

Monocyte Subsets in Heart Failure

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Abstract

Introduction

Monocytes play important roles in inflammation, thrombosis, angiogenesis and tissue repair and may contribute to the pathophysiology of heart failure (HF). Functional diversity is likely to stem from the presence of three distinct monocyte subsets, defined by flow cytometry (FC) as CD14⁺⁺CD16⁻CCR2⁺ (Mon1), CD14⁺⁺CD16⁺CCR2⁺ (Mon2) and CD14⁺CD16⁺⁺CCR2⁻ (Mon3). The aims of this thesis were to study the following parameters in patients with ischaemic HF: 1) monocyte subset numbers, 2) monocyte subset expression of surface receptors for inflammation, angiogenesis, cell adhesion molecules (CAM) and tissue repair, 3) cross-talk between monocytes and platelets in the formation of monocyte-platelet aggregates (MPAs).

Methods

Monocyte subsets were analysed by FC on venous blood samples at baseline in 51 patients admitted with acute HF (AHF), 42 with stable HF (SHF), 44 with stable coronary artery disease (CAD) without HF and 40 healthy controls (HC). Plasma levels of inflammatory cytokines were also measured by flow cytometric bead array technology. In AHF, additional longitudinal samples were taken at discharge and 3 months.

Results

Compared to CAD controls, patients with SHF had higher counts of Mon2 and MPAs associated with Mon2, alongside increased expression of inflammatory markers and CAM receptors on Mon2. Compared to SHF, those with AHF had higher counts of Mon1, Mon2 and MPAs associated with Mon1 and Mon2. Patients with AHF also had increased expression of angiogenic receptors on Mon1 and increased expression of angiogenic receptors, scavenger receptors and CAM receptors on Mon2. After adjusting for confounders, counts of Mon2, MPAs associated with Mon2 and expression of VCAM-1R on Mon2 were associated with clinical outcomes in AHF.

Conclusions

Differences in monocyte subset numbers and cell surface receptor expression are seen in patients with HF. Mon2 appears to have a prognostic role in patients with AHF, however larger studies are required to confirm these findings.

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Abbreviations

AHF: Acute heart failure

AF: Atrial Fibrillation

AMI: Acute myocardial infarction

ANOVA: One way analysis of variance

APC: Allophycocyanin

ATTACH: Anti-TNF Therapy Against Congestive Heart Failure

BD: Becton Dickinson

BMI: Body mass index

BNP: Brain natriuretic peptide

BP: Blood pressure

CAD: Coronary artery disease

CAMs: Cellular adhesion molecules

CCR2: C-C-chemokine receptor type 2

CD: Cluster of differentiation

CRP: C-reactive protein

CT-1: Cardiotrophin-1

CXCR4: C-X-C-chemokine receptor type 4

ECM: Extracellular matrix

EDTA: Ethylene-diamine tetra-acetic acid

eGFR: Estimated glomerular filtration rate

EF: Ejection Fraction

ELISA: Enzyme-linked immunosorbent assay

EPC: Endothelial progenitor cell

ESC: European Society of Cardiology

ESR: Erythrocyte sedimentation rate

HC: Healthy control

HF: Heart failure

HIV: Human immunodeficiency virus

HR: Hazard ratio

hsCRP: High sensitivity C-reactive protein

HSP70: Heat shock protein 70

ICAM: Intercellular adhesion molecule

IHD: Ischaemic heart disease

IL: Interleukin

iNOS: Inducible nitric oxide synthetase

IQR: Interquartile range

LV: Left ventricular

LVEF: Left ventricular ejection fraction

LPS: Lipopolysaccharide

MCP: Monocyte chemoattractant protein

MFI: Median fluorescence intensity

MI: Myocardial infarction

MMP: Matrix metalloproteinases

MPAs: Monocyte platelet aggregates

MRI: Magnetic resonance image

NFKB: Nuclear factor kappa B

NHS: National health service

NK: Natural killer cells

NO: Nitric oxide

NOS: Nitric oxide synthetase

NYHA: New York Heart Association

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate buffer solution

PE: Phycoerythrin

PerCP: peridinin-chlorophyll proteins

PRAISE: Prospective Randomised Amlodipine Survival Evaluation

RA: Rheumatoid arthritis

ROS: Reactive oxygen species

SD: Standard deviation

SHF: Stable heart failure

SOLVD: Study Of Left Ventricular Dysfunction

SOP: Standard operating procedure

STAT3: Signal transducer and activation of transcription -3

STEMI: ST elevation myocardial infarction

TB: Tuberculosis

TF: Tissue factor

TLR: Toll-like receptor

TNF: Tumour necrosis factor

TNFR: Tumour necrosis factor receptor

UK: United Kingdom

VCAM: Vascular cellular adhesion molecule

VEST: Vesnarinone trial

VEGF: Vascular endothelial growth factor

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CHAPTER ONE

INTRODUCTION

1.1 Introduction to heart failure

1.1.1 Epidemiology of Heart Failure

Heart failure (HF) is a general term to describe insufficient cardiac output to meet the requirements of vital organs, or to only do so with elevated cardiac filling pressures. Approximately 900, 000 people in the United Kingdom (UK) have HF, with 1 in 15 of the population aged 75-84 years affected.(1) The incidence is likely to rise due to the combination of an ageing population, improved survival from acute myocardial infarction (MI) and the development of more effective HF therapy, including drugs, cardiac resynchronisation and implantable defibrillators. Unfortunately the mortality rates are still high and one third of newly diagnosed patients will die within the first year and over 50% within 5 years from diagnosis.(2-4) This puts HF at a similar mortality rate as those patients with colon cancer and even worse than those with breast cancer.(5)

The financial impact on the health care system is understandably considerable, and approximately 2% of the National Health Service (NHS) budget is spent on HF.(6) Much of this burden arises from frequent hospital admissions, with an estimated 5% of acute admissions relating to HF and one third of these are re-admitted within 90 days of initial discharge.

1.1.2 Signs and symptoms of heart failure

HF can be sub-categorised in numerous ways, depending on the cardiac chambers involved (right or left HF), chronicity (acute or chronic) and impairment of either systolic or diastolic ventricular function. Historically, HF was regarded as systolic impairment of cardiac function, although more recent data suggest that up to half of all

patients with symptoms of HF have normal ventricular ejection fraction, with the primary problem being abnormal diastolic performance.(7)

Patients with HF typically present with breathlessness, which can be classified according to the New York Heart Association (NYHA) functional class.(8) (**Table 1.1**)

Table 1.1 New York Heart Association classification

Class	Patient Symptoms
Class I (Mild)	No limitation of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation, or dyspnoea.
Class II (Mild)	Slight limitation of physical activity. Comfortable at rest, but ordinary physical activity results in fatigue, palpitation, or dyspnoea.
Class III (Moderate)	Marked limitation of physical activity. Comfortable at rest, but less than ordinary activity causes fatigue, palpitation, or dyspnoea.
Class IV (Severe)	Unable to carry out any physical activity without discomfort. Symptoms of cardiac insufficiency at rest. If any physical activity is undertaken, discomfort is increased.

Other common symptoms include orthopnoea, paroxysmal nocturnal dyspnoea, fatigue and peripheral oedema. Clinical assessment of these patients may demonstrate an array of signs, including widespread respiratory crackles, elevated jugular venous pressure, oedema, heart murmurs and additional heart sounds.

1.1.3 Diagnosing heart failure

Several diagnostic tests can be used in the assessment of patients with HF. Chest X-rays may show cardiomegaly and interstitial oedema and 12-lead electrocardiograms commonly reveal abnormalities, including the presence of bundle branch block, left ventricular (LV) hypertrophy, atrial arrhythmias and changes suggestive of previous MI. Biomarkers may also be used to confirm the diagnosis of HF, with brain natriuretic peptide (BNP) being the most commonly used. BNP is released from the cardiac ventricles during pressure or volume overload and is recognised as an independent prognostic marker in HF, with levels directly correlating with the severity of disease.⁽⁹⁾ However, echocardiography is arguably the most important investigation for diagnosing HF. This ultrasound examination can quantify the ejection fraction (EF), which is the fraction of the end-diastolic volume of blood ejected with each contraction of the ventricle and is reduced in patients with systolic HF. Echocardiography can also identify underlying valvular abnormalities, as well as evidence of underlying ischaemic heart disease. Coronary angiography and ventriculography are also frequently used in the assessment of patients with HF, particularly if there is associated angina and coronary revascularisation is being considered.

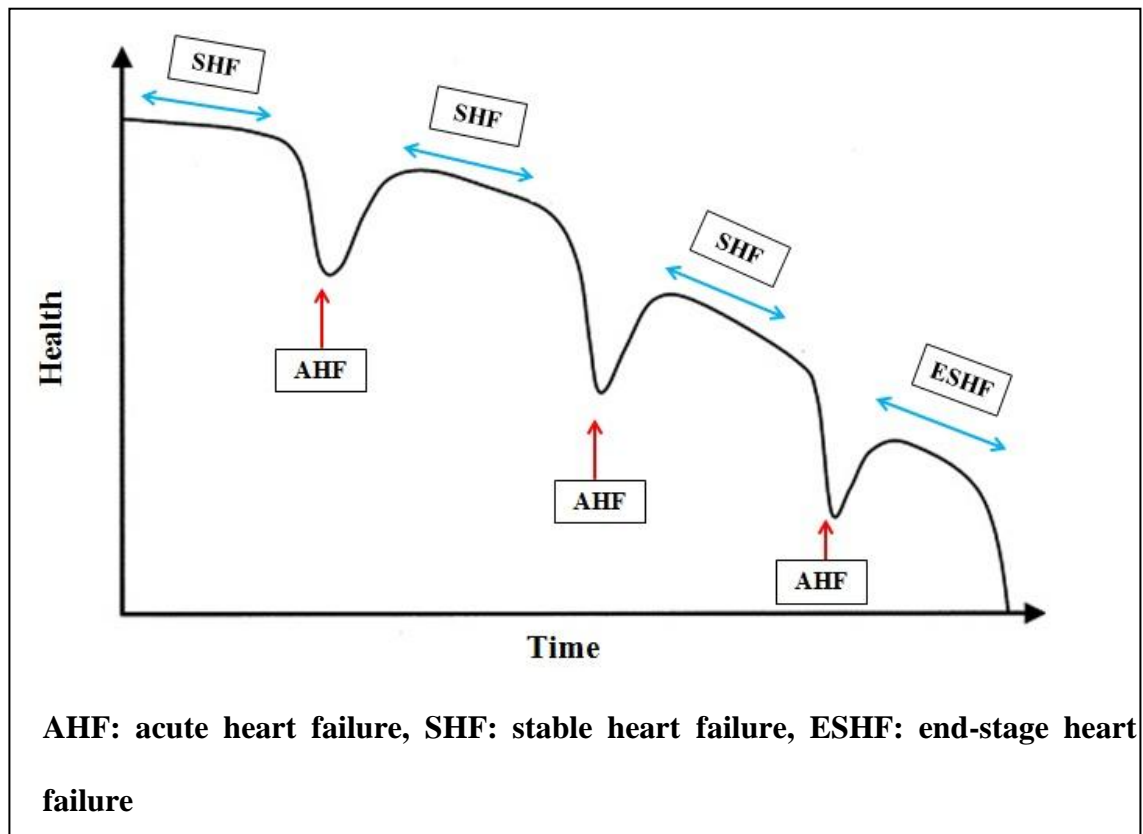
1.1.4 Management and prognosis

The immediate management of acute HF (AHF) is aimed at reducing pulmonary oedema, improving oxygenation and re-establishing tissue-organ perfusion. Drug treatment usually consists of vasodilators, diuretics and opiates and if improvements are not made, inotropes and positive pressure ventilation may also be considered. The chronic management of stable HF (SHF) involves angiotensin-converting enzyme (ACE) inhibitors, beta blockers, diuretics and aldosterone receptor antagonists. Patients

with evidence of dyssynchronous ventricular contraction may also benefit from biventricular pacing as well as implantable cardioverter defibrillators. Ultimately, some patients continue to deteriorate and may be considered for cardiac transplantation.

Patients with HF tend to follow a pattern of disease, with periods of relative stability periodically interrupted by episodes of acute decompensation, often requiring hospital admissions (**Figure 1.1**). Three-quarters of patients with AHF are known to have chronic SHF, with the remaining quarter presenting acutely with a new diagnosis of HF.(10) Triggers for acute decompensation and hospital admission include myocardial ischaemia, poor compliance with medications or failure to adhere to salt and water restriction, arrhythmias and intercurrent infections. A general progressive deterioration in myocardial performance can also lead to admissions. Such disease progression highlights the current limitations in treatment strategies and emphasises the continuing need to develop new approaches to HF therapy.

Figure 1.1 The natural history of heart failure



1.1.5 The aetiology of heart failure

Coronary artery disease (CAD) is the most common underlying aetiology of HF in Western countries. In a large Italian registry of unselected outpatients with HF, the underlying cardiac diagnoses were CAD (40%), dilated cardiomyopathy (32%), valvular heart disease (12%) and hypertensive heart disease (11%). The remaining 5% comprise of infiltrative disorders such as amyloidosis, connective tissue diseases, pharmaceutical drugs (e.g. some chemotherapies) and arrhythmias.(11)

It is now well recognised that HF is not simply a disorder affecting the myocardium, but rather a complex systemic syndrome with interplay between the metabolic, neuroendocrine and immune systems leading to impaired contractility and considerable

patient morbidity and mortality.(12,13) There is growing evidence to support an important role of inflammation in the underlying pathophysiology of HF, underpinned by both animal and human research.(14) Fundamental to the orchestration of inflammation are monocytes, which play a pivotal role in the coordination of the inflammatory cascade and are considered the largest pool of circulating progenitor cells, with the ability to differentiate into dendritic cells and macrophages. Monocytes have characteristics that may make them both beneficial and detrimental to the myocardium, largely due to the existence of diverse monocyte subsets with unique phenotype and function.(15-18) However, scarce data are available on the characteristics of monocyte subsets in HF, and this will be the focus of the work in this thesis.

1.2 Heart failure and the inflammatory paradigm

1.2.1 The role of monocyte-derived cytokines

There is growing evidence to implicate the immune system in the pathophysiology of HF. Markers of inflammation have prognostic significance and in a study of patients following MI, those who developed cardiac pump failure or LV aneurysms had higher peak monocyte counts than in those free of such complications.(19) Monocyte levels were also an independent determinant of readmission with HF, recurrent MI and cardiac death. However, rather than simply representing a marker of myocardial tissue inflammation, monocytes directly influence the disease process. Activated monocytes and macrophages are the major source of cytokines and the increased presence of these inflammatory proteins have formed the basis of the HF inflammatory paradigm.(20) Cytokines are a group of biological protein molecules that serve as intercellular messengers. They are essential for the regulation of target cell proliferation, differentiation and migration as well as controlling further cytokine secretion by such

cells. The most important and well-studied monocyte-derived cytokines implicated in HF are tumour necrosis factor (TNF) and interleukin (IL)-6.

1.2.1.1 Tumour necrosis factor

TNF exerts its biological activity via TNF receptors type 1 (TNFR1) and 2 (TNFR2), with TNFR1 mediating the main effects of initiating cytotoxic and apoptotic responses.(21) Cleavage of pro-TNF (the membrane anchored precursor) with TNF converting enzyme gives rise to the soluble form of TNF which stabilizes the TNF molecule thereby potentiating its activity. TNF is hardly detectable in the normal myocardium and an association between HF and TNF was first made by Levine and colleagues in 1990.(22) They demonstrated elevated circulating levels of TNF in chronic HF patients and numerous animal and human studies have since followed **(Table 1.2).**

TNF levels correlate with NYHA class in chronic HF (23, 24) and appear to be elevated earlier during the process of the disease as compared to classical neurohormones (such as N-type natriuretic peptide) which tend to be elevated only in severe disease.(20, 23) Results from the Vesnarinone trial (VEST), which aimed to assess the immune-inflammatory process in HF (1200 patients with NYHA III/IV), showed that TNF, soluble TNFR1 and TNFR2 were significant predictors of mortality in the study population.(25) The receptors were the most powerful predictors of mortality, perhaps indicating less variability than TNF itself, which only has a half-life of about 30 minutes.(26) Although TNF is also produced by other cells (e.g. neutrophils) monocytes are a major source of the cytokine production.(27) Moreover, monocyte TNF

production (both unstimulated and stimulated with lipopolysaccharide, LPS) parallels the severity of HF, increasing with disease progression.(28)

Table 1.2 The role of TNF in heart failure

Study	Outcome
Animal Studies	
Pagani FD et al(29)	Animal: TNF infusion Direct infusion resulted in impaired systolic and diastolic LV function
Buzkurt B (30)	Animal : TNF infusion Resulted in time dependent depression in LV function which was partially reversible by stopping infusion and giving TNF antagonist
Yokoyama T (31)	Animal: Cultured cardiac myocytes stimulated by TNF Resulted in hypertrophic growth response
Comstock KL, Krown KA (32, 33)	Animal: TNF induced apoptosis in cardiac myocytes
Bryant D (34)	Animal: transgenic mice with over-expression of myocyte TNF Developed biventricular dilatation and reduced ejection fraction. Pathological examination revealed myocyte apoptosis and ventricular fibrosis
Sivasubramanian(35)	Animal: transgenic mice with over-expression of TNF LV remodelling and increased MMP activity

Human Studies	
Levine B (22)	Observational study: circulating levels of TNF increased in CHF and were highest in the most advanced disease
Torre-Amione G (23)	Analysis of pro-inflammatory cytokines in the SOLVD trial. Increased TNF and IL-6 levels correlated with deteriorating functional class. Other than atrial natriuretic factor, no associated with neurohormonal levels seen
Comini L (36)	Serum from HF patients downregulated eNOS expression and increases apoptosis which is linked to the activation of the TNF system.
Deswai A (25)	Largest analysis of cytokines in HF: TNF and IL-6 independent predictors of mortality in advanced heart failure
TNF: Tumour Necrosis Factor; LV: Left Ventricular; MMP: Matrix Metalloproteinase; SOLVD: Study of Left Ventricular Dysfunction; IL: Interleukin; NOS: Nitric Oxide Synthase	

Increased cytokine production in HF is not restricted to the peripheral circulation. Examining hearts explanted at the time of transplantation showed that the failing myocardium produces high quantities of TNF and its receptors.(23) In patients with acute myocarditis, the presence of virus in the myocardium triggers recruitment of monocytes, alongside B and T cells, all of which are capable of producing cytokines.(37) TNF has also been shown to have a direct negative impact on the myocardium and has been implicated in LV dysfunction, remodelling, myocyte apoptosis, endothelial function and activation of inducible form of nitric oxide synthase (iNOS).(38) In a canine model, TNF infusion resulted in impaired LV function (29) and

studies in transgenic mice with over-expression of TNF showed an association with reduced LVEF.(39, 40) As well as directly impairing systolic function, TNF has also been implicated in LV remodelling. TNF stimulates growth and hypertrophy of cultured myocytes (31) and progressive LV dilatation is seen in transgenic mice with over-expression of TNF.(40) A possible mechanism for such dilatation may be due to its effects on myocardial activity of matrix metalloproteinases (MMPs). This family of enzymes are able to degrade matrix proteins and are upregulated in the failing myocardium, as demonstrated by a study using transgenic mice (with selective over expression of TNF in the myocardium) showing an increase in MMP during the phase of LV remodeling.(35)

The precise mechanism of TNF action on the myocardium is unclear, but it may exert a biological effect by impairing coupling of the beta-adrenoceptors-G-protein-adenylcyclase complex, which has a negative inotropic effect (41, 42) and it also stimulates nitric oxide (NO) production, again causing a negative inotropic effect.(43) Activation of the sphingomyelinase pathway and NO-mediated attenuation of beta-adrenergic signalling have also been suggested.(42, 44) A better understanding of the role of monocyte-derived TNF in HF is therefore needed.

1.2.1.2 Interleukin 6

Elevated levels of IL-6 have also been seen in HF and associates with a poor prognosis.(45, 46) IL-6 has pleiotropic effects stimulating B-cell differentiation (47), activation of thymocytes and T cell differentiation (48), activation of macrophages (49) and natural killer (NK) cells as well as stimulating hepatocytes to produce acute phase

proteins (e.g. C-reactive protein, CRP). (50) IL-6 can also cause myocyte hypertrophy, myocardial dysfunction and cachexia, but also inhibits cardiomyocyte apoptosis.(51)

In an analysis from the Prospective Randomised Amlodipine Survival Evaluation (PRAISE) trial, there was a significant trend for higher rates of adverse outcomes in chronic HF with higher levels of IL-6.(52) Analogous with TNF, IL-6 is not just a marker of inflammation and it also exerts direct deleterious effects on the myocardium. Indeed, subcutaneous infusion of IL-6 in rats resulted in cardiac dilatation, reduced end systolic pressure and reduced contractility.(53) Monocytes are an important source of IL-6 and a possible mechanism for IL-6 expression is via stimulation by cardiotrophin-1 (CT-1), which is a member of the IL-6 family of cytokines.(54) In contrast, some have shown that IL-6 induction by CT-1 could be beneficial, with rat infarct model showing reduced infarct size and myocyte apoptosis if IL-6/soluble IL-6R complex was given before coronary artery ligation.(55) These findings highlight the complexity of the HF cytokine theory and provide some understanding as to why previous attempts to influence levels of TNF and IL-6 have had mixed results in clinical trials.(56)

1.2.2 Therapeutic approaches to modify inflammation in HF

In contrast to preclinical research, therapeutic modulation of the cytokine system in clinical trials has been unsuccessful in humans in general and largely disappointing in patients with HF. Much of the work has focused on targeting TNF, either by using recombinant TNF receptors that bind TNF and prevent it from binding to target cell receptors or using monoclonal antibodies to bind to cytokines in the circulation. Etanercept is an example of a drug that acts as a soluble TNF receptor and has been used in two clinical trials for patients with HF.(57) Unfortunately, both trials were

discontinued early as they were unlikely to show a difference in the primary end points of mortality and hospitalization for HF. The anti-TNF Therapy Against Congestive Heart Failure (ATTACH) trial used infliximab (humanised mouse monoclonal antibody against TNF) in patients with moderate to severe symptoms and in fact showed a dose-related increase in death and HF hospitalisations with infliximab compared with placebo.(58) Numerous hypotheses have been proposed to explain why TNF modulation has failed to have any benefit in these clinical trials.(14) One such explanation is that infliximab is toxic to cells expressing TNF, especially at high plasma levels seen in clinical trials.(59) This toxicity may be beneficial in conditions such as rheumatoid arthritis (RA) or Crohn's disease, but may in fact prove deleterious to cardiac myocytes. Another explanation may be that monoclonal antibodies have been shown to have the potential to act as partial agonists for cytokines, thereby potentiating their actions.(60) Finally, low levels of TNF may be (in part) beneficial to the myocardium in such processes as tissue repair and remodeling and blocking its effects may result in worsening HF due to the loss of such benefit. Further attempts to influence the immune system in HF have included immunoglobulin therapy, where some improvement in LV function has been observed. (61, 62) However, only small studies have been performed to date and further data are needed to elucidate the potential risks and benefits of such treatment.

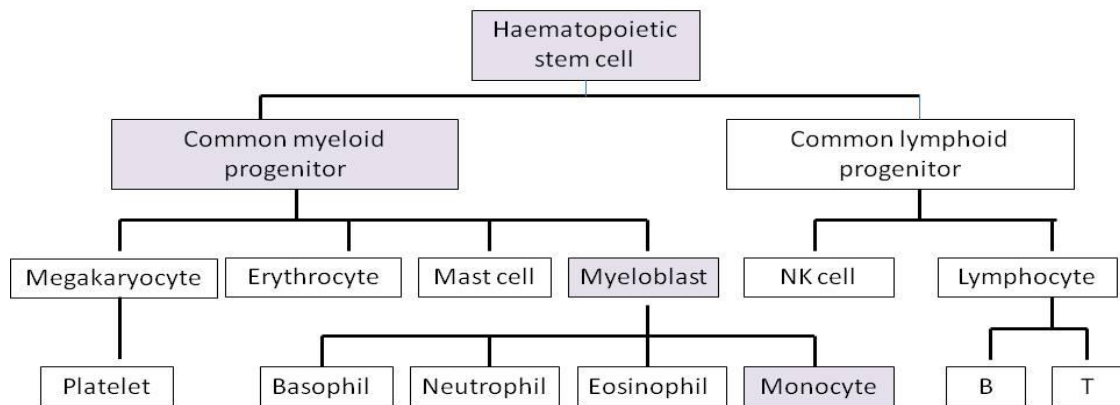
Rather simplistic attempts to suppress the immune system have not been overwhelmingly successful and what emerges following these clinical trials is an appreciation of the complexity of HF and a need for a better understanding of the role of inflammation and the cells involved.

1.3 Monocytes

1.3.1 Monocyte Heterogeneity

Monocytes are pro-inflammatory cells involved in the immune response. They originate from a common myeloid progenitor and account for 3-8% of leukocytes in the peripheral blood.(63) **(Figure 1.2)**

Figure 1.2 The origins of monocytes in humans



Once they leave the bone marrow, monocytes typically circulate or patrol blood vessels for several days before entering tissues and differentiating into macrophages or inflammatory dendritic cells.(64-66) An exception to this are monocytes that reside within the splenic red pulp. In the case of the ischaemic myocardium, this group of monocytes are released into the circulation and account for 40-75% of the monocytes within the myocardium.(67) Under steady state, monocytes do not proliferate but in

response to injury and infection, they rapidly migrate to sites of inflammation under the influence of chemokine receptors and pattern recognition proteins.(68, 69) They have far-reaching functions, including the production of both pro and anti-inflammatory cytokines, MMPs, growth factors, phagocytosis and the regulation of extracellular matrix turnover.(16) Such diverse functionality may arise due to the presence of distinct monocyte subsets.

1.3.1.1 Monocyte subsets in mice

Monocytes are highly diverse cells and the relative expression of surface markers measured by flow cytometry (FC) allows differentiation into distinct subsets. In mice, subsets can be divided into Ly-6C^{hi} and Ly-6C^{lo}, which display differing functional characteristics.(70) Ly-6C^{hi} monocytes express inflammatory cytokines and proteolytic mediators and are rapidly recruited to sites of inflammation.(71) Ly-6C^{lo} monocytes appear to have anti-inflammatory properties and are involved in granulation tissue formation, collagen deposition and healing.(72, 73) Evidence of their diverse functional characteristics comes from the work done by Nahrendorf et al.(74) Using a model of myocardial ischaemic injury in mice, they found that Ly-6C^{hi} monocytes were rapidly recruited to the infarcted myocardium and exhibited phagocytic, proteolytic and inflammatory functions (phase 1). Later on, Ly-6C^{lo} monocytes were recruited to the myocardium to attenuate the inflammatory processes and promote healing by increasing myofibroblast activity, angiogenesis and deposition of collagen (phase 2, between 4 and 7 days post infarction). In the same study, the investigators depleted circulating monocytes during stages 1 and 2 and examined the myocardial response. They found that attenuating phase 1 monocyte recruitment resulted in larger areas of necrotic tissue whereas attenuating monocytes during stage 2 resulted in reduced deposition of

collagen. Whilst Ly-6C^{hi} monocyte infiltration into the myocardium appears to initially confer benefit, there is evidence that continued presence of this subset may produce deleterious effects. In a further mouse study, persistently high levels of Ly-6C^{hi} monocytes within infarct were associated with impaired infarct healing and delayed onset of phase 2 activity.(75) This was associated with greater than three-fold higher myeloperoxidase transcription levels on day 5 post infarct, suggesting hampered transition from an inflammatory to a healing response. These data therefore suggest a need for both subsets to be recruited to the myocardium following cardiac insult, but the timing, extent and duration of involvement is crucial, highlighting the perils of oversimplifying monocyte subsets as being either ‘good’ or ‘bad’.

1.3.1.2 Monocyte subsets in humans

Human subsets were first described by Ziegler-Heitbrock et al in 1988 based on two-colour fluorescence.(18) Initially, the cell-surface marker CD14 (LPS receptor) was used on FC to identify monocytes but for the last 20 years a CD16+ (Fc gamma III receptor) subset has been recognised.(17) Using FC to measure the relative expression of CD14 and CD16, a major subset (~85%) of large-density CD14+CD16- monocytes (equivalent to mouseLy-6C^{hi} subset) and a minor (<15%) subset of smaller, less dense CD14+CD16++ (equivalent to mouseLy-6C^{lo} subset) monocytes were defined. The major set have also been called ‘classical’ monocytes,(27) and the latterly discovered CD16+ monocytes have been termed ‘nonclassical’.(76)

More recently, a further monocyte subset has been recognised by the Nomenclature Committee of the International Union of Immunological Societies, in 2010.(77) This so-

called ‘intermediate’ subset is denoted by CD14⁺⁺CD16⁺. The + sign represents expression that is 10 fold above the isotype control and ++ is 100 fold above.

