2 AIMS AND HYPOTHESES

In this study my aim was to assess numbers of CXCR4+, CD34+, KDR+ cells attributed to the three human monocyte subsets, and monocyte expression of CD204 and CD163 following STEMI. Additionally, their associations with biological and physiological parameters related to myocardial damage and recovery following MI were also assessed. Given the differences in expression of these parameters in healthy subjects previously reported (Shantsila et al, 2011), I hypothesised that the number of CXCR4+ monocytes, CD34+/KDR+ cells and expression of monocyte scavenger receptors is changed in patients following STEMI. Additionally, I hypothesised that these changes are attributable to specific monocyte subsets and are associated with biomarkers of inflammation and convalescent LVEF following STEMI.

7.3 METHODS AND MATERIALS

7.3.1 ST-segment elevation myocardial infarction patients

50 consecutive patients diagnosed with STEMI fulfilling the study criteria were recruited from SWBH NHS Trust between November 2009 and November 2010 (chapter 3.1.1). MI was diagnosed according to the consensus universal definition (Thygesen et al, 2007) (chapter 1.1.3). All STEMI patients underwent PPCI (chapter 1.1.5.1.2) and received standard treatment according to the contemporary ESC guidelines (Van de Werf et al, 2008). The monocyte-derived CXCR4+, CD34+ and KDR+ cells and other biomarkers were assessed at 4 time points following MI onset: within 24 hours of MI, after PPCI (day 1), day 3, day 7 and day 30. 33 STEMI subjects completed follow-up (chapter 5.4.1).

7.3.2 Control group

For a cross-sectional comparison, the 50 STEMI patients were compared to an age- and sex-matched control group of 40 patients with stable CAD (chapter 3.1.2.1). Exclusion criteria are summarised in chapter 3.1.3.

7.3.3 Sample collection

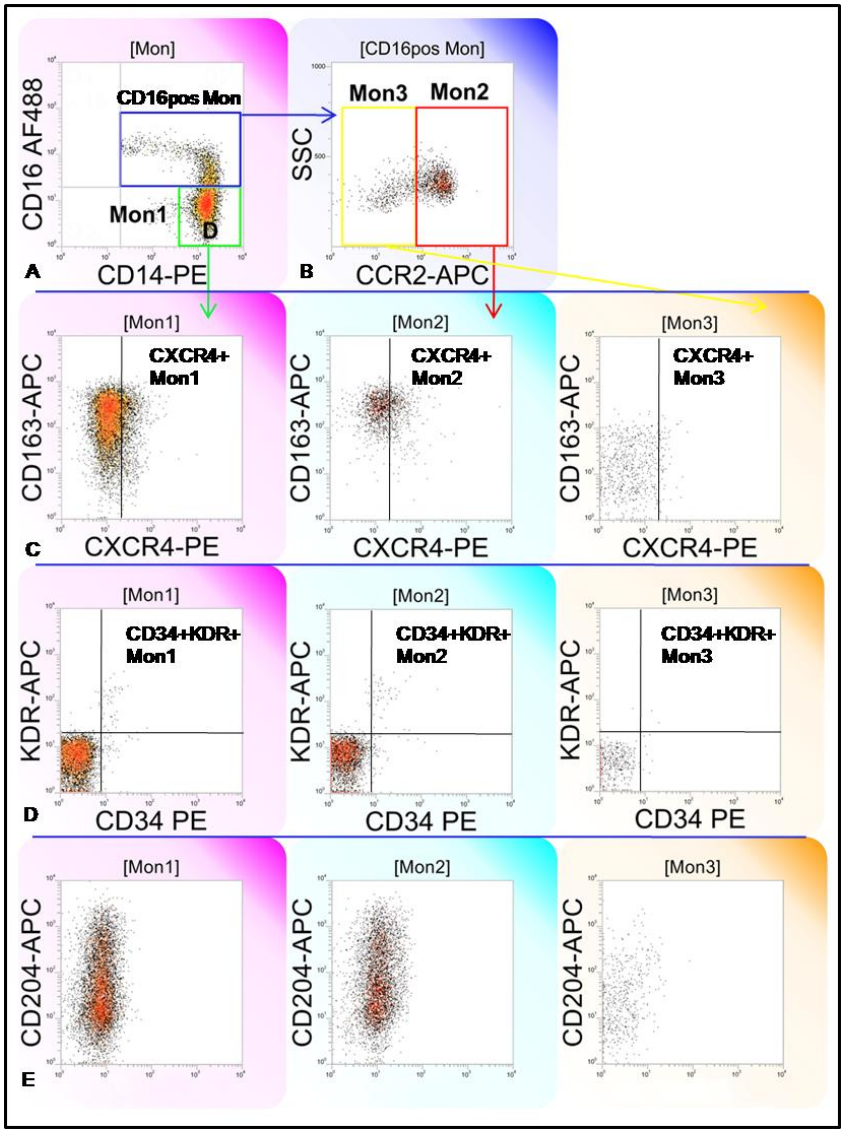
All subjects were asked to refrain from extreme physical exertion for at least 1 hour prior to sample collection (chapter 3.3.1). Peripheral venous blood samples were collected from all participants and processed by flow cytometry within 60 minutes (chapter 3.2). Plasma was separated by centrifugation and stored at -70^{o}C for batched analysis at the end of the study (chapter 3.4). Routine haematology and biochemistry investigations were undertaken by the hospital laboratory (chapter 3.5).

7.3.4 Flow cytometry

Flow cytometric analysis was performed as described in chapter 3.2. The absolute count of monocytes and counts of the three monocyte subsets and associated MPAs were performed as described in chapter 3.2.2. Briefly, monocyte subsets were defined as CD14++CD16–CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2) and CD14+CD16++CCR2– (Mon3) in accordance with contemporary nomenclature (Ziegler-Heitbrock et al, 2010; Shantsila et al, 2011). Monocyte surface expression of CXCR4, CD34, KDR, CD204 and CD163 by each subset was analysed as described in chapter 3.2.3 (Figure 7.1).

Plasma levels of IL-1β, IL-6, IL-10 and MCP-1 were measured by cytometric bead array technology as described in chapter 3.2.5.

Figure 7.1 Flow cytometric analysis of monocyte expression of CXCR4, CD163, CD34, KDR and CD204.



A: Selection of CD14++CD16– monocytes (Mon1) and CD16+ monocytes based on CD14/CD16 expression. B: Separation of CD14++CD16+CCR2+ (Mon2) and CD14+CD16++CCR2– (Mon3) subsets based on their CCR2 expression. C: Measurement of CD163 expression and quantification of CXCR4+ cells related to the individual monocyte subsets (Mon1 [left], Mon2 [centre] and Mon3 [right]). D: quantification of CD34+, KDR+, and CD34+KDR+ cells related to the individual monocyte subsets (Mon1 [left], Mon2 [centre] and Mon3 [right]). E: Measurement of CD204 expression on the monocyte subsets (Mon1 [left], Mon2 [centre] and Mon3 [right]). Mon1, CD14++CD16-CCR2+ ('classical') monocytes ; Mon2, CD14++CD16+CCR2+ ('intermediate') monocytes ; Mon3, CD14+CD16++CCR2- ('non-classical') monocytes

7.3.5 Assessment of left ventricular function

Patients underwent echocardiographic assessment of convalescent LVEF by Simpson's method 6 weeks after STEMI as described in section 3.6.

7.3.6 Statistical analysis

Statistical analyses were performed as described in chapter 3.8. Univariate and multivariate linear regression analyses were used to establish predictive value of the study parameters for LVEF at 6 weeks in STEMI patients.

7.4 RESULTS

7.4.1 Demographics and clinical characteristics of the study population

Fifty patients with STEMI (mean age 58 years, 86% male) and forty patients with stable CAD (mean age 60 years, 83% male) were recruited (Table 7.1). Thirty three STEMI patients had data on CXCR4+, CD34+ and KDR+ monocytes and study parameters at all four time-points (section 5.4.1). The study groups were well matched for most demographic and clinical parameters and medication (Table 7.1); however, glycoprotein IIbIIIa inhibitors were administered to the majority (96%) of STEMI patients but not to CAD patients. Patients with MI had higher rates of clopidogrel or prasugrel usage than subjects with stable CAD ($p<0.001$).

Table 7.1 Demographic and clinical characteristics of the study groups

	STEMI (n=50)	Stable CAD (n=40)	p value
Age, years	58 [12]	60 [11]	0.23
Male, n (%)	43 (86)	33 (83)	0.77
Systolic BP, mmHg	132 [18]	133 [15]	0.84
Diastolic BP, mmHg	78 [14]	75 [9]	0.49
Body mass index, kg/m^2	30 [6]	29 [5]	0.47
Smoking, n (%)	29 (58)	17 (43)	0.28
Stroke, n (%)	3 (6)	5 (13)	0.28
Hypertension, n (%)	24 (48)	20 (50)	0.83
Diabetes, n (%)	16 (32)	8 (20)	0.34
COPD, n (%)	3 (6)	3 (8)	1.00
Creatinine, μmol/l	92 [19]	90 [18]	0.59
Aspirin, n (%)	50 (100)	36 (90)	0.18
Clopidogrel or prasugrel, n (%)	50 (100)	30 (75)	<0.001
Statin, n (%)	48 (96)	34 (85)	0.40
ACE inhibitor, n (%)	45 (90)	29 (73)	0.14
Diuretic, n (%)	2 (4)	5 (13)	0.23
Beta-blocker, n (%)	35 (70)	27 (68)	1.00
Calcium channel blocker, n (%)	8 (16)	11 (28)	0.28

Non-normally distributed data are presented as median [interquartile range]; normally distributed data are presented as mean [standard deviation]. Categorical data are expressed as number (%). ACE, angiotensin-converting enzyme; BP, blood pressure; CAD, coronary

artery disease; COPD, chronic obstructive pulmonary disease; STEMI, ST-elevation myocardial infarction.

7.4.2 Cross-sectional comparison of day 1 after ST-elevation myocardial infarction with coronary artery disease controls

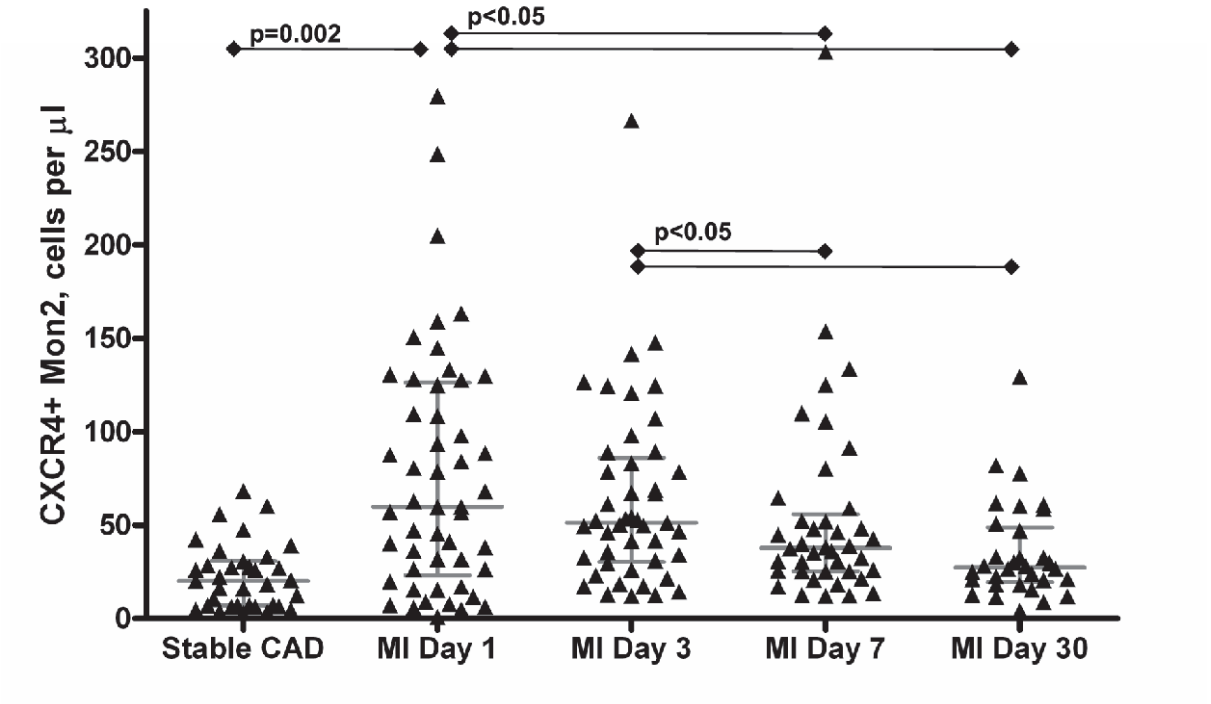
Compared to stable CAD, on day 1 following STEMI there were increased levels of CXCR4+ monocytes associated with the Mon1 (p=0.002) and Mon2 subsets (p=0.002), KDR+ monocytes associated with the Mon1 (p=0.02) and Mon2 subsets (p=0.012) and lower CD163 expression by the Mon3 subset (p=0.008) (Table 7.2, Figure 7.2).

Table 7.2 Angiogenic and reparative cells in ST elevation myocardial infarction compared to subjects with stable coronary artery disease

	STEMI Day 1, n=50	Stable CAD n=40	p value
CXCR4+ Mon1, per μl	389 [296-520]	301 [244-390]	0.002
CXCR4+ Mon2, per μl	60 [25-126]	27 [21-41]	0.002
CXCR4+ Mon3, per μl	25 [13-37]	21 [12-29]	0.18
CD34+ Mon1, per μl	1.9 [1.0-4.2]	1.8 [0.2-3.8]	0.37
CD34+ Mon2, per μl	0.7 [0.2-1.4]	0.4 [0.2-0.7]	0.19
CD34+ Mon3, per μl	1.4 [0.9-2.1]	1.1 [0.4-2.7]	0.43
KDR+ Mon1, per μl	34 [16-71]	19 [6-43]	0.020
KDR+ Mon2, per μl	8.0 [3.0-17]	4.0 [1.5-8.3]	0.012
KDR+ Mon3, per μl	2.7 [1.3-5.0]	2.2 [1.6-4.4]	0.75
CD163 (Mon1), MFI	128 [79-169]	124 [88-163]	0.76
CD163 (Mon2), MFI	240 [173-312]	214 [168-279]	0.22
CD163 (Mon3), MFI	12 [10-16]	16 [12-23]	0.008
CD204 (Mon1), MFI	11 [7-30]	13 [7-31]	0.79
CD204 (Mon2), MFI	29 [15-57]	29 [16-62]	0.87
CD204 (Mon3), MFI	32 [18-49]	27 [19-74]	0.85

Data are presented as median [interquartile range]. Mon1, CD14++CD16–CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2– monocytes; MFI, median fluorescent intensity; STEMI, ST-elevation myocardial infarction; CAD, coronary artery disease.

Figure 7.2 CXCR4+ Mon2 in the study patients with cross-sectional analysis and follow-up in ST-elevation myocardial infarction.



CAD, coronary artery disease; STEMI, ST-elevation myocardial infarction; Mon2, CD14++CD16+CCR2+ monocytes. Bars represent median and inter-quartile range. Horizontal lines represent statistically significant differences between groups, with individual p values.

7.4.3 Longitudinal changes in monocyte parameters following ST-elevation myocardial infarction

Following STEMI, counts of CXCR4+ Mon1 (p=0.009) and CXCR4+ Mon2 (p<0.001) had reduced at 30 days (Table 7.3). CD163 expression on Mon3 (reduced on day 1) did not change significantly, but CD163 expression on Mon1 (p<0.001) and Mon2 (p=0.02) (both unchanged on day 1) reduced significantly during follow-up with lowest values seen on day 3. CD204 expression by the Mon1 subset decreased on day 3 (p=0.025).

Table 7.3 Monocyte parameters during follow up in patients with ST-elevation myocardial infarction

	Day 1	Day 3	Day 7	Day 30	p value
CXCR4+ Mon1, per µl	398 [345-552]‡	372 [322-452]	385 [291-499]	320 [265-485]	0.009
CXCR4+ Mon2, per µl	78 [20-125] †‡	51 [29-107]†‡	37 [25-52]	27 [18-47]	<0.001
CXCR4+ Mon3, per µl	25 [15-37]	20 [12-27]	26 [14-34]	20 [12-29]	0.23
CD34+ Mon1, per µl	2.2 [1.6-4.9]	2.3 [1.3-4.6]	2.5 [1.5-5.1]	2.9 [0.8-9.2]	0.79
CD34+ Mon2, per µl	0.6 [0.3-1.3]	0.8 [0.4-1.6]	0.6 [0.3-1.3]	0.4 [0.3-0.7]	0.11
CD34+ Mon3, per µl	1.6 [0.9-2.1]	1.1 [0.5-2.0]	1.2 [0.7-1.8]	1.2 [0.6-1.8]	0.40
KDR+ Mon1, per µl	42 [18-73]	21 [13-62]	25 [12-81]	44 [21-94]	0.03
KDR+ Mon2, per µl	8.0 [3.1-24]	6.6 [2.4-9.4]	7.2 [2.6-13]	7.5 [2.7-12]	0.44
KDR+ Mon3, per µl	3.8 [1.6-5.5]	2.6 [1.5-4.2]	3.0 [1.8-5.6]	4.0 [2.2-7.2]	0.15
CD163 (Mon1), MFI	131 [77-181]*	92 [57-116]‡	109 [81-156]	121 [90-163]	<0.001
CD163 (Mon2), MFI	251 [180-317]*†‡	198 [150-284]	214 [145-265]	199 [157-269]	0.02
CD163 (Mon3), MFI	12 [10-15]	15 [10-22]	13 [9-20]	15 [12-23]	0.11
CD204 (Mon1), MFI	11 [7-36]*	9 [5-22]	12 [8-28]	10 [7-46]	0.025
CD204 (Mon2), MFI	34 [14-61]	37 [14-59]	37 [17-66]	40 [19-66]	0.11
CD204 (Mon3), MFI	33 [17-49]	28 [17-48]	33 [14-63]	33 [17-53]	0.86

*p<0.05 vs. Day 3, †p<0.05 vs. Day 7, ‡p<0.05 vs. Day 30. Data are presented as median [interquartile range]. Mon1, CD14++CD16–CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2– monocytes; MFI, median fluorescent intensity.

7.4.4 Relations between monocyte parameters, plasma cytokines and myocardial damage

In STEMI patients on day 1, only CXCR4+ Mon2 subset number was found to correlate significantly with peak troponin level ($r=0.35$, $p=0.022$). IL6 levels correlated positively with counts of Mon2-derived CXCR4+ cells ($r=0.63$, $p<0.001$) and KDR+ cells ($r=0.55$, $p<0.001$) and negatively with Mon2 expression of CD204 ($r=-0.29$, $p=0.044$). IL1β levels correlated positively with counts of KDR+ Mon1 ($r=0.34$, $p=0.016$) and Mon2 ($r=0.36$, $p=0.010$). IL10 concentration correlated positively with levels of CXCR4+ Mon2 ($r=0.36$, $p=0.010$) and negatively with KDR+ Mon3 ($r=-0.29$, $p=0.039$) and Mon2 expression of CD204 ($r=-0.28$, $p=0.046$). No significant association was found between MCP-1 and any monocyte parameter. Peak troponin level correlated positively with counts of CXCR4+ Mon2 ($r=0.35$, $p=0.022$), and showed a non-significant trend towards a positive association with CD163 expression on Mon2 ($r=0.28$, $p=0.077$).

7.4.5 Predictive value of monocyte angiogenic and reparative makers for left ventricular ejection fraction six weeks post ST-elevation myocardial infarction

In STEMI patients, reduced CD163 expression by Mon1 on Day 1 and lower counts of CXCR4+ Mon2 (taken as an average of the four time points) were significantly associated with a higher LVEF after adjustment for age, sex, history of diabetes, and troponin levels (Table 7.4).

