A Study of the Adaptive Immune System In Coronary Thromboaspirate during Human Acute Myocardial Infarction

By

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DEDICATION

This thesis is dedicated to my wife Jyotshana whose support and encouragement made this possible.

Presentations

The following presentations have been given at international scientific meetings directly from the work of this thesis:

Chapter 5

Amplified proinflammatory and attenuated immunomudulotory T cells at the culprit site of ST Elevation Myocardial Infarction. G Ghimire, J Spiro, R Kharbanda, M Roughton, A Mitchell, M Mason, C Ilsley, M Rose, M C D Dalby. **Presented at the European Society of Cardiology, Munich 2008**

Chapter 6

Reactive IgM at site of coronary occlusion in patients with ST elevation myocardial infarction. G Ghimire, J Spiro, R Kharbanda, M Roughton, A Mitchell, M Mason, C Ilsley, M Rose, M C D Dalby. **Presented at the American Heart Association, New Orleans 2008**

Chapter 7and 8

Endothelial cell injury induced by intracoronary sera in patients with ST Elevation Myocardial Infarction. G Ghimire, A, McCormack, J Spiro, R Kharbanda, M Rose, M C D Dalby. **Presented at the American College of Cardiology 58th Annual Scientific Session, Orlando, March 2009.**

ABSTRACT

Introduction: This thesis aims to characterize the biology of solid and liquid phase coronary thromboaspirate obtained during therapeutic aspiration at primary angioplasty for ST elevation myocardial infarction (STEMI).

Methods: We studied coronary thromboaspirate obtained from the coronary artery (CA) in STEMI and focussed on three lines of investigation:

- Intracoronary solid phase thrombus was subjected to immunohistochemistry for characterization and quantification of the different leukocyte types and proteins involved in the thrombus.
- Resident cell populations in the liquid phase filtrate from the aspirate including platelets and leukocytes along with their subpopulations were analysed by flow cytometry for phenotypic and activation markers. Comparison was made with cells in the peripheral femoral artery (FA) blood.
- Paired plasma/serum from the CA filtrate and FA blood were analysed for the presence of autoreactive antibodies and cytotoxic potential.

Results:

- Leukocytes were heterogeneously distributed in the ex-vivo thrombus. The granulocyte population was predominant and strongly expressed CD11b and vimentin.
- The CA aspirate filtrate when compared to the paired FA blood contained significantly more leukocyte-platelet conjugates, more activated platelets, and more Vimentin.
- More of the $CD4^+$ T cells in the CA than FA had lost the co-stimulatory molecule CD28 ($p<0.00001$) and they often expressed effector phenotype marker.
- Conversely, the immune-regulatory T cells expressing the FoxP3 were more attenuated in the CA than FA ($p<0.00001$).
- Whilst IgG and IgM antivimentin antibody titres in the CA sera were consistently lower than the titres in FA sera ($p<0.001$), auto-reactive non-HLA IgM were more prevalent in CA sera. The CA sera had increased potential to induce apoptosis than FA sera when incubated with endothelial cells ($p<0.001$).

Conclusions:

- Aspirated intracoronary clot contained granulocytes as the predominant leukocyte component. This investigation of the immune system in the CA during AMI therefore moved on to the adaptive system in the coronary filtrate.
- Increased concentrations of pro-inflammatory immune cells and reduced concentrations of immune-modulating cells were observed in the aspirate from the culprit site of CA occlusion when compared with the FA. The presence of T memory cells implies that this subset may have been released from the ruptured coronary plaque and this phenotypic analysis may help clarify the role of the immune system in the pathogenesis of coronary plaque instability.
- The higher titres of auto-reactive IGM in the CA than FA sera have the potential to activate the complement system and may contribute to myocardial injury following therapeutic restoration of coronary flow in STEMI.

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GLOSSARY OF ABBREVIATIONS

ACS: Acute Coronary Syndrome
(A)MI: (Acute) Myocardial Infarction
APC: Allophycocyanin
CCR: Chemokine Receptor
CD: Cluster of Differentiation
CHD: Coronary Heart Disease
CSA: Chronic Stable Angina
FITC: Fluorescein Isothiocyanate
FSC: Forward Scatter
HLA: Human Leukocyte Antigen
HSP: Heat Shock Protein
IL: Interleukin
Ig: Immunoglobulin
MHC: Major Histocompatibility Complex
NCA: Normal Coronary Artery
NSTEMI: Non ST Elevation Myocardial Infarction
PBS: Physiological Buffer Saline
PCI: Percutaneous Coronary Intervention
PE: Phycoerythrin
PPCI: Primary Percutaneous Coronary Intervention
SSC: Side Scatter
SD: Standard Deviation
TCR: T Cell Receptor
TCM: Central Memory T cell
TEM: Effector Memory T cell

ETHICAL CONSIDERATIONS

The local ethics committee at Royal Brompton and Harefield NHS Trust had prospectively approved all of the work within this thesis. Written informed consent was obtained from all participants. Data obtained from subjects enrolled into the following two research studies were used in this thesis:

1. An Investigation into the Mechanisms of Thromblytic Resistance: **COREC reference number: 06/Q0404/58**
2. Percutaneous Coronary Intervention as a model of clinical inflammation: **COREC reference number: 06/Q0404/55**

PARTICIPANTS

With the supervision of Dr M C D Dalby, Dr R K Kharbanda and Professor M. Rose all the study designs, ethical committee submissions, recruitment of subjects, sample collections, sample preparation and processing, flow cytometric and immunohistochemistry work was performed by Dr G Ghimire. All the studies were performed within the premises of Royal Brompton and Harefield NHS Trust and the Harefield Heart Science centre, Imperial College London.

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CHAPTER 1: INTRODUCTION

Aims of this thesis

This thesis concerns analysis of the cellular and immunological characteristics of coronary thromboaspirate obtained at primary angioplasty (PPCI) for acute ST elevation myocardial infarction (STEMI) and comparison with femoral artery blood of the same subject as well as with patients with stable coronary artery disease. It addresses the cellular composition of the particulate thrombus, the characteristics of the resident cellular component of the aspirate, and finally the toxic potential of the serum component of the aspirate. The significance of this investigation is two fold: firstly it may allow insight into the pathogenesis of the plaque rupture and coronary occlusion process. Secondly it may allow identification of potentially cytotoxic factors which may contribute to the phenomenon of distal myocardial damage often occurring with the therapeutic restoration of coronary flow in STEMI.

Background

Atherosclerotic coronary artery disease continues to be accountable for a significant portion of morbidity and mortality in developed countries despite significant improvement in available therapeutic options. Our understanding of the pathology of this condition is largely based on studies on animal models and post-mortem series; but both methods have significant inherent limitations.

Primary percutaneous intervention (PPCI) for acute ST elevation myocardial infarction (STEMI) is now firmly established as the preferred therapeutic modality to restore the coronary perfusion.[1,2] An increasing body of evidence favours

aspiration thrombectomy as an adjunct to PPCI.[3] This procedure not only appears to improve clinical outcome but also provides a unique opportunity during which athero-thrombotic material can be directly aspirated from the culprit site of the infract related artery and subjected to scientific investigation. A detailed analysis of this ex-vivo specimen may provide novel insights in to the dynamics of plaque instability.

1.2. Historical perspective

Atherosclerosis

The term ‘‘atheroma’’ is derived from Greek ‘‘athera’’ (gruel) and was originally applied to describe a plaque with central yellow lipid core. Although this nomenclature is relatively young, its corresponding entity stretches to antiquity and had been described in mummies of 18th dynasty of the pharaohs of Egypt.[4] The theory of pathogenesis of atherosclerosis has evolved over the last 160 years. In 1844 von Rokitansky proposed the ‘‘encrustation’’ hypothesis proposing that atheroma is derived from healing and resorption of organised mural thrombus.[5] Rudolf Virchow in 1856 refuted this hypothesis by observing that the localised intimal thickening was subendothelial and proposed his infiltrative theory that plasma components (including lipids) elicited an inflammatory response in the arterial wall.[6] This idea was subsequently modified by Antischkow and further included the role of platelets and thrombosis in atherosclerosis.[7] Although Duguid transiently revived the thrombogenic aetiology of atherosclerosis,[8,9] the lipid deposition and inflammation hypothesis became more widely accepted. John French subsequently reported the critical role of structural integrity of endothelial lining in maintaining normal vasomotion and its alteration leading to genesis of

atherosclerosis.[10] On the basis of their observations on hypercholesterolemic animals, Ross and Glomset proposed that injury to the endothelium was the initiating event in atherogenesis[11] and Ross updated his theory a decade later.[12] Atherosclerosis is now considered a chronic inflammatory and proliferative condition which in essence is a response to chronic injury inflicted by noxious stimuli such as chronic hypercholesterolemia,[13] shear stress due to hypertension,[14] potentially microbes,[15,16] and other toxins.[17] This involves interactions between various cell lines including endothelial cells, macrophages, smooth muscle cells, platelets and lymphocytes. [18,19,20]

Myocardial Infarction

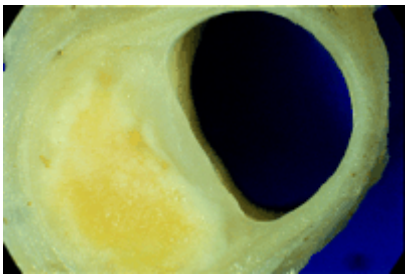
In 1892 Sir William Osler, on the basis of post-mortem studies, described the underlying cause of acute myocardial infarction as "The blocking of one of these vessels by a thrombus or an embolus leads to a condition which is known as anaemic necrosis, or white infarct. This is most commonly seen in the left ventricle and in the septum, in the territory of distribution of the anterior coronary artery."[21] The then prevalent concept that acute myocardial infarction was inevitably a fatal condition was challenged by George Dock when he described the diagnosis of myocardial infarction in a live patient in his paper, `Notes on the coronary arteries`, published in 1896.[22] Subsequently, in a presentation to the Association of American Physicians in 1912, James Herrick correlated the classic signs and symptoms of acute myocardial infarction with acute coronary artery occlusion,[23] refuted the concept that acute myocardial infarction is invariable a fatal condition and made the term coronary thrombosis almost synonymous with acute myocardial infarction. However over the next half a century the cardiology

community was embroiled over the controversy that the coronary thrombosis may be the effect of acute myocardial infarction rather than the cause of it. This controversy began with the publication of a paper by Friedberg and Horn in 1939 [24] who observed coronary thrombosis in only 31% of patients who had evidence of myocardial necrosis on autopsy. They also noted that some patients with classic clinical and electrocardiographic signs of MI had evidence of coronary thrombosis, while others did not. The central role of the thrombus formation in pathogenesis of acute myocardial infarction was unequivocally established by the angiographic studies performed during the first hours of the acute events by De wood et al who demonstrated that 87 percent of patients with acute myocardial infarction within 4 hours had thrombotic occlusion, with the percentage falling to 65 percent when patients were studied 12 to 24 hr after onset of chest pain.[25] The decreasing frequency of thrombi over time is probably explained by endogenous fibrinolysis, fragmentation and distal embolisation with infarct evolution.

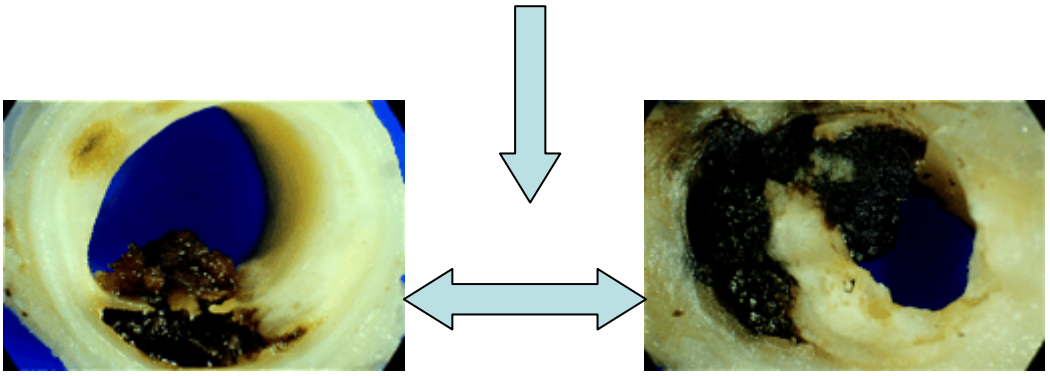
1.3. Acute coronary syndromes

Quantitative morphometric studies have shown that despite progressive growth of an atherosclerotic plaque, luminal narrowing and flow limitation does not occur until the plaque occupies about 40% of the space limited by the internal elastic lamina due to initial positive remodelling.[26] Angiographic studies have shown that the subsequent progression is unpredictable and phasic rather than linear with time.[27] This non-linearity is due intermittent plaque inflammation with consequent disruption leading to superimposed thrombosis and subsequent spontaneous healing. Such phasic episodes may remain asymptomatic if there is no limitation of blood flow or may manifest as unstable angina if there is subtotal thrombotic

occlusion of the coronary artery or as acute myocardial infarction if there is complete thrombotic occlusion of the coronary artery. [28]



Human coronary atherosclerotic plaque with a yellow core of lipid separated from the lumen by a fibrous cap



Intramural non-occlusive thrombus

Unstable Angina

Occlusive intra-coronary thrombus

Acute Myocardial Infarction

Figure 1.1: Intracoronary thrombus

Davies M .Stability and Instability: Two Faces of Coronary Atherosclerosis Circulation. 1996; 94:2013-2020.

Angiographic,[29,30,31] angioscopic,[32] and atherectomy[33] studies had consistently demonstrated the presence of intracoronary thrombus in the majority of patients with unstable acute coronary syndromes (figure 1.1). Retrospective analysis of angiograms performed at variable interval before the index acute coronary

syndrome has indicated that the majority of ACS results from rupture of non-flow limiting intermediate coronary lesions.[34,35] Data from necropsy studies of patients who succumb to acute coronary events suggest plaque composition to be the key determinant of the propensity of the atherosclerotic lesion to rupture and provoke clinical events. The culprit lesions are typically eccentric, thin capped fibroatheroma with large avascular and hypocellular, necrotic lipid core.[36,37,38] Multiple factors may contribute to transformation of the stable plaque into the rupture prone unstable lesion however increasing evidence supports the role of immune cells particularly T lymphocytes in this destabilizing process.

1.4. Thrombus Architecture

Formation of thrombus on disrupted atherosclerotic plaques is the fundamental event in the development of acute coronary syndromes and in the progression of atherosclerosis.[39] The basic component of the thrombus includes polymeric scaffolds of fibrin and enmeshed platelets, erythrocytes and leukocytes. However, published data on the proportional composition of these components is quite variable. One necropsy study showed that only 15% of occlusions consist of pure platelet thrombus, whereas 85% of occlusive thrombi are largely composed of fibrin as well as platelets;[40] another study found that all of the thrombi detected at plaque disruption sites consisted of a mixture of fibrin and platelets.[41] Similarly, fibrin appear to be more abundant than platelets in thrombi on ruptured plaques, while thrombi on eroded plaques were relatively richer in platelets than in fibrin. Anatomically, it has been suggested that the right coronary artery possesses thrombi that are richer in erythrocytes than in thrombi from the left coronary artery.[42] There is significant morphological heterogeneity within a thrombus due to

polarity of distribution of platelets and erythrocytes. The platelets cluster on the "head end" which is in continuity with the plaque crater while the tail end is chiefly composed of erythrocytes and fibrin. Histopathological findings and imaging and experimental studies have highlighted the important role of leukocytes that are actively recruited into growing, platelet-rich arterial thrombi.[43] All thrombi continually undergo propagation, organization, embolisation, lysis, and re-thrombosis, and this dynamic remodelling results in constantly changing compositions.

1.5. T Cell Development

T lymphocytes are derived from $CD7^+$ T cell progenitors which arise from the bone marrow from multi-potential lymphoid stem cells.[44,45] The progenitor cells then migrate to the thymus.[46] Approximately 50-100 progenitor cells enter the thymus daily.[47] This trafficking is a coordinated process orchestrated by various cell adhesion molecules, chemokines and cytokines.[48] In the thymus the T cells rearrange pairs of genes that encode for the heterodimeric T cell Receptor (αβ or γδ) and express it in association with the CD3 complex. At this stage the cells are $CD4^+$ $CD8^+$ and engage antigen –MHC complexes on thymic epithelium or dendritic cells. The clone of cells expressing TCR that have low affinity with the antigens is positively selected, whilst those exhibiting high or no affinity are selectively eliminated by apoptosis. T cells bearing TCR that recognize MHC class I maintain CD8 expression, down regulate CD4 expression and become $CD8^+$ T cells. Conversely, T cells bearing TCR that recognize MHC class II become $CD4^+$ T cells. Only 3% of the immature thymocytes survive the gene rearrangement and

the selection process. These naïve T cells reside in the thymic medulla for 12-14 days before migrating to the periphery.