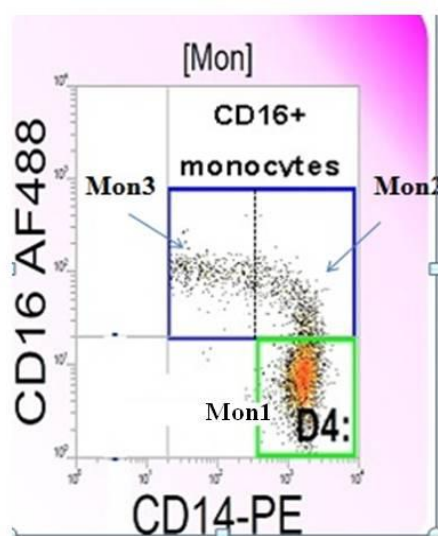
The contemporary nomenclature of monocytes is therefore:

- Classical CD14⁺⁺CD16⁻
- Intermediate CD14⁺⁺CD16⁺
- Nonclassical CD14⁺CD16⁺⁺

For the purposes of this thesis, the following abbreviations will be used (**Figure 1.3**):

- Classical = Mon1
- Intermediate = Mon2
- Nonclassical = Mon3

Figure 1.3 Monocyte subsets defined by the relative expression of CD14 and CD16 on flow cytometry



Mon1: classical monocytes, Mon2: intermediate monocytes,
Mon3: nonclassical monocytes

Recognising these subsets as separate entities is important because they differ not only in their phenotype but also in functionality (**Table 1.3**). However, much of the previous work into monocytes have not applied this further subdivision of CD16⁺ monocytes and this has the potential to affect results when looking at the functionality of these subsets.(78)

CD16⁻ monocytes are inflammatory in that they express C-C chemokine receptor type 2 (CCR2) and can also release myeloperoxidase.(79, 80) Interestingly, CD16⁺ monocytes may also be considered inflammatory, in that they produce TNF and are increased in several inflammatory conditions.

As shown in **Table 1.3**, monocyte subsets express varying surface marker receptors and have differing functionality. It should be noted that whilst the minor subset in murine models is regarded as anti-inflammatory, this cannot necessarily be said for its human counterpart. Mon2 (CD14⁺⁺CD16⁺) produce more of the anti-inflammatory cytokine IL-10 than other subsets and yet also produce similar levels of TNF in response to LPS stimulation. (77, 81-83) In many inflammatory conditions, the CD16⁺ monocytes are up-regulated and this has led to the widespread acceptance of these monocytes being pro-inflammatory (**Table 1.4**). However, it should again be emphasised that the vast majority of previous studies make reference to only two monocyte subsets (CD14⁺CD16⁻ and CD14⁺CD16⁺) without further subdivision of CD16⁺ cells and careful interpretation of such data are needed.

Table 1.3 Functions of monocyte subsets

	CD14⁺⁺CD16⁻ (Mon1)	CD14⁺⁺CD16⁺ (Mon2)	CD14⁺CD16⁺⁺ (Mon3)
Primary role	Phagocytosis, scavenge necrotic debris, release of MMP for remodelling ECM, cytokine production, release of reactive oxygen species	Angiogenesis Anti-inflammatory cytokine production (IL-10)	Collagen deposition, healing, anti-inflammatory effects
Maturity (based on resemblance to surface marker expression on tissue macrophages)	Less mature	Data not available	More mature: reduced CD33 expression IL-10 can induce CD16 expression on monocytes in vivo with CD33 downregulation suggesting that this cytokine drives maturation
Phagocytic activity	High	High	Low
LPS stimulated cytokine production	Potent producers of TNF and IL6	Similar production of TNF to Mon1 Increased production of IL10	No effect on cytokine production Low expression of IL10

Surface expression of angiogenic receptors (VEGFR1, VEGFR2, CXCR4, Tie 2)	Low	High	Low
Adhesion molecules	Medium 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
Chemokine receptor	Mobilisation (via CCR2 receptor) to MCP-1 ligand	Mobilisation (via CX3CR1) to fractalkine	Data not available
Scavenger receptors CD163 (scavenger receptor binding haemoglobin-haptoglobin complex) CD204 (class A scavenger receptor)	Medium Low	High Medium	Low High
IL: Interleukin, LPS:lipopolysaccharideTNF:Tumour Necrosis Factor; VEGF:Vascular Endothelial Growth Factor; CXCR4:C-X-C Chemokine Receptor Type 4; ICAM:Intercellular Adhesion Molecule; VCAM:Vascular Cell Adhesion Molecule; MCP:Monocyte Chemoattractant Protein, CCR2: C-C Chemokine receptor type 2, CD: cluster of differentiation, Mon: Monocyte, MMP: Matrix Metalloproteinases, ECM: Extra cellular Matrix			

Table 1.4 Monocyte subsets in common inflammatory disorders

	CD16-	CD16+	Conclusion
MI (78)	Peak day 3	Peak day 5	Peak levels of CD14+CD16-negatively associated with extent of myocardial salvage on MRI and with recovery of LV function after MI
Stroke (84)	No change	Increased acutely	Peak levels of CD14+CD16- positively correlated with mortality. Peak levels of CD14+CD16+ inversely correlate with mortality
Sepsis (85-87)	Proportional decrease	Increased	Conflicting data on the role of CD14+CD16+ subset: pulmonary TB associated with increased CD16+ monocytes associating with increased levels of TNF (proinflammatory). However another study of erysipelas (beta haemolytic strep infection), showed that increased numbers of CD16+ associated with lower intracellular TNF production
RA (88, 89)		Increased	Increased CD14+CD16+ correlated with ESR and CRP and

			reduce in response to therapy-e.g. glucocorticoids may selectively deplete this subset
HIV (90)		Increased	
Haemodialysis (91)		Increased	
AMI: acute myocardial infarction, MI: myocardial infarction,, MRI:Magnetic Resonance Imaging; LV: Left Ventricular; TB: Tuberculosis; TNF: Tumour Necrosis Factor; ESR: Erythrocyte Sedimentation Rate; CRP: C-Reactive Protein; RA: rheumatoid arthritis, HIV:Human Immunodeficiency Virus			

1.3.2 Monocyte subsets in HF

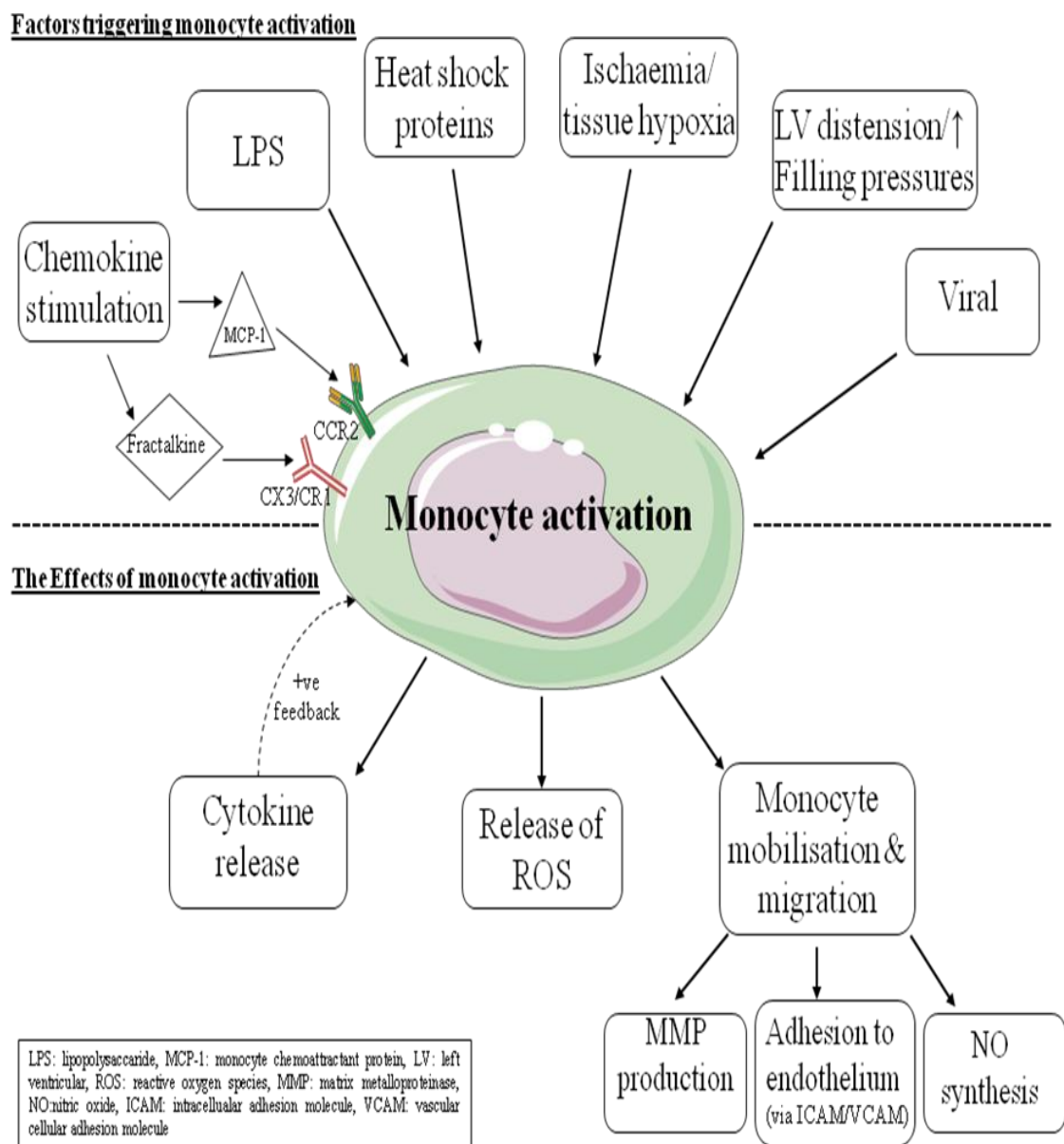
Increased monocyte counts are predictive of all-cause mortality in patients admitted to hospital with HF and are associated with a reduced LVEF following MI.(19, 92) At the time of commencing this thesis, there were very few data on monocyte subsets in HF and no data using the contemporary nomenclature of 3 subsets. However, in 2010, one small study was published looking at 3 monocyte subsets in patients with SHF (n=30) compared to healthy controls (n=26).(93) In this study, the CD14++CD16+ (Mon2) subset was increased in patients with SHF compared to healthy controls and this reflected disease severity, measured by LVEF and BNP. Conversely, the CD14+CD16++ (Mon3) subset appeared to be depleted in HF patients. A criticism of this study is that HF failure patients were compared to healthy individuals, thus raising the possibility that the findings may be attributable, at least in part, to the co-morbidities common to HF patients rather than to HF per se. Furthermore, Mon2 and Mon3 were not separated accurately in the FC analysis which is something that the methodology of this thesis overcame. (See chapter 2, Methods) Nevertheless, the central role of monocytes in the inflammatory process is likely to make these cells important in HF pathophysiology (93) and this concept formed the basis of this thesis.

1.4 Monocyte Activation

The loss of myocardium seen in HF is multifactorial and whilst ischaemic heart disease (IHD) is the most common underlying aetiology, other causes such as viral infection, hypertension or muscle defects play a role. Evidence for monocyte activation in HF has been demonstrated by finding increased plasma levels of neopterin, which is a metabolite of guanosine triphosphate and a specific marker of monocyte activation. (24, 94) Of interest, neopterin levels correlate with plasma TNF levels and monocytes are

therefore likely to be activated as a consequence of multiple, interacting mechanisms.(24) (**Figure 1.4**)

Figure 1.4 Monocyte activation: mechanism and effects (16)



HF is associated with high LV filling pressures, shear stress forces and LV wall distension and alongside hypoxia and tissue ischaemia, they provide various stimuli for monocyte activation.(95) These processes may be important in triggering localised cytokine release within the myocardium itself. Indeed, experimental models have shown a direct association between the degree of LV cavity distension and local TNF production.(21) As the myocardium fails, tissue perfusion is reduced, not only at the myocardial level but also in peripheral tissues (e.g. muscles). Tissue hypoxia is a strong stimulus for pro-inflammatory cytokine production, and also promotes skeletal muscle apoptosis, thereby creating a self-perpetuating cycle.(96)

As well as being a major cellular source of cytokines, monocytes are also one of the main cellular targets of pro-inflammatory cytokines. In HF patients, TNF induces monocyte expression of inducible nitric oxide synthase (iNOS) (36) and macrophages in vivo induce apoptosis via NOS induction.(44, 97) Regardless of whether the initial release of cytokines arises from the myocardium or from peripheral monocytes, a cascade of further monocyte activation and hence recruitment to the failing myocardium creates a vicious cycle.(24)

1.4.1 CD14 activation: bacterial translocation theory

Monocyte activity relies on pattern recognition receptors such as toll-like receptors (TLRs), CD14 and scavenger receptors.(98) One of the most powerful stimuli for monocyte cytokine production is the interaction between CD14 and its ligand LPS.(99) Levels of soluble CD14, monocyte-derived TNF and endotoxin are higher in patients with oedema and moderate-to-severe HF compared to those with mild disease and absence of oedema and these levels reduce after a period of diuretic.(99) This suggests

that increased CD14 expression may play an immunological role in advanced HF.(28, 100)

LPS is the cell wall component of gram negative bacteria which has led researchers to investigate the possible role of bacteria in monocyte activation in HF and has resulted in the endotoxin-cytokine hypothesis. A potential mechanism involves an increased mesenteric pressure from venous congestion causing increased bowel permeability and subsequent bacterial translocation, accompanied by the release of endotoxins into the circulation.(99) Additionally, the process of bacterial adherence to intestinal epithelium may induce mucosal cytokine release which subsequently disrupts the epithelial barrier.(101) Another mechanism resulting in endotoxin translocation relates to the increased sympathetic activity seen in HF, which redistributes blood flow away from the splanchnic circulation causing intestinal ischaemia and increased intestinal mucosal permeability.(102) The gut has abnormal morphology and function in patients with HF and one study showed a 35% increase in intestinal permeability in such patients.(103) In the same study, bowel wall thickness was also significantly greater than matched controls which correlated with blood concentration of leukocytes.

1.4.2 Toll-like receptor 4 and monocyte activation

TLRs are a class of pattern recognition receptors important in innate immunity.(104) TLR4 is expressed in the heart and other organs but is highest in peripheral leukocytes, particularly monocytes.(105) Monocyte TLR4 expression is significantly increased in patients with HF and relates to the severity of disease.(106) One study found enhanced TLR4 staining in the myocardium undergoing remodeling, thus indicating accelerated monocyte recruitment into areas of remodeling within the failing myocardium.(107) Furthermore, a study looking at the significance of TLR4 expression on leukocytes

compared with expression on myocytes showed that only TLR4 expression on leukocytes was associated with myocyte impairment during stimulation with LPS (even if TLR4 was expressed on myocytes).(108) Therefore TLR4 is essential in the monocyte cellular response to bacterial LPS and indeed TLR4-deficient mice have lower tissue inflammation following an ischaemic insult than in those with TLR4.(109, 110)

TLR4 may be activated on monocytes via various mechanisms. It is a co-receptor for CD14 and therefore plays an important role in the CD14-LPS mediated cytokine cascade. Additionally, monocytes can be activated via endogenous stimuli such as heat shock protein 70 (HSP70).(111) HSP70 is a potent activator of the immune system and is a major ligand for the TLR pathway.(112, 113) HSP70 is released by the heart following myocardial ischaemic injury(114) and plasma levels correlate with levels of TNF and IL-6 as well as monocyte TLR4 expression and the degree of subsequent LV impairment.(111)

1.4.3 CRP-Mediated Monocyte Activation

CRP is an acute phase protein secreted by hepatocytes and is elevated in numerous inflammatory conditions. CRP levels are raised in patients with HF and are independent predictors of future adverse events.(115, 116) A difficulty with using standard CRP is that levels in HF often return to those seen in healthy population and high sensitivity CRP (hsCRP) is therefore a better measurement and is increased with severity of disease. Alongside LVEF, hsCRP is an independent predictor for adverse outcomes.(117)

CRP activates monocytes and stimulates their production of inflammatory cytokines in a dose-dependent manner.(118) This enhanced cytokine production is significantly higher in those patients with ongoing myocardial damage (defined by raised cardiac Troponin T) than in those without and is associated with a risk of future cardiac events.(119) Additionally, CRP also plays a regulatory role in the clearance of CRP-opsonized particles by direct binding to Fc-gamma receptors and enhancing phagocytosis via complement receptors. This may suggest a moderated need for CRP which becomes harmful to the myocardium if produced in great quantities, perhaps by enhancing monocyte activity. CRP also attenuates production of NO, which is important in endothelial function, promoting angiogenesis and inhibiting apoptosis.(120, 121) Finally, CRP induces expression of MCP-1 by human endothelial cells, a mechanism important for the migration of monocytes to the myocardium which will be discussed in detail below in section 1.5.(122)

1.5 Monocyte recruitment to the myocardium: the role of Monocyte chemoattractant protein-1

Activated monocytes are able to exert some of their effects on the myocardium by producing pro-inflammatory cytokines in the peripheral circulation, but an increased expression of chemokines by the failing myocardium suggests that they also migrate to the site of inflammation.(123) Chemokines are small peptides capable of mobilizing leucocytes from the bone marrow(124) and are divided into 4 groups depending on the positioning of their cysteine residues in the amino acid sequence.(125) Many chemokines have been implicated in HF and the CXC-chemokines IL-8, growth-regulated oncogene alpha and epithelial neutrophil activating peptide are elevated in

serum of patients with stable HF and are related to the disease severity.(126) MCP-1 has emerged as the most important chemokine responsible for monocyte mobilisation and recruitment to sites of inflammation.(126-128) MCP-1 mediates its effects via the CCR2 receptor(129, 130) and as shown in **Table 1.5**, may have both beneficial and detrimental consequences for the myocardium.

Table 1.5. The role of MCP-1 in heart failure

Beneficial role of MCP-1
Delayed wound repair if MCP-1 blocked (131)
MCP-1 shifts balance towards release of anti-inflammatory cytokines in mouse model of sepsis (132)
MCP gene disruption in mice results in delayed macrophage infiltration into healing myocardium and prolonged inflammation (133)
Detrimental role of MCP-1
Activation of MCP pathway leads to increased adhesion molecules, inflammatory cytokines and MMP (134, 135)
Mice expressing MCP-1 in myocardium results in increased monocyte infiltration, ventricular hypertrophy, dilatation, fibrosis and impaired contractility (136)
MCP-1 directly leads to enhanced generation of ROS in monocytes and LV dysfunction (137)
MCP-1 enhances synthesis of MMP in human fibroblasts (135)
MCP-1 enhances production of inflammatory cytokines (138)
MCP:Monocyte Chemoattractant Protein; MMP:Matrix Metalloproteinase; ROS:Reactive Oxygen Species; LV:Left Ventricular

Mon1 monocytes express CCR2 receptors and therefore migrate in response to MCP-1.(139, 140) This is in contrast with the Mon3 subset which lacks the CCR2 receptor but expresses CX3CR1 and preferentially responds to the chemokine fractalkine (CX3CL1).(139) Increased expression of MCP-1 has been seen in experimental MI within ischaemic segments of the myocardium and reduced and delayed macrophage infiltration into the healing infarct was seen in mouse models where the MCP gene was disrupted.(133, 141) Despite delayed phagocytic removal of dead cardiomyocytes, the MCP deficient mice had attenuated LV remodeling and had a more prolonged inflammatory phase and delayed replacement of injured cardiomyocytes. Circulating levels of MCP-1 inversely correlate with LVEF and are highest in those patients with NYHA IV symptoms.(137) Moreover, the increased MCP-1 levels correlate with increased monocyte activity defined by oxygen- generation by monocytes. Monocytes in particular release high amounts of MCP-1 in HF patients compared to healthy controls and there is enhanced expression of CCR2 in the myocardium.(142-144) A study using macrophage inflammatory protein-1 knock-out mice showed reduced recruitment of activated monocytes in the myocardium, which was associated with reduced cardiac lesions following coxsackie B infection.(145)

MCP-1 may contribute indirectly to HF by recruiting activated monocytes to the myocardium. However, it may also directly lead to LV dysfunction via other mechanisms. For example, MCP-1 may directly act on myocytes which show increased expression of CCR2 in HF.(137) Additionally monocytes of patients with HF generate more reactive oxygen species (ROS) compared to controls and MCP-1 has been implicated in this process.(137) MCP-1 also stimulates the release of inflammatory

cytokines such as IL-1 beta and IL-6 in rat models and has also been implicated in enhancing gene expression and synthesis of MMPs in human fibroblasts. (138, 135)

Finally, hypoxic tissue may provide a stimulus for monocyte recruitment to the failing myocardium and CCR2 has been implicated, although this has not been investigated in HF to date. Exposure to hypoxia causes down-regulation of CCR2 on monocytes, thereby reducing the responsiveness of monocytes to MCP-1 once they are within the hypoxic tissue.(146) Essentially, monocytes become 'trapped' within the hypoxic tissue and are able to differentiate into tissue macrophages and dendritic cells which then exert their biological effects.

1.6 Monocyte-endothelial adhesion

Once monocytes have been attracted towards the failing myocardium down the chemotactic gradient, they must then attach and migrate across the endothelial barrier into the myocardial tissue itself. The expression of cell adhesion molecules (CAMs) is crucial to monocyte recruitment and homing and involves inter-cellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1).(124) Soluble isoforms of CAMs are elevated in patients with HF and reflect enhanced cell surface expression of these molecules.(147, 148) The levels of soluble CAMs increase with severity of disease and are related to clinical outcomes.(147-149) In a study of adhesiveness of peripheral blood mononuclear cells (PBMC) to cultured human arterial endothelial cells, PBMCs from patients with severe HF had greater adhesiveness compared to patients with mild HF and both healthy and disease controls.(150) This suggests that endothelial monocyte adhesiveness may serve as an index of cell activity and increases with severity of HF and has a predictive value for a combined endpoint of

death, transplant and HF readmission. A possible mechanism for endothelial CAM expression is by cytokine activation, once again raising the possibility of monocyte involvement in this pathway.(151, 152)

As discussed previously, fractalkine is a unique cytokine which can function as a chemokine in its soluble form (to enhance migration of Mon3 monocyte subset) but it is also an adhesion molecule in the full-length membrane-bound form.(153) Like its counterpart CCR2, fractalkine is increased in the failing human myocardium and is likely to be important in monocyte subset migration.

1.7 Monocytes and angiogenesis: potential mechanism of tissue repair

Despite the potentially deleterious effects of monocytes on the myocardium, available data indicate a potential role for monocytes in tissue repair, which is clearly important in maintaining LV function. A depletion of macrophages leads to impaired wound healing after myocardial injury with a propensity towards adverse remodeling and increased mortality.(154) In hypertensive rats, a depletion of macrophages leads to an earlier development of myocardial dysfunction.(155) A potential mechanism for tissue repair is angiogenesis, which involves the growth of new blood vessels from pre-existing ones.(19) Vascular endothelial growth factor (VEGF) is a pro-angiogenic growth factor and increased levels have been observed in patients with HF, potentially carrying prognostic significance.(156) Signal transducer and activation of transcription 3 (STAT-3) upregulates VEGF expression(157) and STAT-3 deficiency in mice associates with increased myocardial fibrosis and dilated cardiomyopathy.(158) There

are no data directly implicating monocytes with angiogenesis in HF, but macrophage numbers following MI have positively correlated with the degree of angiogenesis in an animal model.(159) If indeed angiogenesis does occur in HF, this may be a mechanism whereby activated monocytes can infiltrate the myocardium to exact their phagocytic and reparative roles previously discussed.(160)

1.8 Thrombosis in heart failure: formation of monocyte-platelet aggregates

Patients with HF have increased risk of thromboembolism which may contribute to the high morbidity and mortality seen in this condition.(161) Indeed the risk has been quoted at 17.4 fold increased risk of ischaemic stroke within the first month of diagnosing HF.(162) Post-mortum studies have also shown a high incidence of occlusive coronary disease.(163) The reasons for such high risk are multifactorial and have been broadly divided into abnormalities in the vessel wall, abnormal blood flow and abnormalities in blood constituents, giving the so called Vorchow's triad. However, there is currently no evidence to support the use of anticoagulation or antiplatelet therapy in patients with HF, unless there are co-existing indications, such as atrial fibrillation (AF) or underlying CAD.

Monocytes may play a key role in the pro-thrombotic HF condition. Elevated levels of tissue factor (TF) have been observed in patients with HF(164) and monocytes appear to be the major source of TF.(165) Additionally, monocytes promote TF expression on endothelial cells. The adhesion of monocytes to platelets may also represent a link between inflammation and thrombosis. Monocyte platelet aggregates (MPAs) are

elevated in numerous inflammatory conditions, including MI(166), cirrhosis(167), limb ischaemia(168), end-stage renal failure(169) and diabetes.(170) In a recent MI study, the total MPA count and MPAs associated with Mon1 and Mon2 were increased in ST elevation MI (STEMI) patients compared with controls.(166) The increase in MPAs persisted for at least 30 days, despite potent antiplatelet therapy.

The precise role of MPAs is not yet clear but they may represent a process of eliminating activated platelets by process of phagocytosis. One of the precipitants of MPA formation may be exposure to LPS and CRP(171) and it is possible therefore that levels will be elevated in patients with HF. However it is not clear whether MPAs are increased in patients with HF, and furthermore whether MPA formation of specific subsets are affected preferentially.

1.9 Summary

Monocytes are important members of the innate immune system and are implicated in many aspects of cardiovascular disease pathogenesis. The early stages of HF involve host protection with clearance of inflammatory ligands, apoptosis and phagocytosis of necrotic tissue. Continued inflammation may be deleterious as it may eventually lead to adverse remodeling, interstitial fibrosis and impaired myocardial contractility. Monocytes are intimately involved in both tissue damage and repair and an imbalance of this equilibrium in HF is likely to be important in disease progression. Monocytes comprise of distinct subsets with different cell surface markers and functional characteristics but the specific roles of such subsets in HF have not been investigated. It is possible that this heterogeneity allows monocytes to be both detrimental and reparative.

Finally, monocyte activation plays a central role in the inflammatory pathophysiology of HF and occurs via wide-ranging stimuli, many of which are still poorly defined. The subsequent release of inflammatory cytokines, migration to the myocardium, adhesion to the endothelial wall and infiltration into the myocardium are also complex processes involving interplay between many components of the immune system. This degree of complexity may help to explain why the therapeutic modulation of inflammation has not been universally successful in treating HF in clinical trials. Increasing our understanding of the role monocytes play in inflammation and HF pathophysiology may provide the basis to a more targeted approach to therapy in this condition.

1.10 Hypotheses

For this thesis, I hypothesised that patients with ischaemic HF would exhibit abnormal patterns of monocyte subsets compared to controls without HF, with differences also present between AHF and SHF patients:

- i. Patients with HF will have abnormal numbers of monocytes and individual subsets
- ii. In HF, there would be significant changes in monocyte subset expression of surface markers for activation/inflammation, angiogenesis, tissue repair and cell adhesion
- iii. In HF, there would be increased formation of monocyte-platelet aggregates, reflecting changes in both monocyte and platelet activation
- iv. Abnormalities of monocyte parameters in patients with AHF would return to values seen in the stable phase of the disease at follow-up

1.11 Aims and objectives

To test these hypotheses, my objectives for the thesis were:

- i. To study monocyte subset numbers in patients with HF compared to controls without HF
- ii. To measure cell surface marker expression for markers of activation/inflammation (CD14, TLR4, IL-6R), angiogenesis and tissue repair (VEGFR-1, CXCR4 and CD163) and cells adhesion (ICAMR-1, VCAMR-1) in patients with HF.

- iii. To evaluate the interaction between monocytes and platelets in the form of MPA formation in patients with HF
- iv. To assess the prognostic role of monocyte subset parameters on clinical outcomes in patients with AHF (specifically death and rehospitalisation)
- v. To measure circulating plasma biomarkers of HF (BNP, MCP-1 and IL-6) and correlate them with monocyte numbers and surface marker expression
- vi. To study monocyte parameters longitudinally following recovery from AHF

In order to evaluate the impact of having HF on monocyte parameters, a cohort of SHF patients would be compared to a disease control cohort (CAD group, with similar co-morbidities and medications but without HF) in addition to age and sex-matched healthy controls to define 'normality'. In order to evaluate the impact of having AHF, patients with an acute admission of HF would be compared to the SHF cohort.

CHAPTER TWO

METHODS

2.1 Study populations

2.1.1 Cross-sectional study

For the purposes of the cross-sectional study, 4 populations were selected:

- 1) 51 patients with AHF
- 2) 42 patients with SHF
- 3) 44 patients with stable CAD
- 4) 40 HC subjects

The 4 study populations were chosen to represent the spectrum from disease to health. All patients were recruited from cardiology departments located within two hospital trusts based in the West Midlands (Sandwell and West Birmingham Hospitals NHS Trust and Heart of England NHS Foundation Trust). Healthy control participants were recruited from staff, family and friends. All research participants were recruited between the 30th October 2009 and March 2011.

2.1.1.1 Subject selection and inclusion criteria

Heart failure patients

In an attempt to minimise potential confounders, all HF patients recruited into the study (AHF and SHF) were required to have underlying CAD. This inclusion criterion enabled both AHF and SHF patients to be compared to a disease control population (CAD) with similar co-morbidities (e.g. diabetes, hypertension) and medication usage, reducing the number of potential confounders.