Table 7.4 Regression analysis of predictive value of monocyte angiogenic/reparative parameters for left ventricular ejection fraction 6 weeks post ST-elevation myocardial infarction

Model	β	p value
Unadjusted		
CD163 expression on Mon1*	-0.31	0.051
CD163 expression on Mon2*	-0.38	0.015
Counts of CXCR4+ Mon2†	-0.49	0.007
Adjusted for age, sex, diabetes, and troponin levels		
CD163 expression on Mon1*	-0.37	0.044
Counts of CXCR4+ Mon2†	-0.52	0.024

*Measured on Day 1, †Measured as an average of the four time points

β, adjusted regression coefficient; Mon1, CD14++CD16-CCR2+ ('classical') monocytes ; Mon2, CD14++CD16+CCR2+ ('intermediate') monocytes

7.5 DISCUSSION

7.5.1 Myocardial repair and angiogenesis

In this study I describe for the first time significant changes in expression of surface markers associated with repair (CXCR4) and angiogenesis (KDR) following STEMI, attributed to individual human monocyte subsets. Of note, the highly significant increase in CXCR4+ and KDR+ monocyte number was strictly limited to the Mon1 and Mon2 (more prominently) subsets. This parallels higher expression of CXCR4 and KDR by the Mon1 and particularly Mon2 subsets as compared to the Mon3 subset in our group's previous study on healthy subjects (Shantsila et al, 2011). The number of the CXCR4+ Mon2 subset on day 1 after STEMI (but not KDR+ Mon 2 cells) was observed to correlate significantly with peak troponin level. The absence of an association between KDR+ Mon 2 number and peak troponin may be due to a predominant role of the degree of myocardial ischaemia rather than amount of myocardial necrosis per se in their mobilisation.

Although a high count of the CXCR4+ Mon2 subset on day 1 after STEMI was a significant predictor of lower LVEF at 6 weeks, in keeping with the data of Liehn and colleagues (Liehn et al, 2011), it is difficult to speculate on the reasons for this. This relationship could suggest potential detrimental properties of CXCR4+ monocytes or conversely their possible contribution to repair mechanisms in patients with more marked initial myocardial insults (Liehn et al, 2011). The positive correlation between numbers of the CXCR4+ Mon2 subset and both pro- and anti-inflammatory cytokines further suggests that the role of these cells in post-MI processes is complex. Such mechanisms should be explored in the future.

The findings in this study seem to be in accordance with published mouse data on the CXCL12/CXCR4 axis in relation to the myocardium. This pathway appears to be critically involved in embryonic cardiovascular development as CXCR4 or CXCL12 null mice develop lethal vascular malformations. Assessments of its role in cardiac repair in a mouse model of myocardial infarction have produced conflicting findings. Despite evidence implicating CXCR4+ bone marrow-derived cells in reparative processes, $CXCR4^{+/-}$ mice developed smaller post-MI scars compared to wild-type mice expressing normal levels of CXCR4 (Liehn et al, 2011). These opposing observations are likely to reflect the pleiotropic effects of the CXCL12/CXCR4 axis and the diversity of cells mobilised via its activation. CXCR4 deficiency in mice was associated with reduced myocardial infiltration of neutrophils, reduced and delayed infiltration of 'proinflammatory' Gr-1^{high} monocytes but an earlier infiltration with increased numbers of 'reparative' Gr-1^{low} monocytes (Liehn et al, 2011).

Although only limited parallels may be made between characteristics of monocyte subsets in mice and humans, the findings in this study seem to support experimental data showing that CXCR4+ cells derived from the Mon1 subset ('equivalent' to 'inflammatory' mouse monocytes), but not from the Mon3 subset ('equivalent' of 'reparative' mouse monocytes) increase in number after STEMI (Nahrendorf et al, 2007). However, the most prominent changes I observed in CXCR4+ cells were attributed to the Mon2 subset. The function of this subset remains elusive, and there is no direct mouse 'equivalent'. Indeed, according to the current consensus nomenclature, it is called an 'intermediate' subset (Ziegler-Heitbrock et al, 2010). Perhaps Mon2 monocytes could be considered a 'top' subset in many respects, as they have the highest expression of many 'beneficial' reparative markers (such as CXCR4, KDR and CD163).

Of the CD34+KDR+ monocytes in this study, only those derived from the Mon3 subset were increased in number after STEMI. This corresponds to our group's previous observation that the Mon3 subset has the highest expression of CD34 in healthy subjects (Shantsila et al, 2011). Indirectly, these observations support the hypothesis of differential involvement in reparative properties by individual human monocyte subsets following ischaemic injury.

7.5.2 Monocyte scavenger receptor expression

The reparative roles of scavenger receptors expressed by monocytes including CD204 and CD163 are increasingly appreciated (Kobayashi et al, 2007; Chen et al, 2010; Yu et al, 2011). Data from experimental models of myocardial infarction in mice suggest that CD204 deficiency impairs post-MI remodelling via modulation of inflammatory cytokine and MMP-9 production (Tsujita et al, 2007; Hu et al, 2011). Although it was previously shown in peripheral blood films that CD204+ monocytes are increased in number following MI (Emura et al, 2007), my study suggests that this is likely to be due to monocytosis rather than increased monocyte expression of CD204 per se, which did not change significantly. However, following STEMI, CD204 expression by monocyte subsets was associated with plasma levels of inflammatory cytokines, suggesting a relationship with ischaemic myocardial injury. Our group's previous work found that CD204 expression on Mon1 was associated with tPA levels post MI, suggesting an additional relationship with fibrinolytic status (Shantsila et al, 2012).

Of interest, a significant reduction in monocyte CD163 expression following STEMI was observed (on day 1 by Mon3, and later by Mon1 and Mon2). A similar longitudinal trend was seen for CD204 expression by Mon1. Although the mechanistic reasons for this reduction in

scavenger receptor expression after MI is unclear, this may be related to their increased utilisation in the setting of myocardial necrosis, reflecting higher demand for their functions. This may contribute to the independent association between reduced expression of CD163 by the Mon1 subset and higher LVEF 6 weeks after STEMI.

In this study there were no significant correlations between plasma levels of MCP-1 and Mon2-related cells, as may have been expected given the reported role of MCP-1 in monocyte recruitment in humans (Mayr et al, 2009). This suggests that the biology of monocyte recruitment into the circulation is complex and is perhaps modulated by background pathology (STEMI in my study) and chemokine gradients along the vascular bed and within target tissues (Ajuebor et al, 1998). Furthermore, there may be more important signals for monocytes than MCP-1 in the context of STEMI, as suggested by the significant positive correlation between number of CXCR4+ monocytes and peak troponin levels (i.e. degree of myocardial damage). The relationship between MCP-1 levels and monocyte count is also complicated by the fact that monocytes themselves are a major source of MCP-1 and are thus involved in the feedback regulation MCP-1 levels (Yoshimura et al, 1989).

7.6 LIMITATIONS

This study has several limitations reflecting its observational and descriptive nature. The number of participants is relatively small which precludes analysis between the study parameters and clinical outcomes other than convalescent LVEF. Analysis of LVEF at only one time point (6 weeks) does not take into consideration baseline myocardial contractility and repeated high resolution imaging techniques (MRI) could be more robust in this regard.

The observational nature of this study precludes the ability to infer direct mechanistic implications, which should be studied separately. My observations were restricted to examination of peripheral venous blood, and it is likely that monocyte characteristics within the myocardium may differ. However, such experiments were not within the remit of this study.

It was logistically impossible to undertake sample collection at the same time each day. However, the majority of samples were collected from all subjects between 09.00 and 12.00. The remaining influence of diurnal variation on monocyte parameters should be considered as a limitation of my study (Shantsila et al, 2012).

7.7 CONCLUSIONS

There is a significant increase in number of CXCR4+ and KDR+ monocytes in humans following MI, which is only associated with specific monocyte subsets. This is most prominent for the Mon2 subset, previously reported to have distinct pro-angiogenic and pro-reparative phenotypes. Also, MI was associated with reduction in expression of the pro-reparative scavenger receptor CD163 by all monocyte subsets, possibly reflecting it's increased utilisation. The correlation between numbers of the CXCR4+ Mon2 subset with both pro- and anti-inflammatory cytokines highlights the complex interplay of these cells in post-MI reparative processes. Although both low numbers of the CXCR4+ Mon2 subset and low CD163 expression by Mon1 were associated with higher LVEF 6 weeks after STEMI, causative links between these associations are yet to be established. Further research is needed to explore the potential of monocyte-derived cells in the modulation of post-ischaemic myocardial repair. The observations in this study following STEMI suggest that the CD14++CD16+CCR2+ (Mon2) 'Intermediate' monocyte subset may be of particular interest.

CHAPTER 8

RECEPTORS TO INTERLEUKIN 6 AND ADHESION MOLECULES ON CIRCULATING MONOCYTE SUBSETS IN MYOCARDIAL INFARCTION

Contributory publication: Receptors to Interleukin-6 and adhesion molecules on circulating monocyte subsets in acute myocardial infarction. Shantsila E, Tapp LD, Wrigley BJ, Montoro-Garcia S, Lip GY. Thromb Haemost 2013; 110(2): 340-348.

Abstract

The role of individual human monocyte subsets in inflammation and recovery after MI is insufficiently understood. The objective of this study was to evaluate the dynamics of human monocyte subset expression of receptors to VCAM-1, ICAM-1, and IL6 following MI and their relations to inflammatory cytokines and fibrinolytic factors.

Expression of VCAM-1r, ICAM-1r and IL6r by the CD14++CD16-CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2) and CD14+CD16++CCR2- (Mon3) monocyte subsets was quantified by flow cytometry in 50 patients with STEMI and 48 patients with NSTEMI and compared with 40 controls with stable CAD. In STEMI, parameters were measured on days 1 (after PPCI), 3, 7 and 30.

On day 1 after STEMI, VCAM-1r expression was reduced on Mon1 ($p=0.007$), Mon2 ($p=0.036$) and Mon3 ($p=0.005$), whilst in NSTEMI there was a significant increase in VACM-1r expression by Mon2 ($p=0.024$) and Mon3 ($p=0.049$). VCAM-1r expression on Mon1 correlated positively with plasma IL-1β level ($p=0.001$). There was no change in monocyte ICAM-1r expression. IL6r expression was reduced on Mon2 on day 1 after STEMI ($p=0.028$), with increased expression on Mon1 and Mon2 during follow-up. IL6r density on all subsets correlated negatively with plasma levels of tissue-type plasminogen activator ($r=-0.48$, $p=0.0005$ for Mon1, $r=-0.45$, $p=0.001$ for Mon2 and $r=-0.47$, $p=0.001$ for Mon3).

Expression of IL6r and VCAM-1r is reduced on human monocyte subsets involved in inflammatory responses following STEMI. This may represent a regulatory feed-back

mechanism aiming to re-balance the marked inflammation typically present following MI or selective homing of monocytes with higher receptor expression to damaged myocardium.

8.1 INTRODUCTION

The total blood monocyte count in circulation is typically increased in humans following acute MI. Their elevated level is associated with poor cardiac recovery and an increased risk of death (Maekawa et al, 2002). More specifically, the CD14++CD16+ ('intermediate') subset has recently been found to be an independent predictor of future adverse cardiovascular events in high risk patients (Rogacev et al, 2010; Rogacev et al, 2012).

Monocytes exert their biological effects both in circulation and after their migration to the site of tissue injury. Whilst in circulation, monocytes release a range of cytokines which promote systemic inflammatory effects (Shantsila & Lip, 2009). In tissues, monocytes are involved in phagocytosis of necrotic and apoptotic cells, facilitate matrix remodelling by production of MMPs, and have roles in fibroblast formation and angiogenesis (Nahrendorf et al, 2007; Nahrendorf et al, 2010). Monocytes are also involved in pro- and anti-thrombotic processes, including tissue factor expression, contribution to fibrinolysis and phagocytosis of thrombotic debris (Shantsila & Lip, 2009).

Although monocytes are clearly implicated in the pathogenesis of CAD, a disease of inflammatory aetiology, it is perhaps inappropriate and over simplistic to consider monocytes as purely 'damaging' inflammatory cells, as emerging data has demonstrated additional reparative roles for monocytes (chapter 1.2). Such diversity of roles may be attributed to their functional status and the presence of distinct subsets (Nahrendorf et al, 2007). However, little data are available on changes in phenotype of human monocyte subsets following acute MI.

Amongst the receptors expressed on the surface of monocytes, three are of particular relevance to their inflammatory activity and migration to damaged tissues; ICAM-1 receptor, VCAM-1 receptor, and the IL6 receptor. Cell adhesion molecules represent a marker of endothelial cell activation. They play a key role in the recruitment of monocytes to inflamed tissues, particularly by facilitating monocyte adhesion to the endothelium (Bevilacqua et al, 1994; Jang et al, 1994). This is achieved via an interaction between integrin receptors (i.e. ICAM-1r and VCAM-1r) on the surface of monocytes, followed by monocyte adhesion to the endothelium and migration into sub-endothelial tissues (Cotran & Mayadas-Norton, 1998). Despite these important roles, little data is available regarding the expression of these receptors on monocyte subsets after MI and their relations to inflammation, apoptosis and cardiac recovery.

Observational studies have identified that serum IL6 and soluble IL6r levels are elevated in acute MI compared to CAD (Anderson et al, 2013), and that elevation in IL6 levels after ACS correlates with an increased risk of major adverse cardiovascular events (Lee et al, 2005). Compelling genetic and biomarker evidence recently collated in a meta-analysis implicates a causal role for IL6r in the pathophysiological processes of atherosclerosis (Sarwar et al, 2012), a disease hypothesised to result from persistent inflammation. The Asp358Ala allele is a common functional variant of the IL6r. This allele affects proteolytic cleavage of the membrane-bound IL6r, leading to reduced concentrations of membrane-bound IL6r and higher concentrations of the soluble form of IL6r in circulation. This disrupts the IL6-driven inflammatory response by attenuation of IL6r-mediated signalling in hepatocytes and leucocytes, resulting in reduced production of important downstream acute phase proteins including CRP and fibrinogen. Interestingly, Asp358Ala was not associated with IL6r mRNA

production in monocytes and was not associated with IL6 production by monocytes after stimulation by lipopolysaccharide. The data suggest that the 358Ala allele and resultant shedding of IL6r into the circulation (rather than changes in production of IL6 or IL6r) is associated with a clear anti-inflammatory effect and a reduced risk of CAD. This effect was independent of traditional cardiovascular risk factors including dyslipidaemia, cigarette smoking, diabetes, hypertension and obesity. This lead to the conclusion that IL6r pathways have a direct causal relationship in the pathogenesis of CAD (Sarwar et al, 2012).

Of importance, our research group has shown that the three human monocyte subsets defined according to contemporary guidelines (Ziegler-Heitbrock et al, 2010) exhibit significant differences in their expression of ICAM-1r, VCAM-1r and IL6r in healthy humans (Shantsila et al, 2011). However, expression of these receptors on individual subsets in patients with MI has not been described.

8.2 AIMS AND HYPOTHESES

To address these important questions, my objectives in this study were to: (i) examine differences in the expression of ICAM-1r, VCAM-1r and IL6r on the three human monocyte subsets between patients with STEMI, NSTEMI and subjects with stable CAD; (ii) assess dynamics of monocyte expression of these receptors during the acute and recovery phases after STEMI, and (iii) explore associations between these receptors and markers of systemic inflammation, myocardial damage and cardiac functional recovery following STEMI.

8.3 METHODS AND MATERIALS

8.3.1 Study populations

Fifty consecutive patients with STEMI were recruited on admission to SWBH NHS Trust, and forty eight with NSTEMI were recruited on admission to SWBH NHS Trust and Heart of England NHS Foundation Trust between November 2009 and STEMI and May 2011. MI was diagnosed according to the consensus universal definition (Thygesen et al, 2007). STEMI and NSTEMI were managed according to the respective contemporary ESC guidelines (Bassand et al, 2007; Van de Werf et al, 2008). A proportion of STEMI patients did not complete follow-up due to withdrawal of consent, re-infarction or death (chapter 5.4.1).

8.3.2 Control group

For a cross-sectional comparison, patients with STEMI or NSTEMI were separately compared to an age- and sex-matched control group with stable CAD (n=40). Inclusion and exclusion criteria are described in chapter 3.1. No patients had a history of previous MI or LV systolic dysfunction. Comparisons between subjects with STEMI and NSTEMI were not performed due to possible bias related to sample collection after PCI in STEMI, but before PCI in NSTEMI.

8.3.3 Sample collection

Venous blood samples were collected at 4 time points following STEMI: day 1 (within 24 hours after PPCI), and days 3, 7, and 30. In NSTEMI patients blood samples were collected on day 1 following admission (within 24 hours of MI onset), prior to PCI. All NSTEMI patients had received loading doses of dual anti-platelet therapy (aspirin plus clopidogrel) and low-molecular weight heparin prior to venesection (chapter 1.1.6).

All subjects were asked to refrain from extreme physical exertion for at least 1 hour prior to sample collection (chapter 3.3.1). Peripheral venous blood samples were processed by flow cytometry within 60 minutes (chapter 3.2). Plasma was separated by centrifugation and stored at -70^{o}C for batched ELISA analysis (chapter 3.4).

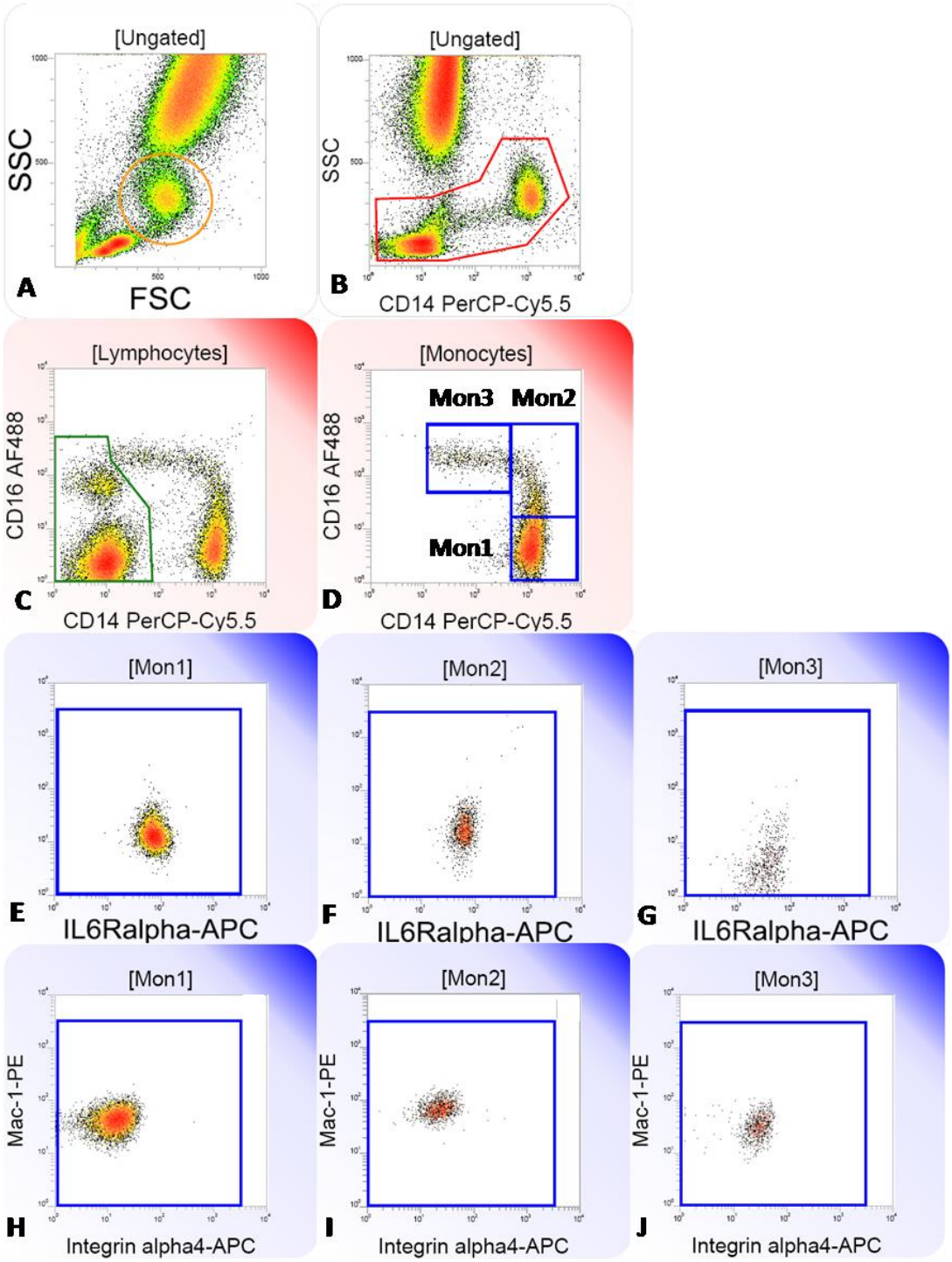
Routine haematology and biochemistry investigations were undertaken by the hospital laboratory (chapter 3.5).