1.6. Recirculation of naïve T cells

The circulation of the naïve T cell circulation is essential for host surveillance. They constitutively migrate to the secondary lymphoid organs where there is maximum probability of encountering its cognate antigen presented on the MHC complex of the APCs. In the lymphoid tissue the T cells extravasate preferentially through high endothelial venules. If they do not encounter any antigen for which they bear specific receptor they re-circulate back to the blood via the efferent lymph.[49]

The leukocyte-endothelial cell interaction during the extrvasation cascade is defined in a multistep paradigm[50,51] which comprises of:

1.6.1. Rolling

The lymphocytes marginate out of the main bloodstream and begin to tether and roll on the surface of the endothelium. In peripheral lymph nodes this is mediated by interaction between L-selectin (CD62L) expressed on the naïve lymphocytes and sulphated sialyl-lewis^x –like sugars which are called peripheral node addressin (PNAd) in high endothelial venules and CD34.[52] The naïve T cell migration to gut-associated lymphoid tissue, on the other hand, is mediated by LPAM-1(α4β7) and mucosal addressin-cell adhesion molecule type 1 (MAdCAM-1).

1.6.2. Activation and firm adhesion

The rolling naïve lymphocytes are exposed to the homeostatic chemokines CCL19 and CCL 21 expressed on the surface of the high endothelial venules[53,54] which

bind to the G-protein coupled receptor CCR7 expressed on the naïve lymphocytes. This interaction activates the lymphocytes inducing conformational changes that increase ligand affinity of the lymphocyte integrin without changing its density which in turn mediate firm adhesion to the endothelium. The integrins are transmembrane molecules composed of noncovalently associated α and β dimers. The integrins expressed on the naive lymphocyte and their cognate ligands expressed on endothelium includes lymphocyte function-associated antigen-1 (LFA-1, αLβ2) and intracellular cell adhesion molecule (ICAM-1,2,3),[55, 56,57] very late antigen-4 (VLA-4, α4β1) and vascular cell adhesion molecule-1 (VCAM-1),[58] α4β7 and MadCAM,[59] and αEβ7 and E-cadherin.[60]

Figure 1.2: T-cell margination

1.6.3. Extravasation

Finally the lymphocyte emigrates through the endothelial cell junction (paracellelular route)[61] although transmigration of the neutrophils through the transcellular route has been observed.[62] The molecular mechanism involved in

the transmigration includes CD31 (platelet endothelial cell adhesion molecule-, PECAM-1), junctional adhesion molecules (JAM A, B and C), CD99 and endothelial cell-selective adhesion molecule (ESAM).

1.7. Development of Memory T cells

Upon recognition of antigenic peptides on APC naïve T lymphocytes proliferate and differentiate in to a variety of effector cells depending on the stimulatory condition and the cytokine milieu.[63] A fraction of the primed T cells persists as dynamic repository of antigen experienced memory T lymphocyte that on secondary encounter with the specific antigen produce a qualitatively different and quantitatively augmented response.[64,65] The naïve and the memory T cells can be identified by the reciprocal expression of the CD45 RA and CD45RO isoforms.[66] On the basis of expression of homing receptor CCR7 and CD62L, the memory T cells can be defined in to two distinct subsets.[67,68] The CCR7- CD45RO+ T cells migrate to peripheral inflamed tissue, display immediate effector function, produce a high amount of IFN-Y and IL-4 upon antigenic stimulation and thus are called effector memory T cell (T_{EM}).[69,70,71] In contrast the CCR7+ memory cells are central memory T cell (C_{EM}) and express lymph node homing receptors, are devoid of immediate effector function, and can differentiate into CCR7- effector cells on secondary stimulation.

1.8. Recirculation of memory T cells

Distinct repertoires of adhesion receptors are expressed by the TEM and CEM endowing them with distinct migratory pattern and thus provide differential immune surveillance. TCM retain the lymph node homing receptors CD62L and the CCR7 and thus are able to home effectively to the secondary lymphoid tissue which

greatly accentuate the chance of re-encountering their cognate antigen. In addition, they can also localize to peripheral tissue and sites of inflammation. Conversely, the TEM lack the CCR7 and thus are unable to home in to the secondary lymphoid tissue. In addition the memory T cell expresses a variety of other homing receptor conferring them tropism for specific tissue. For instance memory T cells homing to the skin express cutaneous lymphocyte antigen (CLA), [72] chemokine receptor CCR4[73] and CCR10.[74] Similarly, the memory T cell migrate to the small intestine expresses LPAM-1 (α4β7)[75] and CCR9 which ligate to the Madcam-1 and CCL25 (TECK) on endothelial cells of gut lamina propria venules respectively.[76,77]

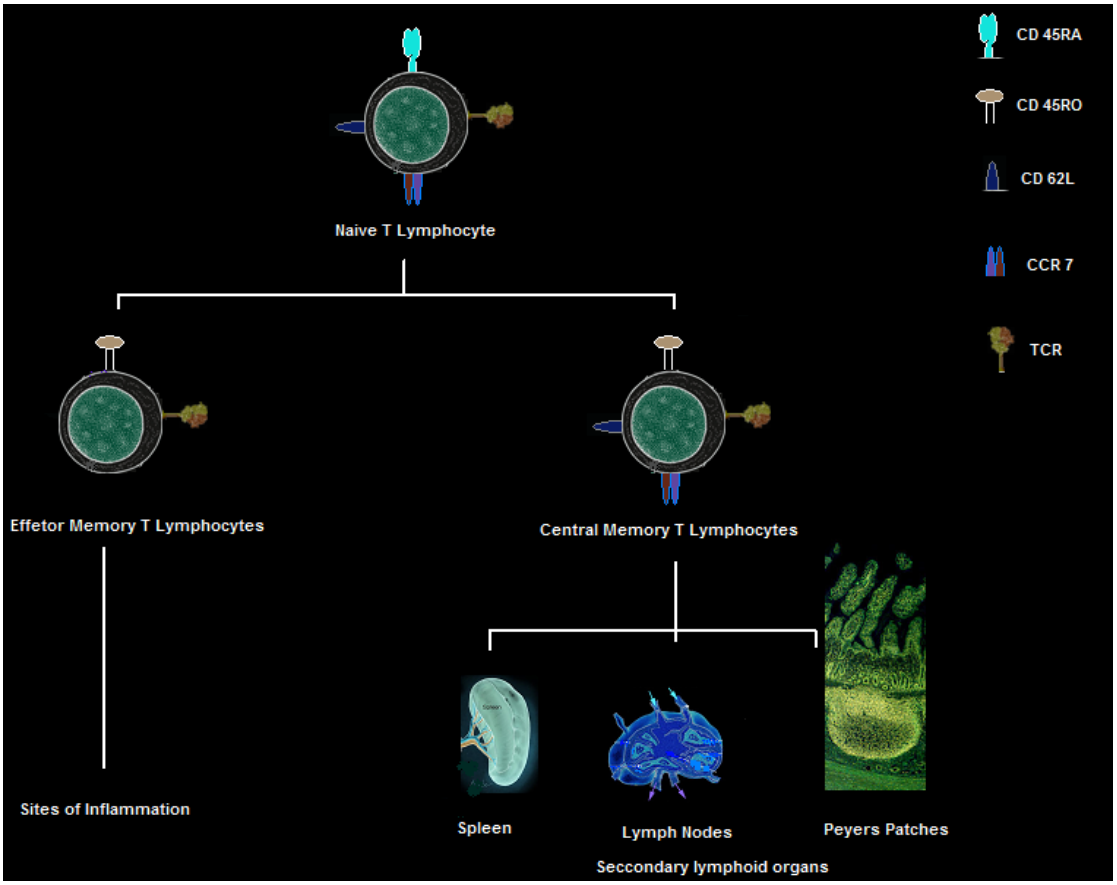


Figure 1.3: T-cell memory subtypes

1.9. The role of immune cells in atherosclerosis

T cells as an important component of atheroma were first demonstrated in 1986.[78] Subsequently it was shown that T cells comprises 10–20% of the cellular population in early atherosclerotic lesions[79,80] and in the absence of T cells, atherosclerotic plaque achieves only 10% of its potential size.[81] Most of the T cells in the atherosclerotic lesion are $CD3^+$ $CD4^+$ cells with αβ T cell receptor.[82] Due to ambient cytokine milieu in the atherosclerotic lesion, the $CD4^+$ T cells are polarised to the pro-atherogenic Th1 lineage.[83] Currently, there is a large body of evidence to incriminate the role of the Th1 response in all stages of atherosclerosis.

However the activation of the immune system including the T cell adaptive response is not confined to coronary lesions. A transient burst of T-cell activation can be detected systemically in patients with acute coronary syndromes.[84,85] Further evidence suggests that the T cell response is antigen driven[86] and is polarised to Th1 lineage.[87] The following discussion focuses on the role of T cells and their pertinent phenotypical subtypes in acute coronary syndromes.

1.9.1. $CD4^+CD28$ null T Cells

CD 28 is homodimer costimulatory receptor expressed on T lymphocytes. This molecule interacts mono-valently with two ligands, CD 80 (B7-1) and CD86 (B7-2) expressed on APC[88] via a conserved amino acid motif MYPPY found on its extracellular domain. In concert with TCR signalling, CD28 enhance T cell activation, cell metabolism, proliferation, and survival.[89,90,91] In addition, CD28:B7 interactions are needed for development and maintenance of $CD4^+CD25^+$ $T_{reg.}$[92]

Although constitutively expressed, the CD 28 molecules can be down regulated with chronic antigenic stimulation[93] as well as replicative and chronological senescence,[94] Functionally the $CD4^+$ $CD28^{null}$ cell are distinct from the classical $CD4^+$ helper T cells.[95] They variably express the Killer Immunoglobulin-Like Receptor (KIR), secrete interferon gamma, can release perforin and granzyme B.[96,97] The KIRs belong to the multigenic family encoded within the leukocyte receptor complex (LCR) on chromosome 19q13.4[98,99] and are normally expressed on NK cells and infrequently on $CD8^+$ T cells.[100] The KIRs recognize MHC class I molecules and circumvent the need for TCR stimulation. Ligation to inhibitory KIRs phosphorylates the cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) which then deactivate substrates critical for cellular activation by dephosphorylation. Conversely, stimulatory KIRs interact with signal transduction chain, DAP12[101], which then activates the protein kinase cascade ultimately leading to degranulation of the cytotoxic granules and release of cytokines. There is evidence that in acute coronary syndrome there is de novo gene expression in the LCR locus leading to cumulative acquisition of KIR2DS2 and its signalling protein DAP12 endowing the $CD4^+CD28^{null}$ cells with cytolytic competence circumventing the need for TCR signalling to initiate endothelial cell lysis.[102]

The lines of evidence for the central involvement of $CD4^+CD28^{null}$ cells include:

1. The circulating $CD4^+CD28^{null}$ cell population is amplified in acute coronary syndromes, constitutes up to 30-50% of total $CD4^+$ T cell population,[103] and their frequency predicts the risk of recurrence of acute coronary events.[104] This expansion is probably related to the degree of systemic inflammation as their frequency correlated with the level of CRP[105] and anti-inflammatory treatment with statin may reduce their frequency[106]

2. Phenotypic and TCR sequence analysis of total RNA in atherosclerotic plaques obtained from patients with fatal myocardial infarction indicated that these oligoclonally expanded population of $CD28^{null}$ $CD4^{+}$ T cells accumulate in culprit lesions and are not present in non-culprit lesions[107] implying they may modulate the stability of the plaque.

1.9.2. $CD40L^{+}$ T Cells

CD40L (also known as CD154 and gp39) is a 39-kD protein of the tumour necrosis factor (TNF) family. It exists both in soluble (sCD40L) and transmembrane form. The transmembrane form is expressed on cells of the immune system (activated $CD4^{+}$ T cells, mast cells, basophils, eosinophils and natural killer cells[108] and on activated platelets[109]). Expression of CD40L, regulated primarily by signalling through the T cell receptor, can be detected on T cells very early (1-2 h) after activation, peaking 6 h after activation and declining over the following 16-24h.[110] Its target CD40, is a 50-kDa homotrimer of the TNF receptor family, constitutively expressed on professional APC and non professional APC like endothelial cells, vascular smooth muscle cells fibroblast platelets and several epithelial cells.[111]

On ligation of CD40L, CD40 recruits adapter molecules TRAFs (TNF Receptor-associated factors) to initiate downstream mediators and effectors. Depending on cell type and TRAF involved the effector function can be diverse. For instance in B lymphocytes, CD40L-CD40-TRAF6 interaction results in isotype switching and IL-6 production while TRAF2/3/5 binding leads to up regulation of B7.1/ B7.2 (CD80/CD86) co-stimulatory molecule.[112] In monocyte and macrophages CD40-TRAF6 interaction leads to activation of Src/ERK1/2 and IKK/NF-kB

proinflammatory pathways.[113] The CD40L- CD40 dyad interaction culminates in the expression of chemokines (monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) and IL-8), pro-inflammatory adhesion molecules (vascular cellular adhesion molecule-1, intracellular adhesion molecule-1 and P-selectin), and tissue factor (TF), and down regulates the expression of thrombomodulin in vascular cells[114,115,116] -all probable participants in atherogenesis and lesion complication.[117] Recent evidence suggests that CD40L mediates the proinflammatory response independent of CD40 via the integrin receptor MAC-1 (CD11b/CD18). [118] Key lines of evidence for this are:

1. Genetic deficiency of CD40 L leads to X-linked hyper IgM syndrome[119] whilst, therapeutic inhibition of CD40L by monoclonal antibody resulted in immune-suppression and increased thromboembolic phenomenon.[120] The thromboembolic tendency is attributable to clot stabilizing effect of CD40L by alpha IIb B3 integrin-dependent mechanism.[121] Hence, inhibition of these interactions by anti-CD40L may render platelet plugs unstable and thus ready to embolise. Alternatively, interaction of the antibody with the platelet Fc receptors may promote platelet aggregation and thrombosis. Ligation of CD40 by CD40L is known to promote the expression of TF[122] and procoagulant leukocyte-derived microparticles expressing TF are a potent source of fibrin deposition on activated platelets.[123,124] Although lack of CD40L may be associated with thrombus fragility, high levels of soluble CD40L, which have been reported in patients with unstable angina,[125] could constitute an increased risk factor for thrombosis especially in regions of stenosis (high shear rate).