Acute heart failure

Consecutive patients admitted to hospital with a primary diagnosis of AHF were recruited. AHF was defined as the rapid onset of symptoms and signs secondary to abnormal cardiac function, in accordance with the European Society of Cardiology (ESC) guidelines.⁽¹⁷²⁾ All AHF patients had documented (LVEF) of $\leq 40\%$ on echocardiography by Simpsons method or left ventriculography.⁽¹⁷³⁾

Stable heart failure

Patients with SHF were recruited from outpatient HF clinics. SHF was defined as LVEF $\leq 40\%$ and no deterioration in clinical condition, admission to hospital or change in medication for the preceding six months

Stable coronary artery disease

This disease control population were recruited from outpatient clinics and were defined as having had a previous MI and/or angiographically proven CAD and LVEF $>50\%$.

Healthy controls

Subjects were identified from interested members of staff and willing family and friends. Subjects were considered healthy based on clinical history and examination.

2.1.1.2 Exclusion criteria

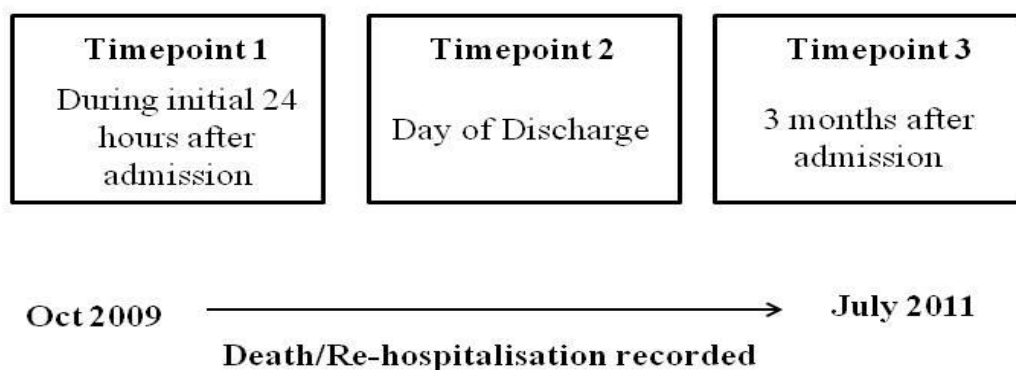
For all study groups, exclusion criteria included factors that could affect monocyte count (infectious and inflammatory disorders, cancer, haemodynamically significant valvular heart disease, renal failure (creatinine $>200 \mu\text{mol/l}$), steroids

or hormone replacement therapy). For AHF in particular, patients were excluded if the precipitation for hospital admission was an acute coronary syndrome.

2.1.2 Longitudinal study

Patients with AHF recruited to the cross-sectional study were recruited to the follow-up part of the study. In order to assess whether monocyte parameters changed over time, assessments were made at the following time points (**Figure 2.1**): (i) during the first 24 hours after admission, (ii) on the day of hospital discharge and (iii) 3 months following hospital admission. Recruitment began on 30 October 2009 and all patients were followed until 30 July 2011 for collection of death and rehospitalisation data only, using hospital and community records where necessary.

Figure 2.1 Follow-up time points in the longitudinal study of AHF



2.2 Ethical approval

This study was performed in accordance with the Helsinki declaration. Ethical approval was granted by the Warwickshire Research Ethics Committee (REC reference: 08/H1211/23) and approval was obtained from the Research & Development departments at Sandwell and West Birmingham Hospitals NHS Trust and Heart of England Foundation NHS Trust. All participants provided written informed consent

2.3 Clinical assessment

At baseline all participants had a full medical history and clinical examination. This approach allowed the collection of detailed information on demographics (age, gender, smoking status and ethnicity), comorbidities (diabetes, hypertension, hyperlipidaemia etc), family history of CAD and medication use. The clinical examination yielded data on peripheral pulse rate, systolic and diastolic blood pressure (BP), height, weight, body mass index (BMI), evidence of valvular disease and co-morbid lung pathology. Baseline characteristics of the four study populations are shown in **Table 2.1**. The four study populations were recruited for comparability of baseline characteristics.

AHF patients underwent two further assessments (**Figure 2.1**). These assessments included a full clinical examination recording details of peripheral pulse rate, systolic BP, diastolic BP, height, weight, BMI, evidence of valvular disease and co-morbid lung disease.

Table 2.1 Demographics and clinical characteristics of the study subjects

	AHF (n=51)	SHF (n=42)	CAD (n=44)	HC (n=40)	p value
<i>Demographics/Clinical characteristics</i>					
Age	72 [11]	71 [10]	68 [9]	67 [7]	0.16
Gender (males), n [%]	36 [71]	35 [83]	30 [68]	23 [58]	0.85
Ethnicity, n [%]					
White	45 [88]	36 [86]	34 [77]	38 [95]	0.12
South Asian	6 [12]	6 [14]	10 [23]	2 [5]	
Hypertension, n [%]	29 [57]	20 [57]	28 [64]	-	0.38
BP (systolic), mmHg	138 [28]	124 [24]	134 [18]	143 [16]	0.082
BP(diastolic), mmHg	80 [17]	73 [12]	70 [11]	78 [8]	0.064
BMI, kg/m ²	28 [5]	28 [4]	28 [4]	26 [3]	0.64
BNP, pg/ml	365 [108-872]	71 [24-256]	-	-	<0.001
LVEF [%]	30 [22-36]†	34 [20-35]†	55 [55-63]	-	0.95‡
MI, n [%]	29 [57]	28 [67]	21 [48]	-	0.25
Smoking, n [%]	25 [49]	23 [55]	14 [32]	2 [5]	<0.001
Stroke, n [%]	5 [10]	7 [17]	6 [14]	-	0.62
Diabetes, n [%]	20 [39]	9 [21]	9 [20]	-	0.083
COPD, n [%]	7 [14]	2 [5]	3 [7]	-	0.28
AF, n [%]	9 [18]	8 [19]	0 [0]	-	0.026
CRT, n [%]	8 [16]	5 [12]	-	-	0.80
eGFR, ml/min	50 [17]*†	62 [16]†	72 [15]	-	<0.001

Medications					
Aspirin, n [%]	37 [73]	34 [81]	38 [86]	-	0.16
Clopidogrel, n [%]	21 [41]	11 [26]	18 [41]	-	0.26
ACE inhibitor/ARB, n [%]	40 [78]	36 [86]	34 [77]	-	0.56
Statin, n [%]	43 [84]	36 [86]	39 [89]	-	0.64
Calcium channel blocker, n [%]	9 [18]	3 [7]	13 [30]	-	0.023
Beta blocker, n [%]	20 [39]*†	32 [76]	34 [77]	-	<0.001
Loop diuretic, n [%]	50 [98]	38 [90]	-	-	0.11
Spironolactone, n [%]	13 [25]	8 [19]	-	-	0.46

Data are presented as mean [Standard deviation] or median [Interquartile range]; *<0.05 vs. SHF, †<0.05 vs. CAD, ‡ difference between AHF and SHF
ACEI: angiotensin-converting enzyme, AHF: Acute heart failure, ARB: angiotensin II receptor blocker, AF: Atrial fibrillation, BMI: Body mass index, BNP: Brain Natriuretic peptide BP: Blood pressure CAD: coronary artery disease, COPD: chronic obstructive airways disease, CRT: cardiac resynchronisation therapy, eGFR: Estimated glomerular filtration rate, HC: healthy controls, LVEF: Left ventricular ejection fraction, MI: Myocardial infarction, SHF: Stable heart failure

2.4 Blood sampling and storage

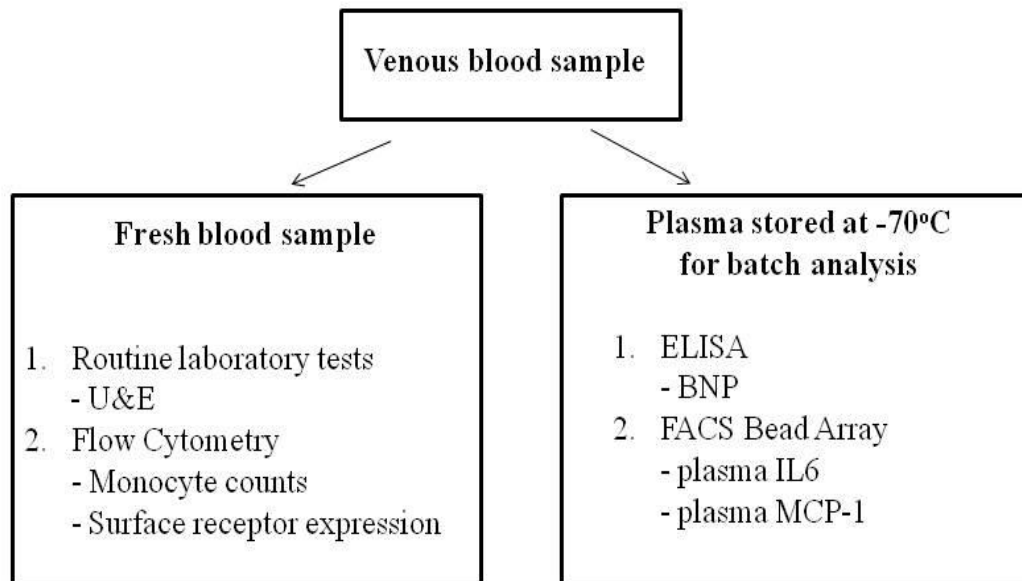
All research participants had a non-fasting blood sample taken and AHF patients undergoing longitudinal assessment had further samples taken at time points 2 and 3 as described in **figure 2.1**.

Prior to venepuncture, patients were rested in chair but were not required to fast. The skin was cleaned with a sterile wipe and a tourniquet applied immediately before commencement of the procedure. The blood samples were obtained by inserting a 21 gauge needle in to a vein located in the anterior cubital fossa of the patients arm. In total, 18ml of non-fasting peripheral venous blood was collected into vacutainer tubes. This consisted of 5mls collected into an ethylene-diamine tetra-acetic acid (EDTA)-containing tube, 8mls in to a citrated tube and 5mls into a serum tube with silicon coated interior. Routine haematological and biochemical tests were performed on the blood samples the same day. In addition, 550µl of fresh EDTA sample was extracted and processed on the Becton Dickinson (BD) FACSCalibur flow cytometer within 60 minutes of collection to yield data on monocyte subsets and monocytes cell surface marker expression. Remaining blood samples were separated by centrifugation and the plasma stored at -70°C for subsequent batched analysis for levels of BNP, plasma IL-6 and MCP-1.

2.4.1 Laboratory measures

An overview of investigations performed on blood samples for all study participants is given in **Figure 2.2** below.

Figure 2.2 Investigations performed on blood samples for each study subject



ELISA: Enzyme-linked immunosorbent assay, BNP: Brain natriuretic peptide,

IL-6: interleukin 6, MCP-1: monocyte chemoattractant protein-1,

U&E: urea and electrolytes.

Biochemistry tests

Routine biochemistry tests included urea, creatinine, sodium and potassium and were performed by the hospital laboratory as per standard protocols.

2.4.2 Markers of systemic inflammation

In order to investigate correlations between monocyte subsets and markers of systemic inflammation and monocyte recruitment, plasma levels of IL-6, MCP-1 were measured by cytometric bead array technology. (**Appendix 1**) The BD FACSCalibur flow cytometer was used for data acquisition, with FCAP Array v2.0.2 software (Burnsville, Minnesota, USA) for data analysis. This technique allows simultaneous quantification of multiple cytokines from the same sample and utilises the fluorescence detection of FC and antibody-coated beads to ‘capture’ cytokines for quantification. The protocol was set up by a specialist from BD and commercially available (BD, Oxford, UK) Human IL-6 Flex Set and Human MCP-1 Flex Set were used according to the manufacturers recommendations.

2.4.3 Markers of heart failure severity

BNP levels were measured using a commercially available enzyme-linked immunoassay (ELISA) set (human BNP-32, Peninsula Laboratories, LLC, CA, USA).

2.5 Specific monocyte assessments

2.5.1 Flow cytometry

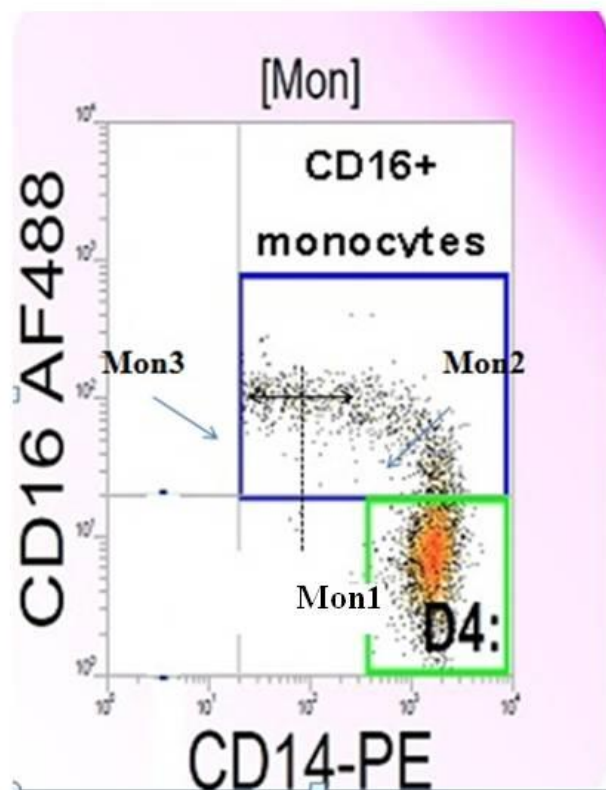
Equipment and software

FC was undertaken using the BD FACSCalibur flow cytometer.

2.5.1.1 Absolute count of monocytes and monocyte subsets

Before publication of the revised nomenclature for monocyte subsets, our research group had been involved in establishing a reliable flow cytometry protocol to allow the accurate enumeration of the 3 monocyte subsets and to discriminate between Mon2 and Mon3, rather than relying on drawing an arbitrary line on the FC plot (**Figure 2.3, Appendix 2**).

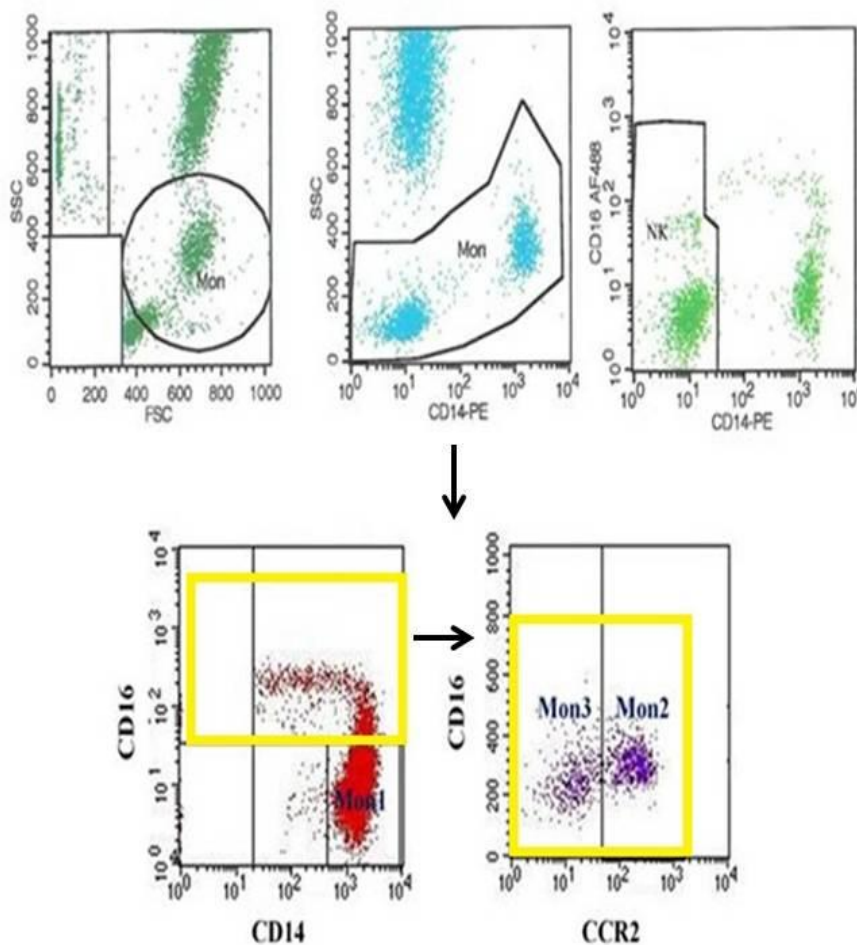
Figure 2.3 Ambiguity in drawing the boundary between Mon2 and Mon3 by using only CD14 and CD16 expression



The additional use of CCR2 expression allows such discrimination, with Mon2 subset strongly expressing the marker whereas Mon3 subset does not (**Figure 2.4**).⁽⁸²⁾

Figure 2.4. Accurate identification of monocyte subsets by flow cytometry using CCR2

Gating strategy using forward and side scatter to select monocytes, side scatter versus CD14 expression to exclude granulocytes and ungated CD14 versus CD16 expression to exclude natural killer (NK) lymphocytes. Subsets were defined as CD14⁺⁺CD16⁻CCR2⁺ (Mon1), CD14⁺⁺CD16⁺CCR2⁺ (Mon2) and CD14⁺CD16⁺⁺CCR2⁻ (Mon3) monocytes.



Mouse anti-human monoclonal fluorochrome-conjugated antibodies anti-CD16-Alexa Fluor 488 (clone DJ130c, AbD Serotec, Oxford, UK), anti-CD14-PE (clone MφP9, BD) and anti-CCR2-APC (clone 48607, R&D systems, Oxford, UK) were mixed with 50µl of fresh EDTA anticoagulated whole blood in TruCount tubes (BD, Oxford, UK) containing a strictly defined number of fluorescent count beads. After incubation for 15 minutes, red blood cells were lysed by 450µl of lysing solution® (BD Oxford, UK) for 15 minutes, followed by dilution in 1.5 ml of phosphate buffer solution (PBS) and immediate flow cytometric analysis. Monocytes were selected by gating strategies based on forward and side scatter to select monocytes, side scatter versus CD14 expression to exclude granulocytes, and ungated CD14 versus CD16 expression to exclude natural killer lymphocytes. Appropriate isotype controls were used and subsets were defined as CD14⁺⁺CD16⁻CCR2⁺ ('classical', Mon1), CD14⁺⁺CD16⁺CCR2⁺ ('intermediate', Mon2) and CD14⁺CD16⁺⁺CCR2⁻ ('non-classical', Mon3) monocytes. Absolute counts of monocyte subsets (cells/µl) were obtained by calculating the number of monocytes proportional to the number of count beads in the TruCount tube according to the manufacturer's recommendations.

2.5.1.2 Expression of surface receptors on monocyte subsets

100µl of whole blood was incubated with mouse anti-human monoclonal fluorochrome-conjugated antibodies for 15 minutes in the dark. **Table 2.2** summarises the surface antigens analysed and their relevant antibodies and fluorochromes. 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') using anti-CD16-Alexa Fluor 488 (clone DJ130c, AbDSerotec, Oxford, UK) and anti-CD14-PerCP-Cy5.5 (clone M5E2, BD) antibodies.

Table 2.2 Fluorochrome-conjugated antibodies used to measure monocyte surface receptor expression

Surface antigen	Fluorochrome	Antibody
CD14	PerCP/Cy5.5	clone M5E2, BD
CD16	Alexa Fluor 488	clone DJ130c, AbDSerotec
TLR4	PE	clone 285219, R&D
IL-6 receptor	APC	clone 17506, R&D
VEGF receptor 1	PE	clone 49560, R&D
CXCR4	PE	clone 12G5, R&D
CD163	APC	clone 215927, R&D
ICAM receptor (integrin β 2/CD18)	PE	clone 212701, R&D
VCAM-1 receptor (integrin α 4/CD49d)	APC	clone 7.2R, R&D
Abbreviation: BD- Becton Dickinson, Oxford, UK, R&D- R&D systems, Oxford, UK, CD- cluster of differentiation, TLR- toll like receptor, IL- interleukin, VEGF- vascular endothelial growth factor, CXCR4- C-X-chemokine receptor 4, ICAM- intercellular adhesion molecule, VCAM- vascular cellular adhesion molecule, PE- phycoerythrin, APC- allophycocyanin, PerCP- peridinin-chlorophyll proteins		

2.6 Validation studies

In the preparation of the laboratory work for this thesis, I took part in several experiments in order to determine optimal sample preparation before recruitment of the study patients in order to improve consistency of results (174):

1. The effect of diurnal variation on monocyte numbers:
 - The hypothesis was that there is variation in monocyte numbers and that where possible, samples should be taken at the same time during the day
2. The effect of exercise on monocyte numbers:
 - The hypothesis was that exercise would change monocyte numbers and that recruits should therefore refrain from exercise before samples were taken
3. The effects of delay in sample preparation:
 - The hypothesis was that a certain delay in sample processing would affect monocyte numbers and therefore study subject samples must be processed within a specific time-frame in order to produce consistent results.

2.6.1 Diurnal variation

The absolute monocyte counts and subsets were assessed in 16 healthy participants at 6 hourly intervals over a 24 hour period (06.00, 12.00, 18.00, 00.00 and 06.00). There were no significant variations in the absolute count or in the counts of Mon1 and Mon3. However, there was significant diurnal variation with Mon2, with values peaking at 18.00 and reaching their nadir at 06.00. It would be impossible to recruit all participants for the thesis at exactly the same time; however I aimed to obtain all samples between 09.00-12.00.

2.6.2 Exercise

12 healthy participants had blood samples taken before and after performing a Bruce protocol exercise test to exhaustion. It was found that 15 minutes post-exercise, the total monocyte count and Mon1 increased significantly, followed by a significant reduction at 1 hour. On the basis of these findings, study participants for the thesis were asked to refrain from physical exercise for at least 1 hour prior to sample collection.

2.6.3 Delay in sample processing

Four samples from healthy participants were analysed immediately and at 1, 2 and 4 hours after collection. Samples were stored at room temperature with slow rotation. The total monocyte count and that of the subsets was not affected by a delay of up to 2 hours, although by 4 hours, a significant increase in the proportion of Mon2 and Mon3 had occurred, with a decrease in the proportion of Mon1. Importantly, the MPA counts at 2 hours had significantly increased (for all three monocyte subsets). Based on the findings of this study, all samples were analysed by FC within 1 hour of collection.

2.6.4 Reproducibility of results

Intra-assay reproducibility was assessed during development of the Standard Operating Procedure (SOP) for studying monocytes and MPAs in the preparatory stages for this project. An SOP is an absolute 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. 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 2**). The SOP was developed by Dr Eduard Shantsila with myself and Dr Luke Tapp.

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 2.3**).

For the plasma markers measured by cytometric bead array, the lower limits of detection were taken from manufacturer's data and were 1.0 pg/ml for IL-6 and 1.3 pg/ml for MCP-1. The inter- and intra-assay CV for all assays was <5%.

BNP was measured using a commercially available enzyme immunoassay set (human BNP-32, Peninsula Laboratories, LLC, CA, USA) according to manufacturer's specifications. The inter- and intra- assay CV was <5%.

Table 2.3 Mean intra-assay coefficients of variation (%) for monocyte parameters

	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
	Subject A Mon1/Mon2/Mon3	Subject B Mon1/Mon2/Mon3	Mean
TLR4	9.3/19.1/6.8	5.8/5.8/2.1	8.1
ILR6R	8.9/7.7/0.9	4.8/3.2/3.1	4.8
ICAM-1R	21.1/12/6/5.6	3.4/2.7/2.1	7.9
VCAM-1R	4.4/3.1/0.9	1.1/1.3/0.002	1.8
CXCR4	9.1/16.2/9.5	13.3/10.4/13.5	12.0
CD163	12.1/11.0/11.6	1.7/1.5/11.4	8.2
VEGFR1	3.2/14.3/10.7	3.2/3.8/6.1	7.0
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			

2.7 Statistical analyses

All data was analysed using SPSS 18.0 for windows (SPSS Inc. Chicago, Illionis). For all statistical analyses, the first step was to identify the distribution of each parameter using the Kolmogorov-Smirnov test. The average of normally distributed data is presented as a mean value [SD] and not-normally distributed data as a median [interquartile range, IQR]. Categorical data are presented as percentages. All analyses considered a p value of <0.05 as statistically significant.

2.7.1 Cross sectional data

- 1) Associations between two categorical variables were assessed using a chi-squared test.
- 2) Associations between a categorical and a continuous variable were analysed using a student's t test or Mann Whitney U test for normally distributed and not-normally distributed data, respectively. For analysis of categorical variables with 3 or more categories, one-way Analysis of variance (ANOVA) was used if the continuous variable was normally distributed and Kruskal-Wallis was be used if the continuous variable was not normally distributed. A post-hoc Tukey test was performed to assess inter-group differences, where appropriate. Arithmetical transformation was performed on non-normally distributed variables prior to post-hoc analysis.
- 3) Correlations between two continuous variables were assessed using Pearson's correlation test when comparing two normally distributed variables. Spearman's correlation test was used to compare two sets of not-normally distributed data or one normally distributed and one not normally distributed variable.

2.7.2 Predictive value of parameters for clinical events

To determine the predictive value of monocyte subset parameters for adverse clinical events in AHF patients, multivariate Cox regression analysis was used. Recognising the relatively small sample size, variables achieving $p < 0.10$ on univariate testing were entered into a multivariate Cox regression analysis to determine the independent predictors of clinical outcome. Age was also included in this analysis due to its well-recognised association with mortality. Kaplan Meier estimates for the distribution of time from index admission to the primary end-point were computed and log-rank analysis was performed to compare event free survival for patients with a monocyte parameter level above and below the median value at admission.

2.7.3 Longitudinal data

Repeated measures ANOVA for normally distributed data and Friedman test for non-normally distributed data were used to analyse longitudinal changes in study parameters.

2.8 Power calculation

Lack of previous studies in this area and the paucity of data on monocytes subsets resulted in difficulty in undertaking an appropriate power calculation. However, based on previous work in our department on healthy subjects, the calculated (from ANOVA) minimum number of participants in each group required to detect an assumption of minimal difference of 0.5 standard deviation in the count of monocytes between the study groups with 80% power ($1 - \beta = 0.8$) with $\alpha = 0.05$ (two-tailed) was $n = 35$. For additional confidence and in anticipation of subject drop-out in the longitudinal study, additional patients were recruited within the pre-defined period of recruitment.

CHAPTER 3

MONOCYTE SUBSET NUMBERS IN HEART FAILURE

Abstract

Introduction: Monocytes play important roles in inflammation, angiogenesis and tissue repair and may contribute to the pathophysiology of heart failure (HF). I examined differences in monocyte subset numbers in patients with acute HF (AHF), stable HF (SHF) and controls and evaluated their impact on clinical outcomes.

Methods: Three monocyte subsets [CD14⁺⁺CD16⁻CCR2⁺ (Mon1), CD14⁺⁺CD16⁺CCR2⁺ (Mon2) and CD14⁺CD16⁺⁺CCR2⁻ (Mon3)] were analyzed by flow cytometry in 51 patients with AHF, 42 patients with SHF, 44 patients with stable coronary artery disease and without HF (CAD) and 40 healthy controls (HC). The prognostic impact of monocyte subsets was examined in AHF.

Results: Patients with AHF had significantly higher Mon1 counts compared to the three control groups ($p < 0.001$ for all). Similarly, Mon2 levels were increased in AHF compared to SHF ($p = 0.004$), and CAD ($p < 0.001$); and increased in SHF vs. CAD ($p = 0.009$). There were no differences in Mon3 counts between the groups. Twenty patients (39.2%) with AHF reached the primary end point of death or re-hospitalisation and after adjustment for confounders, Mon2 count remained negatively associated with a combined end-point of death and re-hospitalisation [hazard ratio (per 10 cells/ μ l 0.79 (confidence interval: 0.66-0.94; $p = 0.009$)].

Conclusions: Mon1 counts are increased in AHF and Mon2 counts are increased in patients with both acute and stable HF. The Mon2 subset was also associated with clinical prognosis in patients with AHF.

3.1 Introduction

As described in the introduction chapter, monocytes play a pivotal role in inflammation and have functional characteristics that may be both detrimental and beneficial to the cardiovascular system, including phagocytosis, cytokine production, collagen synthesis and angiogenesis. Such functional diversity is likely to stem from the presence of distinct monocyte subsets.

Recent attention has been particularly directed towards Mon2 monocytes, which appear to have prognostic significance in cardiovascular disease. This subset is positively associated with an increase in cardiovascular events in patients with chronic kidney disease, including those on dialysis.(175, 176) In contrast, Mon2 levels in patients suffering acute stroke are inversely related to mortality, perhaps indicating diverse roles of this subset within different disease processes.(84) Our understanding of subset functionality has been recently enhanced by genetic expression studies, showing that the Mon2 subset has a distinct gene expression profile compared to the other subsets.(177) They appear to particularly express genes linked to inflammation and angiogenesis, which may be important in tissue remodeling. These findings are also in accordance with recent data demonstrating high expression of cell surface receptors associated with angiogenesis and tissue repair on Mon2 monocytes as well as their abundance in bone marrow.(82) However, little is known about the implications of monocyte subsets in patients with HF.

In this chapter, I aimed to examine: (i) differences in monocyte subset numbers in patients with AHF, SHF and controls, (ii) correlations between subsets, LVEF and plasma levels of MCP-1, IL-6 and BNP in patients with AHF and (iii) associations

between monocyte subsets (particularly Mon2) and cardiovascular outcomes (mortality and re-hospitalisation) in patients with AHF.