8.3.4 Flow cytometry

Expression of ICAM-1r, VCAM-1r, and IL6r by the three monocyte subsets was assessed by flow cytometry as described in chapter 3.2.3 (Figure 8.1). Expression of surface markers was quantified as MFI. Briefly, the three monocyte subsets were defined as CD14++CD16–CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2) and CD14+CD16++CCR2- (Mon3) in accordance with contemporary nomenclature (Ziegler-Heitbrock et al, 2010; Shantsila et al, 2011) (chapter 1.2).

Plasma levels of IL1β and IL6 were measured by cytometric bead array technology as described in chapter 3.2.5.

Figure 8.1 Assessment of monocyte parameters by flow cytometry



Monocyte subsets [D] based on forward scatter, side scatter and CD14 and CD16 expression [A, B, D] with lymphocytes excluded [C]. Individual monocyte subsets were further gated by their expression of IL-6 receptor [E-G], ICAM-1r (MAC-1) and VCAM-1r (integrin alpha 4) [H-J]. FSC, forward scatter; SSC, side scatter; Mon1, CD14++CD16– monocytes; Mon2, CD14++CD16+ monocytes; Mon3, CD14+CD16++ monocytes.

8.3.5 Enzyme-linked immunosorbent assays

The fibrinolytic parameters tPA, PAI-1 antigen and activity were quantified by ELISA of citrated plasma as described in chapter 3.4 using commercially available ZYMUTEST kits.

8.3.6 Assessment of left ventricular function

Patients underwent echocardiographic assessment of LVEF by Simpson's method 6 weeks after STEMI as described in chapter 3.6.

8.3.7 Statistical analysis

Statistical analyses were performed as described in chapter 3.8. Cross-sectional comparisons between STEMI versus CAD and NSTEMI versus CAD (normally distributed data) were analysed by T-test. The Friedman test with Dunn's post-hoc test (where appropriate) was used for follow-up analysis. Univariate and multivariate linear regression analyses were performed to establish predictive value of the study parameters for LVEF 6 weeks post STEMI.

8.4 RESULTS

8.4.1 Subject characteristics

The study groups were well matched for most demographic and clinical parameters and medication (Table 8.1). Glycoprotein IIbIIIa inhibitors were administered to the majority (96%) of STEMI patients but only 10% of NSTEMI patients. Clopidogrel or prasugrel were prescribed more often in subjects with MI than in those with stable CAD ($p<0.001$ for both). As compared to subjects with CAD, those with STEMI more often received angiotensin-converting enzyme inhibitors ($p=0.047$), and those with NSTEMI had higher diastolic blood pressure ($p=0.032$), and were more often treated with statins ($p=0.017$). The median peak troponin T value in patients admitted with STEMI was 2.50μg/l (interquartile range 1.19-5.50 μg/l). Thirty three STEMI patients had data on monocyte phenotype at all four time-points. Mean LVEF 6 weeks after STEMI was 54.1±14.6%.

Table 8.1 Demographic and clinical characteristics of the study groups

	STEMI (n=50)	NSTEMI (n=48)	Stable CAD (n=40)	p value STEMI vs CAD	p value NSTEMI vs CAD
Demographic and clinical characteristics					
Age, years	58 [12]	61 [12]	61 [9]	0.10	0.86
Male, n (%)	43 (86)	38 (83)	30 (83)	0.73	0.93
BP systolic, mmHg	132 [18]	137 [18]	133 [16]	0.82	0.44
BP diastolic, mmHg	78 [14]	81 [10]	75 [9]	0.46	0.032
Body mass index, kg/m2	30 [6]	28 [6]	29 [5]	0.47	0.81
Smoking, n (%)	29 (58)	26 (57)	15 (42)	0.21	0.27
Stroke, n (%)	3 (6)	2 (4)	5 (14)	0.18	0.11
Hypertension, n (%)	24 (48)	27 (56)	19 (53)	0.48	0.89
Diabetes, n (%)	16 (32)	14 (30)	8 (22)	0.40	0.49
COPD, n (%)	3 (6)	3 (7)	3 (8)	0.62	0.70
Creatinine, µmol/l	92 [19]	97 [23]	73 [14]	0.61	0.60
Medications at time of recruitment					
Aspirin, n (%)	50 (100)	48 (100]	33 (92)	0.22	0.24
Clopidogrel/prasugrel, n (%)	50 (100)	48 (100)	18 (47)	<0.001	<0.001
Statin, n (%)	48 (96)	48 (100)	30 (83)	0.28	0.017
ACE inhibitor, n (%)	45 (90)	38 (83)	25 (69)	0.047	0.33
Diuretic, n (%)	2 (4)	2 (4)	5 (14)	0.081	0.11
Beta-blocker, n (%)	32 (70)	34 (71)	25 (69)	0.73	0.70
CCB, n (%)	13 (28)	13 (27)	9 (25)	0.24	0.86

Data are presented as mean [standard deviation], or number (%). ACE, angiotensin-converting enzyme; BP, blood pressure; CAD, coronary artery disease; CCB, calcium channel blocker; COPD, chronic obstructive pulmonary disease; NSTEMI, non-ST-elevation myocardial infarction; STEMI, ST-elevation myocardial infarction.

8.4.2 Interleukin-6 receptor

On day 1 after STEMI, the expression of IL6r on Mon2 was reduced compared to stable CAD ($p=0.028$), with no differences seen for other subsets (Table 8.2). During follow-up, expression of IL6r increased on Mon1 ($p=0.038$ vs. day 1) and Mon2 ($p=0.026$ vs. day 1) by day 30 (Table 8.3). There was no significant difference in IL6r expression between NSTEMI and stable CAD (Table 8.2).

Table 8.2 Monocyte expression of receptors to interleukin-6 and adhesion molecules in ST elevation myocardial infarction and non-ST elevation myocardial infarction compared to stable coronary artery disease

Receptor, MFI	STEMI (n=50)	NSTEMI (n=48)	Stable CAD (n=40)	p value STEMI vs. CAD	p value NSTEMI vs. CAD
IL6r (Mon1)	67 [14]	68 [17]	70 [16]	0.37	0.74
IL6r (Mon2)	57 [12]	61 [13]	62 [15]	0.028	0.80
IL6r (Mon3)	31 [9]	32 [9]	35 [26]	0.25	0.40
VCAM-1r (Mon1)	9 [3]	12 [5]	11 [5]	0.007	0.40
VCAM-1r (Mon2)	19 [8]	29 [10]	23 [10]	0.036	0.024
VCAM-1r (Mon3)	31 [10]	45 [13]	39 [14]	0.005	0.049
ICAM-1r (Mon1)	41 [19]	42 [18]	36 [16]	0.27	0.14
ICAM-1r (Mon2)	69 [33]	75 [28]	65 [25]	0.47	0.10
ICAM-1r (Mon3)	32 [18]	35 [18]	32 [16]	0.85	0.48

Data are presented as mean [standard deviation]. CAD, coronary artery disease; ICAM-1r, receptor to intercellular adhesion molecule-1; IL6r, interleukin 6 receptor; Mon1, CD14++CD16– monocytes; Mon2, CD14++CD16+ monocytes; Mon3, CD14+CD16++ monocytes; MFI, median fluorescent intensity; STEMI, ST-elevation myocardial infarction; NSTEMI, non-ST-elevation myocardial infarction; VCAM-1r, receptor to vascular cell adhesion molecule-1.

Table 8.3 Follow-up monocyte parameters in patients with ST elevation myocardial infarction

Receptor, MFI	Day 1	Day 3	Day 7	Day 30	p value
IL6r (Mon1)	66 [16]‡	68 [15]	66 [17]	73 [17]	0.048
IL6r (Mon2)	56 [12]‡	60 [13]	60 [14]	64 [14]	0.012
IL6r (Mon3)	32 [11]	32 [9]	30 [8]	31 [9]	0.29
VCAM-1r (Mon1)	10 [3]†‡	10 [3]†‡	12 [3]	13 [4]	<0.001
VCAM-1r (Mon2)	20 [7]*†‡	23 [9]	25 [9]	26 [8]	0.009
VCAM-1r (Mon3)	30 [11]*†‡	38 [11]	37 [10]	40 [14]	0.003
ICAM-1r (Mon1)	35 [27-53]	33 [24-50]	42 [30-56]	37 [28-51]	0.40
ICAM-1r (Mon2)	61 [45-90]	62 [41-97]	70 [52-96]	61 [45-76]	0.17
ICAM-1r (Mon3)	25 [18-41]	32 [20-42]	33 [27-45]	31 [21-42]	0.54

*p<0.05 vs. day 3; †p<0.05 vs. day 7; ‡p<0.05 vs. day 30. Data are presented as mean [standard deviation]. CAD, coronary artery disease; ICAM-1r, receptor to intercellular adhesion molecule-1; IL6r, interleukin 6 receptor; Mon1, CD14++CD16– monocytes; Mon2, CD14++CD16+ monocytes; Mon3, CD14+CD16++ monocytes; MFI, median fluorescent intensity; STEMI, ST-elevation myocardial infarction; VCAM-1r, receptor to vascular cell adhesion molecule-1.

8.4.3 Relations between Interleukin-6 receptor parameters, plasma cytokines, fibrinolytic parameters and myocardial damage

In STEMI, monocyte expression of IL6r correlated negatively with plasma tPA levels ($r=-0.48$, $p<0.0005$ for Mon1, $r=-0.45$, $p=0.001$ for Mon2, $r=-0.47$, $p=0.001$ for Mon3) (Table 8.4). There was a non-significant trend towards a positive association between IL6r expression on Mon2 and plasma IL1β levels ($r=0.26$, $p=0.07$). No correlation was found between monocyte IL6r density and peak blood troponin T levels. Monocyte IL6r expression on day 1 after STEMI was not predictive of LVEF at 6 weeks (Table 8.5).

Table 8.4 Correlation analysis between biomarkers and plasma interleukin-6 receptor expression in patients with ST elevation myocardial infarction

	Mon1		**Mon2**		**Mon3**	
	r	p value	r	p value	r	p value
Inflammatory markers						
Interleukin 1β	0.17	0.24	0.26	0.07	0.20	0.17
Interleukin 6	-0.30	0.03	-0.41	0.003	-0.16	0.27
Fibrinolytic factors						
tPA	-0.48	0.0005	-0.45	0.001	-0.47	0.001
PAI-1 antigen	-0.15	0.29	-0.23	0.11	-0.13	0.35
PAI-1 activity	-0.07	0.65	-0.13	0.35	-0.06	0.66
Myocardial damage						
Troponin T	-0.23	0.14	-0.27	0.08	-0.05	0.77

Mon1, CD14++CD16 monocytes; Mon2, CD14++CD16+ monocytes; Mon3, CD14+CD16++ monocytes; tPA, tissue-type plasminogen activation; PAI-1, plasminogen activator inhibitor-1; STEMI, ST-elevation myocardial infarction.

Table 8.5 Regression analysis of predictive value of monocyte parameters for left ventricular ejection fraction at 6 weeks post-ST elevation myocardial infarction

Receptor, MFI	B [SE]	β	p value	B [SE	β	p value
	Univariate analysis			Multivariate analysis*		
IL6r (Mon1)	-0.03 [0.17]	-0.03	0.83	-0.15 [0.16]	-0.14	0.36
IL6r (Mon2)	0.04 [0.21]	0.03	0.87	-0.16 [0.20]	-0.13	0.43
IL6r (Mon3)	-0.06 [0.23]	-0.04	0.80	-0.16 [0.22]	-0.12	0.45
VCAM-1r (Mon1)	-1.50 [0.89]	-0.26	0.10	-1.20 [0.98]	-0.19	0.23
VCAM-1r (Mon2)	-0.03 [0.34]	-0.01	0.94	0.22 [0.37]	0.09	0.56
VCAM-1r (Mon3)	0.01 [0.22]	0.007	0.97	0.18 [0.22]	0.12	0.43
ICAM-1r (Mon1)	-0.33 [0.11]	-0.42	0.007	-0.19 [0.12]	-0.24	0.13
ICAM-1r (Mon2)	-0.17 [0.07]	-0.37	0.016	-0.09 [0.08]	-0.19	0.24
ICAM-1r (Mon3)	-0.23 [0.12]	-0.29	0.066	-0.08 [0.13]	-0.10	0.56

*Adjusted to age and peak troponin T levels (i.e., significant predictors on the univariate analysis). ICAM-1r, receptor to intercellular adhesion molecule-1; IL6r, interleukin 6 receptor; Mon1, CD14++CD16– monocytes; Mon2, CD14++CD16+ monocytes; Mon3, CD14+CD16++ monocytes; MFI, median fluorescent intensity; STEMI, ST-elevation myocardial infarction; VCAM-1r, receptor to vascular cell adhesion molecule-1.

8.4.4 Vascular cell adhesion molecule-1 receptor

8.4.4.1 Vascular cell adhesion molecule-1 receptor expression and monocyte subsets

On day 1 after STEMI, expression of VCAM-1r was significantly reduced compared to stable CAD on all monocyte subsets (p=0.007 for Mon1, p=0.036 for Mon2, and p=0.005 for Mon3) (Table 8.2). VCAM-1r expression increased progressively during follow-up on all monocyte subsets: Mon1 (p=0.024 for day 1 vs. day 7, p=0.001 for day 1 vs. day 30, p=0.018 for day 3 vs. day 7, and p<0.001 for day 3 vs. day 30); Mon2 (p=0.018 for day 1 vs. day3, p=0.017 for day 1 vs. day 7, and p=0.049 for day 1 vs. day 30), Mon3 (p<0.001 for day 1 vs. day3, p=0.001 for day 1 vs. day 7, and p=0.017 for day 1 vs. day 30) (Table 8.3). In NSTEMI, expression of the VCAM-1 receptor was higher than in stable CAD on the Mon2 (p=0.024) and Mon3 (p=0.049) subsets.

8.4.4.2. Relations between Vascular cell adhesion molecule-1 receptor expression, monocyte parameters, plasma cytokines, fibrinolytic parameters and myocardial damage

In patients with STEMI, VCAM-1r expression on Mon1 correlated positively with plasma IL1β levels (r=0.44, p=0.001) (Table 8.6). No correlation was found between monocyte VCAM-1r density, peak blood troponin T levels, or plasma markers of fibrinolysis. Monocyte VCAM-1r expression on day 1 after STEMI was not predictive of LVEF at 6 weeks (Table 8.5).

Table 8.6 Correlation analysis between biomarkers and Vascular cell adhesion molecule-1 receptor expression in patients with ST elevation myocardial infarction

	Mon1		**Mon2**		**Mon3**	
	r	p value	r	p value	r	p value
Inflammatory markers						
Interleukin 1β	0.44	0.001	0.24	0.10	0.06	0.70
Interleukin 6	-0.19	0.20	-0.18	0.20	-0.18	0.20
Fibrinolytic factors						
tPA	0.07	0.63	0.13	0.35	0.31	0.03
PAI-1 antigen	-0.14	0.33	-0.19	0.18	0.08	0.59
PAI-1 activity	-0.12	0.41	-0.05	0.74	0.06	0.66
Myocardial damage						
Troponin T	0.14	0.36	-0.05	0.77	0.12	0.44

Mon1, CD14++CD16 monocytes; Mon2, CD14++CD16+ monocytes; Mon3, CD14+CD16++ monocytes; tPA, tissue-type plasminogen activation; PAI-1, plasminogen activator inhibitor-1; STEMI, ST-elevation myocardial infarction.

8.4.5. Intercellular cell adhesion molecule-1 receptor

There were no differences in monocyte subset ICAM-1r expression on day 1 after STEMI or NSTEMI compared to stable CAD (Table 8.2). There were no significant changes in monocyte subset ICAM-1r expression during follow-up in STEMI. No correlation was observed between monocyte ICAM-1r expression and any of the blood markers tested in STEMI (Table 8.7).

In a univariate analysis, ICAM-1r expression by Mon1 and Mon2 on day 1 after STEMI was significantly negatively predictive of LVEF 6 weeks post-MI (β=-0.42 p=0.007 for Mon1, β=-0.37, p=0.016 for Mon2), with a similar trend also seen for Mon3 (Table 8.5). This parameter lost its predictive value in multivariate analysis after adjustment for age and peak troponin T levels (i.e. the significant predictors of LVEF in the univariate analysis).

Table 8.7 Correlation analysis between biomarkers and intercellular cell adhesion molecule-1 receptor expression in patients with ST elevation myocardial infarction

	Mon1		**Mon2**		**Mon3**	
	r	p value	r	p value	r	p value
Inflammatory markers						
Interleukin 1β	0.03	0.85	0.06	0.69	0.00	0.99
Interleukin 6	-0.09	0.55	-0.09	0.52	-0.04	0.77
Fibrinolytic factors						
tPA	0.01	0.94	0.03	0.81	0.15	0.29
PAI-1 antigen	0.01	0.93	0.00	0.98	0.11	0.46
PAI-1 activity	0.01	0.95	-0.03	0.85	0.02	0.89
Myocardial damage						
Troponin T	0.28	0.07	0.27	0.09	0.14	0.38

Mon1, CD14++CD16 monocytes; Mon2, CD14++CD16+ monocytes; Mon3, CD14+CD16++ monocytes; tPA, tissue-type plasminogen activation; PAI-1, plasminogen activator inhibitor-1; STEMI, ST-elevation myocardial infarction.

8.5 DISCUSSION

This study demonstrates a significant reduction in IL6r expression by the Mon2 subset following STEMI. There was a gradual increase in expression of this receptor on Mon1 and Mon2 at 30 day follow-up. A significant decrease in VCAM-1r density on circulating monocytes after STEMI was observed. One may hypothesise that this may reflect selective homing of monocytes with high VCAM-1r expression to damaged myocardium. Recent data suggests that after an MI, monocytes are recruited to a VCAM-expressing substrate at a rate 50% higher compared to controls at high cardiovascular risk, and that CD14++CD16+ monocytes (ie Mon2) adhere with 7-fold higher efficiency compared to other subsets (Foster et al, 2013).

A recent meta-analysis demonstrated that genetically-determined accelerated shedding of the IL6r into circulation potently protects against atherogenesis in humans, independently of established cardiovascular risk factors. This was thought to be most likely due to disruption of IL6-mediated inflammation (Sarwar et al, 2012). Monocytes have been shown to constitute a major population of circulating IL6r-expressing cell. In healthy subjects, the Mon1 and Mon2 subsets have approximately twice higher IL-6r density than Mon3 (Shantsila et al, 2011). The same pattern was seen in MI patients in this study. Although the precise mechanism underlying IL6r cleavage from monocytes has not been established, it is thought to be modulated by both circulating and cell-bound enzymes (Reich et al, 2007). In this study, the density of IL6r on all monocyte subsets was found to be highly significantly negatively correlated with plasma tPA levels, a fibrinolytic enzyme up-regulated in disorders of thrombosis and endothelial damage and dysfunction such as STEMI. Thus suggests a

possible role for this enzyme in IL6r shedding. Despite the proposition that IL6r remaining on effector cells is thought to be of more importance than that shed into the circulation (Sarwar et al, 2012), no association was observed between IL-6r density on any monocyte subset on day 1 after STEMI and cardiac contractility post-MI.

This is the first study to assess VCAM-1r and ICAM-1r expression on individual monocyte subsets following MI and to explore their relationship to markers of inflammation, cardiac damage and recovery. To allow these results to be interpreted, it should be borne in mind that the presence or absence of changes in receptor expression on circulating monocytes may depend upon a combination of (i) change in receptor expression in response to inflammation, (ii) shedding of receptors into the circulation, and ultimately (iii) speed of monocyte homing to the site of injury. Indeed, accelerated adhesion of leukocytes to endothelial cells is one of the first events in the acute inflammatory response implicated in the pathogenesis of atherosclerosis. Leukocyte surface glycoproteins such as ICAM-1r and VCAM-1r play a major role in this process. Their expression can be up-regulated in response to chemotactic molecules released from sites of inflammation and injury (Mazzone & Ricevuti, 1995).