2. Epidemiologically, soluble CD40L (sCD40L), the 18-kDa extra-cellular portion of the trans-membrane molecule, is considered a predictor of cardiovascular risk.[126, 127,128]

3. Experimental evidence from $LDLR^{-/-}$ and $ApoE^{-/-}$ mice that genetically lacked CD40L or those treated with neutralising anti-CD40l antibodies suggest that CD40L inhibition attenuates atherogenesis, and modifies the plaque in to a phenotypically more stable composition,[129,130] and abrogates progression of pre-existing atherosclerotic lesion.[131,132]

4. T cells expressing CD40L have been observed within atherosclerotic plaque in human subjects.[133] Since the CD40L regulates the expression of interstitial collagenase (MMP1), activated gelatinase (MMP-2), stromelysin (MMP-3) and gelatinase B (MMP-9) in human vascular smooth muscle cells (SMCs)[134] the CD40L-CD40 dyad may promote plaque instability. T cells in peripheral blood of patients with unstable angina had enhanced surface expression of CD40L and increased release of sCD40L on anti-CD3/anti-CD28 stimulation in vitro when compared with patient with stable angina and controls.[135]

1.9.3. HLA-DR^+ T cells

1. HLA-DR^+/IL-$2R^+$ T lymphocytes are present in fatty streaks and fully developed atherosclerotic plaques.[136,137,138] Moreover, HLA-DR^+ T cells are almost a constant finding at rupture sites of thrombosed coronary plaque of patients who have died of acute myocardial infarction.[139]

2. The frequency of HLA-DR^+ T lymphocytes in atherosclerotic lesions is increased as compared to peripheral blood lymphocytes, and the same is true for the activation markers VLA-1, CD26, CD38, and CD45RO.[140]

1.9.4. OX40^{+} T Cell

Ox40 (CD134) are type I transmembrane protein of the TNFR family,[141] which forms trimeric signalling complexes when interacting with its cognate ligand OX40L (GP34). The expression of OX40 on T cell and OX40L on APC is inducible, occurring several days after antigen recognition by the TCR and peaking 2-4 days in to the response.[142,143] The level of expression and its kinetics is augmented by the costimulatory CD 28-CD80/CD86 interaction.[144] This dyad is involved in the generation of memory T cells,[145] enhancing cell survival by up regulating the antiapoptotic proteins BCL-XL and BCL2,[146] cytokine production[147] and T cell migration and tissue infiltration through interaction with OX40L on endothelial cells. [148,149] OX40 - OX40L is expressed in a number of autoimmune conditions.[150,151,152,153,154] Lines of evidence supporting involvement in atheroma and myocardial infarction include:

1. Genetic studies have identified OX40L gene underlying the atherosclerosis susceptibility locus *Ath1* on chromosome 1, and genetic variations in the OX40L locus are associated with myocardial infarction and severity of CAD in man.[155]

2. The quantitative trait locus for MI in human is located on chromosome 4, in the same region as OX40,[156] and genetic polymorphism of the OX40 gene may influence susceptibility to MI.[157]

3. Mice over expressing OX40L showed significantly larger atherosclerotic lesions compared with control, and mice with targeted deletion of this gene (*Tnfsf4*$^{-/-}$) results in significantly smaller lesions compared with control.[155]

4. Inhibition of OX40L in a LDLR$^{-/-}$ mice with anti-OX40L antibody, significantly attenuates the volume of atherosclerosis and cellularity of the plaque.[158]

1.9.5. Antigens for the T cell Response

Chronic phase

The evidence that antigen presentation plays a role in atherogenesis is derived from that fact that professional antigen presenting cells like $CD1a^+$ dendritic cells have been reported in vascular intima.[159,160] As the initial cell type observed infiltrating the arterial intima at a site known to be predisposed to later development of atherosclerotic lesions were lymphoid cells, and not foam cells,[161] and the $CD4^+$ lymphoid cells were HLA-DR and CD25 positive, the expression of the MHC molecules is indicative that antigen presentation has occurred and an immunological reaction is ongoing or has recently happened.[162]

Acute Phase

Progression of atherosclerosis to unstable rupture prone plaque may also be antigen driven as mature DC has been reported amongst the clusters of activated T cells in rupture prone areas of atherosclerotic plaque. [163,164]

The putative antigen recognised by the T cells includes exogenous microbial components or autologous proteins for instance, B-2 GP-1, oxidised LDL (oxLDL), and stress or heat shock proteins (HSP).

1.9.5.1. Heat Shock Protein

HSP are evolutionarily conserved proteins of various molecular weights expressed both in eukaryotes and prokaryotes. They function as molecular chaperones and are responsible for a wide range of physiological function including translocation of oligomeric protein,[165] assembly and folding of nascent protein, cellular signalling, and protein degradation, resolubilize aggregated protein.[166,167] They are expressed constitutively but are inducible in response to various noxious stimuli

for instance, hemodynamic stress,[168] oxidative stress,[169] LDL and oxLDL,[170] via the mitogen activated protein kinase (MAPK) pathway.

HSP 60 is a mitochondrial protein that is translocated in to the cytoplasm in response to stress and have potential to be transported to the cell surface where it seems to provide a danger signal.[171,172] The HSP60 family comprises the human HSP60, hHSP60; mycobacterium homologue, mHSP65; Chlamydia homologue, cHSP60; and E Coli homologue GroEL,[173] the last three exhibiting more than 90% sequence homology amongst themselves and 50%-55% with hHSP60.[174] This degree of phylogenetic conservation confers the opportunity by which immune response directed to microbial HSP60 can be redirected against hHSP60 when it is induced in situ in response to stress and initiate autoimmune damage by process of molecular mimicry.

In experimental studies, immunisation of normocholestrolemic rabbits with Freund`s complete adjuvant (which contains mHSP65) or recombinant mHSP65 resulted in mononuclear cell infiltration of the intima at those arterial site that are known to be subjected to major turbulent hemodynamic stress[175] and peripheral blood showed antibodies and T cells reactive to HSP.[176] Similarly, Wild-type mice (C57BLy6J), which are very resistant to induction of atherosclerosis by a high cholesterol diet alone, also develop aggravated lesions when simultaneously immunized with mHSP65.[177] Immunization of the $LDLR^{-/-}$ mice with HSP65 induced specific T cell reactivity against mHSP65 as well as mammalian HSP60. Transfer of lymphocytes or isolated IgG of immunized mice into non-immunized litter-mates enhanced lesion size.[178]

In addition to expression of HSP60 in the cellular components of atheroma,[179,180] T cells reactive to HSP60 have been localised in human atherosclerotic lesions.[181,182] Further, peripheral T cell reactivity against HSP60 has been shown as an independent predictor of early intima media thickening in young subjects[183] although no such association was found in older subjects.[184] With advanced atherosclerosis, CD4+CD28null cells in the peripheral circulation were found to be reactive to hHSP60 during the acute phase of coronary artery disease and not during the chronic stable phase.[185]

Epidemiological studies have incriminated anti-HSP65 antibodies to be associated with risk of atherosclerosis;[186] their titre correlated with disease severity[187,188] and was shown to predict 5-year mortality.[189] AntimHSP65 antibody titre correlated strongly with human IgA to C. pneumoniae and IgG to H. pylori implying a role for these infections in the production of mHSP antibodies.[190] A subsequent study related elevated human HSP60 IgA antibody levels in conjunction with elevated C. pneumoniae IgA antibody levels and an elevated CRP with a relative risk of 7.0 for coronary events.[191] Experimental studies suggest that in the presence of complement (complement-mediated cytotoxicity) or peripheral blood mononuclear cells (antibody-dependent cellular cytotoxicity), these antibodies can lyse stressed endothelial cells.[192,193]

1.9.5.2. OxLDL

Oxidised LDL (oxLDL) is present in atherosclerotic lesions and comprises a variety of lipid peroxidation products. They can ligate to various macrophage receptors including CD36, SR-B1[194,195] and CD14/TLR4[196] to elicit innate immune response; or behave as immunogenic neoepitopes to elicit adaptive immune

response. [197] T cells isolated from human atherosclerotic plaque were shown to be specifically reactive to oxLDL in MHC class II restricted fashion, [198] and plasma titres of antibodies against oxLDL may accentuate atherosclerosclerosis.[199]

1.9.5.3. ß2 Glycoprotein-I (ß2 GPI)

ß2 GPI is a phospholipid binding protein expressed by platelets, endothelial cells and human atherosclerotic plaque.[200] Adoptive transfer and immunisation of $LDLR^{-/-}$ mice against ß2 GPI accelerated early atherosclerosis.[201,202] This effect may be due to the effect of the antibodies on macrophage leading to enhanced uptake of the oxidised LDL by macrophages.[203]

1.9.5.4. Infections

Seroepidemilogical studies have associated the risk of atherosclerotic cardiovascular disease with serum antibody titres against various microbial pathogens including C. pneumoniae,[204] H. pylori,[205] CMV,[206] EBV,[207] periodontal pathogen[208] and entero virus.[209] However, the serological association is rather disparate.[210,211] Subsequent studies indicated an association of CHD with serum IgA and not IgG.[212] Further, co-infection with multiple pathogens and the cumulative pathogen burden seemed to have synergistic effect in induction of vessel injury.[213,214]

C. pneumoniae has been detected in human plaque.[215,216, 217] T cell lines specific to Chlamydia pneumoniae have been generated from atherosclerotic plaque[218] and they specifically react to cHSP60 and membrane antigen from C pneumoniae.[219] Similarly multiple members of herpes virus family[220] and DNA of several pathogens responsible for periodontal pathology has been isolated from human atherosclerotic plaque[221] and plaque-derived T cells reactive to respective

pathogens have been identified.[222,223] As eluded to earlier, these HSP reactive T cells in turn evoke autoimmune response due to antigenic cross reactivity leading to plaque inflammation.

Acute respiratory infection during the two preceding weeks has been shown to be a risk factor for acute myocardial infarction.[224] Further, in patient with ACS serum levels of antihuman HSP60 IgG antibody and anti-chlamydial IgM but not IgG or IgA were significantly higher when compared with stable CAD and controls.[225] This implicates the role of acute infection in destabilization of atheromatous plaque.

1.9.6. Antigen independent T cell activation

Independent of direct antigen presentation, interleukin-15 exerts pleotropic effects on T cell functions including proliferation, differentiation, cytokine production, apoptosis inhibition and induction of CD40L and CD69 expression.[226,227,228] Plaque macrophages express high level of IL-15 mRNA and its protein especially in lipid rich plaque.[229] Since high lipid content renders plaque vulnerable for rupture, one may speculate this mechanism may be responsible of acute instability of plaque.

1.9.7. Role of cytokines

1.9.7.1. Interferon-γ

Interferon gamma is the principle proatherogenic cytokine expressed by the T cells in atherosclerotic plaque.[230] In ApoE$^{-/-}$ mice, deficiency of interferon-gamma or its receptor attenuates atherosclerosis,[231,232] and conversely, injection of recombinant interferon-gamma accentuates atherosclerosis.[233]

T lymphocytes accumulate at sites where plaques rupture and cause fatal thrombosis;[234] such plaques typically have relative paucity of vascular SMCs and interstitial collagen matrix rendering them vulnerable for rupture. Compared to regions lacking T-cell infiltrates, sites where T-cell accumulates exhibit low levels of interstitial collagen mRNA and protein.[235] Interferon- gamma inhibits interstitial gene expression and protein synthesis in these cells.[236] In addition, apoptosis may contribute to the paucity of SMCs in vulnerable plaques.[237] Indeed, some SMCs in atheroma have fragmented DNA and other features characteristic of programmed cell death.[238] Pro-inflammatory cytokines like interferon-gamma expressed in atherosclerotic plaques can trigger apoptosis.[239] SMCs in the atherosclerotic intima can express fas, while T cells in the atheromata express fas ligand-bearing.[240,241] Thus, the T cells in concert with the inflammatory cytokines like interferon gamma destabilize the plaque leading to acute coronary syndromes.

Clinical studies have revealed interferon-gamma producing CD4 and CD8 T cells are increased in peripheral circulation in patients with an acute coronary syndrome.[242] Although the plasma level of interferon-gamma may not be grossly elevated,[243] the up-regulation of interferon-gamma–inducible genes for transcription of IP-10 and CD64 in circulating T cells is indicative that interferon-gamma activity is augmented in acute coronary syndrome.[244]

Surprisingly LDLR-/- mice transplanted bone marrow from IFN-$\gamma^{-/-}$ mice exhibited larger atherosclerotic lesions than mice that received bone marrow from IFN-$\gamma^{+/+}$mice.[245]

1.9.7.2. Interleukin 12

Interleukin-12 is the principal cytokine to promote Th1 differentiation and atherogenesis by inducing IFN-γ production from Th1 cells. Administration of recombinant IL-12 to ApoE$^{-/-}$ mice could accelerate atherosclerosis with concomitant augmentation of interferon-gamma expression.[246] Conversely, apoE-/-IL12p40-/- mice exhibited reduced plaque burden.[247] In humans, IL-12 mRNAs and protein were significantly expressed at atherosclerotic plaques.[248] This cytokine may be important in progression of atherosclerosis to acute coronary syndrome as elevated plasma level of IL-12 has been detected in unstable angina[249] and acute myocardial infarction.[250]

1.9.7.3. Interleukin 18

IL-18 is pleiotropic cytokine acting in both acquired and innate immunity.[251] It acts synergistically with IL-12 for activation of Th1 and production of IFN-γ.[252] ApoE$^{-/-}$ IL18$^{-/-}$ mice[253] and ApoE$^{-/-}$ mice treated with plasmid DNA encoding for IL-18 binding protein[254] exhibited attenuated atherosclerosis, whilst, IL-18 administration enhanced atherosclerosis in ApoE$^{-/-}$ mice.[255] However, IL-18 administration did not alter atherosclerosis in ApoE$^{-/-}$IFN-γ $^{-/-}$mice implying IL-18 modulates the atherogenic effect by induction of interferon gamma. Increased expression has been reported in human atherosclerotic plaque[256] and increased plasma concentration in AMI.[257] Plasma IL-18 up regulates Fas ligand[258] and concentrations are increased in patients with acute coronary syndromes and correlate with the severity of myocardial dysfunction.[259]

1.9.8. Atheroprotection: Foxp3 T_{reg}s: $CD25^+$ $CD4^+$ T Cells

T_{reg}s are subset of $CD25^+$ $CD4^+$ T cells and express lineage specific marker FOXP3 (Forkhead Box Protein P3), a member of the forkhead winged helix protein family of transcription factors.[260,261] Natural T_{reg} arise in the thymus while Adaptive $Foxp3^+$ T cells can develop from naïve $CD25^-$ $Foxp3^-$ $CD4^+$ T cells in response to antigen and driven by TGF-B.[262,263] High levels of CTLA-4 (cytotoxic T-lymphocyte associated molecule-4), CD103 and GITR (Glucocorticoid-induced TNF receptor) are also expressed on $T_{reg.}$ However, the functional significance of this expression remains to be elucidated. T_{reg}s constitute 10% of peripheral $CD4^+$ T cells[264] and are implicated in inducing and maintaining immune tolerance and the termination of immune responses. Their suppressive effect appears to be contact dependent and probably mediated by TGF-B.[265] Their deficiency leads to inflammatory autoimmune disorder[266] and attenuated peripheral blood $CD4^+CD25^{high}$ T_{reg} have been linked to different types of autoimmune diseases like rheumatoid arthritis, type-1 diabetes, multiple sclerosis and systemic lupus erythematosus.[267,268]

Experimental studies have suggested adoptive transfer of T_{reg} prevented development of plaque in mouse $ApoE^{-/-}$ model of atherosclerosis.[269,270] The natural T_{reg}s are known to produce IL-10[271] which may have a critical role in T_{reg} mediated suppression,[272,273] and IL-10 are associated with attenuation of experimental atherogenesis.[274,275,276] The suppressive effect of T_{reg}s may also be mediated by transforming growth factor-beta as its mRNA can be predominantly detected in the $CD25^+$ $CD4^+$ population.[277] Neutralising anti-TGF-β antibody[278] and soluble TGF-β receptor[279] accelerated atherosclerosis in $ApoE^{-/-}$ mice, and

delivery of activated TGF-β to $LDLR^{-/-}$ mice via adenovirus vector attenuated atherosclerosis.[280]

In Man, T_{reg}s has been demonstrated in peripheral blood of subjects with normal coronary arteries[281] and is decreased in the peripheral blood of patients with acute coronary syndromes.[282,283] Further, the frequency of $FOXP3^+$ T_{reg} in atherosclerotic lesion was found to be attenuated and quantified to be in the range of the 0.5–5% of the total number of $CD3^+$ T cells.[284]

CD 25 is the alpha subunit of the IL-2 trimeric molecule. $CD25^+$ T cells are expressed constitutively in 5-10% of peripheral CD4+ T cells.[285] CD25 expression may not be the specific phenotypic marker of $FOXP3^+$ T_{reg} as CD25 is induced on T cells within 2-24 hours after stimulation by cytokines and persists for few days after diminution of the stimuli.[286,287] Unlike $FOXP3^+$ T_{reg}s, the $CD25^+$ T cells were more prevalent in atherectomy specimens from patients with refractory unstable angina and acute myocardial infarction compared with specimens from patients with stable angina.[288,289]

1.10. Hypothesis for this thesis

The hypothesis for this thesis is that a study of thromboaspirate obtained at PPCI for STEMI in man may allow further elucidation of the immunological mechanisms involved in the processes of atherogeneis, plaque rupture, thrombotic coronary occlusion and microvasular and myocyte damage associated with reperfusion in STEMI.

1.11. Outline plan of investigation

This exploratory investigation will use thromboaspirate obtained at PPCI for STEMI in man. The material obtained will be filtered so that that both solid phase

thrombus and liquid phase filtrate can be analysed. Specifically the solid thrombus will be examined for platelet and leukocyte composition and the liquid phase will be analysed focussing on T cell phenotypes and immunoregulation. In addition the cytotoxicity of coronary sera will be explored in a set of preliminary investigations. For liquid phase assays comparison will be made with chronic stable angina patients and healthy volunteers.

1.12 General statistical and sample size considerations

The work of this thesis explores novel approaches in the investigation of intracoronary biology in patients. Limitations imposed by the nature of the work are discussed in the methods section and individual chapters. Statistical issues have been reviewed in detail with our statistician (MR) and conclusions are limited to the exploratory mechanistic nature of this work. The presentation of results and statistical comparisons is discussed in detail in the methods chapter (2).