3.2 Methods

3.2.1 Study population

The recruitment and data collection for patients with AHF, SHF, CAD and HC are described in detail in chapter 2.

In order to explore the clinical predictive value of subset numbers in patients with AHF, I followed patients up for the following endpoints:

- a. Primary endpoint (the first occurrence of either re-hospitalisation or death)
- b. Secondary endpoint of death alone.

3.2.2 Flow cytometry

Flow cytometric analysis was performed in all study patients as described in detail in chapter 2.

3.2.3 Statistical analysis

Detailed statistical techniques have been described in chapter 2, but additional analyses were performed in this chapter to determine the predictive value of monocyte subset numbers for adverse clinical events in AHF patients. Recognising the relatively small sample size, variables achieving $p < 0.10$ on univariate testing were entered into a multivariate Cox regression analysis to determine the independent predictors of both primary and secondary endpoints in AHF patients. Age was also included in this

analysis due to its well-recognised association with mortality. Kaplan Meier estimates for the distribution of time from index admission to the primary end-point were computed and log-rank analysis was performed to compare event free survival for patients with Mon2 levels above and below the median value at admission.

3.3 Results

3.3.1 Subject characteristics

The study groups had similar baseline demographic and clinical characteristics (**Table 2.1, chapter 2**). Compared to those with SHF, AHF patients had similar values of LVEF but higher levels of BNP. Estimated glomerular filtration rate (eGFR) was lower in AHF than in SHF and CAD ($p=0.001$, and $p<0.001$, respectively); and lower in SHF than in CAD ($p=0.038$).

3.3.2 Cross-sectional analysis

3.3.2.1 Monocyte subsets

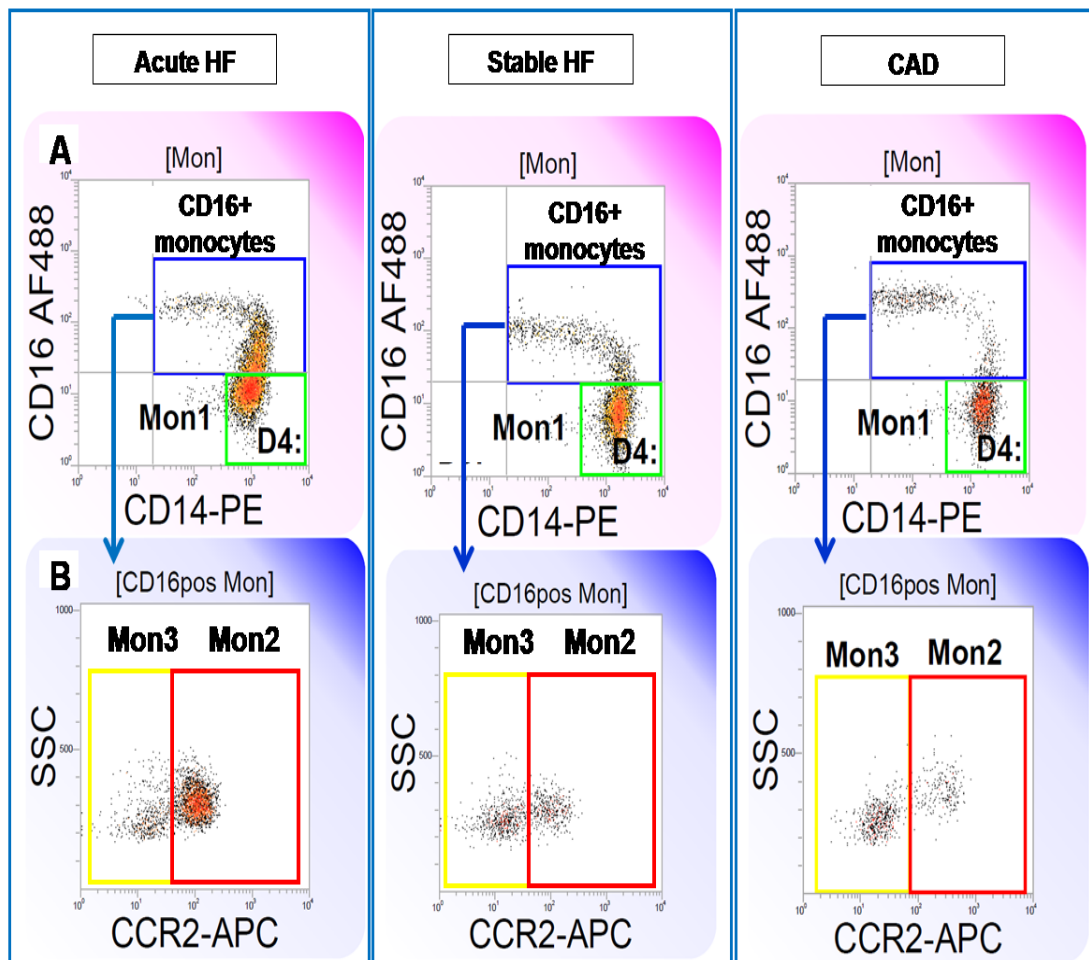
Patients with AHF had significantly higher ‘classical’ Mon1 counts compared to the control groups ($p<0.001$ for all) (**Table 3.1, Figures 3.1 and 3.2**). Similarly Mon2 levels were increased in AHF compared to SHF ($p=0.011$), and CAD ($p<0.001$) and increased in SHF vs. CAD ($p=0.023$). The only difference for Mon3 was a higher count in AHF compared to HC ($p=0.031$).

Table 3.1 Monocyte parameters and plasma markers in cross-sectional analysis

	AHF (n=51)	SHF (n=42)	CAD (n=44)	HC (n=40)	P- value
<i>Monocyte subsets</i>					
Total monocytes, per µl	852 [300]*†‡	646 [172]‡	541 [139]	502 [190]	<0.001
Mon1, per µl	685 [224]*†‡	524 [156]‡	448 [120]	412 [166]	<0.001
Mon2, per µl	60 [42-21]*†‡	43 [30-69]†‡	30 [15-47]	34 [10-53]	0.006
Mon3, per µl	78 [55]‡	71 [33]	60 [28]	55 [29]	0.024
<i>Plasma cytokines</i>					
MCP-1 pg/ml	125 [82]†	136 [99]†	57 [43] ‡	126 [75]	<0.001
IL-6 pg/ml	11 [7-16]*†‡	2.6 [1-4]	1.9 [1-3]	1.7 [0.5-3]	<0.001
<p>AHF: Acute heart failure, SHF: stable heart failure, CAD: coronary artery disease, HC: healthy control, IL-6: Interleukin-6, MCP-1: monocyte chemoattractant protein-1, MFI: median fluorescent intensity. Data are presented as mean[SD] or median [IQR]; *AHF versus SHF, * p<0.05 vs SHF, † p<0.05 vs CAD, ‡ p<0.05 vs HC</p>					

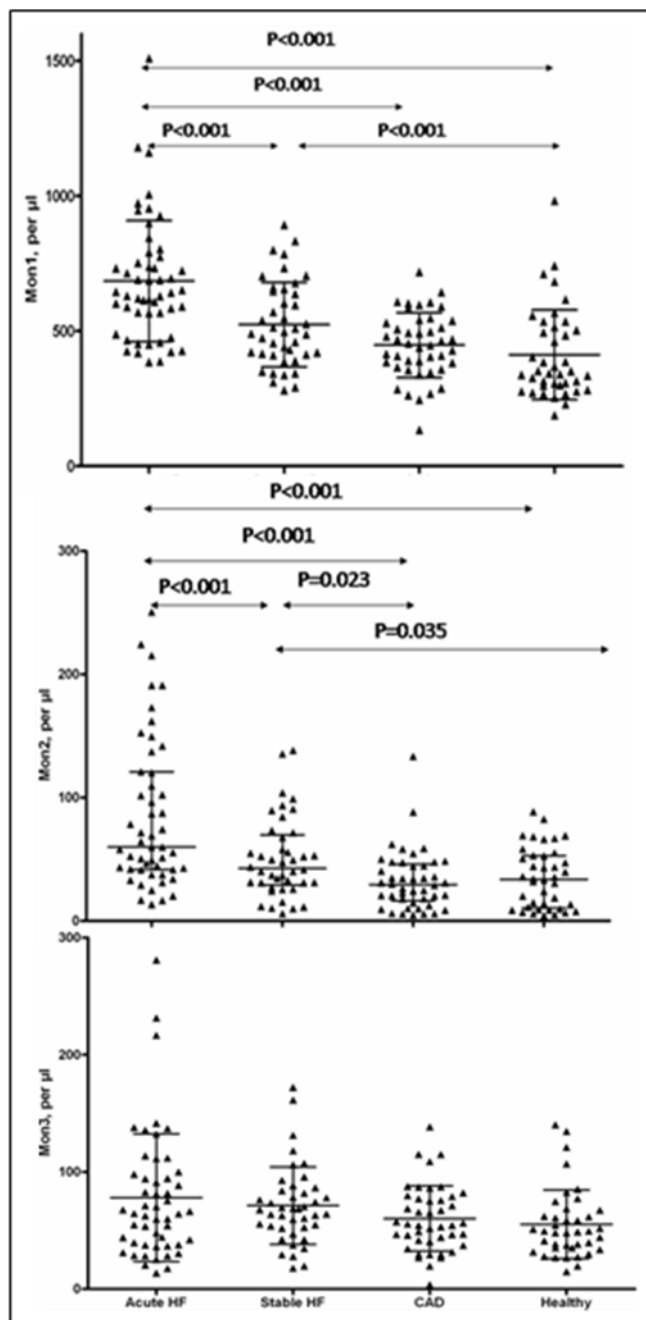
Figure 3.1 Flow cytometric analysis of monocyte subsets

A. Gating of CD14⁺⁺CD16⁻ (Mon1) and CD16⁺ monocytes on the basis of their CD14/CD16 expression: B. Discrimination and gating of CD14⁺⁺CD16⁺CCR2⁺ (Mon2) monocytes and CD14⁺CD16⁺⁺CCR2⁻ (Mon3) monocytes. The number of Mon2 is appreciably higher in AHF compared to SHF and CAD patients.



HF: heart failure, CAD: coronary heart disease, CD: cluster of differentiation, SSC: side scatter, CCR: C-C chemokine receptor

Figure 3.2 Monocyte subset numbers across the study groups



HF: heart failure, CAD: coronary artery disease

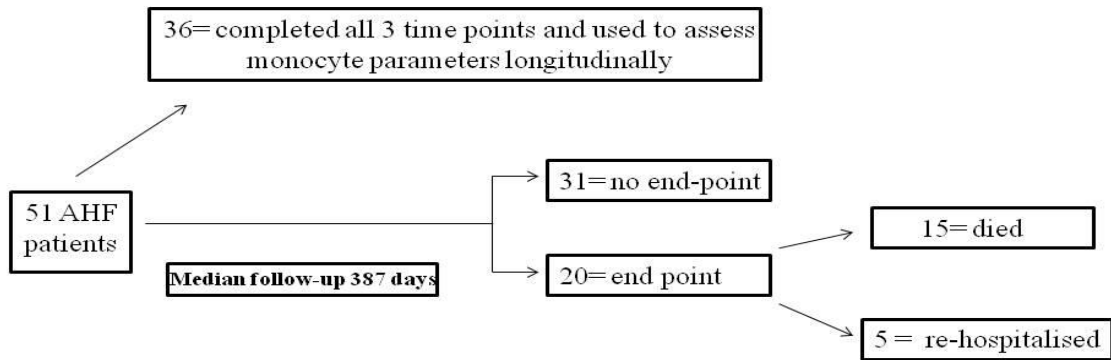
3.3.2.2 Plasma cytokines

Patients with AHF had significantly higher levels of IL-6 compared to other groups ($p < 0.001$ for both, Table 2). No significant differences in IL-6 levels were seen between SHF, and CAD. Patients with AHF and SHF had significantly higher levels of MCP-1 compared to CAD ($p < 0.001$ for both). There were no differences in MCP-1 levels between AHF and SHF.

3.3.3 Longitudinal analysis of study parameters in acute HF

The outcomes of all 51 patients are shown in **Figure 3.3**. Thirty-six AHF patients (71%) completed all three blood test time-points. The median length of hospital stay was 8.5 [5.0-12.8] days. Twenty patients (39.2%) reached the primary end point of death or re-hospitalisation, with a median time to event of 129 (IQR 70-209) days. Fifteen patients (29.4%) reached the secondary end point of death. Thirteen patients died of HF, one died from a ruptured abdominal aortic aneurysm and the cause of death in one patient was unknown. The remaining patients were followed up for a median of 387 [223-550] days.

Figure 3.3 Clinical outcomes in AHF



The cause for hospital admission in all patients was NYHA IV symptoms. Twenty-eight (55%) of patients were already known to have HF and the remaining 23 (45%) patients were admitted with an index episode of breathlessness which was diagnosed for the first time as being secondary to HF. Patients with pre-existing HF were being followed up in outpatient clinics with no indications of poor compliance to medication.

When compared to measurements taken during the first 24 hours of admission, the total monocyte count and all three subsets did not change significantly over 3-months of follow-up (**Table 3.2**). Similarly, IL-6 and MCP-1 levels did not change significantly over time despite a significant fall in levels of BNP ($p=0.031$) and an overall improvement in NYHA status [(NYHA I (n=3), NYHA II (n=15), NYHA III (n=12), NYHA IV (n=6)].

Table 3.2 Longitudinal measurements in AHF at admission, discharge and 3 months

	Admission (n=36)	Discharge (n=36)	Follow-up (n=36)	p value
Total Monocytes ,per µl	893 [336]	853 [255]	858 [331]	0.73
Mon1, per µl	730 [244]	688 [217]	698 [272]	0.60
Mon2, per µl	61 [42-144]	60 [32-111]	59 [25-104]	0.24
Mon3, per µl	79 [63]	91 [54]	90 [48]	0.50
BNP, pg/ml	437 [200-959]*	268 [180-584]	193 [74-342]	0.031
MCP-1, pg/ml	105 [83]	108 [78]	107 [73]	0.96
IL-6, pg/ml	12 [6-16]	8 [4-18]	6 [3-14]	0.60
Data are presented as mean [standard deviation] or median [Interquartile range]; *p< 0.05 vs. follow-up, BNP: brain natriuretic peptide, MCP-1: monocyte chemoattractant protein-1, MFI: median fluorescent intensity, IL-6: interleukin-6				

3.3.4 Predictors of cardiovascular outcome in acute HF

Mon2 counts on admission were significantly lower in patients reaching the primary endpoint of death or re-hospitalisation (46.6 cells/µl IQR 32.0-96.4) compared to those who remained free of events (90.0 cells/µl IQR 50.0-153, p=0.006) The total monocyte count, levels of Mon1 and Mon3 did not differ significantly between patients with or without events.

In univariate Cox regression analysis, BNP (p=0.009), eGFR (p=0.045) and Mon2 count (p=0.02) were significantly associated with the primary end-point, with LVEF showing

a strong trend ($p=0.061$) (**Table 3.3**). In a multivariate Cox regression analysis, there remained a significant association between the Mon2 count and combination of death and re-hospitalisation after adjustments for LVEF, eGFR and BNP, after further adjustment for age (**Table 3.3**). Also Mon2 count was independently and negatively associated with the future risk of death alone (for an increase of 10 cells/ μl , HR 0.808, 95% CI 0.673-0.969, $p=0.021$) after adjustments for age, LVEF, BNP and eGFR.

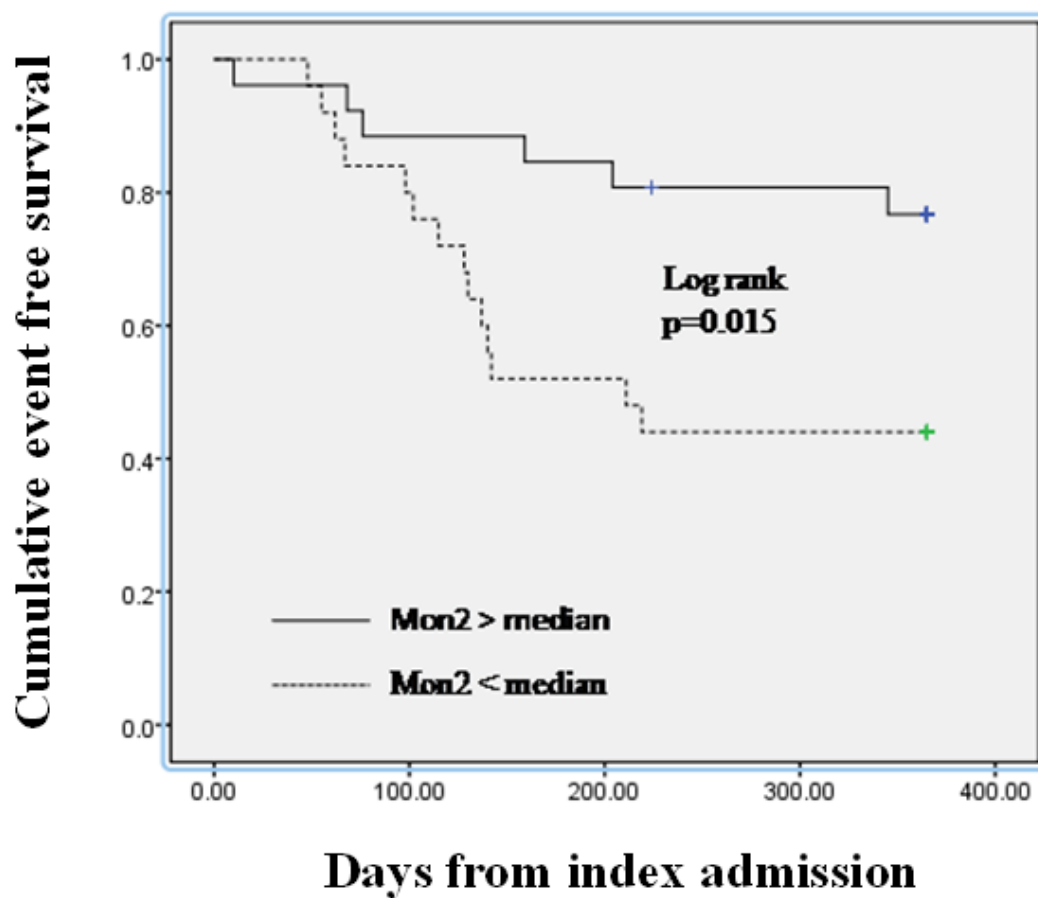
Table 3.3 Cox regression analysis for predictors of combined mortality or re-hospitalisation following admission with AHF

Variable	Hazard ratio	95% CI	p value
<i>Univariate analysis</i>			
<i>Demographics/clinical characteristics</i>			
Age	1.015	0.970-1.061	0.53
Gender (males)	1.048	0.402-2.730	0.92
Hypertension	1.164	0.475-2.852	0.74
Diabetes	1.018	0.412-2.515	0.97
Smoking	1.801	0.734-4.418	0.20
Previous stroke	2.086	0.610-7.129	0.24
BNP, pg/ml	1.001	1.000-1.001	0.009
LVEF, %	0.955	0.909-1.002	0.061
eGFR, ml/min	0.964	0.931-0.999	0.045
<i>Medications</i>			
Aspirin	1.324	0.438-4.002	0.62
Clopidogrel	0.914	0.374-2.238	0.85
ACE inhibitor/ARB	0.750	0.272-2.068	0.58
Beta blocker	1.349	0.556-3.272	0.51
Statin	1.160	0.339-3.962	0.81
<i>Monocyte subsets</i>			
Mon1, per 50 cells / μ l	0.895	0.784-1.023	0.103
Mon2, per 10 cells / μ l	0.862	0.760-0.977	0.020
Mon3, per 10 cells / μ l	0.960	0.868-1.063	0.43
<i>Multivariate analysis (adjusted for eGFR, BNP, LVEF)</i>			
Mon2, per 10 cells/ μ l	0.798	0.669-0.953	0.013
<i>Multivariate analysis (adjusted for eGFR, BNP, LVEF and age)</i>			
Mon2, per 10 cells/ μ l	0.789	0.661-0.943	0.009
ACE: angiotensin-converting enzyme, ARB: angiotensin II receptor blocker, BNP: brain natriuretic protein, eGFR: estimated glomerular filtration rate, LVEF: left ventricular function			

For Kaplan Meier analysis, patients were dichotomised using the median Mon2 value (59.9 cells/ μ l). Patients with Mon2 above median had significantly better outcomes (primary endpoint) compared to those below the median (**Figure 3.4**). There were no significant differences in mortality (secondary endpoint) for patients dichotomised by the Mon2 median value (log rank, $p=0.096$).

Figure 3.4 Kaplan Meier curves of cumulative event-free survival from death/rehospitalisation in AHF

The groups are divided along the median value of Mon2 counts (59.9 cells/ μ l)



3.4 Discussion

In this chapter, I have shown for the first time that Mon2 counts are increased in patients with both acute and stable HF. Secondly, the Mon2 subset was independently associated with mortality and re-hospitalisation following an episode of AHF, with lower counts associating with worse prognosis. Mon1 counts were also significantly increased in patients with AHF compared to those with stable disease, although this did not have any prognostic significance. Whilst Mon3 counts were not affected in AHF, their relative proportion was reduced compared to the control groups.

Of interest, Mon2 counts have previously been shown to be higher in patients with SHF than in healthy subjects, whilst Mon3 counts were lower.⁽⁹³⁾ However, as emphasised in the introduction chapter, one of the major limitations with the study by Barisione et al was the presence of confounders such as CAD, diabetes and background medication in HF compared to healthy controls. In contrast, I tried to reduce potential bias by recruiting HF patients with underlying CAD in order to have a suitable control group of patients with CAD but no LV dysfunction.

Renal impairment is a strong determinant for prognosis in patients with HF.⁽¹⁷⁸⁾ Patients on haemodialysis have elevated Mon2 levels but in patients with chronic renal failure who are not on renal replacement, eGFR does not correlate with the total monocyte count or monocyte subset numbers.^(91, 176) In the present study, patients with AHF had a mean eGFR of 50 ml/min (chronic kidney disease stage 3) and even after adjustment for renal function, Mon2 was still predictive of clinical outcome.

The results in this chapter show that the changes in monocyte subsets (increased Mon1 and Mon2) following acute decompensation of HF persist for at least 3 months, despite improvements in patient symptoms and BNP levels. This is in contrast with a return to baseline values of total monocyte count two weeks following MI.(78) This suggests that the inflammatory process in AHF continues many weeks after the initial event, which is further reflected by persistently elevated plasma IL-6 levels. The prolonged inflammatory response may therefore contribute to the adverse events seen in AHF patients following hospital admission.

Mon2 have been shown to have prognostic relevance in other conditions. Similar to the findings in this chapter, Mon2 counts were increased after stroke, with their higher levels being related to better outcomes.(84) However, elevated Mon2 counts were predictive of future cardiac events in patients with chronic kidney disease.(176) These conflicting data suggest that defining this subset as being either 'beneficial or 'detrimental' is misguided and a better understanding of their functionality within specific disease processes is needed.

However, monocyte numbers in circulation per se may not reflect their corresponding tissue levels and ultimately, their functional state. For instance, potentially detrimental pro-inflammatory effects of Mon2 may be balanced by other, potentially beneficial functions. For example, they appear to be highly phagocytic, which may be important in removal of endogenous ligands produced by the failing myocardium (e.g. apoptotic cells).(177, 179) They also have distinct anti-inflammatory properties (e.g. high production of IL-10) and other features which link them to alternatively polarised M2 macrophages, which have reparative potential.(81) Mon2 also has other features of a

unique pro-reparative phenotype and shows the highest of all monocyte subsets expression of tissue repair markers (e.g. scavenger receptor CD163) and angiogenic surface receptors (including stromal derived factor-1(SDF-1) receptor, angiopoietin receptor and VEGF receptors type 1 and 2).(82) Chapter 5 will focus on analysing surface marker expression for markers of repair and angiogenesis in order to examine any differences in expression amongst HF patients.

3.5 Conclusion

There is a specific up-regulation of Mon1 and Mon2 monocyte subsets in acute decompensated systolic HF. Mon2 levels are also increased in patients with SHF compared to disease-controls. Mon2 appear to have prognostic implications in patients with AHF and merit further evaluation as a prognostic marker and potential therapeutic target. In order begin to understand the potential actions of this subset in HF, it may be important to analyse the surface marker expression of some inflammatory and potentially reparative markers and this will be the focus of subsequent chapters.

CHAPTER 4

SURFACE MARKERS OF ACTIVATION AND INFLAMMATION ON MONOCYTE SUBSETS IN HEART FAILURE

Abstract

Introduction: My aims in this chapter were to measure the relative expression of CD14, TLR4, CCR2 and IL-6R on monocyte subsets in patients with HF and controls, in order to identify differences in markers of cell activation and inflammation.

Methods: Patients with acute heart failure (AHF, n=51) were compared to those with stable HF (SHF, n=42) and stable coronary artery disease (CAD, n=44) without HF and 40 healthy controls (HC). Expression of CD14, TLR4, CCR2 and IL-6R on monocyte subsets was assessed by flow cytometry and expressed as median fluorescence intensity (MFI).

Results: In both AHF and SHF, expression of CD14 on Mon2 was higher compared to CAD ($p=0.022$ and $p=0.017$, respectively). CD14 expression was also significantly higher on Mon3 in AHF compared to all controls. There were no observed differences in TLR4 or IL-6R expression between AHF, SHF and CAD. There were no differences in CCR2 expression on Mon1 between the study groups. Expression on Mon2 was significantly higher in AHF and SHF compared to HC. CCR2 expression on Mon3 was also higher in AHF compared to both CAD and HC. In multivariate Cox regression analysis, IL-6R expression on Mon3 was a significant independent predictor of outcome (HR 1.136, CI 1.05-1.23, $p=0.002$).

Conclusions: Monocyte activation, as defined by expression of CD14, is increased on Mon2 in patients with SHF compared to controls. In patients with AHF, monocyte activation is highest on Mon3, with increased CD14 expression seen on this subset. IL-6R expression on Mon3 is an independent predictor of adverse clinical outcome in AHF patients. TLR4 and CCR2 expression on monocyte subsets are unaffected in HF and alternative markers for cell mobilisation may therefore be more important.

4.1 Introduction

Chapter 3 showed that Mon2 numbers are higher in patients with AHF and SHF compared to controls. Furthermore, this particular subset also appears to have prognostic implications in patients admitted to hospital with AHF. Although absolute numbers of subsets may be important, exploring the phenotype of each subset is also likely to be crucial to the understanding of their function in HF. This chapter therefore aims to assess whether monocyte subset activation and levels of inflammation are also affected in HF.

CD14 is an important surface molecule involved in monocyte activation and the interaction with its ligand, LPS, is one of the most powerful stimuli for monocyte cytokine production.(99) Levels of soluble CD14, monocyte-derived TNF and endotoxin are higher in patients with oedema and moderate-to-severe HF compared to those with mild disease.(99) Therefore, it is possible that increased CD14 expression on monocyte subsets may play a role in the immunological dysbalance seen in advanced HF.(28, 100)

As described in chapter 1, TLRs are a class of pattern recognition receptor important in innate immunity(104) and TLR4 expression is high on peripheral leukocytes, particularly monocytes.(105). Monocyte expression of TLR4 is increased in patients with HF and relates to the severity of disease.(106) One study found enhanced TLR4 staining in the myocardium undergoing remodelling, indicating accelerated leukocyte recruitment into the failing myocardium.(107) Therefore TLR4 is essential in the monocyte cellular response to bacterial LPS although its expression on monocyte subsets in HF has not been examined.

Elevated levels of IL-6 have also been seen in HF and associates with poor prognosis.(45, 46) IL-6 can cause myocyte hypertrophy, myocardial dysfunction and cachexia, as well as inhibiting cardiomyocyte apoptosis.(51) Despite the recognised inflammatory pathophysiology of HF, expression of IL-6 receptors on monocyte subsets has not been examined.

As described in chapter 1, MCP-1 has emerged as one of the most important chemokines responsible for monocyte mobilisation and recruitment to sites of inflammation. In chapter 3, I showed that plasma levels of MCP-1 are elevated in AHF and SHF compared to CAD, but expression of its receptor (CCR2) on monocyte subsets has not been explored and may give insight into a potential mechanism of monocyte recruitment to the failing myocardium.

My aims in this chapter were to measure the relative expression of CD14, TLR4, CCR2 and IL-6R on monocyte subsets in patients with HF and controls, in order to identify differences in markers of cell activation and inflammation.

4.2 Methods

4.2.1 Study population

The recruitment and data collection for patients with AHF, SHF, CAD and HC are described in detail in chapter 2. The baseline characteristics of the study groups are also summarised in **Table 2.1**.