On day 1 after STEMI, a significant reduction in VCAM-1r expression on all three monocyte subsets compared to stable CAD was observed. However, in NSTEMI there was a significant up-regulation of VCAM-1r expression by the Mon2 and Mon3 subsets (with no change for Mon1). These opposing patterns may be related to the difference in degree of myocardial damage and inflammation in STEMI compared to NSTEMI, as a significant positive correlation between VCAM-1r density on the 'proinflammatory' Mon1 subset and blood IL1β levels was observed. In NSTEMI, this may lead to up-regulation of monocyte VCAM-1r

expression. Conversely, in STEMI which is characterised by a much larger degree of myocardial damage and inflammation, accelerated recruitment of monocytes with higher VCAM-1r expression in STEMI could selectively remove such monocytes from the circulation.

There was no change in monocyte ICAM-1r expression on day 1 after either STEMI or NSTEMI compared with CAD, and no change during follow-up in STEMI patients. Previous data showed that in patients with unstable angina (but not in stable controls) monocyte ICAM-1r expression was selectively increased in the coronary circulation but not in the aorta, implying an abundance of highly ICAM-1r expressing monocytes at sites of vascular injury (Mazzone et al, 1993; De Servi et al, 1995). In my study, ICAM-1r expression by Mon1 and Mon2 after STEMI was negatively associated with LVEF at 6 weeks on univariate analysis. This may reflect more pronounced myocardial recruitment of monocytes with higher ICAM-1r expression in patients with a larger amount of infarcted myocardium and hence lower LVEF. Indeed, the predictive value of this parameter for 6 week LVEF lost significance after adjustment for troponin levels, suggesting a relationship with degree of myocardial damage.

Taken together, the findings in this study suggest that the overall net effect on expression of receptors to adhesion molecules by circulating monocytes following MI is complex. Receptor expression may depend upon the rate of monocyte recruitment into damaged myocardium, possibly masking the highest degree of monocyte activation in patients with a larger infarct. Accumulation of activated monocytes could promote local detrimental processes including thrombosis and vasoconstriction, but also therapeutic processes including angiogenesis and removal of debris from sites of injury.

Transgenic mice without adhesion molecule receptors cannot localize leukocytes to the interstitium (Cotran & Mayadas-Norton, 1998). Furthermore, in a canine experimental model of myocardial reperfusion injury, a monoclonal antibody against ICAM-1r significantly attenuated accumulation of inflammatory leukocytes within the myocardium, and potently reduced degree of infarction (Simpson et al, 1988). Accordingly, pharmaceutical manipulation of monocyte expression of adhesion molecules may represent a therapeutic target for modulation of excessive recruitment of these cells in MI. Additionally, although modulation of the pro-inflammatory pathway mediated by membrane-bound IL6r may also represent an attractive therapeutic strategy to reduce cardiovascular risk, IL6 may also bind with soluble IL6r which activates alternative non-pathological signalling pathways. For example, trials of the monoclonal antibody Tocilizumab (a competitive inhibitor of IL6r) have been associated with adverse effects on the lipid profile (Sarwar et al, 2012). This highlights the complexity of targeting discrete pathways, emphasising the fundamental requirement for further investigation into the relationships of monocyte subsets with inflammatory pathways associated with atherosclerosis and MI. The results of phase 3 trials of anti-inflammatory agents in the secondary prevention of cardiovascular disease are awaited with much interest (Ridker, 2009; Ridker et al, 2011).

There are two previous reports describing increased monocyte expression of ICAM-1r and VCAM-1r in MI which appear to contradict my results. The first study (Hillis et al, 2001) only included 5 subjects with non-Q wave MI (approximately equivalent to NSTEMI) and 3 subjects with a Q wave MI (approximately equivalent to STEMI) out of a total of 22 acute coronary syndrome (ACS) patients. This suggests that the majority of patients did not have any degree of myocardial damage. Their study population is therefore pathophysiologically

rather a heterogeneous group. Additionally, none were treated by PCI, thrombolysis or glycoprotein IIb/IIIa inhibitors. There is no data regarding receptor quantification (Hillis et al, 2001). The second study compared 21 patients with ACS (mostly 'unstable angina' rather than myocardial infarction) with healthy controls rather than subjects with stable CAD (Murphy et al, 2003). However, interpretation of the results and direct comparison between these two studies and my findings is limited by significant methodological differences. Firstly, heparinised blood samples were stored at room temperature for up to 4 hours before processing. This may significantly affect monocyte phenotype (Shantsila et al, 2012). Also no specific monocyte markers were used in the study. In the present study carefully defined groups of patients with specific types of MI (i.e. STEMI or NSTEMI) were analysed, and no patients with troponin-negative unstable angina (i.e. no myocardial damage) were included. My study is further strengthened by a validated methodological approach, which allows measurement of accurately defined monocyte subsets according to contemporary nomenclature (Shantsila et al, 2011).

8.6 LIMITATIONS

This study is descriptive in nature and does not provide insight into intra-cardiac processes which may not parallel changes in blood. Therefore, inferences made as to what increases or decreases in circulating monocytes and their expression of receptors may mean for the myocardial tissue itself are purely speculative. Measurements were made on monocytes and biomarkers in circulation, whereas the origin of MI is perhaps a more localised event, with inflammation within an individual culprit atherosclerotic plaque. Mouse data suggest that monocyte counts and activity in circulation may not reflect their accumulation and activity within tissues. However, simultaneous assessment of study parameters in circulation and within the myocardium would be required to confirm the hypotheses proposed, which is clearly outside the constraints of this study. Also, the specific mechanisms responsible for an increase or decrease in expression of monocyte receptors have not been studied; these may include increased expression of a receptor by circulating monocytes versus mobilisation of new monocytes with higher receptor density, and receptor shedding versus internalisation.

Although significant correlations were found between several study parameters and 6 week LVEF, direct pathological links between these findings cannot be made and require further study. The relatively modest reduction in mean LVEF at 6 weeks (mean EF 54.1±14.6%) after STEMI reflects the beneficial effect a PPCI programme has on limiting infarct size. However, this may potentially mask more marked changes in study parameters if patients were to have sustained more myocardial damage. Indeed, the changes in monocyte parameters observed are a cumulative composite of the effects of MI itself, PPCI and medication. This contrasts with animal models where the effects of MI alone can be studied.

Finally, it was logistically impossible to collect the samples at the same time each day. Although the majority of samples were collected between 09.00 and 12.00 and the remaining impact of diurnal variation in monocyte parameters is likely to be minimal, this should be considered as a limitation (Shantsila et al, 2012).

The study population is relatively small which limits assessment of prognostic implications of the changes observed in STEMI. Larger, prospective studies are required to address this issue.

8.7 CONCLUSIONS

The IL6r pathway is thought to have a direct causal role in the pathogenesis of CAD. This study describes specific changes in monocyte IL6r expression and associations related to individual monocyte subsets following STEMI. Circulating monocyte subsets implicated in inflammatory responses appear to exhibit reduced IL6r expression in the acute phase following STEMI, possibly reflecting enzyme shedding. This may represent a regulatory feed-back mechanism aiming to balance the excessive inflammation characteristic of acute MI. The net effect of expression of receptors to adhesion molecules on circulating monocytes after MI may depend upon the rate of monocyte recruitment to damaged myocardium, possible masking monocyte activation in patients with bigger infarcts.

CHAPTER 9

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

9.1 SUMMARY AND OVERALL CONCLUSIONS

The principal aim of this thesis was to provide a detailed phenotypic comparison of differences between the three human monocyte subsets in the setting of CAD and MI, with emphasis on the Mon2 (’Intermediate’) and Mon3 (’Nonclassical’) subsets.

Work in chapter 4, demonstrated multiple significant novel changes in monocyte phenotype in patients with CAD, attributable to specific subsets, and demonstrated significant differences in plasma levels of cytokines in CAD compared to healthy subjects (summarised in table 9.1).

I observed significant reductions in CD14 expression on Mon1 and Mon3, and CD16 expression on Mon1 and Mon2 in patients with CAD.

Patients with CAD express higher Interleukin 6 receptor (IL6r) levels, and my findings suggest that Mon1 and Mon2 may be amongst the effector cells responsible for IL6r-mediated atherogenesis (Sarwar et al, 2012).

My finding of a significantly higher expression of CXCR4 by Mon3 in CAD compared to healthy subjects suggests that these ‘non-classical’ monocytes may be a subset with enhanced CXCR4-dependent mobilisation to tissues in CAD, in keeping with their ‘patrolling’ behaviour (Cros et al, 2010).

The finding of a significant increase in CD34 expression by all three monocyte subsets in CAD may reflect a compensatory mechanism attempting to enhance angiogenic processes in response to low-grade ischaemia in stable CAD.

The negative correlation with plasma IL6 reinforces the anti-inflammatory properties of the haptoglobin scavenger receptor CD163.

The positive correlations between VEGFR1 expression on Mon1 and Mon2 with uPA levels and the negative correlation between plasma PAI-1 level and IL6r expression on Mon2 may reflect a role of fibrinolytic enzymes in the regulation of expression and cleavage of these receptors.

Table 9.1 Summary of expression of surface receptors by monocyte subsets, plasma cytokine levels and correlations with plasma biomarkers in coronary artery disease compared to healthy subjects

	Receptor	**Mon1**	**Mon2**	**Mon3**
Surface expression				
	CD14	↓		↓
	CD16	↓	↓	
	IL6r	↑	↑	
	CXCR4			↑
	CD34	↑	↑	↑
Correlation with IKKβ level				
	CXCR4	+		
	CD34	+		
	VEGFR1	-	-	
	CD42a	+		
Correlations with plasma level				
IL6	CD163	-	-	-
MCP-1	TLR4	+	+	
	VEGFR1		+	
	CD204	-	-	
IL1β	CD42a	+		
uPA	VEGFR1	+	+	
PAI-1 antigen	VEGFR1		+	
	IL6r		-	
PAI activity	IL6r		-	
Plasma cytokines level in CAD				
IL10 ↓ MCP-1 ↓ ↑ IL6 (strong trend)				

Mon1, CD14++CD16-CCR2+ monocytes; Mon2, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2- monocytes; CAD, coronary artery disease; IL, interleukin; IL6r, Interleukin 6 receptor; IKKβ, inhibitory κB kinases; MCP-1, monocyte chemoattractant proten-1; uPA, urokinase type plasminogen activator; PAI, plasminogen activator inhibitor type 1; ↑, increased expression; ↓, decreased expression; +, positive correlation; -, negative correlation

In chapter 5, I described for the first time the differential dynamics of the three human monocyte subsets following STEMI. The findings are summarised in table 9.2. The hypothesis that the Mon2 subset has unique properties is supported by the findings of specific and highly significant changes in their phenotype after STEMI (increased CD14 and CCR2 expression, reduced CD16 expression). Activity of the NFκB pathway was highest in Mon2 on day 1 after STEMI, suggesting a prominent increase in functional activity; the significant reduction on day 7 (compared to other monocytes) suggests that Mon2 are most functionally active in the first few days after STEMI. The significant reduction in Mon2 number on day 7 is in keeping with the increased capacity for Ly-6C^{low} monocytes to migrate into the myocardium at this stage in mice.

The only significant correlations between monocyte parameters and peak troponin level were with Mon2 number and characteristics. Additionally, most monocyte-associated predictors of LVEF at 6 weeks were related to the Mon2 subset, including Mon2 number and number of MPAs associated with Mon2. The only parameter independently predictive of LVEF at 6 weeks was CD16 expression by Mon2 (lower CD16 expression predicted better LVEF). These findings suggest a close relationship between numerous Mon2 subset characteristics and the degree of myocardial damage and recovery following STEMI.

The observation of no change in number of Mon3 after STEMI and little change in phenotype (only increase in CD14 expression) compared to Mon 2 supports the hypothesis that this subset has substantially different physiological roles compared to Mon1 and Mon2.

The complexity of Mon2 biology is suggested by its correlation with levels of both IL6 and IL10 (i.e. both ‘pro’- and ‘anti-inflammatory’ cytokines).

This study demonstrates for the first time that the increase in MPA count persists at 30 days after STEMI, despite ongoing potent dual antiplatelet therapy, and after the monocyte count has returned to a level comparable to stable CAD. This is in accordance with data describing the important regulatory role platelets have in modulating the functional activity of monocytes.

Table 9.2 Summary of changes in monocyte parameters and plasma cytokine levels following ST-elevation myocardial infarction

		Day after STEMI			
		Day1	**Day3**	**Day7**	**Day30**
Monocytes					
Total monocytes	number	↑			↓
Mon1	number	↑			↓
	CCR2 expression	no change			↓
	IKKβ level	↑			↓
Mon2	number	↑ (2.5 fold)		↓	
	proportion	Mon2>Mon3			Mon2<Mon3
	CCR2 expression	↑			↓
	CD14 expression	↑		↓	
	CD16 expression	↓			
	IKKβ level	↑		↓	
Mon3	number	no change	no change	no change	no change
	proportion	Mon3<Mon2			Mon3>Mon2
	CD14 expression	↑		↓	
	CCR2 expression	no change	↑	↓	↓
	CD16 expression	no change	no change	no change	no change
	IKKβ level	↑			↓
Monocyte platelet aggregates					
Total	number	↑			↓ (but higher than CAD
MPA Mon1	number	↑			↓ (but higher than CAD)
	MFI	↑	remained ↑	remained ↑	remained ↑
MPA Mon2	number	↑			trend in ↓(but higher than CAD)
	MFI	↓			
	%	Mon2>Mon1			
MPA Mon3	number	no change	no change	no change	no change
Plasma cytokines					

IL1β	plasma level	no change	↓		same as CAD
IL6	plasma level	↑		↓	same as CAD
	correlations (all positive)	Mon1, Mon2, Total MPAs, MPA-Mon1, MPA-Mon2			
IL10	plasma level	no change		↑	same as CAD
	correlations	positive correlation with Mon2			
MCP-1	plasma level	↓		↓ further	same as CAD

Mon1, CD14++CD16-CCR2+ monocytes; Mon, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2- monocytes; MPA, monocyte platelet aggregates; STEMI, ST elevation myocardial infarction; CAD, coronary artery disease; IKKβ, inhibitory κB kinases, MCP-1, monocyte chemoattractant protein-1; IL, interleukin; ↑, significant increased number; ↓, significant decreased number; MFI, median fluorescent intensity; %, percentage of each subset aggregated with platelets

In chapter 6, changes in monocyte parameters on day 1 following NSTEMI were described. These mirrored previous observations following STEMI (chapter 5), with significant but numerically less prominent increases in Mon1 and Mon2 number and no change in Mon3, and a trend (not statistically significant) towards a reduced relative proportion of Mon3 but increased relative proportion of Mon2.

This study shows for the first time that MI is associated with increased numbers of TLR4+ monocytes, but TLR4 expression (percentage positive cells or MFI) by individual monocyte subsets per se is unchanged. Moreover the increase in TLR4+ monocytes is only attributed to specific human monocyte subsets (Mon1 and Mon2 in STEMI, and Mon2 only in NSTEMI).

The relative clinical importance of monocyte number rather than level of TLR4 expression per se is suggested by the correlation between peak troponin level and TLR4+ Mon2 number (similarly to the correlation with total Mon2 subset count in chapter 5), but not with TLR4 expression on any subset.

There was no association between monocyte TLR4 expression and IKKβ activity, and no association between monocyte TLR4 expression and LVEF 6 weeks post STEMI. Plasma IL6 level correlated positively with TLR4+ Mon2 count but negatively with TLR4 expression on Mon2. This observation is in agreement with data describing the association of this subset with high IL6 levels in chapter 5. In contrast, the negative association with TLR4 expression by other monocytes may reflect partial loss of TLR4 from activated monocytes. These findings suggest that TLR4 expression may not be a reliable marker of monocyte activation in STEMI.

In chapter 7, several significant novel observations were made regarding monocyte subsets and their associations with receptors implicated in reparative properties following STEMI (summarised in table 9.3).

There was a significant increase in number of CXCR4+ and KDR+ monocytes following STEMI, only associated with Mon1 and Mon2 subsets.

The number of the CXCR4+ Mon2 subset on day 1 after STEMI (but not KDR+ Mon 2 cells) correlated significantly with peak troponin level.

The significant reduction in expression of the pro-reparative scavenger receptor CD163 by all subsets following STEMI observed may be related to their increased utilisation in the setting of myocardial necrosis, reflecting higher demand for their functions. This may contribute to the independent association between reduced expression of CD163 by the Mon1 subset and higher LVEF 6 weeks after STEMI.

A high count of the CXCR4+ Mon2 subset and high CD163 expression by Mon1 after STEMI were significant predictors of lower LVEF at 6 weeks. This could suggest potential detrimental properties of CXCR4+ and CD163+ monocytes or conversely their increased utilisation in repair mechanisms in patients with more marked infarction.

The positive correlations between numbers of CXCR4+ Mon2 and both pro- and anti-inflammatory cytokines further suggests that the role of these cells in post-MI reparative processes is complex.

My study suggests the increase in CD204+ monocytes previously seen post-MI is due to monocytosis rather than increased monocyte expression of CD204 per se, which did not change significantly. However, following STEMI, CD204 expression by monocyte subsets was associated with plasma levels of inflammatory cytokines, suggesting a relationship with ischaemic myocardial injury.

Mon2 monocytes could be considered a ‘top’ rather than ‘intermediate’ subset as they have the highest expression of ‘beneficial’ reparative markers (CXCR4, KDR and CD163).

Table 9.3 Summary of changes in monocyte expression of CXCR4, KDR, CD163 and CD204 following ST-elevation myocardial infarction and their relations to plasma cytokines

		Days after STEMI			
		Day1	**Day 3**	**Day7**	**Day 30**
Monocytes					
Mon1	CXCR4+ number	↑			↓
	KDR+ number	↑			
	CD163 expression	no change	↓		
	CD204 expression	no change	↓		
Mon2	CXCR4+ number	↑			↓
	KDR+ number	↑			
	CD163 expression	no change	↓		
Mon3	CD163 expression	↓	no further change		
	CXCR4+ number	no change			
	KDR+ number	no change			
Correlations					
Peak troponin	positive	number of CXCR4+Mon2			
IL1β	positive	number of KDR+Mon1 and KDR+Mon2			
IL6	positive	number of CXCR4+Mon2 and KDR+Mon2			
	negative	Mon2 CD204 expression			
IL10	positive	number of CXCR4+Mon2			
	negative	number of KDR+ Mon3			
	negative	Mon2 CD204 expression			
Peak troponin	positive	number of CXCR4+Mon2			

STEMI, ST-elevation myocardial infarction; Mon1, CD14++CD16-CCR2+ monocytes; Mon, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2- monocytes; MCP-1, monocyte chemoattractant protein-1; IL, interleukin; ↑, significant increased number; ↓, significant decreased number

Chapter 8 described specific changes in IL6r and adhesion molecule expression and associations with cytokines related to individual monocyte subsets following STEMI (summarised in table 9.4). Circulating monocyte subsets implicated in inflammatory responses appear to exhibit reduced IL6r expression in the acute phase following STEMI, possibly reflecting enzyme shedding. This may represent a regulatory feed-back mechanism aiming to balance the excessive inflammation in MI. The density of IL6r on all monocyte subsets correlated negatively with plasma tPA levels, suggesting a possible role for this fibrinolytic enzyme in IL6r shedding.

No association was observed between IL6r density on any monocyte subset on day 1 after STEMI and LVEF at 6 weeks.

VCAM-1r number on all monocyte subsets decreased significantly after STEMI, whereas in NSTEMI there was a significant increase in VCAM-1r expression by Mon2 and Mon3. This may reflect selective homing of monocytes with high VCAM-1r expression to large areas of damaged myocardium in STEMI.