CHAPTER 2: Methods and Materials

2.1. Recruitment of test cohort

I personally attended two hundred primary percutaneous interventions (PPCI) for patients with ST elevation myocardial infarction (STEMI) from November 2006 to May 2008 at Harefield Hospital and recruited 75 subjects. Since the patients at arrival to the hospital are sometimes under the influence of narcotic analgesia impairing their competence for giving informed consent, and the process of prospective consenting could interfere with the clinical care delaying myocardial reperfusion, it was agreed by the ethics committee that retrospective consent be sought for research using samples aspirated for clinical indications.

2.1.1. Inclusion criteria:

- Chest pain of 12 hours duration or less
- ST elevation of at least 2 mm in two consecutive precordial leads or at least 1 mm ST elevation in two consecutive limb leads or new onset LBBB with chest pain
- Consented for PPCI
- Angiographic evidence of thrombotic occlusion of the coronary artery (TIMI-0 flow)
- Thromboaspiration performed on clinical grounds with the sample aspirated available for analysis

2.1.2. Exclusion criteria:

- Refusal or unable to consent
- Acute / subacute stent thrombosis
- Spontaneous TIMI II –TIMI III flow
- Transplant recipients
- Active autoimmune disease
- Known blood dyscriasias
- Immunosuppressive therapy
- Heart Failure

2.2. Recruitment of control cohort

Twenty age and sex matched patients attending Harefield Hospital for the investigation of known coronary artery disease with coronary angiography.

2.2.1. Inclusion criteria

- Known chronic stable angina undergoing coronary angioplasty
- Undiagnosed chest pain undergoing coronary angiography
- Patients undergoing angioplasty restudy / CABG restudy

2.2.2. Exclusion criteria:

- Troponin positive ACS (preceding 3 months)
- Transplant recipient
- Autoimmune disorder
- Heart Failure

2.3. Clinical Characteristics

	STEMI (n=75)	CSA (n=20)	Healthy Volunteer (n=10)
Age: Mean years.	61 (19)	54 (12)	30 (4)
Sex (% Male)	66.6	80	50
Pain to reperfusion time: Minutes(SD)	186(168)	N/A	N/A
Peak Creatinine Kinase U/l(SD)	1315 (1117)	N/A	N/A
Peak Troponin I ug/l (SD)	94 (198)	N/A	N/A
Total Cholesterol mmol/l (SD)	5.9 (1.3)	4.5 (1.4)	N/A
Random Glucose mmol/l (SD)	9.7(6)	7 (2)	N/A
Hypertension %	53.3	60	0
Hypercholesterolemia %	29.4	40	0
Diabetes %	26.7	30	0
Current Smoking %	33	25	0
Family history of premature CAD %	24	25	0
Prior Beta Blocker use %	24	70	0
Prior ACE inhibitor use %	25	65	0
Prior Statin Use %	37.8	90	0
Prior Clopidogrel use %	6.7	25	0

2.4. Plan of investigations

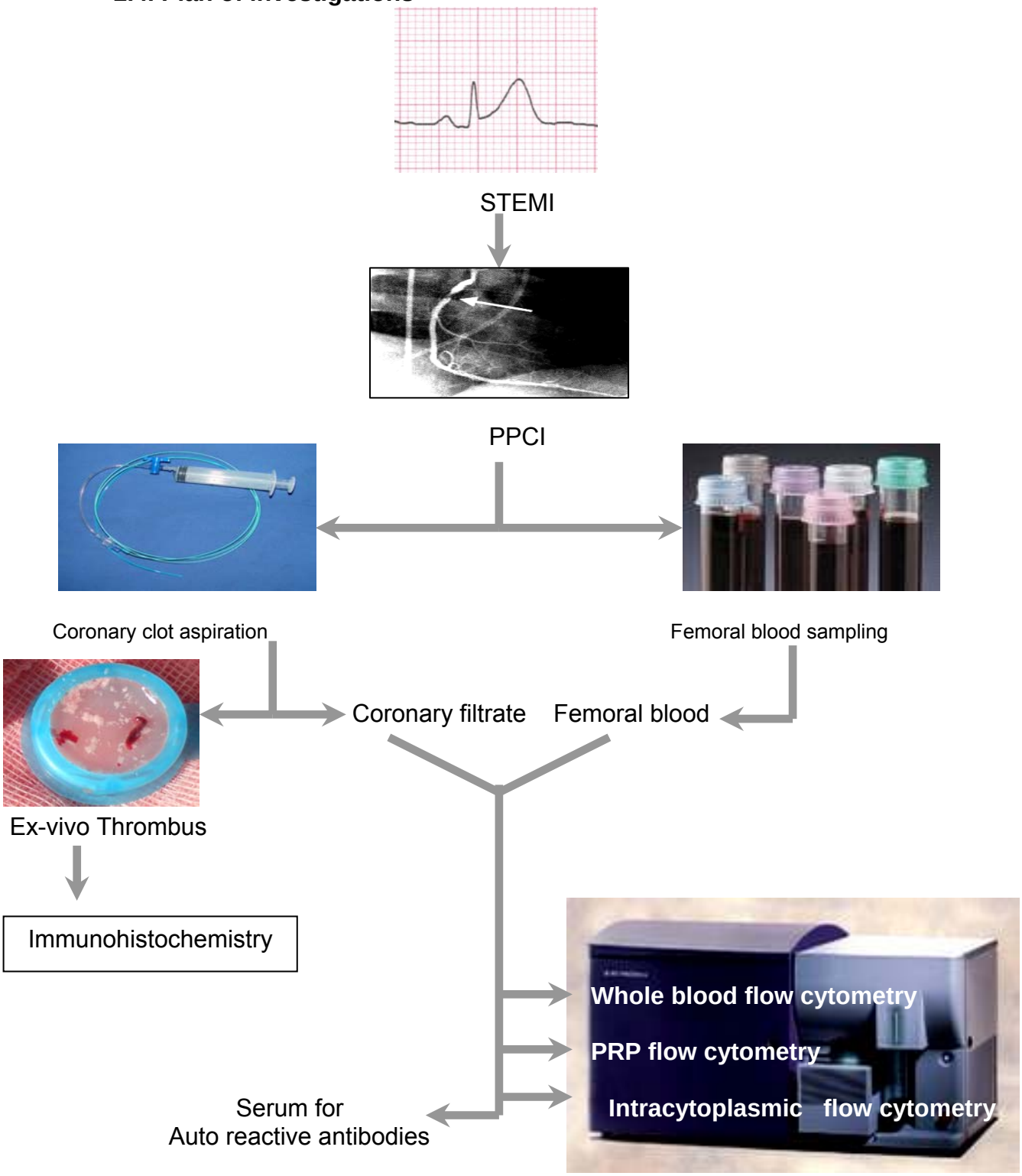


Figure 2.1: Plan of investigation

2.5. Sample collection

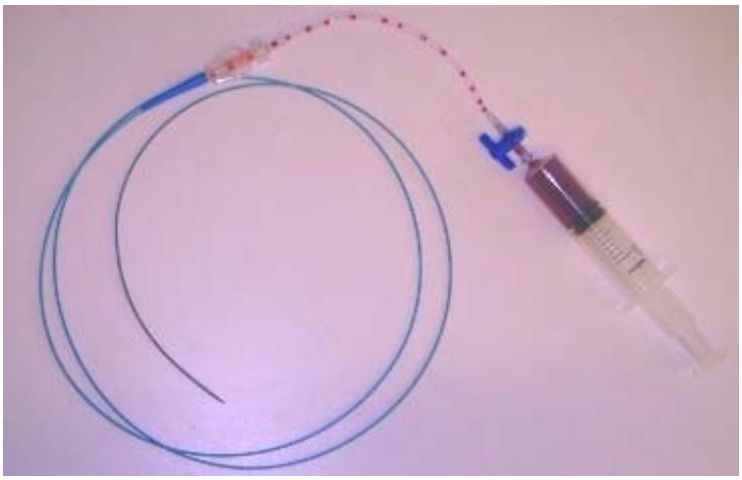
2.5.1. Intracoronary thrombectomy and aspiration

The intracoronary aspiration was performed using a sterile non-pyrogenic aspiration catheter (Export® XT Aspiration catheter, Medtronic, Minneapolis, USA) (figure 2.1-3). The catheter was 140 cm long and has a dual lumen radio-opaque tip with total diameter of 1.73mm. The catheter was advanced over the guide wire under fluoroscopy. Once the tip was advanced to the point just proximal to the site of coronary occlusion a negative pressure was applied using a 20ml syringe and the catheter was advanced forward and backward to obtain the atherothrombotic material. Haemodynamic data provided by the manufacturer (Medtronics, Minneapolis, USA) suggest that the catheter evacuates fluids and debris at a minimum flow rate of 0.5ml/sec. Using the Hagen-Poiseuille formula assuming steady flow, a rigid tube and a parabolic velocity profile, shear rate for the system was $(4Q/\pi R^3)$ 0.05 S^{-1}. This shear rate is small when compared for example with the rate produced within the vacutainer (Becton Dickenson) system commonly used for venepuncture. Assuming similar flow conditions, 4.5ml aspiration would take almost 10 sec giving a flow rate of 0.45ml/sec. Since the internal diameter of 21 gauge needles was 0.5mm, the average shear rate was $4500S^{-1}$. Since the shear rate in the aspiration devise was comparatively low we did not anticipate major activation of platelets induced artefactually by the shear rates.

The aspirate was then filtered through a filter with pore size 0.79 mm to separate the athero-thrombotic material from blood (Figure). The ex-vivo coronary thrombi were embedded in Optimal Cutting Temperature (OCT) compound (Tissue-Tek;

Miles Laboratories Inc., Elkhat, IN), cryofrozen and stored at -80 °C. The coronary filtrate was divided into three aliquots for:

- Whole blood preparation for Leukocyte experiments.
- Centrifuged at 200g for 10 minutes to obtain platelet rich plasma (PRP) for platelets experiments.
- Centrifuged at 3000 rpm for 10 minutes to obtain Platelet poor plasma (PPP). The supernatant PPP was pipetted off and stored at -80 °C.



Export® XT Aspiration catheter, Medtronic, Minneapolis, USA

Figure 2.2: Thrombus aspiration catheter

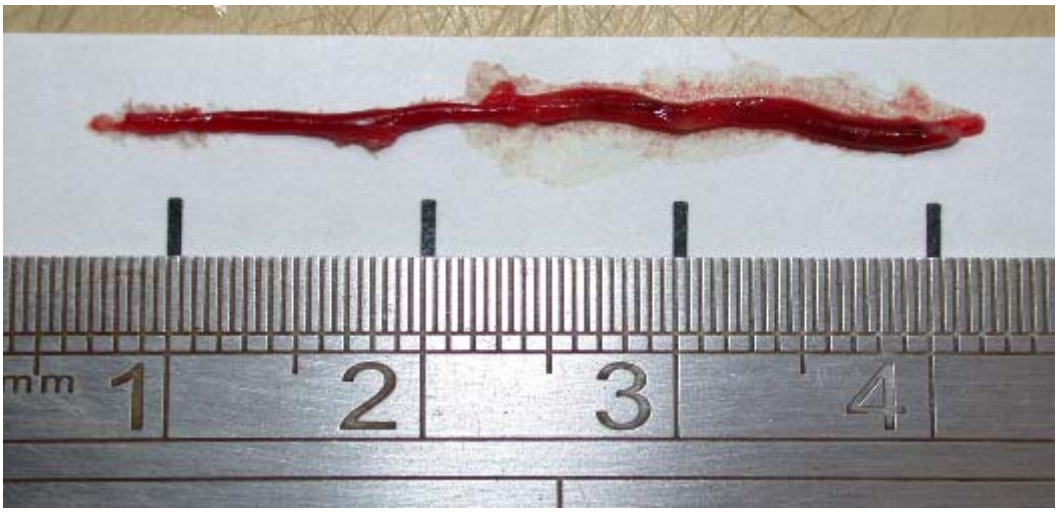


Figure 2.3: Ex vivo intra-coronary thrombus from patient with STEMI

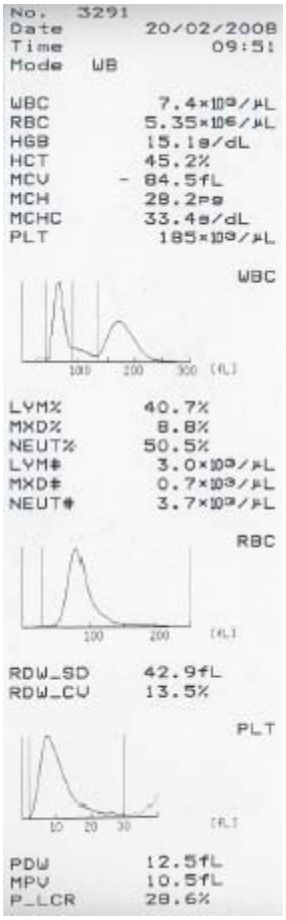
2.5.2. Femoral arterial blood sampling

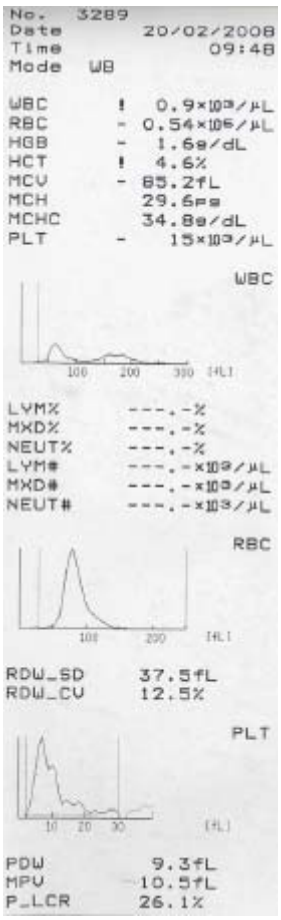
Femoral arterial blood sampling was obtained through the side port of the femoral introducer (Avanti® cordis, Miami, FL, USA) using a 20 ml syringe. The introducer was 11 cm long with 5F-6F (1.65 - 2mm) lumen diameter. The sampling was performed prior to delivery of any anticoagulants or glycoprotein IIb/IIIa inhibitors. The blood was then transferred to EDTA vacutainers without using any needle to minimise the shear rates. The femoral blood was also divided in three aliquots as explained above for paired experiments.

2.6. Sample processing

2.6.1. Whole blood preparation for flow cytometry

2 μL of the whole blood in a 1.5 ml eppendorf tube was diluted 10X with modified Tyrode's solution (composition in mM: NaCl 140, KCl 5.4, CaCl2 1.8, MgCl2 0.5, HEPES 5, D-glucose 5). At this dilution, the approximate number of cells present in each eppendorf tubes provided that the total blood counts in the blood were within normal range would be: WBC: 20,000, with lymphocytes: 8,000, neutrophils: 10,000 and monocytes: 1,600; total platelets: 300,000; and RBC: 10X106. (figure 2.4). The cells were stained with a saturating dose of monoclonal antibodies and incubated at room temperature for 30 minutes. 400 μL of 1X BD FACS™ lysing solution (BD Bioscience, San Jose, USA) was then added and incubated for 30 minutes in the dark at room temperature to lyse the red cells. To label leukocytes, Hoechst 33342 (Molecular Probes, Eugene, OR, USA), diluted in PBS (1 μg/L final concentration), was subsequently added for another 30 min.[290]





Full blood count in whole blood

Full blood count in 10X diluted blood

Figure 2.4: Whole blood preparation for flow cytometry

2.6.2. Buffy coat preparation for flow cytometry

Five ml of the blood sample was centrifuged at 200Xg for 10 minutes. The cells at the interface were pippeted off. Contaminating red cells were removed by cell lysis by osmosis. The protocol used at our centre included addition of 9 ml of distilled water in to the preparation and gentle mixing by inversion for 20 seconds. Adding 1 ml of 10X PBS then reversed the osmosis. The cells were pelleted by centrifugation at 1500 rpm for 6 minutes. The supernatant was discarded and the

pellet washed twice with Hanks (Sigma). Finally the cells were suspended in RPMI-1640 (Sigma).