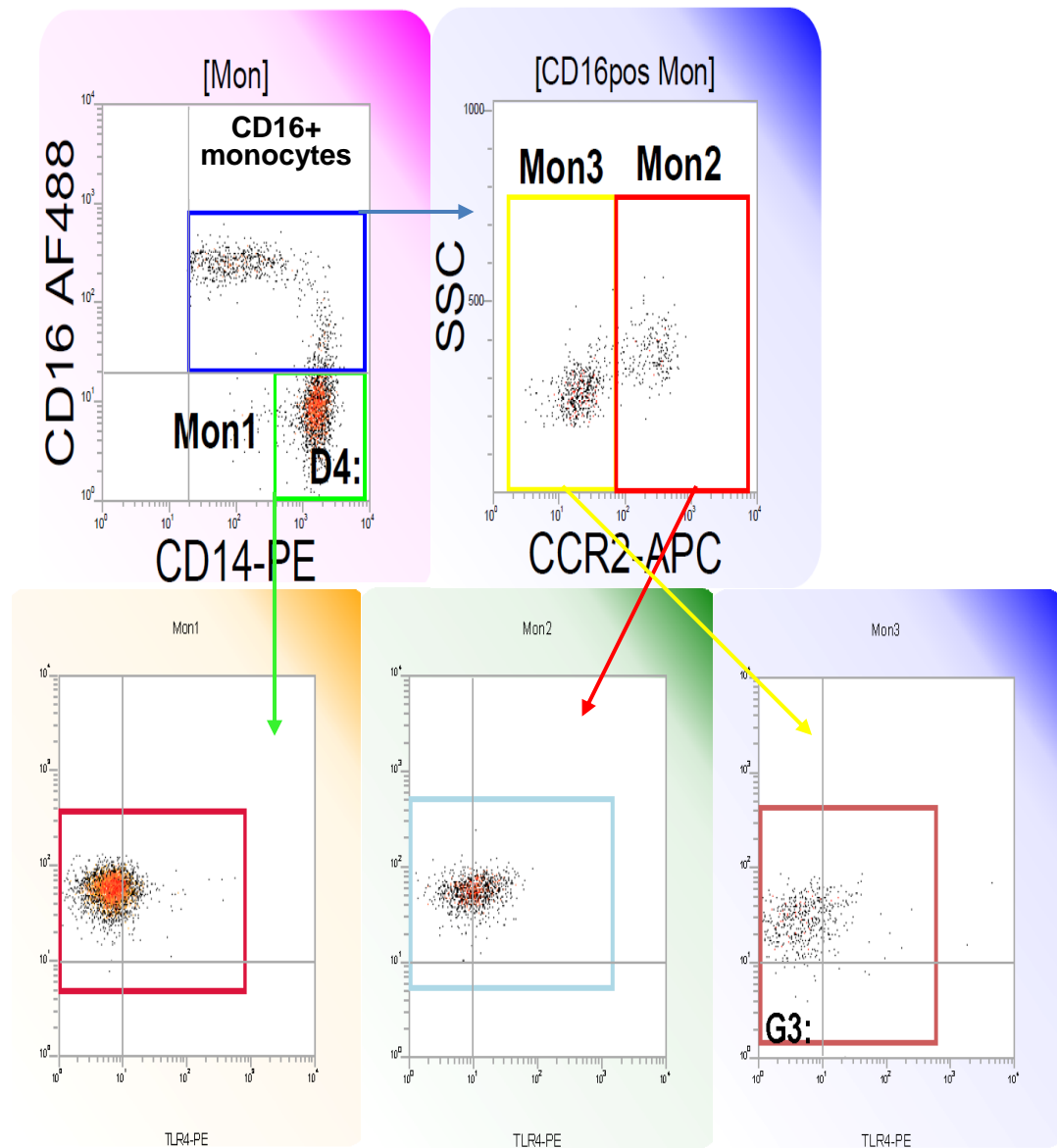
4.2.2 Flow cytometry

Flow cytometry analysis was performed as described in detail in chapter 2. Relative expression of the cell surface receptors on monocyte subsets were quantified as median fluorescence intensity [MFI]. In the example below, relative TLR4 expression is shown. **(Figure 4.1)**

4.2.3 Statistical analysis

Detailed statistical techniques have been described in chapter 2, but additional analyses were performed in this chapter to determine the predictive value of CD14, TLR4, CCR2 and IL-6R for adverse clinical events in AHF patients. Recognising the relatively small sample size, variables achieving $p < 0.10$ on univariate testing were entered into a multivariate Cox regression analysis to determine the independent predictors of adverse outcome, defined as either death or rehospitalisation. Age was also included in this analysis due to its well-recognised association with mortality. Kaplan Meier estimates for the distribution of time from index admission to the primary end-point were computed and log-rank analysis was performed to compare event free survival for patients with IL-6R expression on Mon3 dichotomised around the median value (31.2 MFI) on admission.

Figure 4.1 TLR4 expression on monocyte subsets using flow cytometry



4.3 Results

4.3.1 Subject characteristics

The study groups are summarised in **Table 2.1, chapter 2**.

4.3.2 Cross-sectional analysis

4.3.2.1 Monocyte expression of CD14

In both AHF and SHF, expression of CD14 on Mon2 was higher compared to CAD ($p=0.022$ and $p=0.017$, respectively). CD14 expression was also significantly higher on Mon3 in AHF compared to all controls. (**Table 4.1**).

4.3.2.2 Monocyte expression of TLR4

There were no observed differences in TLR4 expression between all 4 study groups for all 3 monocyte subsets (**Table 4.1**).

4.3.2.3 Monocyte expression of IL-6R

IL-6R expression on Mon1 was significantly higher in SHF and CAD compared to HC. IL-6R expression on Mon2 was significantly higher in AHF, SHF and CAD compared to HC. There were no differences in the expression of IL-6R in patients with AHF compared to SHF or CAD. There were also no differences in expression between SHF and CAD (**Table 4.1**).

4.3.2.4 Monocyte expression of CCR2

There were no differences in CCR2 expression on Mon1 between the study groups. Expression on Mon2 was significantly higher in AHF and SHF compared to HC. CCR2

expression on Mon3 was also higher in AHF compared to both CAD and HC. (Table 4.1)

Table 4.1 Expression of CD14, TLR4, IL-6R and CCR2 on monocyte subsets and plasma cytokine levels

	AHF (n=51)	SHF (n=42)	CAD (n=44)	HC (n=40)	P-value
<i>Expression of monocyte surface markers on subsets</i>					
CD14 (Mon1), MFI	1263 [389]	1411 [424]	1225 [375]	1412 [345]	0.042
CD14 (Mon2), MFI	1481 [473]†	1502 [484]†	1228 [408]	1365 [450]	<0.001
CD14 (Mon3), MFI	236 [116]*†‡	152 [57]	136 [47]	159 [32]	<0.001
TLR4 (Mon1), MFI	5.5 [2.0-7.0]	6.0 [4.9-7.3]	5.5 [4.8-7.2]	5.5 [4.3-6.9]	0.349
TLR4 (Mon2), MFI	9.7 [1.6-14.8]	10.5 [6.2-14.0]	9.6 [7.3-12.5]	9.5 [5.9-15.5]	0.780
TLR4 (Mon3), MFI	3.7 [1.4-5.2]	3.7 [2.9-5.5]	3.8 [3.2-4.3]	3.5 [3.0-4.3]	0.492
IL-6R (Mon1), MFI	65.6 [58.9-73.8]	63.8 [56.9-7.9]‡	70.3 [61.0-83.4] ‡	58.6 [40.5-72.0]	0.036
IL-6R (Mon2), MFI	56.4 [49.5-67.2] ‡	56.8 [51.6-70.7]‡	60.9 [57.4-74.8] ‡	50.7 [31.5-66.9]	0.004

IL-6R (Mon3), MFI	31.2 [24.4-40.1]	28.9 [24.0-36.3]	30.7 [27.4-35.0]	27.8 [23.6-34.2]	0.455
CCR2 (Mon1), MFI	155 [56]	153 [42]	144 [44]	135 [47]	0.22
CCR2 (Mon2), MFI	128 [44]‡	126 [36] ‡	111 [29]	104 [29]	0.005
CCR2 (Mon3), MFI	17 [2.2]†‡	16 [2.5]	15 [2.7]	15 [2.2]	<0.001
<i>Plasma cytokines</i>					
MCP-1 pg/ml	125 [82]†	136 [99]†	57 [43] ‡	126 [75]	<0.001
IL-6 pg/ml	11 [7-16]*†‡	2.6[1-4]	1. 9[1-3]	1.7 [0.5-3]	<0.001
<p>AHF: acute heart failure, SHF: Stable heart failure, CAD: coronary artery disease; HC: healthy control; HF:0 heart failure; MCP-1: monocyte chemoattractant protein-1; MFI: median fluorescent intensity, CCR2: C-C-chemokine receptor type 2, TLR: toll like receptor, CD: cluster of differentiation</p> <p>Data are presented as mean[standard deviation] or median [interquartile range]:</p> <p>* p<0.05 vs SHF, † p<0.05 vs CAD,‡ p<0.05 vs HC</p>					

4.3.3 Longitudinal results for monocyte expression of TLR4, IL6R, CCR2 and CD14 in patients with AHF

The longitudinal results are presented in **Table 4.2**.

Table 4.2 Longitudinal expression of TLR4, IL-6R, CCR2 and CD14 on monocyte subsets

	Admission (n=36)	Discharge (n=36)	Follow-up (n=36)	p value
CD14 (Mon1), MFI	1263 [375]	1297 [354]	1278 [382]	0.91
CD14 (Mon2), MFI	1507[493]	1459 [437]	1427 [375]	0.74
CD14 (Mon3), MFI	242 [112] †	208 [114]	182 [66] §	0.037
TLR4 (Mon1), MFI	4.5 [2.0-6.8] †	4.7 [1.9-7.3] ‡	2.7 [1.7-4.9]	0.002
TLR4 (Mon2), MFI	6.8 [1.6-12.2]	8.6 [1.5-14.7] ‡	3.4 [1.4-10.5]	0.013
TLR4 (Mon3), MFI	3.0 [1.5-4.5] *†	3.8 [1.6-5.4] ‡	1.8 [1.2-3.5]	<0.001
IL-6R (Mon1), MFI	65.5 [58.9-73.7]	67.5 [55.7-80.7]	60.8 [48.7-82.8]	0.54
IL-6R (Mon2), MFI	56.4 [49.5-67.1]	58.0 [49.6-70.3]	53.2 [36.8-75.4]	0.66
IL-6R (Mon3), MFI	31.2 [24.4-40.1]	31.7 [26.5-40.7]	32.0 [24.2-42.8]	0.37
CCR2 (Mon1), MFI	163 [61]	167 [60]	148 [44]	0.12
CCR2 (Mon2), MFI	132 [51]	129 [42]	115 [27]	0.095
CCR2 (Mon3), MFI	17 [2.2] †	17 [1.8] ‡	15 [2.1]	0.014
CD: cluster of differentiation, TLR4: toll-like receptor 4, IL-6R: interleukin receptor-6 receptor, CCR2: C-C-chemokine receptor type 2 MFI: median fluorescent intensity Data are presented as mean [standard deviation] or median [interquartile range] * p<0.05 Admission vs Discharge, †p<0.05 Admission vs Follow-up, ‡p<0.05 Discharge vs Follow-up, §p<0.05 vs. stable HF in cross-sectional analysis				

4.3.4 Correlations between monocyte expression of CD14, TLR4, IL-6R, CCR2 and plasma markers in acute heart failure

There were no significant correlations between any of the surface receptors and plasma IL-6, MCP-1, LVEF and BNP (Table 4.3, Table 4.4).

Table 4.3 Correlations between surface markers and plasma markers

	IL-6		MCP-1	
	r	p-value	r	p-value
TLR4 (Mon1)	-0.06	0.679	0.05	0.757
TLR4 (Mon2)	0.01	0.956	-0.01	0.971
TLR4 (Mon3)	-0.09	0.556	-0.01	0.946
IL-6R (Mon1)	-0.26	0.087	-0.08	0.609
IL-6R (Mon2)	-0.26	0.077	-0.05	0.724
IL-6R (Mon3)	-0.05	0.763	0.04	0.785
CD14 (Mon1)	-0.05	0.729	0.14	0.338
CD14 (Mon2)	-0.02	0.879	0.06	0.693
CD14 (Mon3)	0.20	0.161	0.17	0.248
CCR2 (Mon1)	0.01	0.971	-0.28	0.05
CCR2 (Mon2)	-0.04	0.799	-0.05	0.751
CCR2 (Mon3)	0.10	0.498	0.105	0.466
IL-6: interleukin-6, MCP: monocyte chemoattractant protein-1, TLR4: toll-like receptor 4, IL-6R: interleukin-6 receptor, CCR2: C-C-chemokine receptor type				

Table 4.4 Correlations between surface markers, LVEF and BNP

	LVEF		BNP	
	r	p-value	r	p-value
TLR4 (Mon1)	0.04	0.777	0.06	0.731
TLR4 (Mon2)	0.05	0.731	0.20	0.271
TLR4 (Mon3)	0.12	0.435	0.17	0.361
IL-6R (Mon1)	-0.06	0.718	-0.33	0.067
IL-6R (Mon2)	-0.14	0.363	-0.24	0.195
IL-6R (Mon3)	-0.09	0.556	-0.03	0.882
CD14 (Mon1)	-0.21	0.153	-0.20	0.264
CD14 (Mon2)	-0.14	0.374	-0.14	0.440
CD14 (Mon3)	0.02	0.897	-0.06	0.719
CCR2 (Mon1)	0.02	0.917	0.04	0.805
CCR2 (Mon2)	0.10	0.503	0.06	0.741
CCR2 (Mon3)	0.008	0.959	-0.11	0.527
LVEF: left ventricular ejection fraction, BNP: brain natriuretic peptide, TLR4: toll-like receptor 4, CD: cluster of differentiation, IL-6R: interleukin-6 receptor, CCR2: C-C chemokine receptor type 2				

4.3.5 Predictors of cardiovascular outcome in AHF

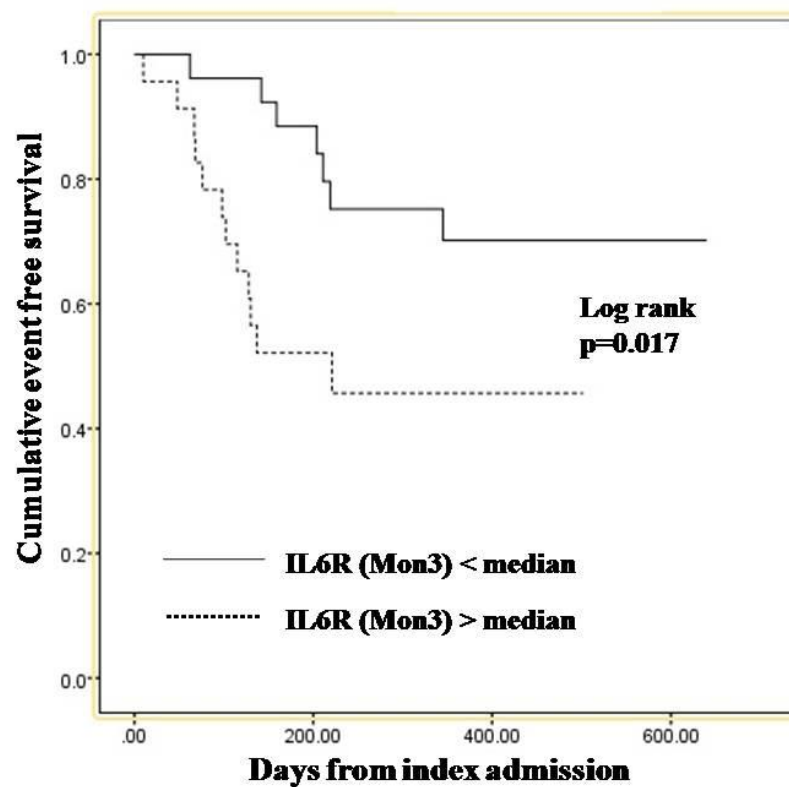
IL-6R expression on Mon3 was an independent predictor of clinical outcomes in patients with AHF (HR 1.034 CI 1.004-1.065, $p=0.028$) (**Table 4.5**). When adjusted for confounders (eGFR, BNP, LVEF and age), IL-6R expression on Mon3 remained a significant independent predictor of outcome (HR 1.136, CI 1.049-1.229, $p=0.002$). When the IL-6R expression on Mon3 was dichotomised around the median value (31.2 MFI), patients with expression above median had significantly worse outcomes compared to those below the median (log rank, $p=0.017$) (**Figure 4.2**).

Table 4.5 Cox regression analysis for predictors of death/rehospitalisation in AHF

Variable	Hazard ratio	95% CI	p value
<i>Univariate analysis</i>			
TLR4 (Mon1)	0.935	0.803-1.089	0.387
TLR4 (Mon2)	0.991	0.936-1.048	0.744
TLR4 (Mon3)	1.010	0.866-1.178	0.898
IL-6R (Mon1)	1.013	0.980-1.047	0.454
IL-6R (Mon2)	1.039	0.998-1.082	0.060
IL-6R (Mon3)	1.034	1.004-1.065	0.028
CD14 (Mon1)	1.000	0.999-1.001	1.000
CD14 (Mon2)	1.000	0.999-1.001	0.992
CD14 (Mon3)	0.996	0.991-1.001	0.104
CCR2 (Mon1)	1.005	0.998-1.011	0.155
CCR2 (Mon2)	1.000	0.990-1.009	0.946
CCR2 (Mon3)	0.957	0.782-1.170	0.667
<i>Multivariate analysis (adjusted for eGFR, brain natriuretic peptide, ejection fraction, and age)</i>			
IL-6R (Mon3)	1.136	1.049-1.229	0.002
TLR4: toll-like receptor 4, CD: cluster of differentiation, IL-6R: interleukin-6 receptor, CCR2: C-C chemokine receptor type 2			

Figure 4.2 Kaplan Meier curves of cumulative event-free survival in patients with AHF for the primary end-point of mortality or re-hospitalization

The groups are divided along the median value (31.2 MFI) of IL-6R expression on Mon3



4.4 Discussion

There are limited data examining the functionality of monocyte subsets in patients with HF. In this chapter, I show that compared to patients with CAD, those with SHF had higher expression of CD14 on Mon2. However, there were no differences in the expression of TLR4, IL-6R and CCR2 on any of the monocyte subsets. Compared to those with SHF, patients with AHF had higher expression of CD14 on Mon3 which may suggest that this subset is more activated (although numbers are not increased as shown in chapter 3). Interestingly, no differences between study groups in the expression of TLR4, IL-6R and CCR2 were observed.. Despite no apparent difference in IL-6R expression between study groups, in patients with AHF, expression on Mon3 had prognostic implications, with higher expression associating with adverse clinical outcomes. This finding appears to exist despite no correlation between IL-6R expression and plasma levels of IL-6, which as shown in chapter 3, are significantly elevated in AHF compared to all controls.

Therefore, in SHF, Mon2 appear to be more activated (reflected by enhanced CD14 expression) whereas in AHF, Mon3 appears to be more activated (enhanced CD14 expression). Furthermore, increased expression of IL-6R (another inflammatory receptor) on Mon3 in AHF is associated with a worse outcome. It is unclear from these data whether enhanced activation of Mon3 in AHF is simply a reflection of disease activity or whether this subset exerts a detrimental effect on the myocardium itself and clearly further studies are needed to explore this pathway.

An interaction between CD14 and LPS is a powerful stimulus for cytokine production and increased LPS levels have been observed in patients with HF, possibly due to mesenteric oedema and increased translocation of gut bacteria into the circulation.

LPS is the cell wall component of gram negative bacteria which has led researchers to investigate the possible role of bacteria in monocyte activation in HF and has resulted in the endotoxin-cytokine hypothesis. A potential mechanism involves an increased mesenteric pressure from venous congestion causing increased bowel permeability and subsequent bacterial translocation, accompanied by the release of endotoxins into the circulation.(99) Additionally, the process of bacterial adherence to intestinal epithelium may induce mucosal cytokine release which subsequently disrupts the epithelial barrier.(101)

Another mechanism resulting in endotoxin translocation relates to the increased sympathetic activity seen in HF, which redistributes blood flow away from the splanchnic circulation causing intestinal ischaemia and increased intestinal mucosal permeability.(102) The gut has abnormal morphology and function in patients with HF and one study showed a 35% increase in intestinal permeability in such patients.(103) In the same study, bowel wall thickness was also significantly greater than matched controls which correlated with blood concentration of leukocytes.

Activation of monocytes with LPS also inhibits apoptosis and prolongs cell survival, compared to stimulation with IL-4 which suppresses monocyte activation and induces apoptosis.(180, 181) Therefore the increased expression of CD14 on monocytes may be a self-preservation response in order to improve cell survival.

CD14 is also involved in cell-cell interaction and anti-CD14 monoclonal antibodies reduce the interaction between monocytes and endothelial cells.(182) Therefore, increased expression of CD14 on Mon2 in SHF and Mon3 in AHF may not only reflect increased cell activation but also provide a mechanism by which monocytes may interact with the endothelium of the heart.

Monocyte activation relies on interactions between specific receptors on the cell surface and pathogen-associated molecular patterns in order to stimulate inflammatory responses. TLR4 is a pattern recognition molecule that plays an important role in the CD14-LPS mediated cytokine cascade. Additionally, monocytes can be activated via endogenous stimuli such as HSP70, which is a ligand for the TLR pathway.

However, attempts to inhibit TLR4 in mouse models of doxorubicin-induced cardiomyopathy resulted in increased inflammation which in turn aggravated cardiac dysfunction.(183) Further evidence of a beneficial role comes from a recent study examining polymorphisms of TLR4 in humans. Compared to patients carrying the wild type gene, those with variant genes had significantly less improvement in LV function with standard treatment.(184)

Recently, our research group demonstrated no changes to TLR4 expression by monocyte subsets following MI,(185) suggesting that TLR4 expression may not be a reliable marker of monocyte activation in this pathology. In that study, there was no correlation between TLR4 expression on monocytes and nuclear factor kappa B (NFkB) activation.

Interestingly, in MI studies using fresh whole blood (compared to prolonged processing of samples), lower proportions of TLR+ monocytes are seen, leading to speculation that cell processing in fact activates these cells.(186) I have demonstrated that TLR4 expression is unaffected in HF and therefore this pattern recognition molecule may not be involved in monocyte activation in HF.

Finally, despite significantly higher plasma levels of MCP-1 in SHF and AHF compared to CAD, expression of CCR2 on all monocyte subsets was similar in all study groups. Although increased expression may have been expected as a reflection of increased cell activation, hypoxia is known to down-regulate CCR2 on monocytes and may account for such findings.(146) Alternatively, MCP-1 may not be so critical to the chemotaxis of monocytes in HF and alternative mechanisms may be more important.

4.5 Conclusion

Monocyte activation, as defined by expression of CD14, is increased on Mon2 in patients with SHF compared to controls. In patients with AHF, monocyte activation is highest on Mon3, with increased CD14 expression seen on this subset. Furthermore, IL-6R expression on Mon3 is an independent predictor of adverse clinical outcome in AHF patients. TLR4 and CCR2 expression on monocyte subsets are unaffected in HF and do not appear to be a marker of cell activation. Alternative markers for cell mobilisation may therefore be more important in this disease process.

CHAPTER 5

SURFACE MARKERS OF REPAIR AND

ANGIOGENESIS ON MONOCYTE

SUBSETS IN

HEART FAILURE

Abstract

Introduction: My aims in this chapter were to explore the possible angiogenic/reparative role of monocyte subsets in heart failure (HF).

Methods: Patients with acute heart failure (AHF, n=51) were compared to those with stable HF (SHF, n=42) and stable coronary artery disease (CAD, n=44) without HF and 40 healthy controls (HC). Expression of VEGF-1 receptor (VEGF-1R), CXCR4 and CD163 on monocyte subsets was assessed by flow cytometry and expressed as median fluorescence intensity (MFI).

Results: Compared to patients with SHF, those with AHF had significantly higher expression of VEGF-1R on Mon1 ($p=0.02$) and Mon2 ($p=0.005$). There were no differences in expression of VEGF-1R between SHF and disease controls for all subsets. Compared to those with SHF, patients with AHF had reduced expression of CXCR4 on Mon1 ($p=0.033$) but similar expressions on Mon2 and Mon3. There were no differences in

expression of CXCR4 between SHF and CAD. CD163 expression on Mon2 was significantly higher on Mon2 in patients with AHF compared to controls. There were no differences of CD163 expression on the remaining subsets and expression did not differ in SHF compared to disease controls. None of the measured monocyte parameters had any prognostic value in patients with AHF.

Conclusions: I have shown for the first time that AHF is associated with changes in the expression of angiogenic markers and scavenger receptors on monocyte subsets. Such changes may be responsible for the reparative and beneficial role of monocytes, especially in Mon2 which appear to be associated with improved clinical outcomes

5.1 Introduction

As discussed in chapter 1, murine models have demonstrated that monocytes are comprised of 2 subsets, with Ly-6Chigh monocytes implicated in inflammation, phagocytosis and the release of proteases (eg. MMPs) whilst Ly-6Clo may be important in the resolution of inflammation and show reparative properties, promote angiogenesis (via VEGF release), myofibroblast recruitment and myocardial remodeling.(74) Such heterogeneity is also present in human monocyte and broadly, monocytes are either CD16- (which have been likened to the inflammatory Ly-6Chigh cells in mice), or CD16+ (which most resemble the reparative Ly-6Clo cells).

As discussed in chapter 3, Mon2 appear to be increased in many inflammatory conditions, including MI, stroke, renal failure and severe asthma and I demonstrated in chapter 3 that they are also increased both acute and stable HF.(187). Moreover, higher Mon2 counts appear to be associated with better prognosis in patients admitted with AHF, which perhaps suggests a beneficial or reparative role for this subset.

In healthy subjects, the Mon2 subset highly expresses receptors for angiogenic factors, such as VEGF receptors type 1 and 2.(82) VEGF is a hormone crucial to angiogenesis and levels are increased in HF.(156) VEGF plays a major role in the chemotaxis of monocytes to sites of inflammation.(188, 189) and a recent study has shown that CD16+ monocytes have reduced expression of VEGFR1 compared to CD16- cells, with reduced chemotaxis towards VEGF-A, PlGF-1 and MCP-1.(190) However, it is unknown whether VEGFR-1 expression on monocyte subsets is altered in patients with HF.

Another chemokine receptor responsible for recruitment of monocytes into tissues and angiogenesis is CXCR4, with elevated levels seen within the failing myocardium on direct staining.(191) CXCR4 plays an important role in the mobilization of haematopoietic precursors and vasculogenesis(192, 193) and circulating levels of its ligand, SDF-1, are elevated in both animals and humans with HF.(144, 194) Activation of CXCR4 by SDF-1 depresses myocardial function in murine models(195) and CXCR4 deficient mice have smaller post-MI scars than those with normal expression.(196)

CD163 is a scavenger receptor for the haemoglobin-haptoglobin complex and is expressed exclusively on monocytes and macrophages.(197) Importantly, it is associated with the down-regulatory phase of inflammation.(198, 199) There is a propensity for upregulation of CD163 in response to IL-6 and IL-10 and stimulation of monocytes by CD163 agonist antibodies results in IL-10 secretion, thereby creating a cycle of activation.(200) Expression of CD163 increases as monocytes differentiate into macrophages and the degree of expression increases with resolution of the inflammatory response and wound healing.(198, 199)

In healthy subjects, monocyte subsets differ significantly in their expression of VEGFR1, CXCR4, CD163 with the highest values seen on Mon2.(82) However, it is unclear whether monocyte subset expression of these receptors is altered in HF.

Given the reparative potential of Mon2 in heart failure, we hypothesised that the expression of chemokine receptors VEGFR-1 and CXCR4 and the anti-inflammatory marker CD163 would be altered in patients with HF compared to controls.

5.2 Methods

5.2.1 Study population

The recruitment and data collection for patients with AHF, SHF, CAD and HC are described in detail in chapter 2.

5.2.2 Flow cytometry

FC analysis was performed as described in detail in chapter 2.

5.2.3 Statistical analysis

Detailed statistical techniques have been described in chapter 2, but additional analyses were performed in this chapter to determine the predictive value of VEGFR-1, CXCR4 and CD163 for adverse clinical events in AHF patients. Recognising the relatively small sample size, variables achieving $p < 0.10$ on univariate testing were entered into a multivariate Cox regression analysis to determine the independent predictors of adverse outcome, defined as either death or rehospitalisation. Age was also included in this analysis due to its well-recognised association with mortality.

5.3 Results

5.3.1 Subject characteristics

The study groups had similar baseline demographic and clinical characteristics (Table 2.1, chapter 2).

5.3.2 Cross-sectional analysis

The cross-sectional results are summarised in Table 5.1.