There was no change in monocyte ICAM-1r expression on day 1 after either STEMI or NSTEMI. However, its expression on Mon1 was reduced at 30 day after STEMI. The negative correlation between ICAM-1r expression by Mon1 and Mon2 after STEMI with LVEF at 6 weeks may reflect more pronounced myocardial recruitment of monocytes with higher ICAM-1r expression in patients with larger infarcts. Indeed, the predictive value of this parameter for 6 week LVEF lost significance after adjustment for troponin levels, suggesting a relationship with degree of myocardial damage.

The findings in this study suggest that the overall net effect on expression of receptors to adhesion molecules by circulating monocytes following MI is complex. This may depend upon the rate of monocyte recruitment into damaged myocardium, possibly masking the highest degree of monocyte activation in patients with a larger infarct. Accumulation of activated monocytes could promote local detrimental processes including thrombosis and vasoconstriction, but also therapeutic processes including angiogenesis and removal of debris from sites of injury.

Table 9.4 Summary of monocyte subsets and their relationships to Interleukin 6 receptor and adhesion molecules following ST-elevation myocardial infarction.

			Day after STEMI			
			Day1	**Day3**	**Day7**	**Day30**
Monocytes						
Mon1	IL6r	number	no change			↑
		expression	negative correlation with tPA			
	VCAM-1r	number	↓ positive correlation with IL1β		↑	↑
	ICAM-1r	number	no change			↓
		expression	negative correlation with LVEF			
Mon2	IL6r	number	↓			↑
		expression	negative correlation with tPA			
			positive correlation IL1β (trend)			
	VCAM-1r	number	↓	↑	↑	↑
	ICAM-1r	number	no change			
		expression	negative correlation with LVEF			
Mon3	IL6r	number	no change			
		expression	negative correlation with tPA			
	VCAM-1r	number	↓	↑	↑	↑
	ICAM-1r	number	no change			

STEMI, ST-elevation myocardial infarction; Mon1, CD14++CD16-CCR2+ monocytes; Mon, CD14++CD16+CCR2+ monocytes; Mon3, CD14+CD16++CCR2- monocytes; IL, interleukin; IL6r, Interleukin6 receptor; ICAM-1r, intercellular cell adhesion molecule-1 receptor; VCAM-1r, vascular cell adhesion molecule-1; tPA, tissue-type plasminogen activator; LVEF, left ventricular ejection fraction at 6 weeks; ↑, significantly increased number; ↓, significantly decreased number

9.2 FUTURE DIRECTIONS

Despite major advances in our understanding of the mechanisms of CAD and the management of MI and consequent LV dysfunction, this disorder remains a major public health concern with much associated morbidity and mortality. This indicates the requirement for ongoing research with the aims of better understanding the complex pathophysiology of this condition, and the development of further targeted therapies. Future research may focus on diagnostic tests to risk stratify patients with CAD to discriminate a benign phenotype from a more aggressive form with propensity to plaque progression, destabilisation, rupture and consequent MI. This may allow for interventions to halt or even reverse plaque progression. Additionally, in the event of MI the emphasis could be on reducing adverse LV remodelling and promotion of LV recovery and contractility.

The work in this thesis is of an exploratory pilot study nature, with the aim of making observations which may be hypothesis generating for future research. Ongoing prospective enrolment of patients to validate the findings in this thesis would be valuable. A prospective study examining the predictive value of monocyte subset count and phenotype to estimate the degree of left ventricular impairment expected in the convalescent recovery phase could be of important clinical utility. This would allow for the rapid identification of post-MI patients at highest risk of complications related to LV dysfunction and therefore appropriate targeting of therapies. It was noted in this thesis that the management of STEMI has greatly improved over the last decade, with more successful reperfusion of the infarct-related artery and consequently improved preservation of left ventricular systolic function. I used simple trans-thoracic echocardiography to measure convalescent LVEF, but it could be more informative

to utilise more sophisticated imaging techniques such as cardiac MRI which has greater spatial resolution and can be used to investigate other important parameters involved in myocardial recovery post MI, such as the extent of microvascular obstruction.

It would be informative to study the effects of interventions known to reduce cardiovascular risk on the three human monocyte subsets. This could include lifestyle measures such as smoking cessation, dietary modification, weight loss, aerobic exercise, along with modification of established risk factors such as blood pressure control and cholesterol lowering. Pleotropic effects including anti-inflammatory properties have been identified for certain therapies such as aspirin and the statin drugs, so it would also be important to study the effects of established medication used in CAD and MI including antiplatelet drugs (aspirin, clopidogrel, prasugrel, ticagrelor), statins and inhibitors of the renin-angiotensin-aldosterone system. Additionally there are major clinical trials of dedicated anti-inflammatory agents investigating the effects of methotrexate (Cardiovascular Inflammation Reduction Trial; CIRT) (Ridker, 2009) and the neutralisation of Il-1β with Canakinumab (Canakinumab Anti-inflammatory Thrombosis Outcomes Study; CANTOS) (Ridker et al, 2011) in atherosclerosis, and it would be informative to investigate the effects of these drugs on the three human monocyte subsets.

It remains unclear whether the 'intermediate' CD14++CD16+CCR2+ subset is a truly biologically distinct cell type or whether these cells are in transition between the other subsets. In vitro studies may be performed to purify and culture the CD14++CD16+CCR2+ subset and identify whether they can develop into 'classical' CD14++CD16-CCR2+ and/or

'non-classical' CD14+CD16++CCR2- monocytes, or give rise to a unique type of macrophage or dendritic cell. It is also unclear whether the differentiation of human monocytes into specific subsets is already complete in the bone marrow and spleen or whether this maturation occurs in the circulation and tissues as has been demonstrated in mice. The Mon1 subset has been reported to show some degree of CD16 expression after stimulation (Passacquale et al, 2011). The changes in phenotype observed may reflect their differentiation towards the Mon2 subset or maturation into CD16+ macrophages. Our previous work demonstrated that all three monocyte subsets are present in bone marrow, which contained relatively higher proportions of Mon2 than found in circulation (Shantsila et al, 2011). A rapid release of the Mon2 subset from bone marrow into the circulation may explain the dramatic increase in Mon2 count on day 1 after STEMI in this study. Further research is required to answer these questions about monocyte subset origins and phenotypic differentiation.

One of the major limitations of the observations made in this thesis is the measurement of monocyte count and phenotype solely in peripheral venous blood. Numerous observations were made in relation to the absolute number of each monocyte subset in peripheral venous blood and their expression of a range of surface markers. It would be informative to be able to quantify monocyte count and study their phenotype within the myocardium during life, as this may be the site at which monocytes maximally exert their roles. In the human setting, direct tissue sampling from the myocardium especially in the context of acute MI would pose major ethical and practical dilemmas. However, the identification of a monocyte 'gradient' may allow inferences to be made about where monocytes are taken up from circulation into tissue. This could be calculated by sequential sampling of blood from multiple sites:

peripheral venous, right atrium, right ventricle, pulmonary artery, left atrium, left ventricle, aortic root, coronary artery ostium and the coronary sinus. Performance of this experiment in an animal model and also humans with CAD and healthy controls would allow for important comparisons between species; the differences between human and animal monocyte subsets has hampered extrapolations between species, and this must be overcome. If this proved successful and reliable it could perhaps be extended to the post-MI setting, firstly in an experimental animal model of MI. Of particular interest in the MI setting would be to examine the subset count, phenotype and function in the occluded artery by means of analysing the material removed from the coronary artery during thrombus aspiration which is widely used during PPCI for STEMI.

Van de Laan and colleagues recently described that 40% of monocytes in the infarct core of subjects who died from MI are CD14+CD16+ (van der Laan et al, 2013). Further delineation of these cells into the Mon2 and Mon3 subsets would be very informative, to further expand our understanding of the temporo-spatial distribution of monocytes following MI which may suggest their functions in this setting.

Non-invasive imaging techniques could also be utilised to allow identification of the three monocyte subsets within tissues including the myocardium. Imaging modalities using labelled nanoparticles including MRI (Sosnovik et al, 2007), PET (Lee et al, 2012b) and fluorescence molecular tomography (Nahrendorf et al, 2007) have been used to track monocytes and their molecular functions in mice. Multi-modal hybrid imaging (PET/MRI) has recently been reported in mice to simultaneously quantify monocyte-associated detrimental myocardial

inflammation and also beneficial remodelling (Majmudar et al, 2013). Ultimately these techniques may be utilised to study human monocytes in the CAD and post-MI settings.

The observations made in the thesis and in other works have further characterised human monocyte subsets. The ultimate aim of developing a detailed understanding of monocyte subset biology and pathophysiology is to investigate the potential to influence their functions for therapeutic benefit. For example this may arrest the development of coronary plaques, rendering them less likely to become large enough to impinge sufficiently on the coronary artery lumen to obstruct normal flow and cause exertional angina. Alternatively, it may be possible to modify the composition of coronary plaques to reduce the likelihood of progression to destabilisation and rupture which precipitates coronary artery thrombosis, obstruction and ultimately myocardial infarction. Such a detailed understanding must travel beyond an over-simplistic labelling of a certain monocyte subset as 'good' or 'bad', 'deleterious' or 'reparative'. The precise functions of monocyte subsets must be understood during each stage of their development, at different anatomical sites, and under healthy conditions and in disease settings. Parallels must be drawn between human and animal monocyte subset biology, as any therapeutic manoeuvre must be studied first in an animal model. The first such step would be to simply enhance or inhibit the function of a particular monocyte subset in healthy controls and subjects with CAD and MI to assess the overall effect on the myocardium. This could be achieved by simply increasing or decreasing the number of a particular subset, or by stimulation/inhibition without altering the number of cells. More specifically, this could focus on specific phenotypes of a subset identified by their expression of surface markers, such as monocytes with 'reparative potential'. The ability of monocyte subsets to modify their phenotype and functionality should be distinguished from

the release of newly synthesised monocytes from storage sites. Pharmacological agents which specifically target individual monocyte subsets are not currently in existence. Clinical studies investigating experimental therapies aimed at manipulating inflammation in CAD and MI have recently been reviewed by Baruch and colleagues (Baruch et al, 2013). Close attention would be vital in monitoring the other known roles of monocytes if they are manipulated, such as innate immunity. The duration of beneficial and also any deleterious effects of such actions should be studied longitudinally, beyond the lifespan of monocytes in existence at the time an agent is introduced to the body.

APPENDICES

APPENDIX 1: Published papers arising from this thesis

The CD14++CD16+ monocyte subset and monocyte-platelet interactions in patients with ST-elevation myocardial infarction. Tapp LD, Shantsila E, Wrigley BJ, Pamukcu B, Lip GY. J Thromb Haemost 2012; 10(7): 1231-41.

TLR4 expression on monocyte subsets in myocardial infarction. Tapp LD, Shantsila E, Wrigley BJ, Montoro-Garcia S, Lip GY. J Intern Med 2013; 273(3): 294-305.

CXCR4 positive and angiogenic monocytes in myocardial infarction. Shantsila E, Tapp LD, Wrigley BJ, Montoro-Garcia S, Lip GY. Thromb Haemost 2013; 109(2): 255-62.

Receptors to Interleukin-6 and adhesion molecules on circulating monocyte subsets in acute myocardial infarction. Shantsila E, Tapp LD, Wrigley BJ, Montoro-Garcia S, Lip GY. Thromb Haemost 2013; 110(2): 340-348.

Monocyte subsets in coronary artery disease and their associations with markers of inflammation and fibrinolysis. Shantsila E, Tapp LD, Wrigley BJ, Pamukcu B, Apostolakis S, Montoro-Garcia S, Lip GY. Atherosclerosis 2014; 234(1): 4-10.

APPENDIX 2: Published abstracts arising from this thesis

Dynamics of monocyte subsets over 30 days in ST elevation myocardial infarction treated by primary percutaneous coronary intervention. LD Tapp, BJ Wrigley, B Pamukcu, GYH Lip, E Shantsila. Presented at the Arteriosclerosis, Thrombosis & Vascular Biology Scientific Sessions, Chicago, 2011.

Immunophenotypic characterisation of an immature human monocyte subset with angiogenic potential. Eduard Shantsila, Luke Tapp, Ben Wrigley, Stavros Apostolakis, Mark T Drayson, Gregory YH Lip. Presented at the Arteriosclerosis, Thrombosis & Vascular Biology Scientific Sessions, Chicago, 2011.

Counts and relative proportion of human monocyte subsets in ST-elevation and non-ST-elevation myocardial infarction. L Tapp, BJ Wrigley, B Pamukcu, GYH Lip, E Shantsila. Presented at the European Society of Cardiology Congress, Paris, 2011.

Nuclear factor kappa beta activation in monocyte subsets in patients with ST-elevation myocardial infarction. B Pamukcu, LD Tapp, BJ Wrigley, AS Jaipersad, GYH Lip, E Shantsila. Presented at the European Society of Cardiology Congress, Paris, 2011.

Dynamics of the three human monocyte subsets over 30 days in ST-elevation myocardial infarction. LD Tapp, BJ Wrigley, B Pamukcu, GYH Lip, E Shantsila. Presented at the British Cardiovascular Society Annual Conference, Manchester, 2012.

Expression of receptors to interleukin 6 and adhesion molecules on circulating monocyte subsets in myocardial infarction. Tapp L, Shantsila E, Wrigley BJ, Lip GY. Presented at the British Cardiovascular Society Conference, London, 2013.

Monocyte subset phenotype in coronary artery disease and their associations with markers of inflammation and fibrinolysis. Tapp L, Shantsila E, Wrigley BJ, Montoro-Garcia S, Lip GY. Presented at the British Cardiovascular Society Conference, London, 2013.

Receptors to Interleukin 6 and adhesion molecules on circulating monocyte subsets in acute myocardial infarction. LD Tapp, E Shantsila, BJ Wrigley, S Montoro-Garcia, GYH Lip. Presented at European Society of Cardiology Congress, Amsterdam, 2013.

Monocyte subset phenotype in coronary artery disease and their associations with markers of inflammation and fibrinolysis. LD Tapp, E Shantsila, BJ Wrigley, S Montoro-Garcia, GYH Lip. Presented at European Society of Cardiology Congress, Amsterdam, 2013.

APPENDIX 3: Standard Operating Procedure 201; Monocyte subsets and monocyte platelet aggregates by flow cytometry

STANDARD OPERATING PROCEDURE 201

MONOCYTE SUBSETS

Monocyte platelet aggregates by flow cytometry
SOP written by Eduard Shantsila and Andrew Blann

N.B. Use of the flow cytometry is forbidden
Without having been officially trained

Required pre-training

1. SOPs on venepuncture and on good clinical practice
2. SOP 195 – General operation of the flow cytometer

Contents

1. Introduction

Monocytes are large mononuclear cells (MNCs) derived from the bone marrow but on transit to the tissues where they seem likely to become semi-resident macrophages. Traditionally, they have been defined by glass-slide morphology, size, and scatter, but we now have the ability to define monocytes by cell surface molecules, using the FACS. For example, CD14 is a receptor for LPS present on monocytes, macrophages and neutrophils. CD16 is an antigen found on the Fc receptors and is present on natural killer cells, neutrophil polymorphonuclear leukocytes, monocytes and macrophages. So leukocytes populations can be further classified by the density of the expression of these markers, for example….

- M1 = CD14 strong CD16 negative
- M2 = CD14 strong CD16 strong
- M3 = CD14 weak CD16 strong

A further characteristic of monocytes in chemotaxis, such as to the chemokine monocyte chemoattractant protein-1 (MCP-1), a cytokine involved in monocyte infiltration in inflammatory diseases such as rheumatoid arthritis as well as in the inflammatory response against tumors. CCR2, short for chemokine (C-C motif) receptor 2, is a chemokine receptor for MCP-1 CCR2 has also recently been designated CD192.

Platelets are anucleate fragments of the cytoplasm of the megakaryocyte. They form thrombi when self-aggregating but more so in the presence of fibrin. However, platelets may also bind to monocytes. Cell surface markers of platelets include CD42a, also known as GpIX. It follows that dual labelling of blood with a monocyte marker (CD14/CD16/CCR2) and a platelet marker (CD42a) will identify monocyte-platelet aggregates (MPAs).

This SOP describes enumeration of monocyte subsets (dependent on expression of CD14, CD16 and CCR2) and their participation in the formation of MPAs. And of course you will need a platelet count for the project, derived from the full blood count, from the Advia (see SOP 171).

2. Materials and Supplier contact details:

Micro-reagents are kept in the fridge behind the door or on nearby shelves. Bulk fluids in boxes on other shelves and beneath the benches.

1) BD "FACS Flow" Running solution [Becton Dickinson {BD}, Catalogue No. 342003] 10L containers.

2) 3 ml BD Falcon tubes [BD Catalogue No. 352054]

3) BD"FACS Clean" Cleaning Solution [BD Catalogue No. 340345]

4) BD Lysing solution [BD Catalogue No. 349202]

5) Sterile Phosphate Buffered Saline solution, 0.5L bottles [Invitrogen Ltd, Catalogue No 20012-068]

6) CD14 -PE conjugated monoclonal antibody - 100 tests [BD Catalogue No. 555398]

7) CD16 – Alex-flour 488 conjugated monoclonal antibody - 100 tests [ABD Serotec, Cambridge]

8) CD42a-PerCP conjugated monoclonal antibody [BD Catalogue No. 340537]

9) CCR2-APC conjugated monoclonal antibody [R&D Systems Europe Ltd, Cat No. FAB151A]

   [n.b. this combination of antibodies constitute a **Mastermix**: See ADB, ES]

10) Clear pipette tips [Alpha Laboratories Limited Catalogue No FR1250 1250ul Fastrak Refill NS]

11) Yellow pipette tips [[Alpha Laboratories Limited Catalogue No FR1200 200ul Fastrak Refill NS]

12) Count beads [BD (Trucount tubes)]. This is a crucial aspect as it will give us the number of monocytes/ml of venous blood. The product tube has a statement of the number of beads in each tube and so from this you can work out beads/mL.

Remember to dispose of all material thoughtfully.

3. Detailed method

3.1 General Preparation

3.1.1 Lysing solution.

Make from 50ml concentrate 10x FACS Lysing Solution (kept at room temperature). Dilute with 450ml distilled water in ½ litre bottle. This solution should not be used if it is older than a month (kept at room temperature).

3.2 Blood sample preparation

1. Add 12.5µL of Mastermix Absolute Monocyte Count (which includes CD14 2.5 µL, CD16 2.5µL, CD42a 5µL and CCR2 2.5 µL fluorochrome labelled antibodies) with an electronic micropipette. Just place into the tube below a metal grid without touching the pellet.

2. Gently vortex the EDTA blood sample. Take 0.05 mL (=50 µL) of whole blood with electronic pipette and add to a Trucount tube.

3. Do not touch the pellet (this is critical!). Mix the tube gently with the vortex (3 sec). Incubate for 15 minutes in the dark, room temperature, shaking with horizontal shaker (set at 500 units). Add 0.45 ml (=450 µL) pre-diluted BD FACS Lyse solution (see 3.1.1) with a clear tip using the 1ml pipette. Incubate for 15 minutes on shaker as above.

4. Add 1.5 ml of PBS solution without touching the sample, followed by gentle vortex to ensure thoroughly mixed

3.3 Start up procedure [See SOP 195 on General Operation]

Part 1 – restoring reagents and preparation

1. Switch on Flow Cytometer by pressing the green switch on the right hand side. The Apple Macintosh computer must also be switched on, but only 15 secs **after** the Flow Cytometer, or the link will not be recognized. Open the reagent panel on the left hand

side (LHS) by pulling the lid towards you. On the left is the sheath fluid reservoir, in the middle are switches and tubes, and on the right is the waste reservoir.

2. Carefully unscrew the top of the sheath fluid reservoir and fill with sheath fluid (in large box on shelf at head height – use plastic tube) to the level indicated on the top right hand corner of the reservoir (little plastic bar).

3. Carefully disconnect/unscrew the waste container and empty contents down sink with plenty of water. Add approximately 40ml concentrated household bleach along with 360 ml of distilled water and reconnect container (plastic tubes available).

4. Pressurise the unit (takes about 20-30 sec) by moving the black toggle switch "Vent Valve" switch to the down/front position. It is located at the rear of the middle section between the sheath fluid tank and the waste container.

5. Air must be excluded from the tubing system by flushing it out. Any excess air trapped in the sheath filter can, if necessary, be cleared by venting through the bleed tube (the dead-ended rubber tube with a cap).