2.6.3. Mononuclear cells by density gradient centrifugation for flow cytometry

Five ml of the whole blood was diluted with iso-volume of phosphate-buffered saline (PBS). The mixture was layered over 15 ml of Histopaque-1077 (Sigma, St. Louise, Missouri, USA). Mononuclear cells were collected from the interface after density centrifugation at 1500 rpm for 20 minutes. The cells were washed three times with Hanks (Sigma) after centrifuging at 1500 rpm for 5 minutes. The pellet was suspended in 1 ml RPMI-1640 (sigma). A haemocytometer was used to calculate the number of cells per ml. The cell suspension was appropriately diluted to obtain 1 million cells per ml.

2.6.4. Cryo-sectioning of the thrombus

The ex-vivo frozen thrombi were subjected to serial cryotomy at -19°C to -22°C to obtain 5-micron sections. The sections were mounted on glass slides, incubated at 37°C for 30 minutes, and fixed with acetone for 5 minutes. The slides were wrapped with cling film and stored at -80°C.

2.6.5. Immunohistochemistry

Cryostat sections were fixed with acetone for 5 minutes and incubated with 5% normal goat serum and 1% bovine serum albumin (BSA) for 30 minutes to block the non-specific binding sites. A panel of primary antibody suspended in 1% BSA

was add in to the spots and incubated at room temperature for 1 hour. The sections were washed thrice in PBS for 5 minutes, incubated with secondary antibody for one hour and again washed thrice in PBS.

The sections were visualized using a three-color immuno-microscope (Nikon, Japan). Individual cells were quantified by measuring the intensity the specific fluorphore using Lucia-G software (Laboratory Imaging Ltd., Prague, Czech Republic). Ten random high power fields were analysed in each section numbers of positive cells/high power fields.

2.7. Flow Cytometry

2.7.1. Principles

Flow cytometry was used to analyze the optical properties (light scattering and flurochrome emission) of 0.2-150 μ particles or cells in mono-disperse suspension, hydro-dynamically focused in to single file and subjected to the laser intercept to generate specific multi-parametric data pertaining to its physical and chemical composition. Forward-scattered light (FSC) is an index of mostly diffracted light and is detected just off the axis of the incident laser beam in the forward direction by a photodiode and correlates with cell volume. Orthogonal or side-scattered light (SSC) is a measurement of refracted and reflected light that occurs at any interface within the cell where there is a change in refractive index. SSC is collected perpendicular to the laser beam and is proportional to cell granularity or internal complexity. Two parameter histogram-correlating measurements of FSC and SSC can allow for differentiation of cell types in a heterogeneous cell population (figure 2.5).

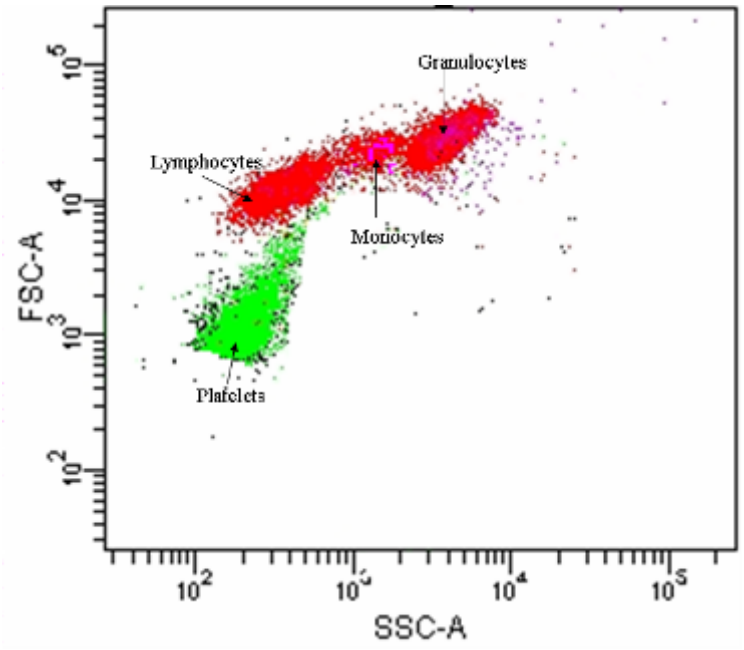


Figure 2.5: Whole blood FACS after red cell lysis using FSC and SSC

2.7.2. Optics

When excited with a laser beam of a particular wavelength, a fluorochrome specific for that wavelength absorbs light and elevates electrons to a higher level of energy. The excited electron quickly decays to its ground state, emitting excess energy as photon of light of specific wave length. Monoclonal antibodies against both extra-cellular and intra-cellular antigens can be conjugated with different flurochromes and particular cell type can be identified. Combinations of fluorophores with varying emission spectra can be used for detailed phenotyping of a particular cell of interest or in heterogenous cell populations to distinguish separate subpopulations. The following illustration depicts the absorption and emission spectra of various flurochromes used in our experiments (figure 2.6).

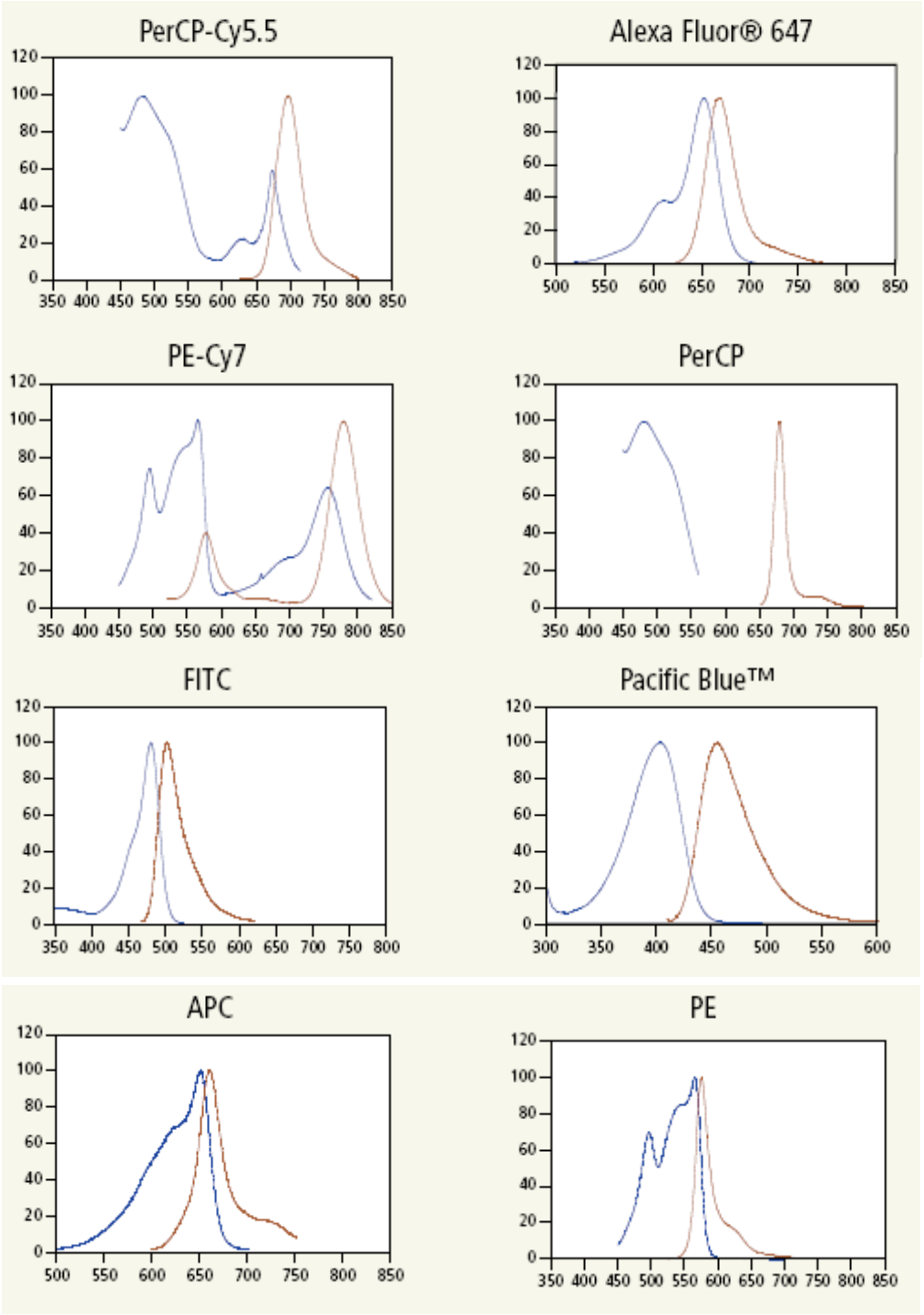


Figure 2.6: Absorption and emission fluorochrome spectra

The emitted fluorescence and SSC signals are diverted to the photomultiplier tubes (PMTs), and a photodiode collects the FSS signals. These light signals are

converted to electronic signals (voltages) by photo detectors and then the voltage pulse is assigned a digital value by the Analog-to-Digital Converter (ADC) and is displayed in an appropriate position on the data plot.

2.7.3. Methodology

In our centre flow cytometric analysis was performed on the BD fascaria equipped with three lasers at 488 nm (argon), 633 nm (Helium-Neon), and 405 nm (Diode). Analysis was performed within two hours of sample collection. The multiparametric resolution of the side and forward scatter was optimised for resolving lymphocytes, monocyte and granulocyte population. Appropriate electronic threshold parameters were set to eliminate debris and cell fragments. Non-specific staining with the isotype-matched control monoclonal antibodies was kept below 1%. The intrinsic spectral overlap of the different fluorochromes while performing simultaneous multicolour flow cytometry could cause spurious events being recorded in inappropriate detectors in multi-colour contour plots leading to false positive population. This effect was mitigated by electronic subtraction of unwanted emission to remove the effects of spectral spill-over by using appropriate single and double stained control samples.

2.8. Investigation of potential confounding factors

2.8.1. Contrast media effect

Iodinated angiographic contrast media can influence haemostasis; and both prothrombotic[291,292] and anticoagulant[293,294] effects have been previously been described. This effect is probably mediated by the effect of the contrast media on

the platelets which degranulate in response to the low osmolar contract media leading to expression of CD62P, CD63 (GP53), release of α-granules (PF4) and dense granules (5-HT). Since coronary blood aspiration was performed subsequent to intracoronary injection of contrast media in to an occluded coronary artery, it was possible that the difference observed on coronary aspirate when compared with femoral arterial sample (sampled prior to contrast) could be the spurious effect of the contrast media. Therefore, to identify the effect of contrast media on different cell lines, femoral arterial blood from 5 controls (patients with stable coronary artery disease undergoing angiography) was admixed in serial dilutions (figure 2.7) with the contrast media in-vitro and then subjected to standard whole blood flow cytometry.

For platelet experiments, as per the standard practice, the 1.5 ml Eppendorf tubes are centrifuged at 200g for 10 minutes to obtain platelet rich plasma. As shown in the figure platelet rich plasma was obtained consistently on aliquot 1 and 2. Aliquot 3 also separates into plasma but was contaminated with RBC. Since the coronary aspirate on all test samples separated into plasma without any RBC contamination, one can infer that the maximum concentration of the contrast in the coronary aspirate has to be less than 250 μL in 1.5 ml. Furthermore when the coronary aspirate was obtained hardly any residual contrast was visualised inside

Blood+CM	Aliquot 1	Aliquot 2	Aliquot 3	Aliquot 4	Aliquot 5	Aliquot 6	Aliquot7
Contrast Media	Nil	125μL	250μL	375μL	500μL	750μL	1000μL

the coronary artery on the fluoroscope.

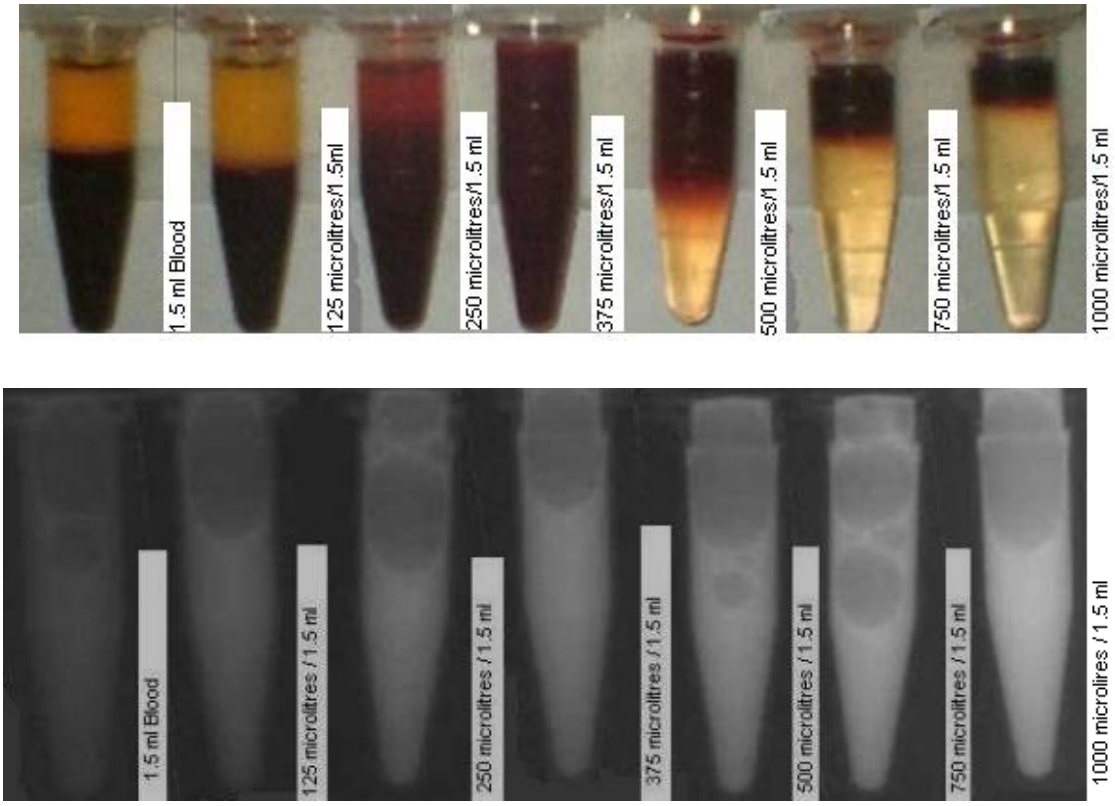


Figure 2.7: Controlling for contrast media effect

The PRP obtained from the whole blood, as well as the serial dilution at 125μL contrast /1.5 ml of blood and 250μL contrast /1.5 ml blood were stained with the same panel of antibodies as the test samples to evaluate the effect of the contrast media.

2.8.2. Effects of shear rates

As discussed earlier in section 2.5.1, the hemodynamic profile of the aspiration device, the Export® XT, allows a minimum flow rate of 0.5ml/sec engendering minimum shear rates of 0.05 S^{-1}. Although this shear rate is small, the total

duration for which the cells are exposed to this shear rates is significantly higher than the vacutainer as the length of the Export® XT devise is 140 cm. To evaluate whether the observed difference between coronary aspirate and femoral blood was attributable to the shear rates in the Export® XT devise, femoral arterial blood obtained from 5 patients undergoing coronary angiography was aspirated in vitro using the Export® XT devise. The paired femoral and the post aspiration samples were subjected to staining procedure similar to the test samples and analysed in the flow cytometer.

2.8.3. Reliability

To evaluate the repeatability (test retest) of the overall procedure (venessection, collection and flow cytometry) serial peripheral venous samples from 10 healthy volunteers were obtained at an index time and 2 hours later. The whole blood and PRP obtained from both samples were stained as per standard per standard protocol and subjected to flow cytometry. The data was analysed to obtain the mean difference.

2.9. Statistical considerations

These experiments have been performed on relatively small numbers of patients. All raw data has been analysed for normality of distribution and a degree of skewing was seen in some of the distributions, following statistical review it was concluded that the data should be described using nonparametric parameters in terms of median and quartile range and this is the format used throughout the text. Unless iterated to the contrary therefore, the numerical immunological data is presented as median with 1st and 3rd quartiles in brackets. Statistical analysis are

performed using wilcoxon signed rank test while analysing one subject two sample measures and Mann-Whitney test while comparing two different cohorts like STEMI versus chronic stable angina patients. In the figures however the graphs are presented as the median (solid line) interquartile range (shaded box) and range (whiskers) to give the reader a clear picture of the totality of the data including the range. Correlations between continuous variables are assessed with linear regression analysis, and the corresponding r squared value and Pearson correlation coefficients are calculated. Statistical significance is accepted as conventional 2-tailed $p < 0.05$ and is mentioned where it is significant. Where p=ns is used, the p-value is greater than 0.05. Given the small sample size, interpretation of 40

the p-values is conservative in this text, and the differences for which true significance is claimed are statistically robust and reach significance with both parametric and non-parametric statistical methodology.