Table 5.1 Expression of reparative/angiogenic receptors on monocyte subsets

Monocyte parameter, MFI	AHF (n=51)	SHF (n=42)	CAD (n=44)	HC (n=40)	P value
VEGFR-1 (Mon1)	7.0 [5.4-8.4] *†	5.6 [4.8-6.5]	6.0 [5.0-7.3]	6.2 [5.2-7.3]	0.025
VEGFR-1 (Mon2)	17.4 [11.0-23.8] *†‡	11.9 [9.2-16.5]	12.8 [9.4-17.2]	13.4 [8.9-17.4]	0.015
VEGFR-1 (Mon3)	4.8 [3.5-7.5] ‡	4.2 [3.3-5.9]	4.0 [3.3-4.8]	3.5 [2.0-4.7]	0.028
CXCR4 (Mon1)	11.8 [9.9-13.6] *†	13.5 [12.6-15.9]	16.0 [13.0-18.9]	13.3 [11.3-15.8]	<0.001
CXCR4 (Mon2)	17.8 [15.2-23.2] †	20.7 [17.3-27.2]	23.5 [17.3-30.5] ‡	19.3 [14.7-26.0]	0.014
CXCR4 (Mon3)	7.8 [5.7-9.4] ‡	7.8 [5.4-10.1] ‡	8.0 [5.5-10.5] ‡	5.1 [3.7-7.1]	<0.001
CD163 (Mon1)	129 [83.3-163]	120 [89.4-174]	121 [85.6-179]	132 [106-153]	0.957
CD163 (Mon2)	307 [219-416] *†‡	222 [166-290]	214 [160-297]	214 [185-262]	<0.001
CD163 (Mon3)	18.1 [10.1-28.2]	14.5 [10.2-18.8]	15.5 [10.9-21.4]	13.3 [8.4-23.5]	0.421
AHF: acute heart failure, SHF: stable heart failure, CAD: coronary heart disease, HC: healthy controls, CD: cluster of differentiation, CXCR4: C-X-C chemokine receptor type 4, VEGFR: vascular endothelial growth factor receptor, MFI: medium fluorescence intensity * p<0.05 vs SHF, † p<0.05 vs CAD, ‡ p<0.05 vs HC					

5.3.2.1 Monocyte expression of VEGF-1 receptor

Compared to patients with SHF, those with AHF had significantly higher expression of VEGF-1R on Mon1 ($p=0.02$) and Mon2 ($p=0.005$). There were no differences in expression of VEGF-1R between SHF and disease controls for all subsets. VEGF-1R expression was significantly higher on Mon3 in AHF compared to HC.

5.3.2.2 Monocyte expression of CXCR4

Compared to those with SHF, patients with AHF had reduced expression of CXCR4 on Mon1 ($p=0.033$) but similar expressions on Mon2 and Mon3. There were no differences in expression of CXCR4 between SHF and CAD. Expression of CXCR4 was significantly higher on Mon2 in patients with AHF and CAD compared to HC and expression on Mon3 was higher in AHF, SHF and CAD compared to HC.

5.3.2.3 Monocyte expression of CD163

CD163 expression on Mon2 was significantly higher on Mon2 in patients with AHF compared to controls. There were no differences on the remaining subsets and expression did not differ in SHF compared to disease controls.

5.3.3 Longitudinal results

The longitudinal results are presented in **Table 5.2** below.

Table 5.2 Expression of reparative/angiogenic receptors on monocyte subsets in AHF at admission, discharge and 3 months

Monocyte parameter, MFI	Admission (n=36)	Discharge (n=36)	Follow-up (n=36)	p value
VEGFR-1 (Mon1)	7.02 [4.65-8.63]	6.81 [5.90-10.7]	6.41 [4.74-7.81]	0.185
VEGFR-1 (Mon2)	17.9 [9.20-26.5]	16.8 [10.9-24.7]	13.9 [9.79-18.3]	0.150
VEGFR-1 (Mon3)	4.72 [3.43-7.29]	5.83 [3.37-8.14]	4.00 [3.29-4.37]	0.026
CXCR4 (Mon1)	11.8 [9.9-13.6]	12.1 10.0-14.4]	11.5 [9.6-14.3]	0.254
CXCR4 (Mon2)	17.8 [15.2-23.2]	19.9 [16.4-26.9]	18.7 [13.1-24.3]	0.085
CXCR4 (Mon3)	7.8 [5.7-9.4]	8.06 [6.4-11.4]	6.9 [5.0-9.6]	0.121
CD163 (Mon1)	129 [81.8-160] †	75.0 [59.0-133.6]	70.1 [49.2-114]	0.006
CD163 (Mon2)	274.2 [210.5-415.1] †	220.3 [147.9-296.6] ‡	159.6 [108.6-209.7]	<0.001
CD163 (Mon3)	17.6 [9.65-31.4]	19.4 [11.9-26.2]	12.3 [8.63-18.2]	0.079
CXCR4: C-X-C chemokine receptor type 4, VEGFR: vascular endothelial growth factor receptor, MFI: medium fluorescence intensity, CD: cluster of differentiation †p<0.05 Admission vs Follow-up, ‡p<0.05 Discharge vs Follow-up				

5.3.4 Correlations between monocyte expression of VEGF-1 receptor, CXCR4, CD163, LVEF, BNP, IL-6 and MCP-1

VEGF-1R expression on Mon1 and Mon2 inversely correlated with LVEF ($r = -0.38$, $p = 0.001$ and $r = -0.33$, $p = 0.031$ respectively). CXCR4 expression on Mon2 and Mon3 significantly correlated with IL-6 ($r = 0.41$, $p = 0.008$ and $r = 0.41$, $p = 0.007$ respectively).
(Table 5.3)

5.3.5 Predictors of cardiovascular outcome in AHF

None of the analysed surface markers had any significant predictive value in clinical outcomes (Table 5.4).

Table 5.3 Correlations between surface markers and plasma markers

	LVEF		BNP		IL-6		MCP-1	
	r	p-value	r	p-value	r	p-value	r	p-value
CXCR4 (Mon1)	-0.12	0.48	-0.12	0.53	0.16	0.30	-0.01	0.93
CXCR4 (Mon2)	-0.12	0.46	0.32	0.09	0.41	0.008	0.01	0.93
CXCR4 (Mon3)	0.11	0.49	0.23	0.24	0.41	0.007	0.12	0.42
CD163 (Mon1)	-0.16	0.33	0.12	0.51	-0.26	0.09	-0.13	0.38
CD163 (Mon2)	0.05	0.74	0.25	0.18	0.00	0.98	-0.16	0.29
CD163 (Mon3)	-0.38	0.012	-0.35	0.06	0.01	0.94	0.15	0.33
VEGFR1 (Mon1)	-0.48	0.001	-0.12	0.52	0.02	0.89	0.07	0.65
VEGFR1 (Mon2)	-0.33	0.031	0.12	0.53	0.19	0.21	0.01	0.94
VEGFR1 (Mon3)	-0.27	0.08	0.09	0.62	0.14	0.36	-0.03	0.83
IL-6: interleukin-6, MCP-1: monocyte chemoattractant protein-1, LVEF: left ventricular ejection fraction, BNP: brain natriuretic peptide, CXCR4: C-X-C chemokine receptor type 4, CD: cluster of differentiation, VEGFR1: vascular endothelial growth factor receptor								

Table 5.4 Cox regression analysis for predictors of death/rehospitalisation in AHF

Variable	Hazard ratio	95% CI	p value
<i>Univariate analysis</i>			
CXCR4 (Mon1)	0.996	0.837-1.184	0.961
CXCR4 (Mon2)	1.028	0.983-1.075	0.232
CXCR4 (Mon3)	1.013	0.958-1.070	0.655
CD163 (Mon1)	1.001	0.995-1.008	0.754
CD163 (Mon2)	1.000	0.998-1.003	0.828
CD163 (Mon3)	1.007	0.982-1.034	0.578
VEGFR1 (Mon1)	0.975	0.890-1.068	0.586
VEGFR1 (Mon2)	1.004	0.971-1.039	0.809
VEGFR1 (Mon3)	1.029	0.894-1.185	0.692
CI: confidence interval, CXCR4: C-X-C chemokine receptor type 4, CD: cluster of differentiation, VEGFR: vascular endothelial growth factor receptor			

5.4 Discussion

In this chapter, I have demonstrated differences in the expression of markers for both repair and angiogenesis in patients with AHF compared to SHF. On Mon1, there was increased expression of VEGFR-1 and a reduced expression of CXCR4. On Mon2, there was increased expression of VEGFR-1 and CD163. No differences were observed between patients with SHF and CAD.

Angiogenesis is a process characterised by the growth of new blood vessels from pre-existing ones.(201) Arteriogenesis has more recently been introduced to define the outgrowth of pre-existing arterioles into larger conductance collateral vessels.(201) VEGF is perhaps the best characterised pro-angiogenic factor and levels are increased in patients with HF.(156) Although increased VEGF levels are not considered direct proof of enhanced angiogenesis in HF, there is direct evidence of impaired angiogenesis leading to HF.(158) STAT3-deficient mice have reduced myocardial capillary formation with subsequent interstitial fibrosis and LV dysfunction. Another study showed that disruption of angiogenesis contributes to the progression from adaptive cardiac hypertrophy to the development of HF.(202) VEGF improved collateral circulation and improved cardiac contractility in animal model.(203) Macrophage depletion delays wound healing and impairs cardiac remodelling.(154) Macrophages isolated from sites of tissue injury can induce angiogenesis in vitro, largely by the production of VEGF.(204, 205) In this chapter, VEGF-1R expression on Mon1 and Mon2 in AHF inversely correlated with LVEF, which perhaps reflects attempts at angiogenesis and repair in response to significantly impaired systolic function.

There is no direct evidence to link monocytes with endothelial progenitor cells (EPCs) but in vitro studies have shown that CD14⁺ cells can differentiate into endothelial-like cells with endothelial characteristics.(206) Under the influence of VEGF, monocytes can develop endothelial phenotype surface markers and can even form tubular-like structures in vitro.(207) Also, in response to MCP-1, macrophages infiltrate the myocardium to form erythrocyte-containing vascular-like tubes.(208) Furthermore, injection of CD14⁺ cells has been shown to improve vascularisation and healing in mouse models of ischaemic limbs.(209)

SDF-1 has been shown to induce therapeutic angiogenic cell homing and improve cardiac function following MI and has been regarded as being beneficial to the myocardium in this setting.(210, 211) Although SDF-1 and its main receptor CXCR4 may have a reparative potential, there are some conflicting data that suggest detrimental effects on injured myocardium. For instance, selectively blocking CXCR4 using AMD3100 reduces scar formation and in fact improves cardiac performance following MI.(212) Furthermore, reduced myocardial scarring is seen in CXCR4 deficient mice following MI with an associated reduction in pro-inflammatory monocyte infiltration and increased recruitment of reparative monocytes into the heart.(196) Interestingly however, LV function did not improve, possible due to a concomitant reduction in neovascularisation. Therefore the role CXCR4/SDF-1 axis is finely balanced with increased levels causing beneficial remodeling and neovascularisation but deficiencies also limiting infarct size and improving adaptation to hypoxic stress. In this chapter, reduced expression of CXCR4 was seen on Mon1 in AHF, which may reflect a response to myocardial damage (analogous to MI models) and a shift towards reparative

monocyte subset infiltration into the failing myocardium. Further studies will be needed to see whether this is the case.

5.5 Conclusions

I have shown for the first time that AHF is associated with changes in the expression of angiogenic markers and scavenger receptors on monocyte subsets. Such changes may be responsible for the reparative and beneficial role of monocytes, especially in Mon2 which in appear to be associated with improved clinical outcomes

CHAPTER 6

EXPRESSION OF CELL ADHESION MOLECULE RECEPTORS ON MONOCYTE SUBSETS IN HEART FAILURE

Abstract

Introduction: The objective of this chapter was to evaluate the expression of cell adhesion molecule (CAM) receptors on monocyte subsets in heart failure (HF) and examine their prognostic implication. Increased circulating levels of soluble CAMs have been observed in patients with HF but the precise mechanism of monocyte adhesion to the vascular endothelium remains unknown.

Methods: Patients with acute heart failure (AHF, n=51) were compared to those with stable HF (SHF, n=42) and stable coronary artery disease (CAD, n=44) without HF and 40 healthy controls (HC). Expression of intercellular adhesion molecule-1 receptor (ICAM-1R) and vascular CAM-1 receptor (VCAM-1R) on monocyte subsets was assessed by flow cytometry and expressed as median fluorescence intensity (MFI).

Results: Compared to patients with SHF, those with AHF had significantly higher expression of ICAM-1R on Mon2 ($p=0.015$). Compared to those with stable CAD, patients with SHF had a significantly higher expression of ICAM-1R on Mon2 ($p=0.040$). Compared to SHF, patients with AHF had a similar expression of VCAM-1R on both Mon1 and Mon3 but significantly higher expression on Mon2 ($p=0.037$). There were no significant differences between SHF and CAD in monocyte expression of VCAM-1R. In multivariate Cox regression analysis, VCAM-1R expression on Mon2 was associated with adverse clinical outcome (death or rehospitalisation) in AHF [HR 1.07 (1.01-1.14), $p=0.029$].

Conclusions: HF is associated with increased monocyte expression of surface receptors to both ICAM-1 and VCAM-1, being particularly linked to Mon2 subsets. Expression of VCAM-1R on Mon2 may have prognostic value in patients with AHF.

6.1 Introduction

CAMs represent a marker of endothelial cell activation and vascular inflammation and have been implicated in numerous inflammatory conditions, including allograft rejection, atherosclerosis and vasculitis.(213-215) CAMs have also been found to be increased in patients with HF.(147) CAMs mediate adhesion of monocytes to the vascular endothelium via interactions with integrin receptors found on the surface of monocytes and leads to monocyte penetration through the endothelial layer into areas of inflammation.(216) ICAM-1 and VCAM-1 are the two key CAMs belonging to the immunoglobulin gene superfamily and represent the most abundant leukocyte surface glycoproteins.(216) ICAM-1 is the ligand for integrin lymphocyte function associated antigen 1 and is composed of a beta subunit (CD18) and alpha subunit (CD11a). VCAM-1 is the ligand for very late antigen-4 and is composed of a beta subunit (CD29) and an alpha-4 subunit (CD49d). Patients with HF have increased accumulation of macrophages and an upregulation of CAMs within the failing myocardium.(124, 152) Circulating soluble CAMs (believed to be cleaved from the endothelial membrane) are also elevated in HF and correlate with severity of symptoms.(148, 152)

In patients with MI, increased ICAM-1 receptors are seen on monocytes compared to controls, providing a possible mechanism for increased monocyte-endothelial adherence in this disease process.(217) However, the precise mechanism of monocyte adhesion to the vascular endothelium in HF remains unknown and it is unclear whether expression of CAM receptors on monocyte subsets is affected in HF. The prognostic role of monocyte CAM receptor expression as a measure of their mobilisation into the site of inflammation is also unknown, but may be of importance.

In this chapter, my objectives were as follows: (i) to examine differences in the expression of ICAM-1 receptors (ICAM-1R) and VCAM-1 receptors (VCAM-1R) on monocyte subsets in patients with AHF, SHF and HF-free controls; and (ii) to evaluate whether expression of these receptors is associated with clinical outcomes (mortality and rehospitalisation) in patients with AHF.

6.2 Methods

6.2.1 Study population

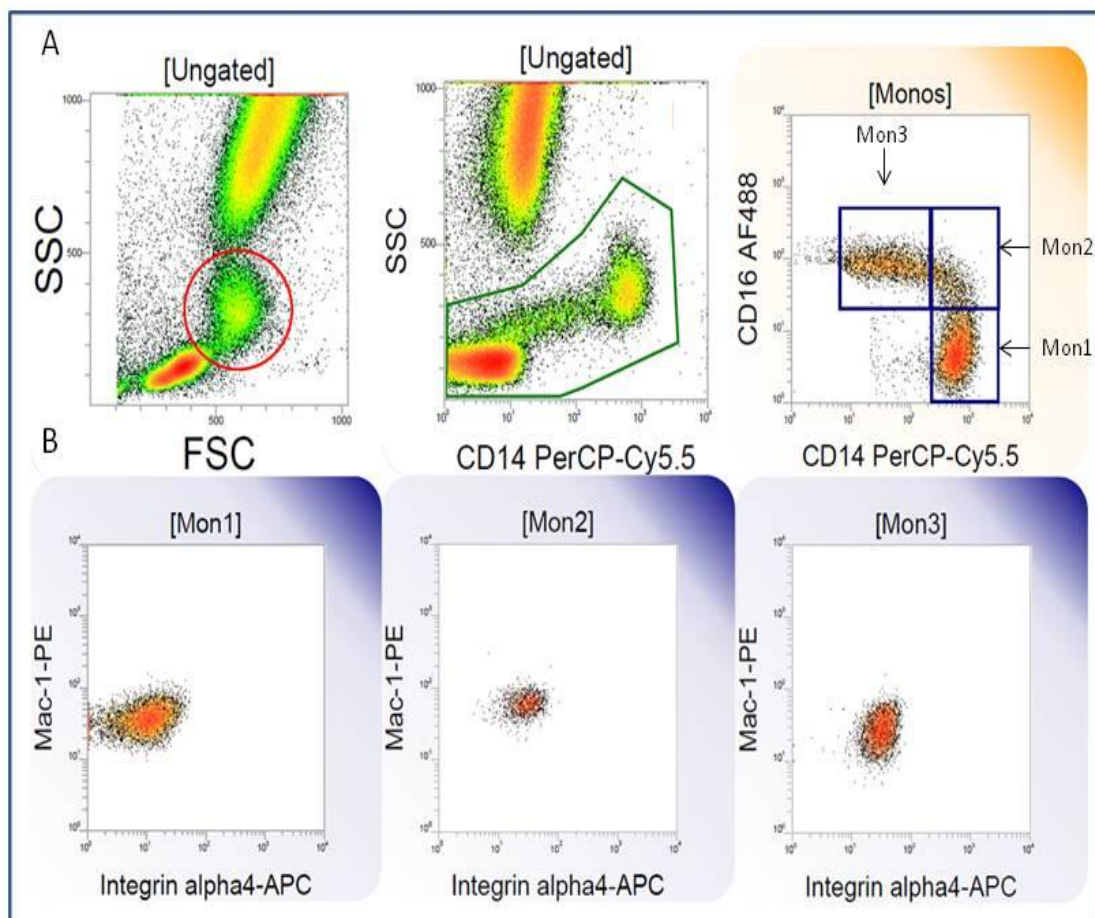
The recruitment and data collection for patients with AHF, SHF, CAD and HC are described in detail in chapter 2.

6.2.2 Flow cytometry

Flow cytometry analysis was performed as described in detail in chapter 2 (**Figure 6.1**).

Figure 6.1 Flow cytometric measurements of CAM receptor expression on monocyte subsets

- A. Selection of CD14⁺⁺CD16⁻CCR2⁺ ('classical', Mon1), CD14⁺⁺CD16⁺CCR2⁺ ('intermediate', Mon2) and CD14⁺CD16⁺⁺CCR2⁻ ('non-classical', Mon3) monocytes.
- B. Measurement of ICAM-1R (Mac-1-PE) and VCAM-1R (Integrin alpha4-APC) expression on individual monocyte subsets. Axes are measurements of median fluorescent intensity



6.2.3 Statistical analysis

Detailed statistical techniques have been described in chapter 2, but additional analyses were performed in this chapter to determine the predictive value of ICAM-1R and VCAM-1R on monocyte subsets for adverse clinical events in AHF patients. Recognising the relatively small sample size, variables achieving $p < 0.10$ on univariate testing were entered into a multivariate Cox regression analysis to determine the independent predictors of adverse outcome, defined as either death or rehospitalisation. Age was also included in this analysis due to its well-recognised association with mortality. Kaplan Meier analyses of cumulative event-free rates, stratified into two groups on the basis of median MFI of Mon2 VCAM-1R, were compared. The differences between event-free curves were tested by a log rank test.

6.3 Results

6.3.1 Subjects characteristics

The study groups had similar baseline demographic and clinical characteristics (Table 2.1, chapter 2).

6.3.2 Cross-sectional analysis

6.3.2.1 Monocyte expression of ICAM-1 receptor

Compared to patients with SHF, those with AHF had significantly higher expression of ICAM-1R on Mon2 ($p = 0.015$) and a trend towards higher expression on Mon1 ($p = 0.083$) and Mon3 ($p = 0.073$) (Table 6.1, Figure 6.1). Compared to those with stable CAD, patients with SHF had a significantly higher expression of ICAM-1R on Mon2 ($p = 0.040$) and a trend towards a higher expression on Mon1 ($p = 0.072$).

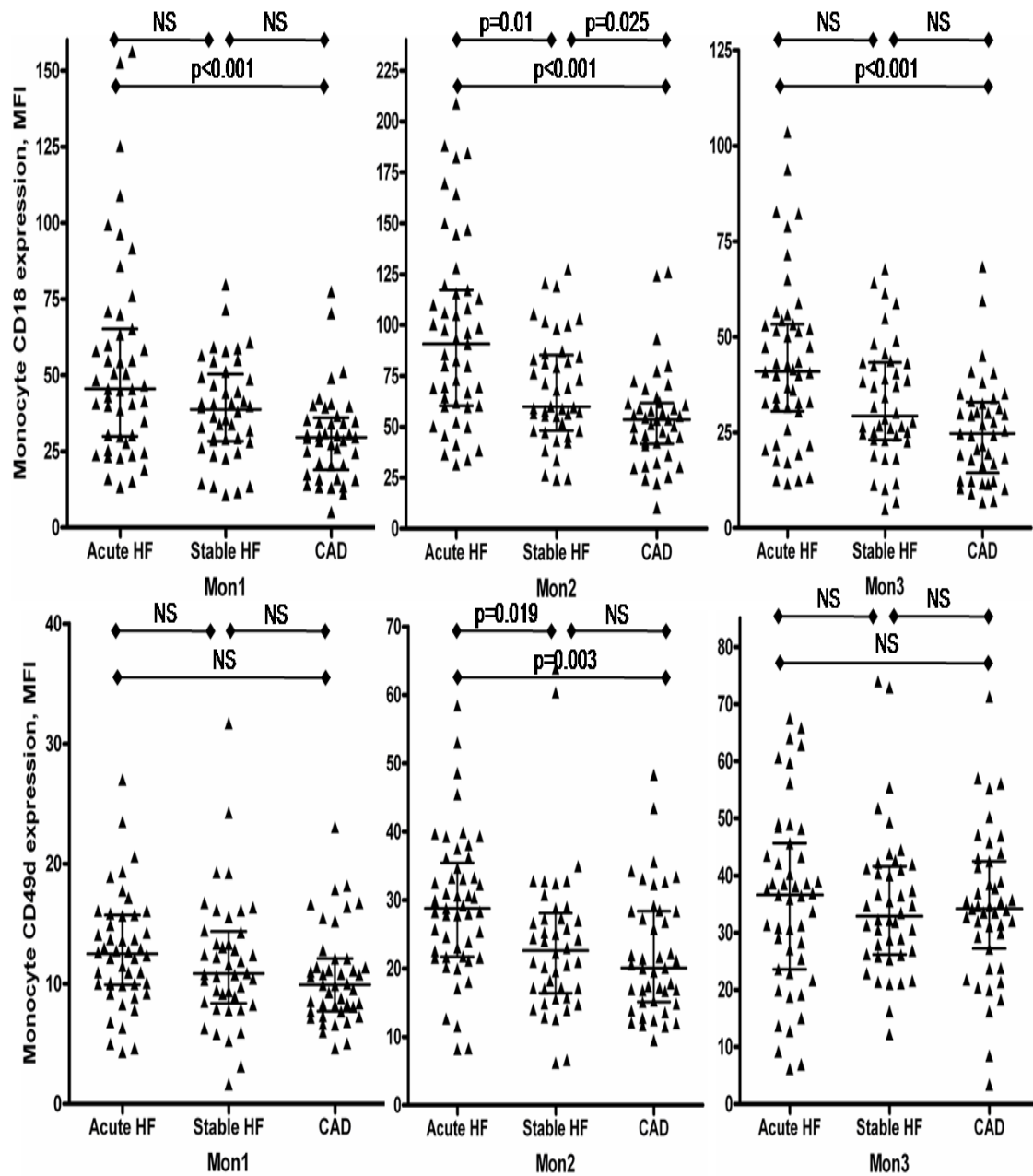
6.3.2.2 Monocyte expression of VCAM-1 receptor

Compared to SHF, patients with AHF had a similar expression of VCAM-1R on both Mon1 and Mon3 but significantly higher expression on Mon2 ($p=0.037$) (**Table 6.1, Figure 6.1**). There were no significant differences between SHF and CAD in the expression of VCAM-1R on any of the monocyte subsets. There were no differences in the expression of VCAM-1R on Mon3 across the study groups.

Table 6.1 CAM receptor expression on monocyte subsets in the study groups

	AHF (n=51)	SHF (n=42)	CAD (n=44)	HC (n=40)	P value
ICAM-1R (Mon1), MFI	46 [30-66] †	39 [29-50]	30 [18-36]	34 [24-41]	<0.001
ICAM-1R (Mon2), MFI	88 [66-116] *†‡	60 [48-85] †	54 [41-62]	56 [44-75]	<0.001
ICAM-1R (Mon3), MFI	41 [29-54] †‡	29 [23-43]	25 [13-33]	30 [19-36]	<0.001
VCAM-1R (Mon1), MFI	13 [10-16]	11 [9-14]	10 [8-12]	11 [7.9-14]	0.100
VCAM-1R (Mon2), MFI	29 [22-35] *†	23 [17-28]	20 [15-28]	19 [16-31]	0.002
VCAM-1R (Mon3), MFI	37 [25-46]	33 [26-41]	34 [27-43]	33 [26-45]	0.971
<p>Data are presented as mean [Standard Deviation] or median [Interquartile range]; *<0.05 vs. SHF, †<0.05 vs. CAD, ‡ p<0.05 vs HC AHF: acute heart failure, SHF: stable heart failure, HC: healthy controls CAD: coronary artery disease, HF: heart failure, Mon1: CD14++CD16- monocytes, Mon2: CD14++CD16+CCR2+ monocytes, Mon3: CD14+CD16++CCR2- monocytes, MCP-1: monocyte chemoattractant protein-1, MFI: median fluorescent intensity</p>					

Figure 6.2 CAM receptor expression across the disease groups



HF: Heart failure, CAD: coronary heart disease, MFI: median fluorescence intensity.

CD18: ICAM-1R expression, CD49d: VCAM-1R expression

6.3.3 Longitudinal analysis of study parameters in AHF

In patients with AHF, there were no significant changes in ICAM-1R expression during follow-up (**Table 6.2**). In patients with AHF, VCAM-1R expression on Mon3 reduced significantly during follow-up ($p<0.001$), whilst VCAM-1R expression on Mon1 and Mon2 did not show any changes.

Table 6.2 CAM receptor expression on monocyte subsets in AHF at admission, discharge and 3 months

	Admission (n=36)	Discharge (n=36)	Follow-up (n=36)	p value
ICAM-1R (Mon1), MFI	45 [29-70]	54 [34-82]	35 [27-52]	0.60
ICAM-1R (Mon2), MFI	90 [60-132]	95 [70-137]	67 [51-101]	0.30
ICAM-1R (Mon3), MFI	41 [28-57]	47 [33-65]	34 [21-58]	0.75
VCAM-1R (Mon1), MFI	12 [10-16]	13 [9-16]	10 [6-14]	0.38
VCAM-1R (Mon2), MFI	30 [23-36]	29 [23-35]	25 [14-34]	0.17
VCAM-1R (Mon3), MFI	38 [23-49]	43 [33-53] ‡	30 [18-45]	<0.001
Data are presented as mean [Standard deviation] or median [Interquartile range], ICAM-1R: intercellular cell adhesion molecule-1 receptor, VCAM-1R: Vascular cell adhesion molecule-1 receptor, MFI: median fluorescent intensity, Mon1: CD14++CD16- monocytes, Mon2: CD14++CD16+ monocytes, Mon3: CD14+CD16++ monocytes, MFI: median fluorescent intensity, ‡p<0.05 Discharge vs Follow-up				

6.3.4 Correlations between monocyte expression of ICAM-1 and VCAM-1 receptors and plasma markers in AHF

ICAM-1R expression on Mon1 and Mon3 were significantly and negatively correlated with BNP levels (**Table 6.3**). Interleukin-6 significantly correlated with BNP values ($r=0.45$, $p=0.008$). There were no significant associations between monocyte ICAM-1R/VCAM-1R expression and IL-6 and MCP-1 concentrations.

Table 6.3. Correlation analysis between surface markers and plasma markers in AHF

	ICAM-1R						VCAM-1R					
	Mon1		Mon2		Mon3		Mon1		Mon2		Mon3	
	r	P	R	p	r	p	r	p	r	p	r	p
BNP	-0.46	0.009	-0.29	0.11	-0.39	0.029	-0.06	0.76	0.11	0.58	-0.24	0.22
IL6	-0.20	0.19	-0.11	0.46	-0.20	0.18	0.05	0.74	0.15	0.34	-0.10	0.52
MCP-1	0.12	0.44	0.12	0.45	0.13	0.39	-0.03	0.87	-0.16	0.30	-0.17	0.29
BNP: brain natriuretic peptide, ICAM-1R: intercellular cell adhesion molecule-1 receptor, IL-6: interleukin-6, MCP-1: monocyte chemoattractant protein-1, VCAM-1R: vascular cell adhesion molecule-1 receptor												

6.3.5 Predictors of cardiovascular outcome in AHF

In univariate Cox regression analysis, higher VCAM-1R expression on Mon2, measured within the first 24 hours of admission, was associated with a higher incidence of combined death and rehospitalisation [Hazard ratio (HR) 1.05, 95% CI 1.01-1.09, $p=0.018$] (**Table 6.4**). After adjustment for potential confounders including age, BNP

levels, LVEF and eGFR, VCAM-1R expression on Mon2 remained a predictor of adverse outcome [HR 1.07 (1.01-1.14), p=0.029].