6. Close the drawer

Part 2 - Cleaning the machine

7. Ensure that a 3 ml Falcon tube (labelled 1) approximately 1/3 full of distilled water is positioned over the sample injection port (SIP) – a needle sheath - and that the swing arm is positioned under this tube. Press the prime button on the panel. When the system enters "standby" with in 30 seconds then press the "prime" button again. When the standby and low buttons comes on again then remove tube 1. We will re-use tube 1 in the shut down procedure.

8. Prepare a second falcon tube (labelled 2) with FACS-clean (should contain about 2750 microlitres so that when inserted on to the sip it doesn't touch the **O** ring). This is a smaller box on a shelf at above head height and above the bigger box of sheath flow fluid

9. Present tube 2 to the SIP and place support arm underneath it. Press the buttons "run" and "high" on the panel at the same time, and run the FACS-clean in falcon tube 2 with supporting arm to left or right open for one minute. Then return the supporting arm to underneath the tube and "run-high" five minutes. This process ensures the machine is clean prior to running samples and helps minimise blockages.

10. Return to falcon tube 1 with distilled water. Repeat the above step 9 with this distilled water tube.

11. Press the '**STANDBY**' and '**LOW**' button on the system.

12. The machine is now ready to run samples.

3.4 Running blood samples.

Note. This must be learned from an experienced operator and you must seek scientific staff support to clear queries as the intricacies of the Cell Quest software are complex.

1. Open CellQuest Pro software
2. Click 'File' – 'Open'
3. Click on the 'Monocyte Protocols' folder within 'Data 1' folder.
4. Click on the 'Monocyte Absolute Count'. This will open study protocol.

5. Click '**Connect to Cytometer**', located under the 'Acquire' menu.

6. Under the '**Cytometer**' menu, click '**Instrument Settings**'. The window appears displaying the compensations and threshold. Change settings by clicking on the **open** icon on the window which displays the folders select 'Monocyte Protocols' folder with in the 'Data 1' folder and click on the 'Monocyte Absolute Count' instrument settings in this folder. This will update the system settings to the preferred settings for the acquisition. Click '**Set'** on the window and by clicking '**Done'** the windows disappears. Make sure to click '**Set**' prior to clicking '**Done**'.

7. Click the '**Acquire'** menu once more and click '**Show browser**'.

8. Click directory-'**Change'** in order to specify the location folder.

9. Initial user must create new folder by clicking on '**New folder'** and by entering the title of the folder and choose that folder.

10. Change the custom suffix to the preferred title and number for data and click '**OK'**.

11. Untick the setup box (by clicking on it) in the browser Acquisition window. Now insert your sample and press "RUN" and "HIGH".

12. Open swing arm at bottom right of the cytometer and replace the Falcon tube with the sample to be run. Replace the swing arm under the Falcon tube.

13. Press the buttons '**Run**' and '**High**' on the control panel of the cytometer.

14. Click '**Acquire'** on the browser menu. The sample will now run for ~ 12 mins. Cell events will be displayed on the screen throughout the process (n.b. the higher the cell density, the more rapidly the cells will be acquired).

15. Click on '**Counters'** under the '**Acquire'** and observe the events per second which varies from 1000 to 8000 depending on various factors. The objective is to acquire 10,000 count beads for analysis.

16. Observe the acquisition closely since the system may get blocked (which happens very rarely) and the plots may not show any progress and the counters may not show any events per second.

17. Click pause on the acquisition window and replace the sample from the SIP with sterile PBS and run for 20/30 seconds minutes (clicking acquire wouldn't change the results) and then continue acquisition with your sample on the SIP. If the problem still persists please inform the senior scientific staff and seek assistance.

18. After attaining the target events the analysis stops and the file number changes automatically. Click on '**print'** under the '**files'**. Confirmation window appears again click on print.

19. Vortex your next sample gently. Re-programme the software with a new sample number, and repeat the step 11.

20. If the cytometer is not ready message appears open the drawer and check the fluids level which may need refilling or emptying. The system may run out of Sheath fluid if there are more samples.

21. Be absolutely sure you have downloaded your results on to paper. Keep this paper safe. Do not assume the computer will keep the results safe, even if you have directed it to do so. Obtain all the raw data (cell numbers) and apply them into the specific spreadsheet you have designed for your project. The same spreadsheet should have the WBC and platelet count results from the Advia

3.5 Shut-down procedure [See SOP 195 on General Operation]

1. In this section we re-use tubes 1 and 2 with distilled water and FACS clean respectively. Install FACS Clean tube 2 over the SIP needle. Press button '**High'** and '**Run'** on the panel. Leave the support arm out at 90 degrees for approximately 1 minute. This cleans the outer portion of the aspiration sheath. The fluid will be rapidly aspirated, so ensure that the tube doesn't empty completely.

2. Now replace the side arm under the Falcon tube and allow it to run for approximately 5 minutes. This cleans the inner portion of the aspiration sheath and the FACS machine itself.

3. Repeat steps 2 and 3 with the distilled water tube 1. Once step 3 is complete, Leave the sheath in falcon tube 1 containing distilled water and press '**STANDBY'.**

4. Open the reservoir draw and depressurize the machine by moving the "Vent Valve" toggle switch to the up/rear position. The machine will hiss as it depressurizes.

5. Leave the machine on for a further 5 minutes to allow the laser lamp to cool. Turning the machine off prematurely will result in the lamp cracking.

6. Finally power down the FACScalibur (green button) and Apple Mac, and then clean up!

7. Note: * IF THE SYSTEM IS TO BE USED AGAIN ON THE SAME DAY…

LEAVE THE SYSTEM ON STANDBY and then
DEPRESSURISE THE SYSTEM.

4. Interpretation of plots

For the first couple of analyses you will need to have all this explained to you by Dr Blann or Dr Shantsila. These numbers refer to the illustrative plot and nine individual plots…

TOP THREE PLOTS

1. The top left initial plots show the FSC/SSC plot (forward and side scatter, all in green). This is needed to gate the presumed monocytes. Be generous at this stage, include all monocytes. Contamination by granulocytes and lymphocytes will be removed during the next stage.

2. Immediately to the right (i.e. centre) is a plot of the cells stained with CD14 (light blue) which further gates the monocytes to separate them from granulocytes. Note a large residual proportion of granulocytes at the top of the SSC index.

3. Top right is plot of CD14/CD16 events (red/brown). Four gates have been drawn to define different populations of monocytes. M1 defines CD14strong/CD16-ve, whilst M4 defines cells expressing a lot of CD16. The latter will be sub-typed shortly.

CENTRE THREE PLOTS

4. Centre left is a plot of the Count beads (green), which are sampled at a concentration of, for example, 50,000 beads/tube. From this you will get monocytes/mL and thus MPAs/mL. The CD14-PE horizontal axis is irrelevant.

5. Centre middle is (green) plot of CD16 versus CD14, which allows you to gate and exclude lymphocytes from analysis. Note that pattern is a bit like the upper right box, but with CD14-ve/CD16-ve events present.

6. Centre right is a plot derived from Gate 4. It shows events (cells) that express high and low levels of CCR2 according to side scatter. There is a gating line down the middle of this plot to give cells staining high and low staining for CCR2. Gate 5 is cells staining weakly for CCR2 (=M3) whilst Gate 6 is cells staining strongly for CCR2 (=M2).

LOWER THREE PLOTS (all CD42a versus CCR2)

7. Lower left is a plot of CD42a versus CCR2 on population M1. MPAs are to the right of the line

8. Lower middle is a plot of CD42a versus CCR2 in M2. MPAs are to the right of the line

9. Lower right is a plot of CD42a versus CCR2 in M3. MPAs are to the right of the line

Other numbers on the sheet (1- 12) refer to mathematical analyses, not to plots, as, follows….

5. Interpretation of results (numbers)

This is complicated, so pay attention. There are 12 analyses – the first 4 are raw data:

1. The total number of events counted and the acquisition date are given top left of the numbers section (i.e. 60,964 on 08-Apr-10).

2. On the far right is number of count beads (9127) used to quantify events to cells/μL

3. On the left is some maths from the opening plots showing number of total events collected in this particular analysis and the proportion that are monocytes.

4. Below this is the maths from Gates 5 and 6 (SSC and CCR2, middle right plot). So there are 667 M2 events and 871 M3 events, giving you relative proportions. This data is used to calculate the absolute count of subsets M2 and M3.

From these analyses numbers 1 – 4 the machine works out for you (given the count bead number in analysis 2 i.e. 9127) the percentage and numbers of monocytes and monocyte subsets, and these are given as numbers 5 – 12 as follows….

5. Mon is the total number of monocytes per μl, i.e. 582.95 cells/μL.
6. Mon 1 is the number of M1 monocytes per μl, i.e. 409.5 cells/μL.
7. Mon 2 is the number of M2 monocytes per μl, i.e. 98.23 cells/μL
8. Mon 3 is the number of M3 monocytes per μl, i.e. 75.22 cells/μL

The machine has also worked out the % of each subset immediately below.

Next – for MPAs…

9. MPA is the total number of MPAs per μL, i.e. 102.86 cells/μL
10. MPA1 is the total number of MPAs in the M1 population, i.e. 71.29 cells/μL
11. MPA2 is the total number of MPAs in the M2 population, i.e. 19.28 cells/μL
12. MPA3 is the total number of MPAs in the M3 population, i.e. 12.29 cells/μL

From this you can work out the proportions given a calculator. It follows that since you have the platelet count from the Advia, you can also work out how many of the total platelet pool are bound to monocytes. But this is for a separate analysis.

Conclusion

Using this dataset as a template, the numbers that need to go into your spreadsheet are as follows…

Total monocyte count = 582.95 cells/μL

Subsets: M1 count = 409.50 cells/μL (70.25%)
M2 count = 98.23 cells/μL (16.85%)
M3 count = 75.22 cells/μL (12.9%)

Total MPA count = 102.86 cells/μL

Subsets: MPA1 count = 71.29 cells/μL
MPA2 count = 19.28 cells/μL
MPA3 count = 12.29 cells/μL

Once in the spreadsheet, you can easily do the arithmetic for conversion to %

6. Validation and quality control

The intra-assay reproducibility of the methods was assessed on six samples of blood; one set of three from a healthy male and second set of three from a woman with a history of renal and ovarian cancer. The inter-assay reproducibility was derived 'x' samples from a healthy middle aged man.

Intra-assay coefficients of variation (CV) (%) were as follows:

	Subject A	Subject B	Mean
Mon1	0.9	1.4	1.15
Mon2	10.6	9.9	10.25
Mon3	3.9	4.7	4.3
Total Mon	0.6	0.4	0.5
MPA1	3.4	5.2	4.3
MPA2	14.1	9.7	11.9
MPA3	8.2	6.8	7.5
Total MPA	3.2	4.5	3.85
		Median intra assay CV	**4.6%**

Inter-assay coefficients of variation (CV) (%) were as follows:

Signed off……Andrew Blann…… 2010……..

Figures not electronic: illustrative plot (see ADB): Raw analyses available from ADB

APPENDIX 4: Standard Operating Procedure 210; Cytometric bead array

STANDARD OPERATING PROCEDURE 210

Cytometric Bead Array

Dr. Eduard Shantsila and Dr. Silvia Montero-Garcia.

N.B. Use of the flow cytometry is forbidden
Without having been officially trained

Required pre-training

3. SOPs on venepuncture and on good clinical practice

4. SOP 195 – General operation of the flow cytometer

Contents

1. Introduction

The cytometric bead array (CBA) assay combined with flow cytometry (FC) can be used to measure multiple soluble analytes with a particle-based immunoassay in a single tube. This technique may supplant a conventional ELISA with less volume sample for multiple markers (less dilution too), less time and lower costs. And even more, it seems more reliable than conventional ELISA since each bead can be considered as individual test and in one sample you are counting hundreds of beads for each marker instead of only duplicate wells. We will use as many kits as markers (up to 30 markers with the FACScalibur).

Samples are incubated, in batch, with beads bearing a specific antibody, then with PE-conjugated antibodies to form sandwich complexes (like in ELISA), shortly washed and acquired by FC in couple minutes. Easy and fast! The FC data will be then collected and analyze in CBA software (bought license). This software enables linear regression analyses using the standard curves of known concentration.

This SOP is relevant for IL6, MCP-1 and fractalkine.

Measurement of IL-1 and IL-10 failed quality control

2. Materials and Supplier contact details:

2.1 BD “FACS Flow” Running solution [Becton Dickinson, Catalogue No. 342003]10L containers.

2.2 BD “FACS Clean” Cleaning Solution [Becton Dickinson, Catalogue No. 340345]

2.3 3 ml BD Falcon tubes [Becton Dickinson, Catalogue No. 352054]

2.4 15 ml Falcon tube

2.5 Clear pipette tips [Alpha Laboratories Limited, Catalogue No FR1250 1250 ul Fastrak Refill NS]

2.6 Yellow pipette tips [Alpha Laboratories Limited Catalogue No FR1200 200 ul Fastrak Refill NS]

2.7 Human IL-1β Flex Set [Becton Dickinson, Catalogue No. 558279]

2.8 Human IL-6 Flex Set [Becton Dickinson, Catalogue No. 558276]

2.9 Human IL-10 Flex Set [Becton Dickinson, Catalogue No. 558274]

2.10 Human MCP-1 Flex Set [Becton Dickinson, Catalogue No. 558287]

2.11 Human Fractalkine Flex set [Becton Dickinson]

2.12 Human Soluble Protein Master Buffer Kit [Becton Dickinson, Catalogue No.558264]

3. Detailed Method

Bring all reagents to room temperature before use (they are stored at +4^{o}C, generally in the ‘fridge in the flow cytometer room)

3.1. Preparation of the standards (standard curves)

3.1.1 In a 15 mL falcon tube labelled as “TOP STANDARD” put the 4 standards together (all the beads from each kit standard vial) and mix carefully with 4 mL of RPMI-diluent buffer. DO NOT VORTEX, but wait 15 min until all the beads are fully dispersed.

3.1.2 Prepare 10 clean falcon tubes, label them with the following dilutions names (top row table 1) and add 0.5 mL of RPMI-diluent buffer to each (middle row Table 1).

3.1.3 Then make serial (double) dilutions by taking 0.5 mL of the more concentrated dilution to the less concentrated. Mix by pipetting up and down three or four times, again do not vortex. Total: 11 standard tubes with the following concentrations of each marker per tube (bottom row Table 1).

3.1.4 Although BD only recommends storage at 4˚C for one week, the tubes below have been frozen at -70˚C in order to use them in the next assays.

3.1.5 Prepare 11 new FC tubes, label them with the same concentrations and add 50 µL of the below dilutions. These are the tubes for the standard curve that we are going to process by FC. Run the tubes from the least concentrated (0 pg/mL) up to the most (2500 pg/mL).

Table 1

	Top standard	1/ 2	1/ 4	1/ 8	1/ 16	1/ 32	1/ 64	1/ 128	1/ 256	1/ 512	0 (negative control)
RPMI-diluent buffer (mL)	4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Final concentration (pg/mL)	2500	1250	625	312.5	156	80	40	20	10	5	0

3.2. Preparation of the sample tubes

3.2.1 Mark one FC tube per sample
3.2.2 Add 50 µL of the serum or plasma (sample) to each tube.
3.2.3 We are not doing replicates!!

3.3. Master Mix beads preparation

3.3.1 Each kit has a blue tab tube which contains specific capture beads for the chosen marker (50X). We will use 1 µL of this solution (beads-Ab) per marker and sample

3.3.2 For the Master Mix (MM) it is convenient to prepare 2 samples more than we need in order not to run out of beads. We will use these capture beads for:

- Standards
- Samples
- 2 extra samples

3.3.3 Vortex each capture beads vial for 15 seconds to resuspend the solution
3.3.4 Transfer the required volume of each marker bead (1 µL x number of sample), mix all together in a FC tube labelled "MM beads"
3.3.5 Add 0.5 mL of wash buffer (which is basically PBS)
3.3.6 Centrifuge at 300 g, 5 min
3.3.7 Carefully discard the supernatant, do not touch the pellet (beads)
3.3.8 Calculate the volume of diluent required to dilute the beads, taking into account that we are mixing 4 marker beads. Each bead must be diluted in 50 µL with the **diluent for serum/plasma**. 50 minus 4 = 46, hence 46 µL of diluent per sample x number of samples
3.3.9 Incubate "MM beads" for 15 min at RT prior to use
3.3.10 Add 50 µL of "MM beads" to each sample: standards and samples
3.3.11 Leave the samples in the rotator (slowly) for a couple of minutes, in order to mix well.
3.3.12 Then, incubate at RT for **1hour** in darkness

3.4. Preparation of PE-detection reagent

3.4.1 Protect PE Detection Reagent (PE-R) from prolonged exposure to light
3.4.2 At this point we have samples incubating with the specific markers beads, but we need to stain the beads-bound to analytes with PE
3.4.3 Each kit contains its own PE-Detection Reagent (50X).
3.4.4 Like in the case of beads, we will transfer 1 µL/sample of each PE-R to a new FC tube labelled "PE-Detection reagent mixture", and mix the 4 PE-R together.
3.4.5 These reagents do not need to be washed
3.4.6 Calculate the volume of diluent required to dilute the PE-R, taking into account that we are mixing 4 PE-R. Each PE-R must be dilute in 50 µL with the **detection dilution buffer**. Example : 50 – 4 = 46, hence 46 µL of diluent per sample x number of samples
3.4.7 Add 50 µL of "PE-Detection reagent mixture" to each 1 hour incubated sample: standards and samples
3.4.8 Leave the samples in the rotator (slowly) for a couple of minutes, in order to homogenate.
3.4.9 Then incubate at RT for **2 hours** in darkness

3.5. Running samples in flow cytometer

3.5.1 Add to each incubated sample (1 hour with beads and 2 hours with PE-R), 1 mL wash buffer to stop the incubation
3.5.2 Centrifuge 300 *g*, 5min
3.5.3 Discard supernatant carefully without touching the beads
3.5.4 Add 300 µL wash Buffer to each sample
3.5.5 Switch on the flow cytometer (SOP 195 – General operation of the flow cytometer)
3.5.6 Open CBA protocol and CBA instrument settings in CBA folder (or a copy of it in your folder)
3.5.7 Vortex slowly the tubes to resuspend beads before FC
3.5.8 Run FC "CBA Array Protocol" at **low speed** (acquisition will be completed automatically once 1200 beads are collected).
3.5.9 Run first standards from bottom to top concentration
3.5.10 Run the samples
3.5.11 Copy folder with results in a USB.
3.5.12 NB!!! You do not need to make any printouts
3.5.13 Switch off the flow cytometer (SOP 195 – General operation of the flow cytometer)
3.5.14 Go to a computer with the FACArray 2.0 software to calculate concentrations of the cytokines in your samples. The results will be presented in pg/mL.
3.5.15 Print a report to have a hard copy of your results (an example is attached).
3.5.16 Export data into a spreadsheet for statistical analysis.

SOP 210: Cytometric Bead Array analysis by flow cytometry

Signed off……..

Appendices: Becton Dickinson kit insert of MCP-1 protocol

Validation data x 2

Assay performance

	Lower limit of sensitivity	Intra-assay CV
IL-6	1 pg/mL	19.8%*
IL-1 beta	1 pg/mL	Not possible*
IL-10	1 pg/mL	Not possible*
MCP-1	1 pg/mL	12.2%*
Fractalkine (February 2012)	10 pg/mL (the point at which left tail of standard curve flattens out)	3.1% (n=10)

*n=8, **Analyte undetectable in the plasma, so assay unvalidated

REFERENCES

Ajuebor M.N., Flower R.J., Hannon R. et al (1998). Endogenous monocyte chemoattractant protein-1 recruits monocytes in the zymosan peritonitis model. **J Leukoc Biol**, 63: 108-116.

Ancuta P., Rao R., Moses A., et al (2003). Fractalkine preferentially mediates arrest and migration of CD16+ monocytes. **J Exp Med**, 197: 1701–7.

Ancuta P., Wang J., Gabuzda D. (2006). CD16+ monocytes produce IL-6, CCL2, and matrix metalloproteinase-9 upon interaction with CX3CL1-expressing endothelial cells. **J Leukoc Biol**, 80: 1156 – 64.