CHAPTER 3: SOLID PHASE CORONARY THROMBUS

3.1. Background

Formation of thrombus on disrupted atherosclerotic plaques is the fundamental event in the development of acute coronary syndromes and in the progression of atherosclerosis.[295] The basic component of the thrombus includes polymeric scaffolds of fibrin and enmeshed platelets, erythrocytes and leukocytes. However, as discussed in the introduction, published data on the proportional composition of these components is variable. There is significant morphological heterogeneity within a thrombus due to polarity of distribution of platelets and erythrocytes. The platelets cluster on the "head end" which is in continuity with the plaque crater while the tail end is chiefly composed of erythrocytes and fibrin as shown schematically in the following figure (3.1).

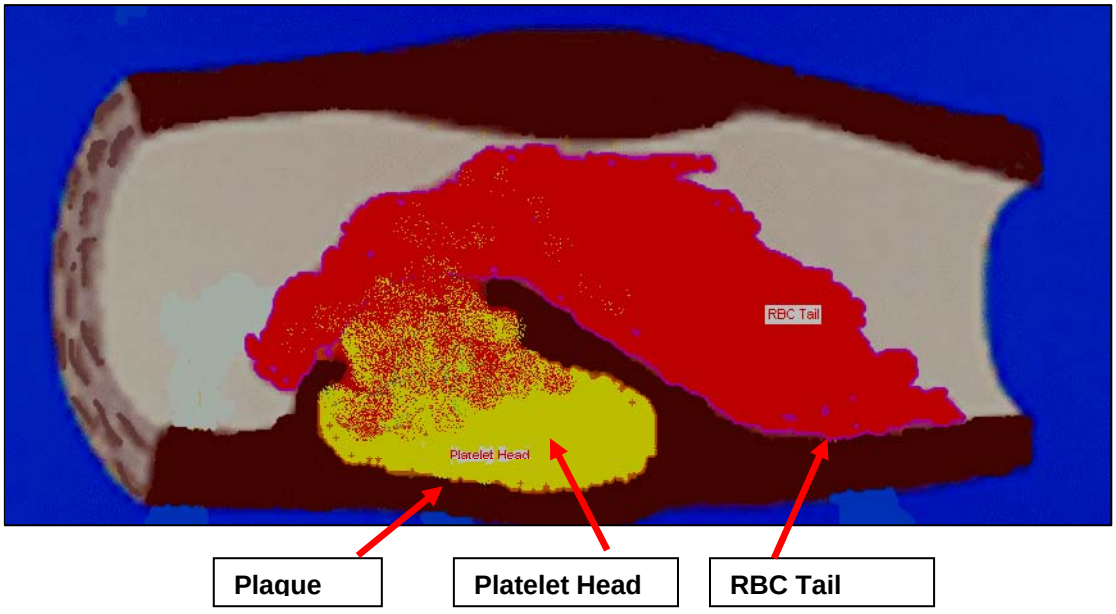


Figure 3.1: Illustration of thrombus orientation

Much of the available data relating to coronary thrombus composition has been obtained from necropsy, which has its inherent limitations. A detailed compositional analysis of the ex vivo thrombi aspirated during STEMI however may provide more clinically relevant data.

Despite administration of multiple antiplatelet and antithrombotic therapies, recurrence of coronary events sometimes occurs in clinical practice, which may indicate involvement of other cell types in acute coronary events. Therefore in this chapter we aim to focus on the presence of leukocytes and subpopulations in the coronary thrombus.

3.2. Method

Five μm cryostat sections of the 15 ex-vivo coronary thrombi were subjected to immuno-staining as described in chapter 2 using the following panel of antibodies:

	Primary antibody	Secondary antibodies
Platelets	CD42a-PerCP CD41a-PerCP PAI-1-FITC CD41a	Alexa Fluor- 350
Leukocytes	DAPI CD14- PE-Cy5 CD11b-PE-Cy5 CD3-FITC CD20-FITC	Vimentin - Alexa Fluor-647
Fibrin	Fibrin-FITC	

The sections were visualized using three-color immuno-microscope (Nikon, Japan). Individual cells were quantified by measuring the intensity the specific fluorphore using Lucia-G software (Laboratory Imaging Ltd., Prague, Czech

Republic). Because of heterogenous morphology of the thrombus, area of interest was identified by presence of nuclear signal DAPI (6-diamidino-2-phenylindole). Ten high power fields were analysed in each section. Using a grid provided in the software the total number of leucocytes and their subpopulation were counted and their proportion of the total leukocyte calculated.

3.3. Results

Clinical characteristics:

	N=15
Age: Mean years.	57 (14)
Sex (% Male)	60
Pain to reperfusion time: Minutes(SD)	185 (164)
Peak Creatinine Kinase U/l(SD)	1348 (11150)
Peak Troponin I ug/l (SD)	98 (209)
Total Cholesterol mmol/l (SD)	5.9 (1.1)
Random Glucose mmol/l (SD)	9.5 (4.5)
Hypertension %	60
Hypercholesterolemia %	40
Diabetes %	20
Current Smoking %	40
Family history of premature CAD %	20
Prior Beta Blocker use %	40
Prior ACE inhibitor use %	60
Prior Statin Use %	40
Prior Clopidogrel use %	0

3.3.1. Heterogeneity of Thrombus

As discussed in section 10.1 the heterogeneity of the thrombus morphology had been well described in post-mortem studies where the orientation of the thrombus inside the coronary artery is preserved and can be studied in situ. In our study, the spatial orientation of the thrombi is lost during aspiration thrombectomy. However, as illustrated below, we observed distinct polarisation of the distribution of platelets. Some areas of the thrombus revealed dense aggregates of platelets and some fibrin while other areas had sparse platelets and abundant fibrin (fig 3.2-3).

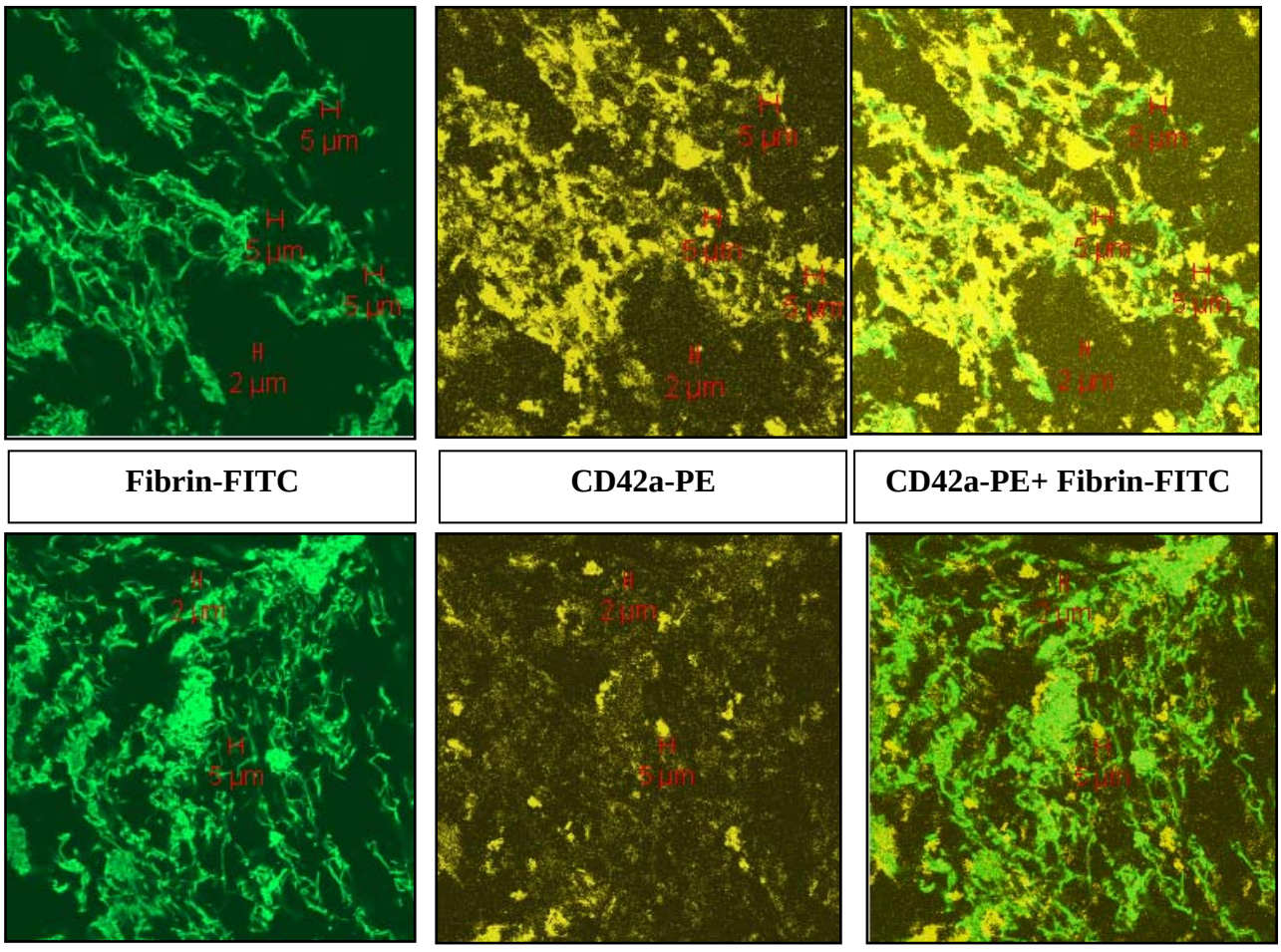
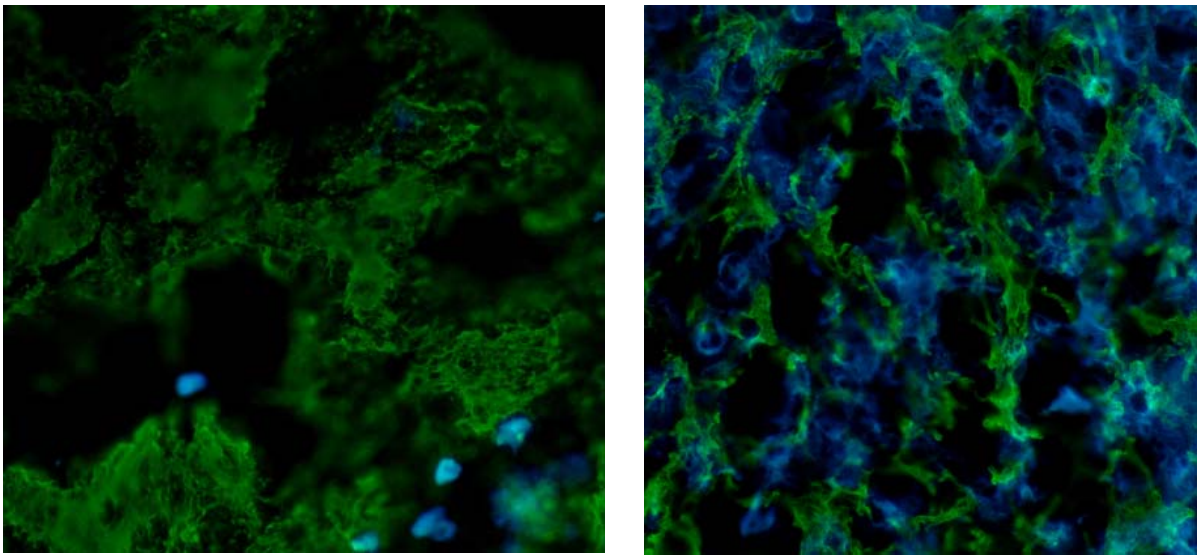


Figure 3.2: Ex-vivo coronary thrombus & platelet-fibrin heterogeneity: Upper tier Platelet rich, lower tier Fibrin rich

Intriguingly, the compositional heterogeneity was not restricted to the fibrin and platelets alone. We observed that the nucleated white cells also distributed heterogeneously along the thrombus. The areas in close proximity to the platelet aggregates also demonstrated aggregates of nucleated white cells. Other regions of the thrombus, on the other hand, revealed paucity of nucleated cells and presence of dense mesh of fibrin.



Green: Fibrin (FITC), Blue: Nuclear signal (DAPI)

Figure 3.3: Ex vivo coronary thrombus

3.3.1. Phenotyping the white cells involved inside the thrombus

Since leukocytes are the only cellular component of the thrombus that contains DNA, staining the thrombi with the nuclear stain DAPI (4', 6-diamidino-2-phenylindole) that specifically binds to the DNA identified the leukocyte component. The monocytes were identified by expression of the marker CD14, the

T lymphocytes by CD3, the B-lymphocytes by CD20 and the neutrophil population identified by exclusion (figure 3.4).

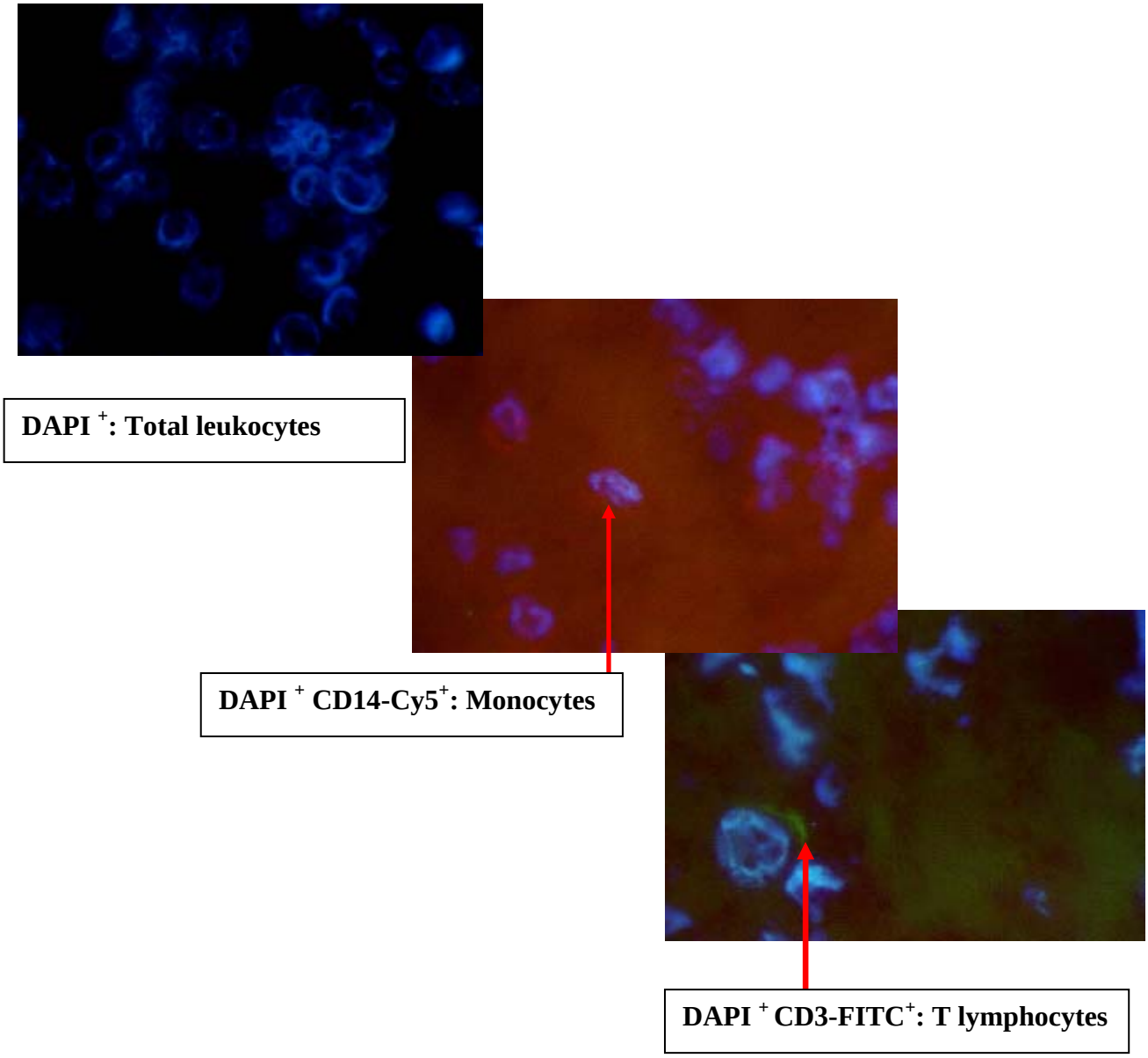
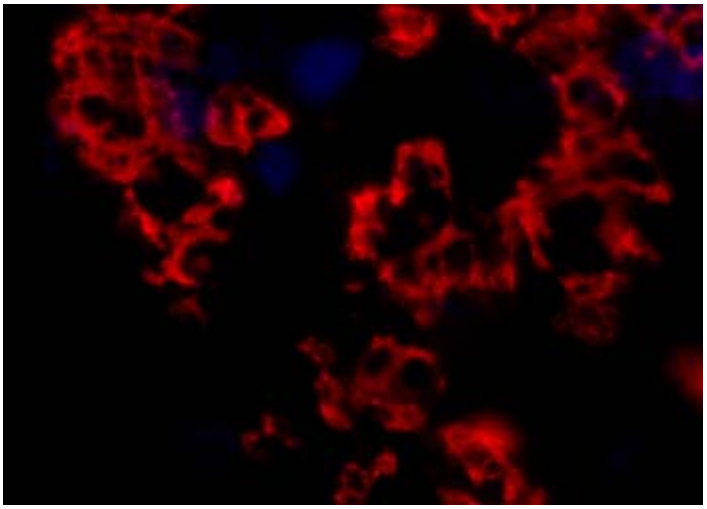