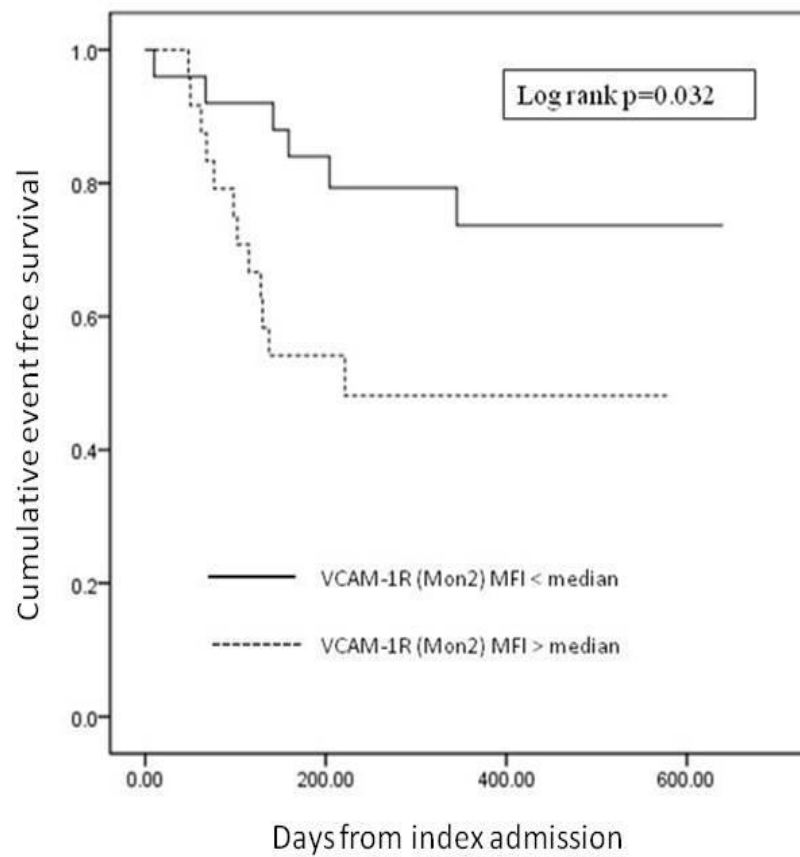
Patients were divided into 2 groups on the basis of the median expression of VCAM-1R on Mon2 (MFI 29). Kaplan-Meier survival analysis found that high VCAM-1R expression on Mon2 (i.e. above the median) was associated with worse outcomes (log rank test, p=0.032) (**Figure 6.2**).

Table 6.4 Cox regression analysis for predictors of death/rehospitalisation in AHF

Parameter	Unadjusted Hazard Ratio	p value	*Adjusted Hazard Ratio	p value
ICAM-1R (Mon1), MFI	0.99 (0.98-1.01)	0.371	1.00 (0.94-1.05)	0.86
ICAM-1R (Mon2), MFI	1.00 (0.99-1.01)	0.498	1.00 (0.98-1.02)	0.97
ICAM-1R (Mon3), MFI	0.99 (0.96-1.01)	0.284	0.97 (0.916-1.02)	0.25
VCAM-1R (Mon1), MFI	1.06 (0.97-1.15)	0.225	1.13 (0.98-1.29)	0.09
VCAM-1R (Mon2), MFI	1.05 (1.01-1.09)	0.018	1.07 (1.01-1.14)	0.029
VCAM-1R (Mon3), MFI	1.00 (0.97-1.03)	0.839	0.95 (0.89-1.00)	0.07
*Adjusted for age, brain natriuretic peptide, left ventricular ejection fraction and estimated glomerular filtration rate, ICAM-1R: intercellular cell adhesion molecule-1 receptor, VCAM-1R: vascular cell adhesion molecule-1 receptor, MFI: median fluorescence intensity				

Figure 6.3 Kaplan Meier curves of cumulative event-free survival from death/rehospitalisation in AHF

The groups are divided along the median MFI value of VCAM-1R on Mon2 (=29)



6.4 Discussion

I have shown for the first time that HF is associated with increased expression of both ICAM-1R and VCAM-1R on the Mon2 subset. Patients with AHF have a higher expression of both studied CAM receptors on Mon2 compared to those with SHF, with high VCAM-1R expression related to higher rates of death and rehospitalisation.

During vascular inflammation, an initial low affinity interaction between leukocytes and endothelial cells is mediated by selectins (another group of CAMs), followed by ICAM-1 and VCAM-1 facilitating their firm adhesion and migration through the endothelial barrier.(216) Although ICAM-1 is expressed on the surface of inactivated cells, exposure to inflammatory cytokines (e.g., TNF and IL-1) leads to deregulated expression. Conversely, VCAM-1 is hardly expressed on unstimulated endothelium but is markedly increased following cytokine stimulation and also takes part in monocyte adherence and migration.

Circulating levels of soluble CAMs are increased in HF and play an important role in mediating cell-cell interaction during vascular inflammation and endothelial dysfunction.(147) Moreover, high levels associate with adverse clinical outcomes and correlate negatively with LVEF.(149) Monocytes play an important role in the immune response associated with HF and a recent study showed increased numbers of Mon2+ monocytes in patients with stable HF compared to healthy controls.(93) As well as elevated circulating monocytes, significantly higher numbers of macrophages have been found within the myocardium of HF patients, suggesting a migration of monocyte precursors to the site of inflammation.(218) Indeed, monocytes from patients with severe HF have greater adhesiveness to cultured human aortic endothelial cells

compared to controls or those with mild HF, which correlates with clinical outcomes.(150)

Until now, the potential mechanisms for monocyte adhesion to the endothelium in HF remained unclear. In this study, we have shown that monocyte expression of CAM receptors is enhanced in HF, which may provide an insight into the mechanisms of monocyte-endothelial interaction.

In both AHF and SHF, ICAM-1R expression was only increased on Mon2. This subset has been shown to be elevated in AHF and SHF and appears to correlate with disease severity. In contrast, VCAM-1R expression was only increased on Mon2 in AHF and not in SHF. One possible explanation for this apparent discrepancy is that levels of systemic inflammation in AHF are higher than in SHF, as reflected by higher plasma IL-6 levels. Studies have shown that persistent ICAM-1 expression on the endothelium is characteristic of chronic inflammation, whereas expression of VCAM-1 indicates endothelial damage and more acute inflammation.(150, 213, 219, 220) Thus, up-regulation of VCAM receptors on monocytes may be induced by the acute inflammatory response associated with AHF rather than the more chronic state. In support of this, analysis of myocardium from patients undergoing cardiac transplantation revealed high expression of endothelial ICAM-1, whereas VCAM-1 was not expressed, suggesting an absence of acute inflammation in such patients.(124)

Increased ICAM-1R and VCAM-1R expression on Mon2 may result in preferential adhesion to the myocardial endothelium and thence into the heart itself compared to the other monocyte subsets. It remains unclear whether this would be beneficial or detrimental to the myocardium given the diversity in phenotype and functionality within

the subsets (as discussed in earlier chapters). In healthy subjects, Mon2 in particular appears to be highly phagocytic, which may be important in removing apoptotic cells and endogenous ligands from the myocardium.(177) Mon 2 is also a potent producer of IL-10, an anti-inflammatory cytokine which may be important in tissue repair as well as strongly expressing angiogenic surface markers (e.g. VEGF).(82)

In healthy subjects, Mon2 also releases TNF (compared to Mon1, which releases IL-1 β and IL-6), which has been shown to be harmful and leads to increased CAM expression on the endothelial surface, creating a cycle of activation and inflammation.(221) Furthermore, TNF has also been shown induce ICAM-1 expression on myocytes in rat models which may provide a potential mechanism for direct access of monocytes to myocytes per se.(222)

6.5 Conclusions

I have shown for the first time that HF is associated with increased monocyte expression of surface receptors to both ICAM-1 and VCAM-1, being particularly linked to the Mon2 subset. This may provide some insight into the important interaction between monocytes and the vascular endothelium in HF. Enhanced CAM receptor expression is greatest on Mon2 and VCAM-1R expression on this subset appears to have prognostic value in patients with AHF. There are currently no direct data to suggest that Mon2 is recruited to the myocardium more readily than the other subsets in HF. However, given the fact that absolute Mon2 numbers are higher in HF alongside enhanced CAM receptor expression on this subset, further investigation is warranted on its pathophysiological implications.

CHAPTER 7

FORMATION OF

MONOCYTE-PLATELET AGGREGATES

IN HEART FAILURE

Abstract

Introduction: Cross-talk between monocytes and platelets is reflected by the formation of monocyte-platelet aggregates (MPAs), which are a sensitive marker of both monocyte and platelet activation. It is not known whether MPAs are affected in HF.

Methods: MPAs were analysed by flow cytometry for the 3 monocyte subsets in 51 patients with AHF, 42 patients with SHF, 44 patients with stable CAD and 40 HC. The prognostic impact of MPAs was examined in patients with AHF.

Results: The median total MPA count was significantly higher in AHF (133 per μl , IQR 99-180) compared to SHF (81 per μl , IQR 55-117, $p<0.001$), CAD (70 per μl , IQR 49-98, $p<0.001$) and HC (52 per μl , IQR 35-76, $p<0.001$). MPAs associated with Mon1 and Mon2 were also significantly increased in AHF compared to the three control groups ($p<0.001$). The median proportion of Mon1 aggregated with platelets was increased in AHF (17% IQR 12-23) compared to SHF (12%, IQR 10-18, $p=0.033$), CAD (13%, IQR 10-16, $p<0.001$) and HC (11%, IQR 7-13, $p<0.001$). A higher percentage of Mon3 aggregated with platelets was also seen in AHF compared to SHF ($p=0.012$), and HC ($p<0.001$) but not compared to CAD ($p=0.647$). MPAs associated with Mon2 were significantly lower in patients who experienced adverse clinical outcomes of death or re-hospitalization (median 12.8 cells/ μl IQR 7.1-19.1) compared to those who remained free of events (median 16.1 cells/ μl IQR 11.2-27.7, $p=0.03$). MPAs associated with Mon2 count remained an independent negative predictor of combined death and re-hospitalisation (for an increase of 5 cells/ μl) 0.581, 95% CI 0.343-0.984; $p=0.043$].

Conclusion: MPA formation in patients with both acute and stable HF is increased and appears to be confined to monocytes from Mon1 and Mon2 subsets. MPAs associated with Mon2 are also negatively predictive of a worse prognosis in AHF.

7.1 Introduction

HF is both an inflammatory and pro-thrombotic condition as evidenced by elevated levels of circulating cytokines and an increased risk of thromboembolic events.(223) Platelets are the major cellular component of thrombosis(224) and patients with HF have enhanced platelet activation as reflected by increased whole blood aggregation(225), high mean platelet volume(226) and increased platelet P-selectin surface exposure.(227) In addition to roles in haemostasis and thrombosis, platelets are also able to regulate the activity of other cell types and cross-talk between monocytes and platelets is reflected by formation of MPAs. MPAs have been shown to be a more sensitive marker of platelet activation than platelet surface P-selectin, since degranulated platelets rapidly shed P-selectin but still function in the circulation.(228, 229) Monocyte aggregation with platelets is accompanied by monocyte activation, resulting in increased cytokine production, expression of cell-adhesion molecules and the release of MMPs, all of which may be important in collagen breakdown and LV dysfunction.(230)

Even in the absence of platelet interaction, monocytes play an important role in inflammation and thrombosis, performing vital functions such as phagocytosis, cytokine production and tissue repair(16) as well as being a major source of blood tissue factor.(165)

MPAs are increased in numerous conditions associated with ischaemia and thrombosis, such as MI, limb ischaemia(231) and stroke.(232) However, an interaction between monocytes (and their individual subsets) and platelets has not been studied in HF and the possible impact on clinical outcome is unknown.

In this chapter, I aimed to compare levels of MPAs in patients with AHF, SHF and control subjects with normal LV function, as well as dynamics of monocyte-platelet interactions and their association with clinical outcomes in patients with AHF.

7.2 Methods

7.2.1 Study population

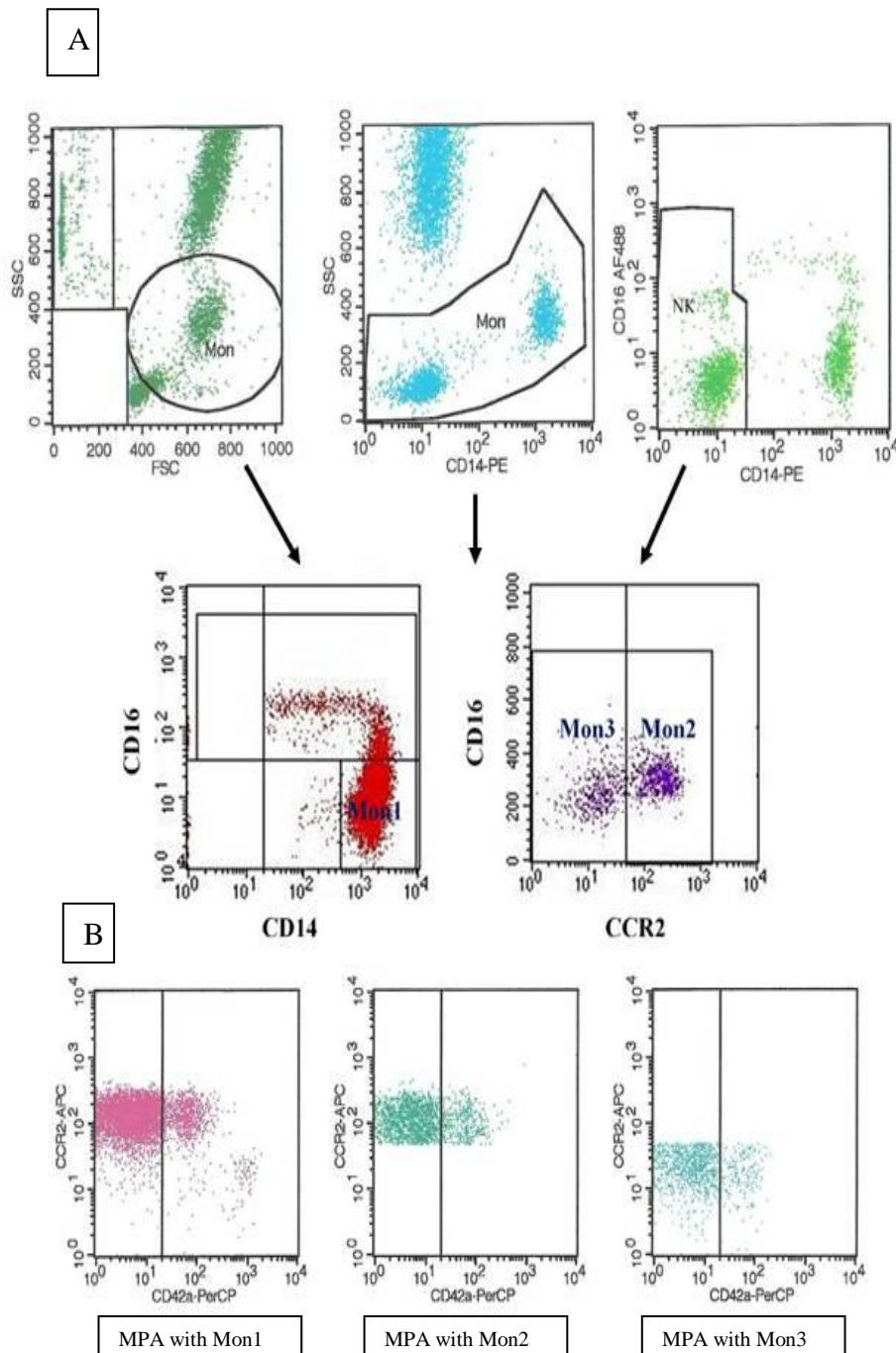
The recruitment and data collection for patients with AHF, SHF, CAD and HC are described in detail in chapter 2.

7.2.2 Flow cytometry

Flow cytometry analysis was performed as described in detail in chapter 2. Monocytes were selected by gating strategies based on forward and side scatter to select monocytes, side scatter versus CD14 expression to exclude granulocytes, and ungated CD14 versus CD16 expression to exclude natural killer lymphocyte, MPAs were defined as events positive to both monocyte markers and the platelet marker CD42a (glycoprotein IX) (**Figure 7.1**). The number of events collected was at least 400 events for each monocyte subset and 10000 count beads.

Figure 7.1 Identification of monocyte-platelet aggregates by flow cytometry

- (A) Gating strategy using forward and side scatter to select monocytes, side scatter versus CD14 expression to exclude granulocytes and ungated CD14 versus CD16 expression to exclude natural killer (NK) lymphocytes
- (B) Monocyte-platelet aggregates defined as monocytes expressing the platelet surface marker CD42a



7.3.3 Statistical analysis

Detailed statistical techniques have been described in chapter 2, but additional analyses were performed in this chapter to determine the predictive value of MPAs for adverse clinical events in AHF patients. Recognising the relatively small sample size, variables achieving $p < 0.10$ on univariate testing were entered into a multivariate Cox regression analysis to determine the independent predictors of both primary and secondary endpoints in AHF patients. Age was also included in this analysis due to its well-recognised association with mortality. Kaplan Meier estimates for the distribution of time from index admission to the primary end-point (death or rehospitalisation) were computed and log-rank analysis was performed to compare event free survival for patients with MPAs associated with Mon2 above and below the median value at admission.

7.3 Results

7.3.1 Subjects characteristics

The baseline characteristics of the study groups are also summarised in **Table 2.1**, **chapter 2**.

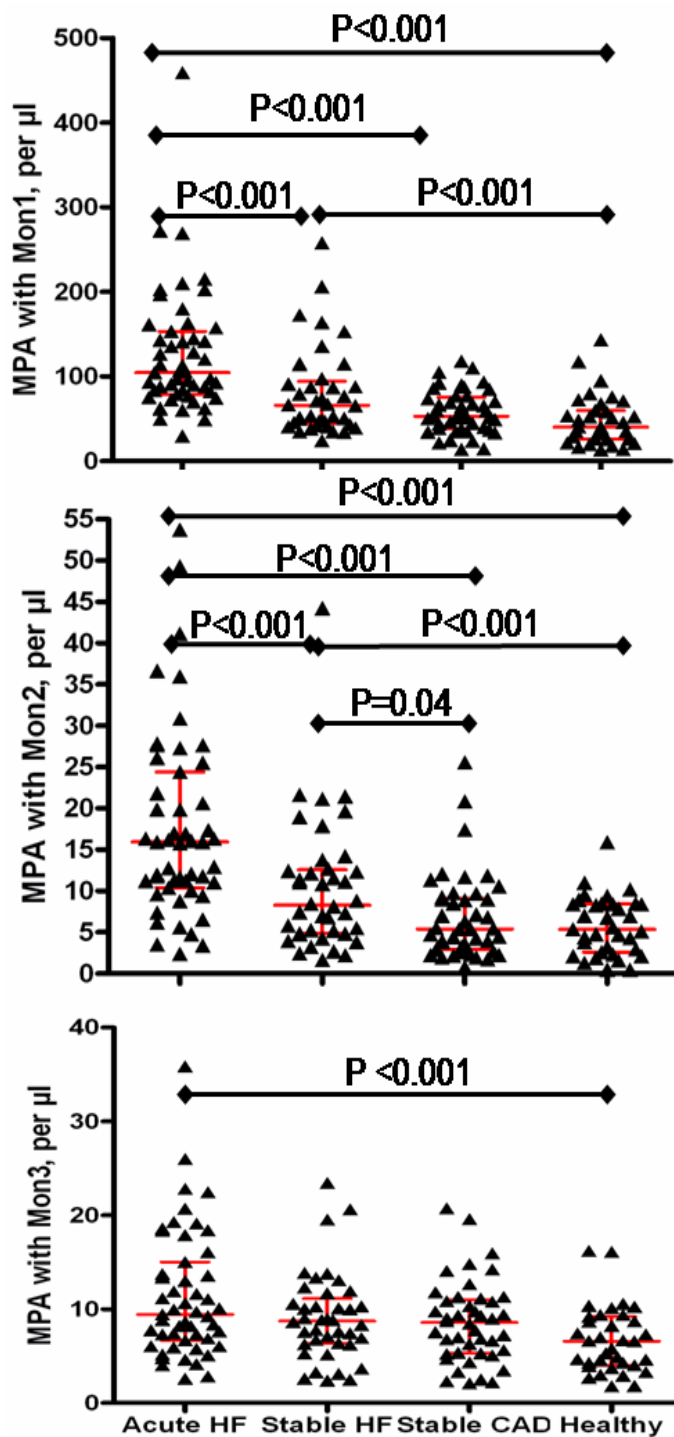
7.3.2 Cross-sectional analysis

The total MPA count and MPAs associated with Mon1 and Mon2 were significantly increased in AHF compared to the three control groups (**Table 7.1**, **Figure 7.2**). MPAs associated with Mon3 were higher in AHF compared to HC ($p < 0.001$) but similar to SHF and CAD.

Table 7.1 Monocyte-platelet aggregates in the cross-sectional analysis

	AHF (n=51)	SHF (n=42)	CAD (n=44)	HC (n=40)	p value
Total MPA, per μ l	133 [99-180]*†‡	81 [55-117]	70 [49-98]	52 [35-76]	<0.001
MPA with Mon1, per μ l	105 [79-153]*†‡	66 [44-96]	53 [38-76]	40 [26-60]	<0.001
MPA with Mon2, per μ l	16 [10-24] *†‡	8 [5-13] §	5 [3-9]	5 [3-8]	<0.001
MPA with Mon3, per μ l	9 [7-15] ‡	9 [6-11]	9 [5-11]	7 [4-9]	0.003
Mon1 with MPA, %	17 [12-23]*†‡	12 [10-18]	13 [10-16]	11 [7-13]	<0.001
Mon2 with MPA, %	24 [15-29]	20 [15-26]	21 [15-27]	19 [13-26]	0.536
Mon3 with MPA, %	15 [13-19]*†	13 [11-14]	13 [12-16]	12 [9.7-14]	<0.001
Monocytes, per μ l	852 [300]*†‡	646 [172]	541 [139]	502 [190]	<0.001
Platelets, per μ l	253 [90]	227 [51]	240 [65]	267 [76]	0.20
<p>Normally distributed data are presented as mean [standard deviation], non-normally distributed data are presented as median [inter-quartile range]. AHF: acute heart failure, SHF: stable heart failure, CAD: coronary artery disease, HC: healthy control, Mon1: CD14++CD16- monocytes, Mon2: CD14++CD16+CCR2+ monocytes, Mon3: CD14+CD16++CCR2- monocytes, MFI: median fluorescent intensity, MPA: monocyte platelet aggregates.* p<0.05 AHF vs SHF § p<0.05 SHF vs CAD † p<0.05 AHF vs CAD p<0.05 SHF vs HC, ‡ p<0.05 AHF vs HC</p>					

Figure 7.2 Monocyte-platelet aggregate counts for the 3 monocyte subsets



HF: heart failure, CAD: coronary artery disease, MPA: monocyte platelet aggregate,

The proportion of Mon1 aggregated with platelets was increased in AHF compared to SHF ($p=0.03$), CAD ($p<0.001$) and HC ($p<0.001$). There were no differences in the proportion of Mon2 aggregated with platelets across the study groups. A higher percentage of Mon3 aggregated with platelets was seen in AHF compared to SHF ($p=0.012$), and HC ($p<0.001$) but not compared to CAD ($p=0.22$).

Patients with SHF had significantly higher total MPA count compared to HC ($p=0.02$) and there was a trend towards higher counts compared to CAD ($p=0.07$). Similarly, MPAs associated with Mon1 were significantly higher in patients with SHF compared to HC ($p<0.001$) but there were no differences when compared to CAD ($p=0.10$). MPAs associated with Mon2 were higher in SHF compared to CAD ($p=0.04$) and HC ($p=0.004$). There were no differences between MPAs associated Mon3 when comparing SHF with CAD and HC.

The proportion of Mon1 aggregated with platelets was higher in SHF compared to HC ($p=0.01$) but not different compared to CAD ($p=0.59$). The proportion of Mon2 and Mon3 aggregated with platelets did not differ between SHF, CAD and HC.

7.3.3 Longitudinal analysis

When compared to measurements taken during the first 24 hours of admission, the total MPA count and MPAs associated with Mon1, Mon2 and Mon3 did not change significantly over 3-months of follow-up (**Table 7.2**), despite an overall improvement in NYHA status [(NYHA 1 ($n=3$), NYHA 2 ($n=15$), NYHA 3 ($n=12$), NYHA 4 ($n=6$)]. The only parameter to change was a reduction in the percentage of Mon3 aggregated to platelets at 3 months compared to on admission ($p=0.009$).

Table 7.2 Monocyte-platelet aggregates in AHF at admission, discharge and 3 months

	Admission (n=36)	Discharge (n=36)	Follow-up (n=36)	p value
Monocytes, per μ l	893 [336]	853 [255]	858 [331]	0.73
Platelets, per μ l	249 [92]	271 [105]*	201 [59]	0.011
Total MPA, per μ l	133 [99-180]	133 [96-172]	124 [95-197]	0.43
MPA with Mon1, per μ l	105 [79-153]	106 [73-150]	94 [68-150]	0.40
MPA with Mon2, per μ l	16[10-24]	13 [7.5-19]	16 [6.6-29]	0.51
MPA with Mon3, per μ l	9 [7-15]	11 [8-15]	10 [6-18]	0.61
Mon1 with MPA, %	17 [12-23]	17 [13-23]	14 [11-20]	0.33
Mon2 with MPA, %	24 [15-29]	22 [17-27]	19 [16-27]	0.78
Mon3 with MPA, %	15 [13-19] †	15 [11-18] ‡	13 [8.4-17]	0.013
<p>Normally distributed data are presented as mean [standard deviation], non-normally distributed data are presented as median [inter-quartile range]. Mon1: CD14+CD16-monocytes, Mon2: CD14++CD16+CCR2+ monocytes, Mon3: CD14+CD16++CCR2-monocytes, MPA: monocyte platelet aggregates.</p> <p>* discharge vs. follow-up, p=0.003; † admission vs. follow-up, p=0.009; ‡discharge vs. follow-up, p=0.049</p>				

7.3.4 Correlation between MPAs, plasma markers and LVEF.

In patients admitted with AHF, plasma concentration of IL-6, MCP-1, and BNP were 11 [7-16] pg/ml, 125 [7-16] pg/ml, and 365 [108-872] pg/ml, respectively. Counts of MPA with Mon3 significantly correlated with MCP-1 levels ($r=0.32$, $p=0.022$). There was no correlation between MPAs and IL-6 or BNP levels (**Table 7.3**). The percentage of Mon1 aggregated with platelets negatively correlated with LVEF measured during hospital admission ($r=-0.30$, $p=0.046$) (**Table 7.4**)

Table 7.3 Correlation analysis between monocyte-platelet aggregates, plasma IL-6 and MCP-1 in AHF

	IL-6		MCP-1	
	r	p-value	r	p-value
Total MPA, per μ l	-0.01	0.95	0.05	0.73
MPA with Mon1, per μ l	0.01	0.92	-0.01	0.94
MPA with Mon2, per μ l	-0.12	0.40	0.25	0.078
MPA with Mon3, per μ l	0.04	0.76	0.32	0.022
Mon1 with MPA, %	-0.03	0.86	-0.28	0.49
Mon2 with MPA, %	-0.02	0.91	-0.12	0.42
Mon3 with MPA, %	0.03	0.82	-0.03	0.86
Mon1: CD14+CD16- monocytes, Mon2: CD14++CD16+CCR2+ monocytes, Mon3: CD14+CD16++CCR2- monocytes, MPA: monocyte-platelet aggregates, MCP-1: monocyte chemoattractant protein-1, IL-6: interleukin-6				

Table 7.4 Correlation analysis between monocyte-platelet aggregates, plasma IL-6 and MCP-1 in AHF

	LVEF		BNP	
	r	p-value	r	p-value
Total MPA, per μ l	-0.26	0.087	-0.26	0.14
MPA with Mon1, per μ l	-0.27	0.068	-0.25	0.16
MPA with Mon2, per μ l	-0.06	0.70	-0.22	0.22
MPA with Mon3, per μ l	-0.16	0.30	-0.16	0.36
Mon1 with MPA, %	-0.30	0.046	-0.09	0.62
Mon2 with MPA, %	-0.18	0.24	-0.02	0.93
Mon3 with MPA, %	-0.12	0.41	0.05	0.79
Mon1: CD14+CD16- monocytes, Mon2: CD14++CD16+CCR2+ monocytes, Mon3: CD14+CD16++CCR2- monocytes, MPA: monocyte-platelet aggregates, LVEF: left ventricular ejection fraction, BNP: brain natriuretic peptide, MPA: monocyte platelet aggregates				

7.3.5 Predictors of cardiovascular outcome in AHF

MPAs associated with Mon2 were significantly lower in patients who experienced adverse clinical outcomes of death or re-hospitalisation (12.8 cells/ μ l IQR 7.1-19.1) compared to those who remained free of events (16.1 cells/ μ l IQR 11.2-27.7, $p=0.03$). The total MPA count, levels of MPAs with Mon1 and Mon3 did not differ significantly between patients with or without events.