Anderson D.R., Poterucha J.T., Mikuls T.R. et al (2013). IL-6 and its receptors in coronary artery disease and acute myocardial infarction. **Cytokine**, 62(3): 395-400.

Antman E.M., Cohen M., Bernink P.J.et al (2000). The TIMI risk score for unstable angina/non-ST elevation MI: a method for prognostication and therapeutic decision making. **JAMA**, 284:835–842.

Arslan U., Kocaoğlu I., Balci M.M. et al (2013). Monocyte heterogeneity in myocardial infarction with and without ST elevation and its association with angiographic findings. **Coronary Artery Disease**, 24: 404-411.

Asehnoune K., Strassheim D., Mitra S. et al (2004). Involvement of reactive oxygen species in Toll-like receptor 4-dependent activation of NF-kappa B. **J Immunol**, 172(4): 2522-2529.

Auffray C., Fogg D., Garfa M. et al (2007). Monitoring of blood vessels and tissues by a population of monocytes with patrolling behaviour. **Science**, 317: 666-670.

Auffray C., Sieweke M.H., Geissmann, F. (2009). Blood monocytes: development, heterogeneity, and relationship with dendritic cells. **Annu Rev Immunol**, 27: 669-692.

Azeredo E.L., Neves-Souza P.C., Alvarenga A.R. et al (2010). Differential regulation of toll-like receptor 2, toll-like receptor 4, CD16 and human leucocyte antigen-DR on peripheral blood monocytes during mild and severe dengue fever. **Immunology**, 130(2): 202-216.

Baruch A., van Bruggen N., Kim J.B. et al (2013). Anti-Inflammatory Strategies for Plaque Stabilisation after Acute Coronary Syndromes. **Curr Atheroscler Rep**, 15:327.

Bassand J.P., Hamm C.W., Ardissino D. et al (2007). Guidelines for the diagnosis and treatment of non-ST-segment elevation acute coronary syndromes. **Eur Heart J**, 28:1598–1660.

Belge K.U., Dayyani F., Horelt A. et al (2002). The proinflammatory CD14+CD16++DR++ monocytes are a major source of TNF. **J Immunol**, 168: 3536–3542.

Benson V.L., McMahon A.C., Khachigian L.M. et al (2013). Acute local elevation in monocyte chemoattractant protein-1 (MCP-1), distal to the culprit lesion in acute ST elevation myocardial infarction. **Int J Cardiol**, doi: 10.1016/j.ijcard.2013.03.078.

Bernard S.A., Gray T.W., Buist M.D. et al (2002). Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. **N Engl J Med**, 346: 557–563.

Bevilacqua M.P., Nelson R.M., Mannori G. et al (1994). Endothelial-leukocyte adhesion molecules in human disease. **Annu Rev Med,** 45: 361-378.

Bocan T.M. (2002). Pleiotropic effects of HMG-CoA reductase inhibitors. **Curr Opin Investig Drugs**, 3(9): 1312-1317.

Buechler C., Ritter M., Orso E. et al (2000). Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and anti-inflammatory stimuli. **J Leukoc Biol**, 67(1): 97-103.

Buffon A., Biasucci L.M., Liuzzo G. et al (2002). Widespread coronary inflammation in unstable angina. **N Engl J Med**, 347:5–12.

Cannon C.P., Braunwald E., McCabe C.H. et al (2004). Intensive versus moderate lipid lowering with statins after acute coronary syndromes. **N Engl J Med**, 350: 1495–1504.

Carlin L.M., Stamatiades E.G., Auffray C. et al (2013). Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. **Cell**, 153: 362-375.

Chen Y., Wermeling F., Sundqvist J. et al (2008). A regulatory role for macrophage class A scavenger receptors in TLR4-mediated LPS responses. **Eur J Immunol**, 40: 1451-1460.

Chen Z.M., Jiang L.X., Chen Y.P. et al (2005). Addition of clopidogrel to aspirin in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial. **Lancet**, 366: 1607–1621.

Cooper D.L., Martin S.G., Robinson J.I., et al (2012). FcγRIIIa expression on monocytes in rheumatoid arthritis: role in immune-complex stimulated TNF production and non-response to methotrexate therapy. **PLoS One**, e28918. doi: 10.1371/journal.pone.0028918.

Cotran R.S., Mayadas-Norton, T (1998). Endothelial adhesion molecules in health and disease. **Pathol Biol (Paris)**, 46: 164-170.

Cros J., Cagnard N., Woollard K. et al (2010). Human CD14dim Monocytes Patrol and Sense Nucleic Acids and Viruses via TLR7 and TLR8 Receptors. **Immunity**, 33(3): 375-386.

De Luca G., Suryapranata H., Stone G.W. et al (2005). Abciximab as adjunctive therapy to reperfusion in acute ST-segment elevation myocardial infarction: a meta-analysis of randomized trials. **JAMA**, 293: 1759–1765.

De Palma M., Venneri M.A., Galli R. et al (2005). Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. **Cancer cell**, 8(3): 211-26.

De Servi S., Mazzone A., Ricevuti G. et al (1995). Clinical and angiographic correlates of leukocyte activation in unstable angina. **J Am Coll Cardiol**, 26: 1146-1150.

Dresske B., El Mokhtari N.E., Ungefroren H. et al (2006). Multipotent cells of monocytic origin improve damaged heart function. **Am J Transplant,** 6: 947-958.

Dutta P., Courties G., Wei Y. et al (2012). Myocardial infarction accelerates atherosclerosis. **Nature,** 487(7407): 325-329.

Edfeldt K., Swedenborg J., Hansson G.K. et al (2002). Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. **Circulation**, 105(10): 1158-1161.

Emura I., Usuda H., Fujita T. et al (2007). Increase of scavenger receptor A-positive monocytes in patients with acute coronary syndromes. **Pathol Int**, 57: 502-508.

European Society of Cardiology/American College of Cardiology Committee (2000). Myocardial infarction redefined — A consensus document of the Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction. **Eur Heart J**, 21: 1502–1513; **J Am Coll Cardiol**, 36: 959–969.

Febbraio M., Podrez E.A., Smith J.D. et al (2000). Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. **J Clinical Invest**, 105(8): 1049-1056.

Foster G.A., Gower R.M., Stanhope K.L. et al (2013). On-chip phenotypic analysis of inflammatory monocytes in atherogenesis and myocardial infarction. **Proc Natl Acad Sci U S A**, 110(34): 13944-13949.

Fox K.M. (2003). Efficacy of perindopril in reduction of cardiovascular events among patients with stable coronary artery disease: randomised, double-blind, placebo controlled, multicentre trial (the EUROPA study). **Lancet**, 362: 782–788.

Fox K.A , Dabbous O.H., Goldberg R.J. et al (2006). Prediction of risk of death and myocardial infarction in the six months after presentation with acute coronary syndrome: prospective multinational observational study (GRACE). **BMJ**, 333 (7578): 1091.

Fox K., Garcia M.A., Ardissino D.J et al (2006). Guidelines on the management of stable angina pectoris: executive summary: the task force on the management of stable angina pectoris of the European society of cardiology. **Eur Heart J**, 27(11): 1341-1381.

Fox K.A., Eagle K.A., Gore J.M. et al (2010). The Global Registry of Acute Coronary Events, 1999 to 2009 - GRACE. **Heart**, 96: 1095–1101.

Fox K.A., Clayton T.C., Damman P. et al (2010). Long-term outcome of a routine versus selective invasive strategy in patients with non-ST-segment elevation acute coronary syndrome a meta-analysis of individual patient data. **J Am Coll Cardiol**, 55: 2435–2445.

Furman M.I., Barnard M.R., Krueger L.A. et al (2001). Circulating monocyte-platelet aggregates are an early marker of acute myocardial infarction. **J Am Coll Cardiol**, 38(4): 1002-1006.

Geissmann F., Jung S., Littman D.R. (2003). Blood monocytes consist of two principal subsets with distinct migratory properties. **Immunity**, 19(1): 71-82.

Gerrity R.G. (1981). The role of the monocyte in atherogenesis: II. Migration of foam cells from atherosclerotic lesions. **Am J Pathol**, 103(2): 191-200.

Gordon S. and Taylor P.R. (2005). Monocyte and macrophage heterogeneity. **Nat Rev Immunol**, 5(12): 953-964.

Gonzalez P., Alvarez R., Batalla A. et al (2001). Genetic variation at the chemokine receptors CCR5/CCR2 in myocardial infarction. **Genes Immun**, 2:191–195.

Gown A.M., Tsukada T., Ross R. (1986). Human atherosclerosis. II. Immunocytochemical analysis of the cellular composition of human atherosclerotic lesions. **Am J Pathol**, 125(1): 191-207.

GUSTO investigators (1993). An international randomized trial comparing four thrombolytic strategies for acute myocardial infarction. The GUSTO investigators. **N Engl J Med**, 329: 673–682.

Hamm C.W., Bassand J.P., Agewall S. et al (2011). ESC guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: The task force for the management of acute coronary syndromes (ACS) in patients presenting without persistent st-segment elevation of the european society of cardiology (ESC). **Eur Heart J**, 32: 2999-3054.

Heberden (1772). Some account of a disorder of the breast. **Med Transact R Coll Phys Lond** , 2: 59.

Heine G.H., Ulrich C., Seibert,E., et al (2008). CD14(++)CD16+monocytes but not total monocyte numbers predict cardiovascular events in dialysis patients. **Kidney Int**, 73, 622–629.

Hillis G.S., Dalsey W.C., Terregino C.A. et al (2001). Altered cd18 leucocyte integrin expression and adhesive function in patients with an acute coronary syndrome. **Heart**, 85: 702-704.

Holzer M on behalf of the Hypothermia after Cardiac Arrest Study Group (2002). Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. **N Engl J Med**, 346: 549–556.

Hristov M., Leyendecker T., Schuhmann C. et al (2010). Circulating monocyte subsets and cardiovascular risk factors in coronary artery disease. **Thromb Haemost,** 104(2): 412-414.

Hristov M., Schmitz S., Nauwelaers F (2012). A flow cytometric protocol for enumeration of endothelial progenitor cells and monocyte subsets in human blood. **J Immunol Methods**, 381: 9-13.

Hu Y., Zhang H., Lu Y. et al (2011). Class A scavenger receptor attenuates myocardial infarction-induced cardiomyocyte necrosis by suppressing M1 macrophage subset polarisation. **Basic Res Cardiol**, 106: 1311-1328.

Ikejima H., Imanishi T., Tsujioka H. et al (2010). Upregulation of fractalkine and its receptor, CX3CR1, is associated with coronary plaque rupture in patients with unstable angina pectoris. **Circ J,** 74(2): 337-345.

Imanishi T., Ikejima H., Tsujioka H. et al (2010). Association of monocyte subset counts with coronary fibrous cap thickness in patients with unstable angina pectoris. **Atherosclerosis**, 212(2): 628-635.

Ishikawa Y., Satoh M., Itoh T. et al (2008). Local expression of Toll-like receptor 4 at the site of ruptured plaques in patients with acute myocardial infraction. **Clin Sci (Lond)**, 115(4): 133-140.

ISIS-2 (Second International Study of Infarct Survival) Collaborative Group (1998). Randomised trial of intravenous streptokinase, oral aspirin, both, or neither among 17187 cases of suspected acute myocardial infarction: ISIS-2. **Lancet**, 8607: 349-360.

Jang Y., Lincoff A.M., Plow E.F. et al (1994). Cell adhesion molecules in coronary artery disease. **J Am Coll Cardiol**, 24: 1591-1601.

Jolly S.S., Yusuf S., Cairns J. et al (2011). Radial vs. femoral access for coronary angiography and intervention in patients with acute coronary syndromes (RIVAL): a randomised, parallel group, multicentre trial. **Lancet**, 377: 1409–1420.

Jonasson L., Holm J., Skalli O. et al (1986). Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. **Arteriosclerosis**, 6(2): 131-138.

Julian D.G. (1961). Treatment of cardiac arrest in acute myocardial ischaemia and infarction. **Lancet**, 7207: 840-4.

Julian D.G.(1968). Coronary Care and the Community. **Ann Intern Med**, 69(3): 607-613.

Jung K., Kim P., Leuschner F. et al (2013). Endoscopic time-lapse imaging of immune cells in infarcted mouse hearts. **Circ Res**, 112: 891-899.

Kashiwagi M., Imanishi T., Tsujioka H. et al (2010). Association of monocyte subsets with vulnerability characteristics of coronary plaques as assessed by 64-slice multidetector computed tomography in patients with stable angina pectoris. **Atherosclerosis**, 212(1): 171-176.

Kashiwagi M., Imanishi T., Ozaki Y. et al (2012). Differential expression of Toll-like receptor 4 and human monocyte subsets in acute myocardial infarction. **Atherosclerosis**, 221(1): 249-253.

Kastrati A., Dibra A., Spaulding C. et al (2007). Meta-analysis of randomized trials on drug-eluting stents vs. baremetal stents in patients with acute myocardial infarction. **Eur Heart J**, 28: 2706–2713.

Keeley E.C., Boura J.A., Grines C.L. (2003). Primary angioplasty versus intravenous thrombolytic therapy for acute myocardial infarction: a quantitative review of 23 randomised trials. **Lancet**, 361: 13-20.

Kim W. K., Sun Y., Do H, et al (2010). Monocyte heterogeneity underlying phenotypic changes in monocytes according to SIV disease stage. **J Leukoc Biol**, 87: 557-567.

Kobayashi H., Sakashita N., Okuma T. et al (2007). Class A scavenger receptor (CD204) attenuates hyperoxia-induced lung injury by reducing oxidative stress. **J Pathol**, 212: 38-46.

Kong P.K., Connolly D., Varma C. et al (2009). High-risk myocardial infarction patients appear to derive more mortality benefit from short door-to-balloon time than low-risk patients. **Int J Clin Pract**, 63(12): 1693-701.

Kuckleburg C.J., Yates C.M., Kalia N. et al (2011). Endothelial cell-borne platelet bridges selectively recruit monocytes in human and mouse models of vascular inflammation. **Cardiovasc Res**, 91(1): 134-141.

Lawler P.R., Filion K.B., Eisenberg M.J. (2011). Efficacy of exercise-based cardiac rehabilitation post-myocardial infarction: a systematic review and meta-analysis of randomized controlled trials. **Am Heart J**, 162(4): 571–584.

Lee K.W, Lip G.Y.H., Blann A.D. (2004). Plasma Angiopoietin-1, Angiopoietin-2, Angiopoietin Receptor Tie-2, and Vascular Endothelial Growth Factor Levels in Acute Coronary Syndromes. **Circulation**, 110: 2355-2360.

Lee K.W., Lip G.Y.H., Tayebjee et al (2005). Circulating endothelial cells, von Willebrand factor, interleukin-6, and prognosis in patients with acute coronary syndromes. **Blood**, 105: 526-532.

Lee S., Vinegoni C., Feruglio P.F. et al (2012). Real-time in vivo imaging of the beating mouse heart at microscopic resolution. **Nat Commun**, 3: 1054.

Lee W.W., Marinelli B. van der Laan A.M. et al (2012). PET/MRI of inflammation in myocardial infraction. **J Am Coll Cardiol**, 59: 153-163.

Leuschner F., Rauch P.J., Ueno et al (2012). Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopoiesis. **J Exp Med**, 209: 123-137.

Libby P. (1995). Molecular bases of the acute coronary syndromes. **Circulation**, 91: 2844-2850.

Libby P., Nahrendorf M., Pittet M.J. et al (2008). Diversity of denizens of the atherosclerotic plaque: not all monocytes are created equal. **Circulation**, 117(25): 3168-3170.

Liehn E.A., Tuchscheerer N., Kanzler I., et al (2011). Double-edged role of the CXCL12/CXCR4 axis in experimental myocardial infarction. **J Am Coll Cardiol**, 58: 2415-2423.

Lo Celso C., Fleming H.E., Wu J.W. et al (2009). Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. **Nature,** 457(7225): 92-96.

Maekawa Y., Anzai T., Yoshikawa T. et al (2002). Prognostic significance of peripheral monocytosis after reperfused acute myocardial infarction: a possible role for left ventricular remodeling. **J Am Coll Cardiol**, 39: 241-246.

Majmudar M.D., Keliher E.J.. Heidt T.H. et al. Monocyte-directed RNAi targeting CCR2 improves infarct healing in atherosclerosis-prone mice. **Circulation**, 127(20): 2038-2046.

Mandelzweig L., Battler A., Boyko V. et al (2006). The second Euro Heart Survey on acute coronary syndromes: characteristics, treatment, and outcome of patients with ACS in Europe and the Mediterranean Basin in 2004. **Eur Heart J**, 27: 2285–2293.

Mayr F.B., Spiel A.O., Leitner J.M., et al (2009). Influence of the Duffy antigen on pharmacokinetics and pharmacodynamics of recombinant monocyte chemoattractant protein (MCP-1, CCL-2) in vivo. **Int J Immunopathol Pharmacol**, 22: 615-625.

Mazzone A., Ricevuti G. (1995). Leukocyte cd11/cd18 integrins: Biological and clinical relevance. **Haematologica**, 80: 161-175.

Mazzone A., De Servi S., Ricevuti G et al (1993). Increased expression of neutrophil and monocyte adhesion molecules in unstable coronary artery disease. **Circulation**, 88: 358-363.

Medzhitov (2007). TLR-mediated innate immune recognition. **Semin Immunol**, 19(1): 1-2.

Mehta N.N., Li M., William D. et al (2011). The novel atherosclerosis allele at 10q11 regulates plasma CXCL12 levels. **Eur Heart J**, 32(8): 963-971.

Methe H., Kim J.O., Kofler S. et al. Expansion of circulating Toll-like receptor 4-positive monocytes in patients with acute coronary syndrome. **Circulation**, 111(20): 2654-2661.

Michel J.B., Virmani R., Arbustini E. et al (2011). Intraplaque haemorrhages as the trigger of plaque vulnerability. **Eur Heart J**, 32(16): 1977-1985.

Milonas C., Jernberg T., Lindbäck J. et al (2010). Effect of Angiotensin-converting enzyme inhibition on one-year mortality and frequency of repeat acute myocardial infarction in patients with acute myocardial infarction. **Am J Cardiol**, 105(9): 1229-34.

Montalescot G., Zeymer U., Silvain J. et al (2011). Intravenous enoxaparin or unfractionated heparin in primary percutaneous coronary intervention for ST-elevation myocardial infarction: the international randomised open-label ATOLL trial. **Lancet**, 378: 693–703.

Montoro-Garcia S., Shantsila E., Orenes-Pinero E. et al (2012). An innovative flow cytometric approach for small-size platelet microparticles: Influence of calcium. **Thromb Haemost**, 108: 373-383.

Morimoto H., Takahashi M., Shiba Y. et al (2007). Bone marrow-derived CXCR4+ cells mobilized by macrophage colony-stimulating factor participate in the reduction of infarct area and improvement of cardiac remodelling after myocardial infarction in mice. **Am J Pathol**, 171(3): 755-766.

Muntinghe F.L., Verduijn M., Zuurman M.W., et al (2009). CCR5 deletion protects against inflammation-associated mortality in dialysis patients. **J Am Soc Nephrol**, 20:1641–1649.

Murphy R.T., Foley J.B., Crean P. et al (2003). Reciprocal activation of leukocyte-endothelial adhesion molecules in acute coronary syndromes. **Int J Cardiol**, 90: 247-252.

Murray C.J., Lopez A.D. (1997). Alternative projections of mortality and disability by cause 1990–2020: Global Burden of Disease Study. **Lancet**, 349: 1498–1504.

Nahrendorf M., Swirski F.K., Aikawa E. et al (2007). The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. **J Exp Med,** 204: 3037-3047.

Nahrendorf M., Sosnovik D.E., Waterman P. et al (2007). Dual channel optical tomographic imaging of leukocyte recruitment and protease activity in the healing myocardial infarct. **Circ Res**, 100: 1218-1225.

Nahrendorf M., Pittet M.J., Swirski F.K. (2010). Monocytes: Protagonists of infarct inflammation and repair after myocardial infarction. **Circulation**, 121: 2437-2445.

Nakamura K., Miyagi K., Koguchi Y. et al (2007). Toll-like receptor 4 signal transduction inhibitor, M62812, suppresses endothelial cell and leukocyte activation and prevents lethal septic shock in mice. **Eur J Pharmacol**, 569(3): 237-243.