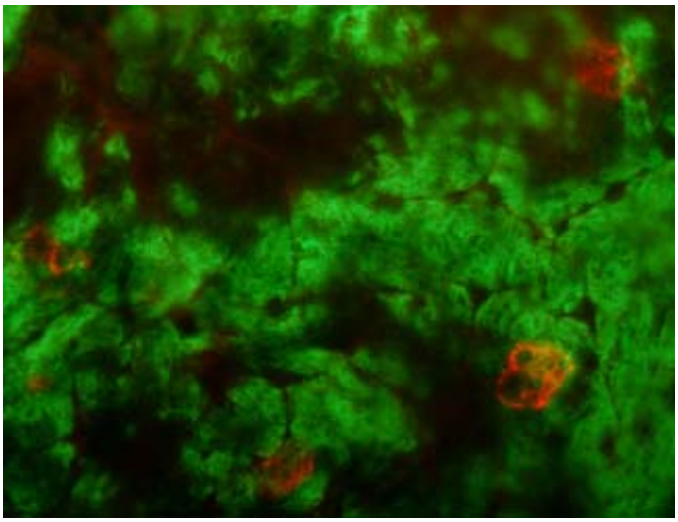
Figure 3.4: Cell types in thrombus

In the 15 ex vivo coronary thrombi studied granulocytes ($DAPI^+$ $CD14^-$ $CD3^-$/ $CD20^-$) accounted for 89.5 percent of the leucocytes. The monocytes ($DAPI^+$ $CD14^+$) accounted for 10.5 percent of the leukocytes. $CD3^+$ was only very scarcely present and no $CD20^+$ B lymphocytes were observed.

The thrombi were stained for expression of CD11b (MaC-1) on the surface of the leukocytes. Intense expression of CD11b was observed on almost all leukocytes. The leukocytes also stained well for vimentin (figure 3.5).



CD11b-PE-Cy5 (Red) + CD41a-Alexa Fluor-350 (Blue)



Vimentin-Alexa Fluor-647 (Red) + Fibrin-FITC (Green)

Figure 3.5: Vimentin expression

3.4. Conclusion

The leukocytes comprise an integral component of the thrombus. Like the platelets, the leukocyte distribution within the thrombus was heterogeneous. Phenotypically, the leukocytes population resident on the thrombus was overwhelmingly granulocytic and less commonly monocytic. They strongly expressed CD11b and the intracytoplasmic intermediate filament vimentin. Since CD11b can function as a fibrin receptor, the strong expression of this molecule by the thrombi leukocytes suggest that these cells may have an import role in thrombus formation and stabilisation.

Although lymphocytes contribute to 30-40% of the total leukocyte population in the circulation there was relative paucity of their presence within in the thrombus. The under-representation of lymphocytes in the thrombus in this phenotypic analysis suggests that the presence of leucocytes is not merely a passive process of trapping in the fibrin mesh. Given this lack of evidence however of involvement of the cellular lymphocyte adaptive immune system in to the ex vivo thrombus, the work of this thesis moved on to focus on the filtrate component of the thromboaspirate.

CHAPTER 4: LIQUID PHASE: PLATELETS AND PLATELET LEUKOCYTE CONJUGATES

4.1. Background

Increased platelet activity is central to acute athero-thrombotic clinical events[296,297,298] and is a predictor of cardiac outcomes after myocardial infarction (MI).[299] Patients with ACS are treated with empirical dual or triple anti-platelet therapy, however there are no fully validated clinical tools in routine clinical use to measure in vivo platelet function and guide therapy accordingly at this time. This empiric antiplatelet strategy has improved the clinical prognosis; however, patients with ACS still continue to have a significant incidence of major cardiac events at 4 to 6 months after hospital discharge.[300]

Platelets are endowed with the potential for homotypic interaction with other platelets and heterotypic interaction with leukocytes. This heterotypic interaction depends on ligation of CD62p on platelets with PSGL on leukocytes[301,302] and is subsequently strengthened by GPIb–CD11b ligation,[303] fibrinogen bridging of platelet GPIIb/IIIa and leukocyte CD11/CD18,[304,305] and mutual bridging of CD36 thrombospondin.[306] Monocytes and granulocytes have a greater propensity to form conjugates with platelets when compared with the potential of lymphocytes.[307,308] Activation of platelets can augment the interaction with monocytes and neutrophils further[309] and circulating platelet–monocyte and platelet–neutrophil aggregates are a surrogate marker of platelet activation in vivo.[310,311] In patients with acute myocardial infarction circulating monocyte - platelet aggregates can be potentially considered as an early marker of

myocardial infarction.[312] Relative to the systemic circulation, more monocyte and neutrophil-platelet aggregates have been observed at the culprit site of coronary artery occlusion during myocardial infarction.[313]

On the other hand less than 3% of circulating T lymphocytes is engaged in the heterotypic interaction with platelets. Activation of the platelets does not seem to enhance this interaction which conversely is augmented by lymphocyte activation.[311] There is accumulating evidence to suggest that platelets and lymphocytes reciprocally modulate each other's function to promote atherogenesis. Platelets facilitate the transmigration of the lymphocyte across the endothelium, immobilized platelets enhance lymphocyte adhesion on the vessel wall[314,315,316] and platelets promote the infiltration of lymphocytes into the inflammatory sites.[317] Furthermore, the platelet derived chemokine RANTES enhances T cell infiltration[318] and is pro-atherosclerotic.[319, 320] However, there is a paucity of data regarding platelet-lymphocyte conjugates in coronary blood in acute myocardial infarction.

In this chapter we aim to

1. Evaluate platelet activation in patients with STEMI at the site of plaque rupture and in the peripheral circulation.

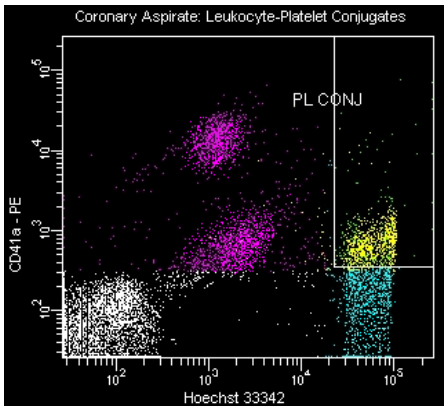
2. Evaluate the formation of conjugates with leukocytes in patients with STEMI at the site of plaque rupture and in the peripheral circulation.

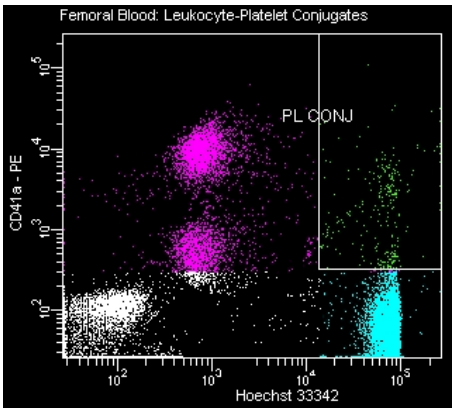
4.2. Method

Twenty paired platelet rich plasma samples were obtained from coronary aspirate and femoral arterial blood from patients with STEMI. The platelets were stained with monoclonal antibodies against following antigens: CD41a-PerCP (BD

Biosciences), CD62P-APC (BD Biosciences) and CD40L-Cy5 (BD Biosciences) using parallel isotype control IgG1-PerCP (BD Biosciences), and IgG1-APC (BD Biosciences), and IgG1-Cy5 (BD Biosciences) respectively. The mouse anti-human vimentin (Abcam, MA USA) and its isotype control, mouse antihuman IgG1 was secondarily conjugated with fluorophore Alexa fluor 647 (Invitrogen) and used along with rabbit anti-human PAI-1-FITC and its isotype control rabbit antihuman IgM-FITC to evaluate the expression of the vimentin-PAI-1 binary complex. At least 30,000 platelets events were analysed and collected using an appropriate density neutral filter.

Similarly, paired coronary aspirate and femoral arterial blood (n=20) were obtained from patients with STEMI while undergoing PPCI. Control sample which included femoral arterial blood (n=18) from patients with chronic stable angina undergoing coronary angiography or PCI. The decalcified whole blood (EDTA prevents in vitro L-P conjugation as CD62P ligation with PSGL-1 is Ca_2^{++} dependent) was diluted 10X in modified Tyrodes buffer and stained with CD41a-PE (BD Bioscience), CD 11b-PC5, CD14-FITC, CD3 FITC and nuclear stain Hoechst 33342. The RBCs were lysed using lysing solution (BD San Jose, CA, USA). Since the experiments were performed and analysis completed within approximately 90 minutes of obtaining the samples and the lysing solution contained formaldehyde in it, further glutarldehyde fixation was not used to stabilize the platelet-leukocyte conjugates. Total leukocyte-platelet conjugates were identified as Hoechst positive nucleated cells co-localizing with surface expression of CD 41a. At least 5000 Hoechst positive events were analysed and collected in each sample.





A **B**

Figure 4.1: Co-localization of mono-nucleated white blood cells

(Hoechst positive events) with platelets (CD41a positive), ***A****: coronary aspirate,* ***B:*** *femoral arterial blood.*

The leukocyte subpopulations were identified by forward and side scatter characteristics, and by using monoclonal antibodies against the surface antigen including CD14 as a monocyte marker, and CD3 as a pan T cell marker. Flow cytometric analysis as detailed earlier was performed within 1.5 hours of sample collection.

4.2.1. Effect of shear rates

As discussed in section 2.5.1, the shear rate in the aspiration devise was comparatively low we did not anticipate major activation of platelets induced artefactually by the shear rates. To evaluate the effect of shear on formation of conjugates, femoral arterial blood samples from 5 subjects with chronic stable angina undergoing coronary angiography were obtained and subjected to in-vitro aspiration through the Export® XT Aspiration Catheter. The pre and post

aspiration samples were stained with CD41a-PE (BD) and Hoechst 33342. The total leukocyte- platelets conjugate along with leukocyte subpopulation identified on the basis of scatter characteristics were analysed.

Results:

The mean difference observed between the two sets of experiments was calculated and based on this the 95% limits of agreement.

	Pre aspiration (mean)	Post aspiration (mean)
Leukocyte-Platelet conjugates	5.5	5.4
Monocyte Platelet Conjugates	12	12.0
Lymphocyte-Platelet Conjugates	1.2	1.2

As can be seen from the table there was no relevant difference attributable to the aspiration process.

	Mean Difference	95% L o A	
Leukocyte-Platelet conjugates	0.08	-0.30	0.46
Monocyte-Platelet Conjugates	-0.04	-0.90	0.82
Lymphocyte-Platelet Conjugates	-0.02	-0.28	0.24

4.2.2. Effect of contrast media

The effect contrast media on formation of leukocyte-platelets conjugate was evaluated by serial dilution (125 µl/1.5 ml, 125 µl/1.5 ml, 250 µl/1.5 ml and so forth till 1000 µl/1.5 ml) and the leukocyte (and its subpopulations) -platelet conjugates were analysed as shown in the figure. Overall there was no significant effect of the contrast media on the conjugate formation (figure 4.2).

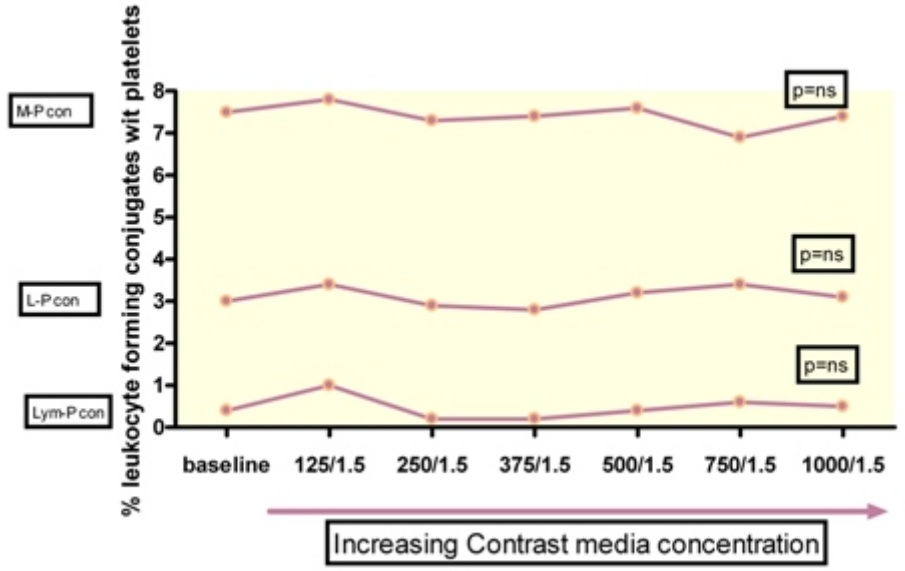


Figure 4.2: Controlling for contrast media

4.2.3. Reproducibility

The reliability in measuring leukocyte-platelet conjugates (including the vensection and analysis), was evaluated by performing these experiments at an index time and repeating the experiments on fresh sample of blood from the same healthy volunteers after 2 hours later.

	Index (mean %)	2 hours (mean %)
Total leukocyte-platelet conjugates	4.6	4.7
Lymphocyte-platelet conjugates	0.5	0.5
Monocyte-platelet conjugates	6.2	6.1
Granulocyte-platelet conjugates	4.6	4.3

The mean difference observed between the two sets of experiments was calculated and based on this the 95% limits of agreement. As depicted in the table below there was no biologically relevant between the index case and the repeat at 2 hours.

	Mean Difference	95% L o A	
Total leukocyte-platelet conjugates	-0.08	-1.02	0.86
Lymphocyte-platelet conjugates	0.01	-0.62	0.64
Monocyte-platelet conjugates	0.16	-1.42	1.73
Granulocyte-platelet conjugates	0.28	-1.00	1.55

4.3. Results

Clinical characteristics:

	STEMI (n=20)	CSA (n=18)
Age: Mean years.	62 (17)	56 (12)
Sex (% Male)	75	77
Pain to reperfusion time: Minutes(SD)	168 (180)	N/A
Peak Creatinine Kinase U/l(SD)	1500 (1380)	N/A
Peak Troponin I ug/l (SD)	98 (239)	N/A
Total Cholesterol mmol/l (SD)	5.1 (1.4)	4.5 (1.4)
Random Glucose mmol/l (SD)	9 (5)	7 (2)
Hypertension %	60	50
Hypercholesterolemia %	30	38
Diabetes %	40	22.2
Current Smoking %	35	27.7
Family history of premature CAD %	10	27.7
Prior Beta Blocker use %	45	72.2
Prior ACE inhibitor use %	20	61
Prior Statin Use %	40	88
Prior Clopidogrel use %	10	27.7

4.3.1. Platelet Activation

Relatively fewer CD41a positive events were observed in the coronary aspirate in comparison with femoral arterial blood (p=0.043) when comparatively equivalent number of Hoechst 33342 positive mononuclear cells were identified and recorded.

Of the CD41a positive events, there was no difference in surface co-expression of CD41a and P-selectin (CD62P) on the platelet between the coronary aspirate and the femoral samples (p=0.471).