In a univariate Cox regression analysis, BNP ($p=0.009$), creatinine ($p=0.013$) and MPA associated with Mon2 ($p=0.042$) were predictors of clinical outcome, with LVEF

showing a strong trend ($p=0.061$) (Table 4). In a multivariate Cox regression analysis, MPAs associated with Mon2 remained an independent negative predictor of combined death and rehospitalisation after adjustment for age, LVEF, creatinine and BNP (**Table 7.5**).

For Kaplan-Meier analysis, patients were grouped by quartiles of MPAs associated with Mon2 (**Figure 7.3**): Quartile 1: ≤ 10 cells/ μl , Quartile 2: $>10 - 16$ cells/ μl , Quartile 3: $>16 - 24$ cells/ μl , Quartile 4: >24 cells/ μl . Patients with the lowest counts of MPAs from Mon2 (Quartile 1) had significantly worse clinical outcome compared to those with the highest counts (Quartile 4) (Log rank test, $p=0.037$). There were no significant differences between quartile 1 and quartile 4 with regards to antiplatelet therapy, with 81.8% of quartile 1 and 92.3% of quartile 4 taking at least one of these medications ($p=0.527$).

Figure 7.3 Kaplan Meier curves of cumulative event-free survival from death/rehospitalisation by quartiles of MPAs associated with Mon2 monocytes in AHF

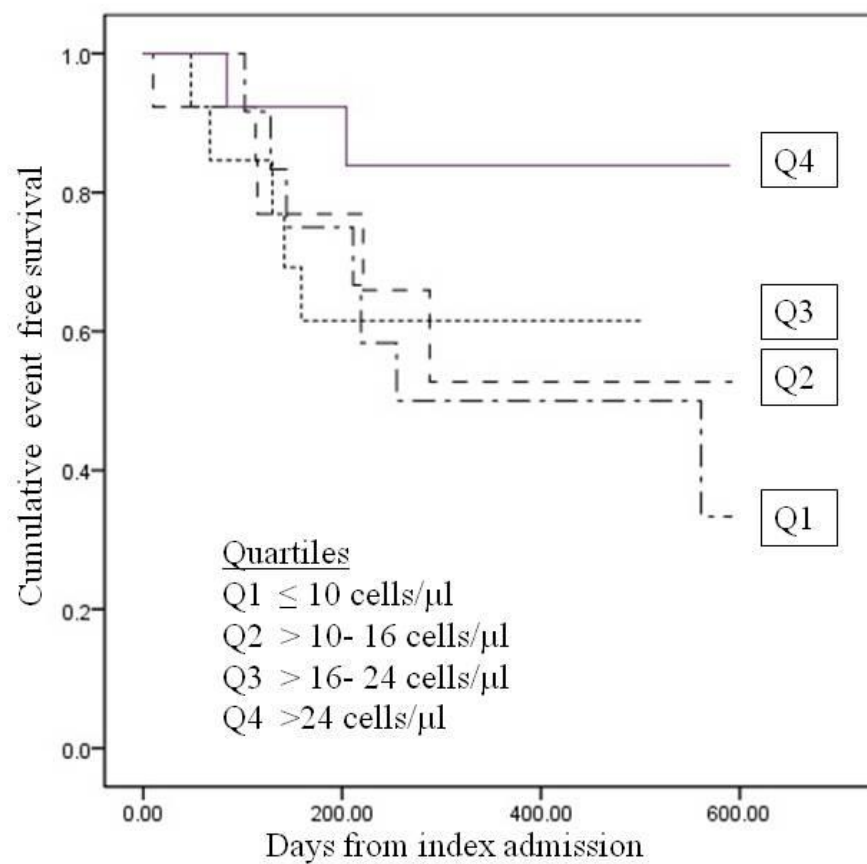


Table 7.5 Cox regression analysis for predictors of death/rehospitalisation in AHF

Variable	Hazard ratio	95% CI	p value
<i>Univariate analysis</i>			
<i>Demographics/clinical characteristics</i>			
Age	1.015	0.970-1.061	0.53
Gender (males)	1.048	0.402-2.730	0.92
Hypertension	1.164	0.475-2.852	0.74
Diabetes	1.018	0.412-2.515	0.97
Smoking	1.801	0.734-4.418	0.20
MI	1.069	0.437-2.617	0.88
Stroke	2.086	0.610-7.129	0.24
BNP, pg/ml	1.001	1.000-1.001	0.009
LVEF, (%)	0.955	0.909-1.002	0.061
Creatinine, $\mu\text{mol/l}$	1.017	1.004-1.031	0.013
<i>Medications</i>			
Aspirin	1.324	0.438-4.002	0.62
Clopidogrel	0.914	0.374-2.238	0.85
ACEI/ARB	0.750	0.272-2.068	0.58
Beta blocker	1.349	0.556-3.272	0.51
Statin	1.160	0.339-3.962	0.81
<i>Monocyte subsets</i>			
Total MPA, per μl	0.999	0.993-1.004	0.62
MPA with Mon1, per μl	0.999	0.993-1.005	0.80

MPA with Mon2, per μ l	0.945	0.894-0.998	0.042
MPA with Mon3, per μ l	0.989	0.933-1.070	0.99
Mon1 with MPA, %	1.028	0.979-1.080	0.27
Mon2 with MPA, %	1.035	0.998-1.074	0.067
Mon3 with MPA, %	1.045	0.984-1.109	0.15
Platelets, per μ l	0.998	0.991-1.004	0.46
<i>Multivariate analysis</i>			
MPA with Mon2, per 5 cells/ μ l	0.581	0.343-0.984	0.043
CI: confidence interval, MPA: monocyte platelet aggregates, BNP: Brain Natriuretic peptide, LVEF: left ventricular ejection fraction, ACEI: angiotensin converting enzyme inhibitor, ARB: angiotensin receptor blocker, MI: myocardial infarction, Mon1: CD14+CD16- monocytes, Mon2: CD14++CD16+CCR2+ monocytes, Mon3: CD14+CD16++CCR2- monocytes			

7.4 Discussion

MPAs represent a sensitive marker of both monocyte and platelet activation and I have shown for the first time that the total MPA count is increased in patients with both AHF and SHF compared to controls with normal LV function. Importantly, increased formation of MPAs in AHF is confined to monocytes from the Mon1 and Mon2 subsets and not from the Mon3 subset. I have also shown that MPAs from Mon2 are inversely predictive of adverse outcome in patients admitted to hospital with AHF, independent of age, LVEF, BNP and renal function. The cause of death in the majority of patients was due to worsening of HF and no patients experienced stroke or MI during follow-up. Thus, an association between clinical outcome and the formation of MPAs appears to be in addition to the acknowledged increased risk of thrombo-embolism in HF patients.

It remains unclear why MPAs from Mon2 are increased in AHF compared to controls and yet patients who have the worst outcomes have the lowest numbers of such complexes. One might speculate that MPAs from this subset of monocytes have beneficial or reparative properties in the myocardium and a failure to form MPAs in AHF results in a greater insult to the myocardium and hence, a worse prognosis. Alternatively, circulating MPA levels may not reflect their abundance within the myocardium itself and low levels of circulating MPAs from Mon2 in those patients who have the worst prognosis may reflect a rapid migration and uptake of these complexes into the myocardium. Indeed, the transendothelial migration of MPAs is associated with a dissociation of the aggregate.⁽²³³⁾ Nonetheless, the observational nature of this study does not allow mechanistic insights into the specific roles of MPAs in HF and further data on the prognostic role of MPAs are required.

Mon2 are increased in inflammatory conditions and the formation of MPAs may reflect another marker of their pro-inflammatory activity. In acute coronary syndrome, MPAs represent a link between inflammation and thrombosis, with increased numbers formed in patients with troponin positive coronary events, perhaps reflecting instability of coronary plaques and vascular inflammation.(234-236) In HF, the precise trigger for MPA formation is less clear, although contributing factors are likely to include haemodynamic changes, activation of the renin-angiotensin system, endothelial dysfunction and increased catecholamines.(237)

As well as simply representing a marker of platelet activation, a recent study has shown that the formation of MPAs results in significant upregulation of circulating CD16+ monocytes (mainly Mon2) which adhere to human umbilical vascular endothelial cells more than CD16- cells (238), thereby giving rise to a potential mechanism for monocyte migration into the failing myocardium. Furthermore, CD16+ monocytes have higher pro-inflammatory activity than CD16- cells and in-vivo studies have demonstrated higher cytokine production by monocytes (including IL1-Beta, IL-8, MCP-1) in response to MPA formation.(239-241) Consequently, MPA formation is also associated with promoting a greater inflammatory phenotype in monocytes.(238, 242)

Phagocytosis is one of the most important functions of monocytes and studies have shown that this activity is particularly marked in Mon1 and Mon2 monocytes.(82) The increased formation of MPAs in these subsets (in both acute and stable HF) might also reflect a mechanism of eliminating activated platelets from the circulation by phagocytosis. It is therefore possible that the high number of MPAs in HF may reflect the increased number of activated platelets found in this condition. In addition to the

direct formation of MPAs, monocytes and platelets may also interact with each other to augment individual intrinsic function. For example, tissue factor release by monocytes in HF interacts with P-selectin on the surface of platelets which results in enhanced fibrin formation.(237, 243)

Patients with HF have been shown to have increased plasma MCP-1.(244, 245) In this chapter there was a significant correlation between the plasma levels of MCP-1 and counts of MPA associated with 'non-classical' Mon3. This is perhaps in accordance with previous evidence showing the intimate role platelets play in the regulation of monocyte recruitment to tissues.(233) Additionally, these observations once more indicate very complex and context-dependent patterns of monocyte-platelet interaction. For example, the P-selectin mediated pathway has been shown to be pivotal for monocyte aggregation with platelets in the settings of atherothrombosis, but not in endotoxemia.(246)

The results of this chapter show that increased MPA formation in Mon1 and Mon2 following acute decompensation of HF persists for at least 3 months, despite improvements in patient symptoms. Our findings suggest that the inflammatory mechanisms that activate monocytes and platelets continue many weeks after the initial event, which may contribute to the considerable morbidity and mortality seen in this patient group. Therapeutic modulation of thrombotic substrates in HF is an attractive concept, particularly in patients with AHF in whom inflammation and thrombosis are greatest. Reductions in MPAs have been demonstrated following administration of monoclonal antibodies against PSGL-1 and the blockade of P-selectin(247) A large proportion of AHF patients recruited in this study were taking antiplatelet medication,

with 73% on aspirin and 41% on clopidogrel. Antiplatelet therapy has been shown to reduce the numbers of circulating monocyte-platelet aggregates in patients with stable CAD and this reflects a reduction in both platelet and monocyte activation (248). Conversely, anticoagulation appears to have little effect.(249) and at present, there are no convincing clinical trial data to suggest a beneficial role on mortality of either anticoagulation or antiplatelet therapy in patients with HF, unless there are concomitant indications, such as CAD or AF.

7.5 Conclusion

For the first time, I have shown that MPA formation is increased in patients with HF and this may provide some preliminary evidence of an interaction between inflammation and thrombosis in HF. In AHF, the increase in MPAs appears to be confined to monocytes from Mon1 and Mon2 subsets, which have been shown in other studies to be pro-inflammatory and highly phagocytic. Also, MPAs with Mon2 are also negatively predictive of a worse prognosis in AHF, perhaps reflecting a beneficial role for these complexes during acute decompensation.

CHAPTER 8

DISCUSSION

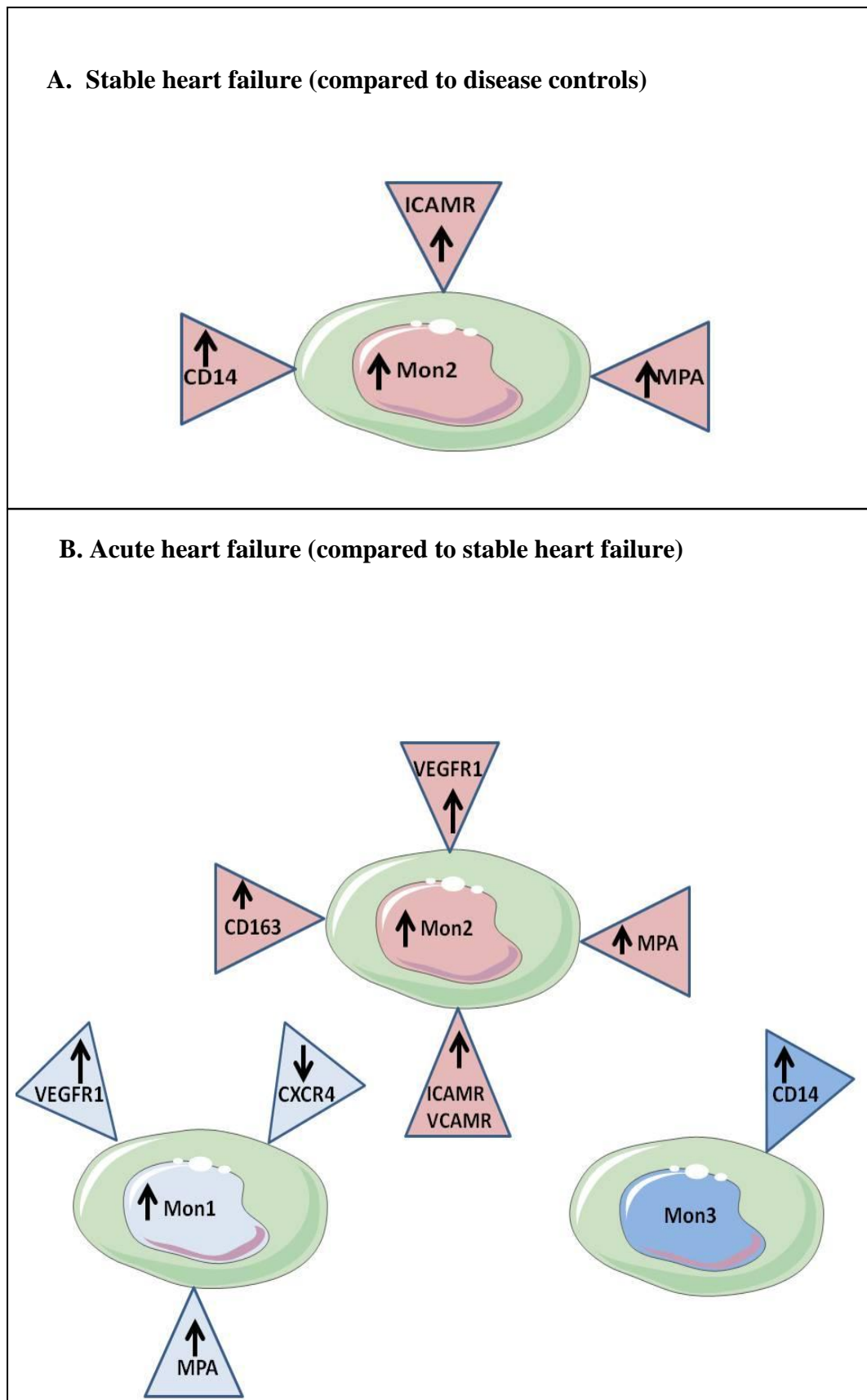
8.1 Summary of key findings

Much of the work presented in this thesis has already been discussed in the individual results chapters (see chapters 3-7). The aim of this chapter is to bring all of these findings together in the context of the original hypotheses described in chapter 1 and to discuss how this work may lead on to future research.

The overall key findings in this thesis are:

- i. Patients with HF have abnormal numbers of monocytes and individual subsets compared to controls without HF (chapter 3)
- ii. HF is associated with differences in monocyte subset surface expression of markers for activation/inflammation (chapter 4), angiogenesis (chapter 5), tissue repair (chapter 5) and cell adhesion (chapter 6).
- iii. HF is associated with increased formation of MPAs (chapter 7)
- iv. The majority of abnormalities in monocyte subset parameters seen on admission in patients with AHF persist during short-term follow-up (chapters 3-7)
- v. The Mon2 subset appears to have a prognostic role in patients with AHF, with counts of Mon2 (chapter 3), MPAs associated with Mon2 (chapter 7) and expression of VCAM-1R on Mon2 (chapter 6) associating with clinical outcomes

Figure 8.1 Surface marker expression in patients with acute and stable heart failure



8.2 General discussion

The specific findings in the cross-sectional study for patients with SHF and AHF are shown in **Figure 8.1**. In patients with SHF, there was an increase in counts of Mon2 and MPAs associated with Mon2, alongside increased expression of inflammatory markers (CD14) and cell adhesion receptors (ICAM-1R) on Mon2. In patients with AHF, there was an increase in counts of Mon1 and Mon2 and MPAs associated with Mon1 and Mon2. There was an increase in the expression of angiogenic receptors on Mon1 (VEGF-1R) and an increase in expression of angiogenic receptors (VEGF-1R), scavenger receptors (CD163) and CAM receptors (ICAM-1R and VCAM-1R) on Mon2. Expression of the chemokine receptor CXCR4 was reduced on Mon1 in AHF. The only significant difference on Mon3 was enhanced expression of CD14.

Interestingly, the majority of monocyte parameters did not change significantly during follow up in the longitudinal study. Absolute subset numbers remained the same, but some markers of inflammation did change, with TLR4 expression on Mon1 and Mon2 falling significantly at follow-up. TLR4 expression on Mon3 was increased at discharge compared to admission, with subsequent reductions seen at follow-up. CCR2 and VCAM-1R expression on Mon3 also fell during follow-up. The scavenger receptor CD163 also fell significantly on Mon1 and Mon2 during follow-up. It remains unclear how long it would take for many of the monocyte parameters to ‘normalise’ to baseline SHF values in patients admitted with AHF. During the design of the study, it was felt that 3 months follow-up would allow adequate recovery and the majority of patients did indeed have improvements in NYHA class and concurrent reductions in BNP by this time. Furthermore, given the high rates of recurrent decompensation following AHF admissions, it was felt that too many patients would have been re-admitted before any

‘stable’ blood samples could be taken if the follow-up had been longer. Nevertheless, despite clinical improvements, plasma levels of IL-6 and MCP-1 were unchanged at 3 months. This suggests that systemic levels of inflammation were still high and may explain why many monocyte parameters did not change significantly at follow-up. These data may support the idea that AHF is a separate disease process compared to the stable condition, with important differences in the immune response and therefore potential therapeutic targets.

One of the most interesting aspects of the study is the impact of monocyte parameters on clinical outcomes. Unfortunately, patients with HF often have a poor prognosis and this is reflected in the findings from this study, with more than one third of patients admitted with AHF having a clinical event (either death or rehospitalisation) during the follow-up period. It should be acknowledged that the number of AHF patients recruited was relatively small, therefore evaluating the impact of monocyte parameters on prognosis should be regarded as hypothesis generating rather than definitive, with larger scale studies clearly needed. However, the Mon2 subset appeared to have a prognostic role in patients with AHF, with higher numbers associating with improved clinical outcomes. These findings are similar to results seen in stroke patients, with elevated numbers of Mon2 also associating with improved outcomes.(84) Conversely, high Mon2 in patients with chronic renal failure predicted future cardiac events(176) and Mon2 also independently predicted cardiovascular events in patients referred for elective coronary angiography.(250)

A simplistic interpretation of these findings might be that in stable CAD and chronic renal failure, the Mon2 subset is detrimental and leads to MI, stroke and death as a

result of vascular inflammation. Conversely, in AHF and stroke, the Mon2 subset is in some way beneficial and ‘protects’ against adverse sequelae. Adrenaline has been shown to increase the number of CD14⁺⁺ monocytes(251) and the acute nature of both stroke and AHF are mediated by a catecholamine driven stress response. One hypothesis may be that adrenaline in the acute setting induces a more ‘protective’ phenotype and functionality of the Mon2 subset but further research is required to test this hypothesis.

In order to investigate the apparent ‘benefits’ of Mon2 in AHF, the phenotype of these cells were evaluated by measuring surface marker expression. I discovered that Mon2 highly expressed VEGFR-1 which negatively correlated with LVEF, which may suggest an involvement in angiogenesis. Although MCP-1 is known to be a powerful chemokine for monocyte migration (by its interaction with CCR2 receptor), CCR2 expression was unchanged which suggests that this pathway may not be the principle mechanism for monocyte recruitment in AHF. Instead, enhanced expression of VEGFR-1 may be more important in migration, as VEGF is also known to be involved in monocyte chemotaxis to sites of inflammation.

Further evidence of the ‘reparative’ potential for Mon2 was the discovery in chapter 5 that CD163 expression also increased in AHF on this subset. CD163 is a scavenger receptor known to be involved in the down-regulatory phase of inflammation.(198, 199) Activation of the CD163 receptor has been shown to enhance IL-10 release, which is an anti-inflammatory cytokine and may reduce inflammation in the acute setting.

Increased VCAM-1R expression on Mon2 was seen in AHF compared to SHF, whereas lower expression associated with better outcomes. One might speculate that in patients with severe HF (and who go on to have worse clinical outcomes), increased receptors to VCAM-1 on Mon2 leads to enhanced monocyte recruitment into the failing myocardium (in an attempt to limit damage) and consequently fewer numbers seen in the peripheral circulation (as seen in chapter 3 where lower Mon2 numbers associated with worse outcomes). Further work looking at monocyte subsets within the myocardium itself may add to our understanding.

Another novel finding was the increased formation of MPAs from Mon1 and Mon2, with higher numbers from Mon2 associating with improved clinical outcomes. MPAs represent a marker of both platelet and monocyte activation and data have shown that formation of such aggregates upregulates CD16⁺ monocytes, which adhere to endothelial cells more than CD16⁻ monocytes. There may therefore be a link between MPA formation, cell adhesion (increased VCAM-1R expression) and chemotaxis (increased VEGFR-1 expression) on Mon2 which results in increased recruitment into the failing myocardium.

8.3 Limitations

The study has a number of limitations which need to be considered when interpreting the results. Although many of the statistical tests resulted in significant p-values at conventional levels, it should be noted that numerous monocyte markers were analysed during the study which may result in type 1 error in the statistical analyses. A Bonferroni correction was not applied to the statistical analyses which should be regarded as a limitation of the study. Furthermore, the number of subjects recruited was

relatively small, particularly in the AHF cohort where the prognostic role of monocyte parameters was assessed. Although more than one third of AHF patients reached the end point of death or rehospitalisation, further studies with a greater number of patients are required to confirm the influence of such parameters on clinical outcomes. Moreover, outcome data were only collected for patients with AHF and further studies will be needed to assess whether monocyte parameters have any prognostic role in SHF patients. Although the number of patients required for future studies will depend on the hypothesis to be addressed, an estimate of sample size for the multivariate Cox regression analysis can be made based on the findings in this study. Assuming the event rate (death or rehospitalisation) remains at 33% with 4 predictor variables in the analysis, 102 AHF patients would be required to confirm the predictive value for Mon2 subset counts on clinical outcomes.

I specifically recruited HF patients with underlying CAD in order to draw comparisons with an appropriate control group. Whilst baseline characteristics were similar between patient groups (**table 2.1**), more patients had AF in HF than CAD and fewer AHF patients were taking a beta-blockers than controls. Therefore residual confounding from co-morbidities may still exist between groups. Furthermore, the findings in this thesis should be evaluated in patients with HF due to other aetiologies, such as dilated cardiomyopathy or diastolic HF.

One of the major limitations of this study is its observational design which does not allow detailed insight into the functional mechanisms of monocyte action in HF. It remains unclear whether monocyte subset numbers and phenotype are simply a reflection of the underlying disease process in HF or whether they directly contribute to

its pathophysiology and monocyte counts and activity in the peripheral circulation may not reflect their abundance or functionality within the myocardium itself.

8.4 Implications for future research

This thesis has allowed me to systematically answer many questions relating to the role of monocytes in HF but in doing so, has generated many more which still require attention. The prognosis of patients with HF is unfortunately still poor despite advances in current therapy, and identifying ‘markers’ of poor prognosis may allow clinicians to plan the management for such patients more effectively, either in terms of utilising more aggressive therapies or even planning end of life pathways. Therefore further larger scale studies are required to evaluate the prognostic role of monocytes in HF.

It is still unclear from the work in this thesis whether the functions of specific monocyte subsets are beneficial or detrimental to the myocardium. I have demonstrated increased expression of markers for inflammation and activation on the Mon2 subset, which may lead to harmful effects as previously discussed. However, I have also demonstrated that this subset has increased expression of reparative and angiogenic markers which may result in attenuation of myocardial damage and lead to tissue repair. It is therefore unclear whether reducing or indeed enhancing specific subsets would improve clinical outcomes. An exciting future direction would be to manipulate subset levels and even phenotype in animal models using pharmaceutical agents in order to assess the effects on myocardial performance and ultimately clinical outcomes. Another direction of animal research might be to separate subsets and introduce them into the host in a HF model, either into the peripheral circulation or into the heart itself in order to assess the response to individual subsets.

Although monocyte subset numbers and phenotype addressed in this thesis may be important, assessing their functionality is likely to be crucial in furthering our understanding of their role in HF. For example, it would be interesting to evaluate cytokine production, phagocytosis and cell adhesion for the different subsets in vitro as well as utilizing novel approaches of non-invasive imaging to track monocyte subsets in vivo.

Finally, the work in this thesis focuses on monocytes collected from the peripheral circulation and these may not reflect levels within the myocardium itself. An obvious future direction would be to assess samples of myocardium taken at the time of explantation during cardiac transplantation in order to compare monocyte subsets with blood samples taken simultaneously from the peripheral circulation. This would enable testing of the hypothesis that subsets with highest expression of CAM receptors migrate to the myocardium and the numbers in the peripheral circulation are consequently lower.

The number of potentially interesting and exciting studies leading on from this thesis is not limited to the brief outline given above and it should be acknowledged that the understanding in this field of work is very much in its infancy.

8.5 Conclusions

The work described in this thesis has significantly added to our understanding of the role monocyte subsets play in HF. There are significant differences in subset numbers as well as cell surface receptor expression which may be important in the pathophysiology of this complex disease. However, this work also highlights several deficiencies in our understanding which need to be explored in future research.

Appendix 1: Standard Operating Procedure for cytometric bead array

STANDARD OPERATING PROCEDURE 210

Cytometric Bead Array

Dr. Eduard Shantsila and Dr. Silvia Montero-Garcia. January 2012
Updated February 2012 for Fractalkine

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

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Introduction

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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-1 β 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°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 μ L with the **diluent for serum/plasma**. $50 - 4 = 46$, hence 46 μ L 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 μ L 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 μ L/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 μ L with the **detection dilution buffer**. Example : $50 - 4 = 46$, hence 46 μ L of diluent per sample x number of samples
- 3.4.7 Add 50 μ L 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 μ L 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.

Appendix 2: Standard Operating Procedure for monocyte subsets, 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

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

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Interpretation

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” within 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.

NB: FACS COMP may need to be run

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]

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)

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

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

Next – for MPAs...

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

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.

SOP 201: Enumeration of monocytes subsets and
monocyte platelet aggregates by flow cytometry

Signed off.....Andrew Blann..... 2010.....

Appendix 3. Publications arising from this thesis

Much of the work arising from this thesis has now been published in peer review journals:

Original manuscripts

1. Increased expression of cell adhesion molecule receptors on monocyte subsets in ischaemic heart failure

Wrigley BJ, Shantsila E, Tapp LD, Lip GY

Thromb Haemost. 2013;110:92-100

2. CD14⁺⁺CD16⁺ monocytes in patients with acute ischaemic heart failure

Wrigley BJ, Shantsila E, Tapp, LD, Lip GY

Eur J Clin Invest. 2013;43:121-30

3. Increased formation of monocyte-platelet aggregates in ischaemic heart failure

Wrigley BJ, Shantsila E, Tapp LD, Lip GY.

Circ Heart Fail. 2013;6:127-35

4. The effects of exercise and diurnal variation on monocyte subsets and monocyte-platelet aggregates

Shantsila E, Tapp LD, Wrigley BJ, Montoro-Garcia S, Ghattas A, Jaipersad A, Lip GY.

Eur J Clin Invest 2012;42:832-9

Literature review

1. The role of monocytes and inflammation in the pathophysiology of heart failure

Wrigley BJ, Lip GY, Shantsila E.

Eur J Heart Fail. 2011;13:1161-71.

Published abstracts

1. Unique characteristics of CD14⁺⁺CD16⁺ monocytes in patients with acute heart failure and implications for clinical outcome

Wrigley BJ, Shantsila E, Tapp LD, Lip GY

British Cardiovascular Society, Manchester

*Heart.*2012;98:A64-A65

2. Abnormal monocyte subsets in ischemic heart failure

Wrigley BJ, Tapp LD, Pamukcu B, Shantsila E, Lip GYH

European Society of Cardiology Heart Failure Congress, 2011, Gothenburg, 60505

3. Increased formation of monocyte-platelet aggregates in ischemic heart failure
Wrigley BJ, Tapp LD, Pamukcu B, Shantsila E, Lip GYH
Atherosclerosis, Thrombosis and Vascular Biology Congress, 2011, Chicago
Arterioscler Thromb Vasc Biol. 2011 Abstracts supplement, P673:208

4. Immunophenotypic characterisation of an immature human monocyte subset
with angiogenic potential
Shantsila E, Tapp L, Wrigley B, Apostolakis S, Drayson M, Lip GYH.
Arteriosclerosis, Thrombosis and Vascular Biology Congress, 2011, Chicago
Arterioscler Thromb Vasc Biol. 2011 Abstracts supplement, P 653:203

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