Napoli C., D'Armiento F.P., Mancini F.P. et al (1997). Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. Intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions. **J Clin Invest,** 100(11): 2680-90.

Noman A., Ahmed J.M., Spyridopoulos I. et al (2012). Mortality outcome of out-of-hours primary percutaneous coronary intervention in the current era. **Eur Heart J**, 33(24): 3046-3053.

Pai J.K., Kraft P., Cannuscio C.C., et al (2006). Polymorphisms in the CCchemokine receptor-2 (CCR2) and -5 (CCR5) genes and risk of coronary heart disease among US women. **Atherosclerosis**, 186: 132–139.

Panizzi P., Swirski F.K., Figueiredo J.L. et al (2010). Impaired infarct healing in atherosclerotic mice with Ly-6C(hi) monocytosis. **J Am Coll Cardiol**, 55(15): 1629-1638.

Park S.Y., Lee .J.H., Kim Y.K. et al (2005). Cilostazol prevents remnant lipoprotein particle-induced monocyte adhesion to endothelial cells by suppression of adhesion molecules and monocyte chemoattractant protein-1 expression via lectin-like receptor for oxidized low-density lipoprotein receptor activation. **J Pharmacol Exp Ther**, 312(3): 1241-1248.

Parry C.H. (1799). An inquiry into the symptoms and causes of syncope anginosa, commonly called angina pectoris. Edinburgh, London: Bryce, Murray and Callow; 1799.

Passlick B., Flieger D. & Ziegler-Heitbrock H.W. (1989). Identification and characterization of a novel monocyte subpopulation in human peripheral blood. **Blood**, 74(7): 2527-2534.

Pitt B., Remme W., Zannad F. et al (2003). Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. **N Engl J Med**, 348:1309-1321.

Ray K.K., Cannon C.P., McCabe C.H. (2005). Early and late benefits of high-dose atorvastatin in patients with acute coronary syndromes: results from the PROVE IT-TIMI 22 trial. **J Am Coll Cardiol**, 46(8): 1405-1410.

Reich D., Patterson N., Ramesh V. et al (2007). Admixture mapping of an allele affecting interleukin 6 soluble receptor and interleukin 6 levels. **Am J Hum Genet**, 80: 716-726.

Ridker P.M., Danielson E., Fonseca F.A. et al (2008). Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. **N Eng J Med**, 359: 2195-2207.

Ridker P.M. (2009). Testing the inflammatory hypothesis of atherothrombosis: scientific rationale for the cardiovascular inflammation reduction trial (CIRT). **J Thromb Haemost** 2009; **7** (suppl 1)**:** 332-339.

Ridker P.M., Thuren T., Zalewski A. et al (2011). Interleukin-1β inhibition and the prevention of recurrent cardiovascular events: rationale and design of the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS). **Am Heart J**, 162: 597-605.

Rijken D.C. & Lijnen H.R. (2009). New insights into the molecular mechanisms of the fibrinolytic system. **J Thromb Haemost**, 7(1): 4-13.

Robbins C.S., Chudnovskiy A., Rauch P.J. et al (2012). Extramedullary haematopoiesis generates Ly-6C (high) monocytes that infiltrate atherosclerotic lesions. **Circulation**, 125: 364-374.

Rogacev K.S., Ulrich C., Blomer L., et al (2010). Monocyte heterogeneity in obesity and subclinical atherosclerosis. **Eur Heart J**, 31: 369-376.

Rogacev K.S., Seiler S., Zawada A.M. et al (2011). CD14++CD16+ monocytes and cardiovascular outcome in patients with chronic kidney disease. **Eur Heart J**, 32(1): 84-92.

Rogacev K.S., Cremers B., Zawada A.M. et al (2012). CD14++CD16+ monocytes independently predict cardiovascular events. **J Am Coll Cardiol**, 60(16): 1512-1520.

Roger V.L., Go A.S., Lloyd-Jones D.M. et al (2012). Executive summary: heart disease and stroke statistics-2012 update: a report from the American Heart Association. **Circulation**, 125: 188–197.

Sarwar N., Butterworth A.S., Freitag D.F. et al (2012). Interleukin-6 receptor pathways in coronary heart disease: A collaborative meta-analysis of 82 studies. **Lancet**, 379: 1205-1213.

Satoh M., Shimoda Y., Maesawa C. et al (2006). Activated toll-like receptor 4 in monocytes is associated with heart failure after acute myocardial infarction. **Int J Cardiol**, 109(2): 226-234.

Schaer C.A., Schoedon G., Imhof A. et al (2006). Constitutive endocytosis of CD163 mediated hemoglobin-heme uptake and determines the non-inflammatory and protective transcriptional response of macrophages to haemoglobin. **Circ Res**, 99: 943-950.

Schauer D. ,Starlinger P., Reiter, C., et al (2012). Intermediate monocytes but not TIE2-expressing monocytes are a sensitive diagnostic indicator for colorectal cancer. **PLoSONE** 7:e44450. doi:10.1371/journal.pone.0044450

Schirmer S.H., Fledderus J.O., van der Laan A.M. et al (2009). Suppression of inflammatory signalling in monocytes from patients with coronary artery disease. **J Mol Cell Cardiol**, 46(2): 177-185.

Schlitt A., Heine G H , Blankenberg S. et al (2004). CD14+CD16+ monocytes in coronary artery disease and their relationship to serum TNF-alpha levels. **Thromb Haemost**, 92:419-24.

Shah P.K., Falk E., Badimon J.J. et al (1995). Human monocyte-derived macrophages induce collagen breakdown in fibrous caps of atherosclerotic plaques. Potential role of matrix-degrading metalloproteinases and implications for plaque rupture. **Circulation**, 92(6): 1565-1569.

Shantsila E., Lip G.Y. (2009). Monocytes in acute coronary syndromes. **Arterioscler Thromb Vasc Biol**, 29: 1433-1438.

Shantsila E., Lip G.Y. (2009). The role of monocytes in thrombotic disorders. Insights from tissue factor, monocyte-platelet aggregates and novel mechanisms. **Thromb Haemost**, 102: 916-924.

Shantsila, E., Lip G.Y.H. (2009). Monocyte diversity in myocardial infarction. **J Am Coll Cardiol**, 54(2): 139-142.

Shantsila E., Wrigley B., Tapp L. et al (2011). Immunophenotypic characterization of human monocyte subsets: Possible implications for cardiovascular disease pathophysiology. **J Thromb Haemost**, 9: 1056-1066.

Shantsila E., Montoro-Garcia S., Tapp L.D. et al (2012). Fibrinolytic status in acute coronary syndromes: Evidence of differences in relation to clinical features and pathophysiological pathways. **Thromb Haemost**, 108: 32-40.

Shantsila E., Montoro-Garcia S., Lip G.Y. (2014). Monocytes circulate in constant reversible intercation with platelets in a [Ca2+]-dependent manner. **Platelets**, 25(3): 197-201.

Shantsila E., Tapp L.D., Wrigley B.J. et al (2013). CXCR4 positive and angiogenic monocytes in myocardial infarction. **Thromb Haemost**, 109(2): 255-62.

Shantsila E., Tapp L.D., Wrigley B.J. et al (2013). Receptors to Interleukin-6 and adhesion molecules on circulating monocyte subsets in acute myocardial infarction. **Thromb Haemost**, 110(2): 340-348.

Shantsila E., Tapp L.D., Wrigley B.J. et al (2014). Monocyte subsets in coronary artery disease and their associations with markers of inflammation and fibrinolysis. **Atherosclerosis**, 234(1): 4-10.

Sheu J.J., Chang L.T., Chiang C.H. et al (2008). Prognostic value of activated toll-like receptor-4 in monocytes following acute myocardial infarction. **Int Heart J**, 49(1): 1-11.

Shiraki R, Inoue N., Kobayashi S. et al (2006). Toll-like receptor 4 expressions on peripheral blood monocytes were enhanced in coronary artery disease even in patients with low C-reactive protein. **Life Sci**, 80(1): 59-66.

Silverstein R.L., Febbraio M. (2000). CD36 and atherosclerosis. **Cur Opin Lipidol**, 11(5): 483-491.

Simon D.I., Ezratty A.M., Francis S.A. e al (1993). Fibrin(ogen) is internalized and degraded by activated human monocytoid cells via Mac-1 (CD11b/CD18): a nonplasminfibrinolytic pathway. **Blood**, 82(8): 2414-2122.

Simpson P.J., Todd R.F. 3rd, Fantone J.C. et al (1988). Reduction of experimental canine myocardial reperfusion injury by a monoclonal antibody (anti-mo1, anti-cd11b) that inhibits leukocyte adhesion. **J Clin Invest**, 81:624-629.

Skrzeczyńska-Moncznik J., Bzowska M., Loseke S. et al (2008). Peripheral blood CD14high CD16+ monocytes are main producers of IL-10. **Scand J** Immuno, 67(2): 152-159.

Sosnovik D.E., Nahrendorf M. Deliolanis N. et al. Fluorescence tomography and magnetic resonance imaging of myocardial macrophage infiltration I infarcted myocardium in vivo. **Circulation**, 115: 1384-1391.

Steg G., James S.K., Atar D. et al (2012). ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation. **Eur Heart J**, 33: 2569–2619.

Steppich B., Dayyani F., Gruber R. et al (2000). Selective mobilization of CD14(+)CD16(+) monocytes by exercise. **Am J Physiol Cell Physiol**, 279: C578–586.

Stone G.W., Witzenbichler B., Guagliumi G. et al (2008). Bivalirudin during primary PCI in acute myocardial infarction. **N Engl J Med**, 358: 2218-2230.

Stone G.W., Maehara A., Lansky A.J. et al (2011). A prospective natural-history study of coronary atherosclerosis. **N Engl J Med,** 364: 226–235.

Stoneman V., Braganza D., Figg N. et al (2007). Monocyte/macrophage suppression in CD11b diphtheria toxin receptor transgenic mice differentially affects atherogenesis and established plaques. **Circ Res**, 100(6): 884-893.

Sulicka J., Surdacki A., Mikola-jczyk T., et al (2013). Elevated markers of inflammation and endothelial activation and increased counts of intermediate monocytes in adult survivors of childhood acute lymphoblastic leukaemia. **Immunobiology** doi:10.1016/j.imbio.2012.09.003

Sunderkötter C., Nikolic T., Dillon M.J. et al (2004). Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. **J Immunol** 172(7): 4410-4107.

Swirski F.K., Libby P., Aikawa E. et al (2007). Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. **J Clinical Invest**, 117(1): 195-205.

Swirski F.K., Nahrendorf M., Etzrodt M. et al (2009). Identification of splenic reservoir monocytes and their deployment to inflammatory sites, **Science**, 325: 612-616.

Swirski F.K., Nahrendorf M (2013). Leukocyte behaviour in atherosclerosis, myocardial infarction, and heart failure. **Science**, 339: 161-166.

Svilaas T., Vlaar P.J., van der Horst I.C. et al (2008). Thrombus aspiration during primary percutaneous coronary intervention. **N Engl J Med** 2008; 358:557–567.

Tabas I.(2005). Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. **Arterioscler Thromb Vasc Biol**, 25(11):2255-64.

Tacke F., Alvarez D., Kaplan T.J. et al (2007). Monocyte subsets differentially employ CCR2, CCR5 and CX3CR1 to accumulate within atherosclerotic plaques. **J Clin Invest**, 117: 185-194.

Takarada S., Imanishi T., Kubo T. et al (2009). Effect of statin therapy on coronary fibrous-cap thickness in patients with acute coronary syndrome: assessment by optical coherence tomography study. **Atherosclerosis**, 202(2): 491-497.

Tallone T., Turconi G., Soldati G. et al (2011). Heterogeneity of human monocytes: an optimized four-color flow cytometry protocol for analysis of monocyte subsets. **J Cardiovasc Transl Res**, 4(2): 211-219.

Tapp L.D., Shantsila E., Wrigley B.J. et al (2012). The CD14++CD16+ monocyte subset and monocyte-platelet interactions in patients with ST-elevation myocardial infarction. **J Thromb Haemost**, 10(7): 1231-41.

Tapp L.D., Shantsila E., Wrigley B.J. et al (2013). TLR4 expression on monocyte subsets in myocardial infarction. **J Intern Med**, 273(3): 294-305.

Teupser D., Stein O., Burkhardt R. et al (1999). Scavenger receptor activity is increased in macrophages from rabbits with low atherosclerotic response: studies in normocholesterolemic high and low atherosclerotic response rabbits. **Arterioscler Thromb Vasc Biol**, 19(5): 1299-1305.

Thompson A., Gao P., Orfei L. et al (2010). Lipoprotein-associated phospholipase A(2) and risk of coronary disease, stroke, and mortality: collaborative analysis of 32 prospective studies. **Lancet**; 375: 1536–1544.

Thygesen K., Alpert J.S., White H.D. (2007). Universal definition of myocardial infarction. **Eur Heart J**, 28: 2525–2538; **Circulation**, 116: 2634–2653; **J Am Coll Cardiol,** 50: 2173–2195.

Thygesen K., Alpert J.S., White H.D. et al (2012). Third universal definition of myocardial infarction. **Euro Heart J**, 33: 2551–2567.

Todt J.C., Hu B., Curtis J.L. et al (2008). The scavenger receptor SR-A I/II (CD204) signals via the receptor tyrosine kinase Mertk during apoptotic cell uptake by murine macrophages. **J Leukoc Biol**, 84: 510-518.

Tsujioka H., Imanishi T., Ikejima H. et al (2009). Impact of heterogeneity of human peripheral blood monocyte subsets on myocardial salvage in patients with primary acute myocardial infarction. **J Am Coll Cardiol**, 54(2): 130-138.

Tsujioka H., Imanishi T., Ikejima H. et al (2010). Post-reperfusion enhancement od CD14(+)CD16(-) monocytes and microvascular obstruction in ST-segment elevation acute myocardial infarction. **Circ J**, 74(6): 1175-82.

Tsujita K., Kaikita K., Hayasaki T. et al (2007). Targeted deletion of class A macrophage scavenger receptor increases the risk of cardiac rupture after experimental myocardial infarction. **Circulation**, 115: 1904-1911.

Vabulas R.M., Ahmad-Nejad P. Ghose S. et al (2002). HSP70 as endogenous stimulus of Toll/interleukin-1 receptor signal pathway. **J Biol Chem**, 276(33): 15107-15112.

Valgimigli M., Biondi-Zoccai G., Tebaldi M. et al (2010). Tirofiban as adjunctive therapy for acute coronary syndromes and percutaneous coronary intervention: a meta-analysis of randomized trials. **Eur Heart J**, 31: 35–49.

Van de Werf F., Bax J., Betriu A et al (2008). Management of acute myocardial infarction in patients presenting with persistent st-segment elevation: The task force on the management of ST-segment elevation acute myocardial infarction of the european society of cardiology. **Eur Heart J**, 29: 2909-2945.

Van der Laan A.M., ter Horst E. N., Delewi R. et al (2013). Monocyte subset accumulation in the human heart following acute myocardial infarction and the role of the spleen as monocyte reservoir**. Eur Heart J,** doi:10.1093/eurheartj/eht331

Van Furth R., Cohn Z.A. (1968). The origin and kinetics of mononuclear phagocytes. **J Exp Med**, 128: 415-435.

van Gils J.M., da Costa Martins P.A., Mol A. et al (2008). Transendothelial migration drives dissociation of platelet-monocyte complexes. **Thromb Haemost**, 100(2): 271-9.

Venneri M.A., De Palma M., Ponzoni M., et al (2007). Identification of pro-angiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. **Blood,** 109: 5276–5285.

Virmani R., Kolodgie F.D., Burke A.P et al (2005). Atherosclerotic plaque progression and susceptibility to rupture: angiogenesis as a source of intraplaque haemorrhage. **Arterioscler Thromb Vasc Biol**, 25(10): 2054-2061.

Wallentin L., Becker R.C., Budaj A. et al (2009). Ticagrelor versus clopidogrel in patients with acute coronary syndromes. **N Engl J Med**, 361: 1045–1057.

Watanabe T., Hirata M., Yoshikawa Y. et al (1985). Role of macrophages in atherosclerosis. Sequential observations of cholesterol-induced rabbit aortic lesion by the immunoperoxidase technique using monoclonal antimacrophage antibody. **Lab Invest** 53(1): 80-90.

WHO Fact sheet N8310, updated June 2011, http://www.who.int/mediacentre/factsheets/fs310/en/index.html.

Widimsky P., Wijns W., Fajadet J. et al (2010) Reperfusion therapy for ST elevation acute myocardial infarction in Europe: description of the current situation in 30 countries. **Eur Heart J,** 31: 943–957.

Wiviott S.D., Braunwald E., McCabe C.H. et al (2007). Prasugrel versus clopidogrel in patients with acute coronary syndromes. **N Engl J Med**, 357:2001-2015.

Wong K.L., Tai J.J., Wong W.C. et al (2011). Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. **Blood,** 118(5), e16-31.

Woollard K.J., Geissmann F. (2010). Monocytes in atherosclerosis: subsets and functions. **Nat Rev Cardiol**, 7: 77-86.

Wrigley B.J., Shantsila E., Tapp L.D. et al (2013). CD14++CD16+ monocytes in patients with acute ischaemic heart failure. **Eur J Clin Invest**, 43(2): 121-30.

Wyss C.A., Neidhart M., Altwegg L. et al (2010). Cellular actors, Toll-like receptors, and local cytokine profile in acute coronary syndromes. **Eur Heart J**, 31(12): 1457-1469.

Xie P., Cao Y.S., Su P. et al (2010). Expression of toll-like receptor 4, tumour necrosis factor-alpha, matrix metalloproteinase-9 and effects of benazepril in patients with acute coronary syndromes. **Clin Med Insights Cardiol**, 4:89-93.

Xu X.H., Shah P.K., Faure E. et al (2001). Toll-like receptor 4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidised LDL. **Circulation**, 104(25): 3103-3108.

Yeh R.W., Sidney S., Chandra M. et al (2010). Population trends in the incidence and outcomes of acute myocardial infarction. **N Engl J Med**, 362: 2155–2165.

Ylitalo R., Oksala O., Ylä-Herttuala S. et al (1994). Effects of clodronate (dichloromethylene bisphosphonate) on the development of experimental atherosclerosis in rabbits. **J Lab Clinical Med**, 123(5): 769-776.

Yona S., Kim K.W., Wolf Y (2013). Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. **Immunity**, 38: 79-91.

Yonekawa K., Neidhart M., Altwegg L.A. et al (2011). Myeloid related proteins activate Toll-like receptor 4 in human acute coronary syndromes. **Atherosclerosis**, 218(2): 486-492.

Yoshimura T., Robinson E.A., Tanaka S., et al (1989). Purification and amino acid analysis of two human monocyte chemoattractants produced by phytohemagglutininstimulated human blood mononuclear leukocytes. **J Immunol**. 142: 1956-1962.

Yu X., Yi H., Guo C. Et al (2011). Pattern recognition scavenger receptor CD204 attenuates Toll-like receptor 4-induced NF-kappaB activation by directly inhibiting ubiquitination of tumour necrosis factot (TNF) receptor-associated factor 6. **J Biol Chem**, 286: 18795-18806.

Zawada A.M., Rogacev K.S., Rotter B. Et al (2011). SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset. **Blood**, 118(12): e50-61.

Zeymer U., Margenet A., Haude M. et al (2010). Randomized comparison of eptifibatide versus abciximab in primary percutaneous coronary intervention in patients with acute ST-segment elevation myocardial infarction: results of the EVA-AMI Trial. **J Am Coll Cardiol**, 56: 463–469.

Ziegler-Heitbrock, H.W., Passlick B., Flieger D. (1988). The monoclonal antimonocyte antibody My4 stains B lymphocytes and two distinct monocyte subsets in human peripheral blood. **Hybridoma**, 7(6): 521-527.

Ziegler-Heitbrock L., Ancuta P., Crowe S. et al (2010). Nomenclature of monocytes and dendritic cells in blood. **Blood**, 116: e74-80.

Ziegler-Heitbrock L. and Hofer T.P.J. (2013). Toward a refined definition of monocyte subsets. Front Immunol, 4:23. doi: 10.3389/fimmu.2013.00023.