In contrast, the platelets expressing CD41a and co-localizing with CD40L, PAI-1, and vimentin-PAI-1 binary complex were respectively 32 (13.2, 60.1), 40 (29, 47.5) and 3.15 (1.4, 10.4) in the coronary aspirate and 22(8.8, 35) (p=0.005), 35 (27, 46) (p=0.0034) and 1 (0.5, 2.97) (p=0.0001) in the femoral artery (figure 4.3).

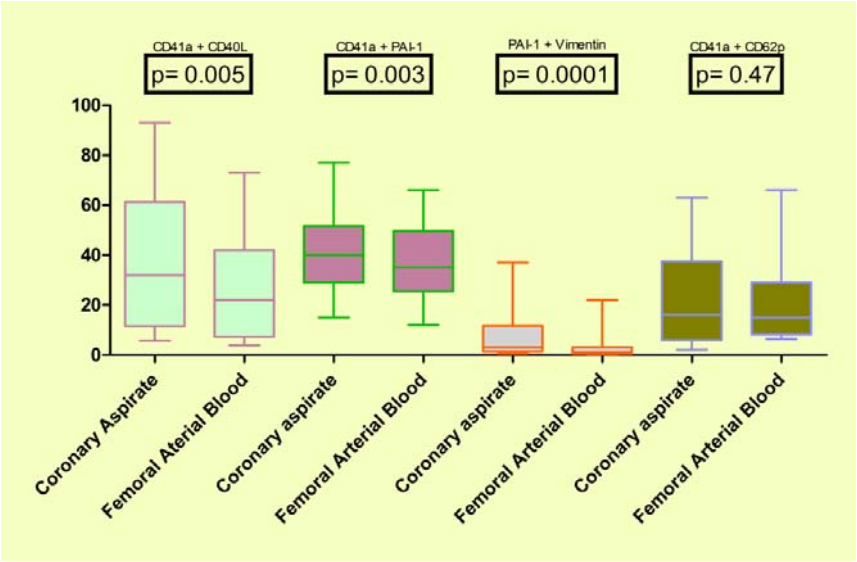


Figure 4.3: Comparison of platelet activation markers *including **CD40L**, **PAI-1**, **PAI-1-Vimentin** complex and **CD62 P** between coronary aspirate and femoral arterial blood*

4.3.2. Total platelet leukocyte conjugates

The following table depicts the total platelet-leukocyte conjugates and relative subpopulation analysis of the leukocytes colocalizing with mononuclear white cells across the spectrum of coronary disease.

		N	Total L-P Conjugates	Monocyte-platelet conjugates	Granulocyte-Platelet Conjugates	Lymphocyte-Platelet Conjugates
STEMI	Intracoronary	20	15.1 (9, 25)	42.4 (33, 60)	4.4 (2.6, 9)	20 (13, 30)
	Femoral	20	3.9 (2, 7.2)	23.6 (19, 35)	2 (1.35, 2.85)	10 (7, 15)
Chronic stable Angina		18	3.9 (2.9, 5)	14 (10, 25)	1.4 (.55, 2)	1.1 (1, 1.4)

4.3.2.1. ST elevation myocardial infarction (platelet-all leukocytes).

Of the mononuclear cells positive for nuclear stain Hoechst, 15.1 (9, 25) cells in the coronary aspirate and 3.95 (2, 7.2) cells in the femoral blood co-localized with CD41a, p=0.0001 (figure 4.4).

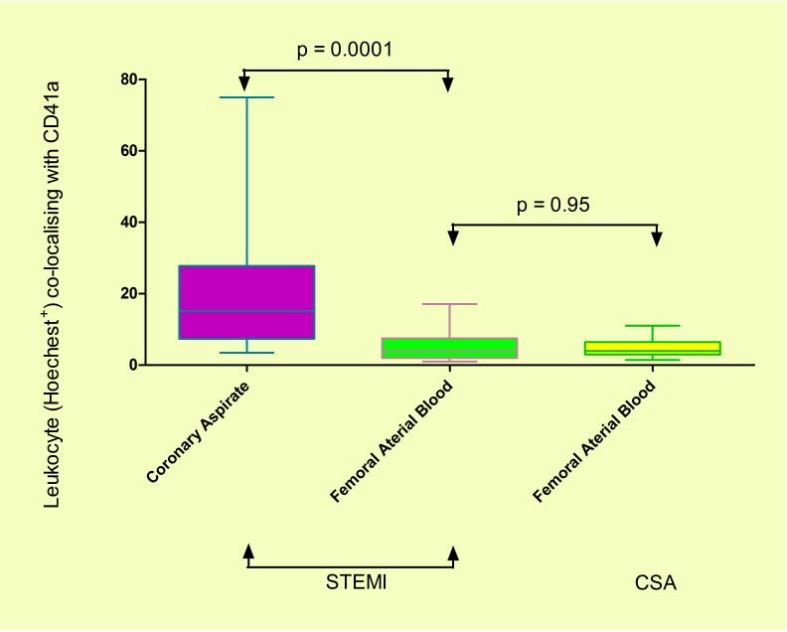
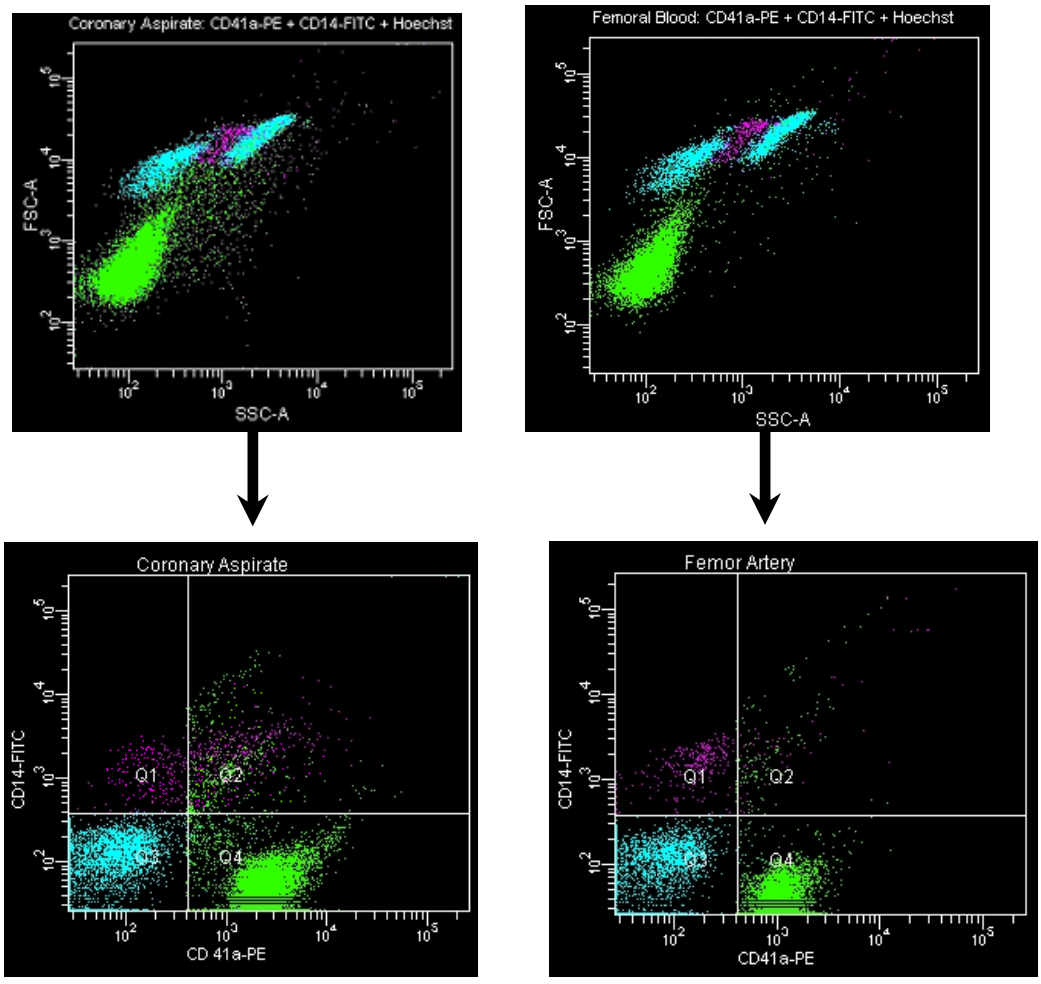


Figure 4.4: Total platelet leukocyte conjugates

4.3.1.3. Chronic stable angina (platelet-all leukocytes)

The percentage of the leukocytes in peripheral circulation engaged in forming conjugates with platelets was 3.9 (2.9, 5). This was not significantly lower than the degree of conjugate formation in femoral arterial blood of patients with STEMI (figure 4.4)

4.3.3. Platelet monocytes Conjugates



A **B**

Figure 4.5: Platelet monocyte conjugates (FACS)

*(**Q2** population) in Coronary Aspirate **A**, Femoral Artery **B**: the monocytes are identified as the purple coloured cell population on the scatter plot.*

4.3.3.1. ST elevation myocardial infarction (platelet-monocyte)

There was no difference in percentages of mononuclear cells expressing the monocyte phenotype marker, CD14, between the coronary aspirate and femoral blood (p=0.55)). In the coronary aspirate, 42 (33, 60) of the monocytes co-localized with platelets expressing CD41a, in contrast to the 23.6 (19, 35) in femoral artery (p = 0.0003) (figure 4.6).

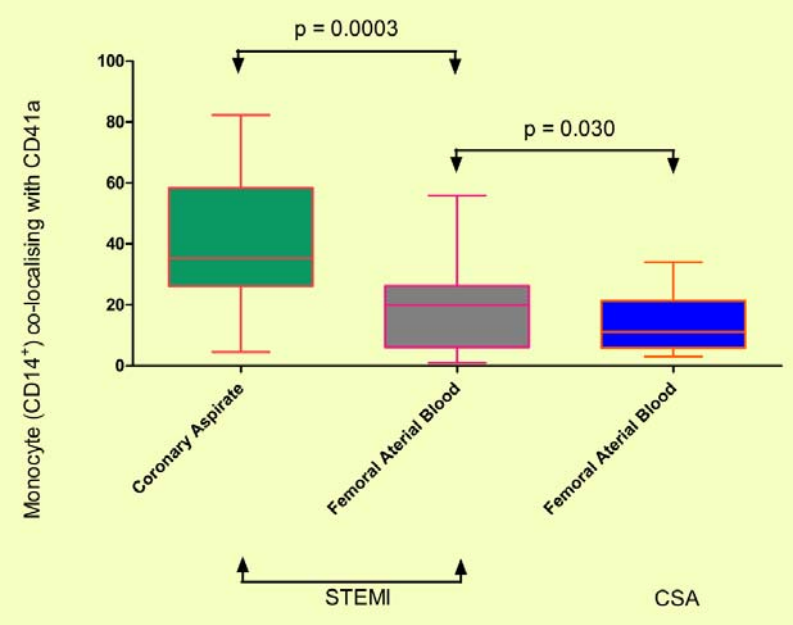


Figure 4.6: Platelet monocyte conjugates (summary)

4.3.3.3. Chronic stable angina (platelet-monocyte)

In stable coronary disease, 14 (10, 25) of the monocytes in peripheral arterial circulation formed aggregates with platelets in contrast to the 23.6 (19, 35) in the femoral arterial blood of patient with STEMI, p=0.03 (figure 4.6).

4.3.4. Granulocyte-Platelet Conjugates

4.3.4.1. ST elevation myocardial infarction (platelet-granulocyte)

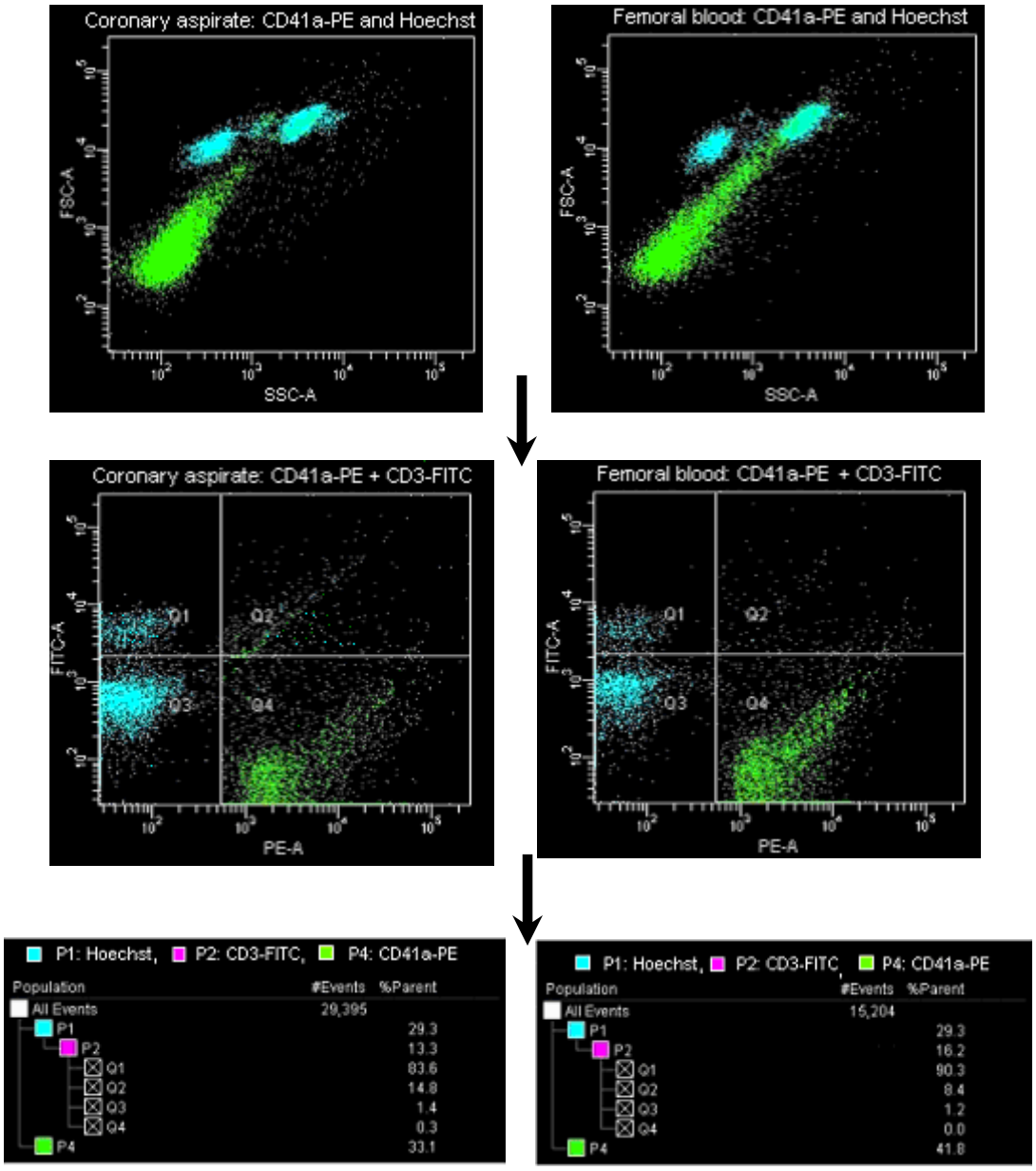
The granulocytes were identified by forward and side scatter characteristics and by exclusion of CD3 and CD14 positive mononuclear cells. There was no difference in total percentages of the granulocytes between the coronary aspirate and femoral blood (p= 0.8). In the coronary artery, 4.4 (2.6, 9) of the granulocytes formed conjugates with platelets expressing CD41a, compared with 2(1.3, 2.8) in femoral artery. (p= 0.0005).

4.3.4.3. Chronic stable angina (platelet granulocyte)

In CSA, 1.4 (0.55, 1.9) of the granulocytes formed aggregates with platelets in peripheral arterial circulation.

4.3.5. Lymphocyte-Platelet Conjugates

Coronary Blood Femoral Blood



A **B**

Figure 4.7: Lymphocyte-platelet conjugates (FACS)

(Q2 population) in Coronary Aspirate A, Femoral Artery B: the lymphocytes are identified as the discreet cell population on the top left hand corner in the scatter plot

4.3.5.1. ST elevation myocardial infarction (platelet-lymphocyte)

There was no difference in percentages of mononuclear cells expressing the pan T cell marker CD3 between the coronary aspirate and femoral blood (13.5 versus 14.9 p=0.17). However, 20 (13.5, 30) of T lymphocytes in the coronary artery, and 10 (7, 15.5) in the femoral artery co-localized with CD41a (p=0.0003) (figure 4.8).

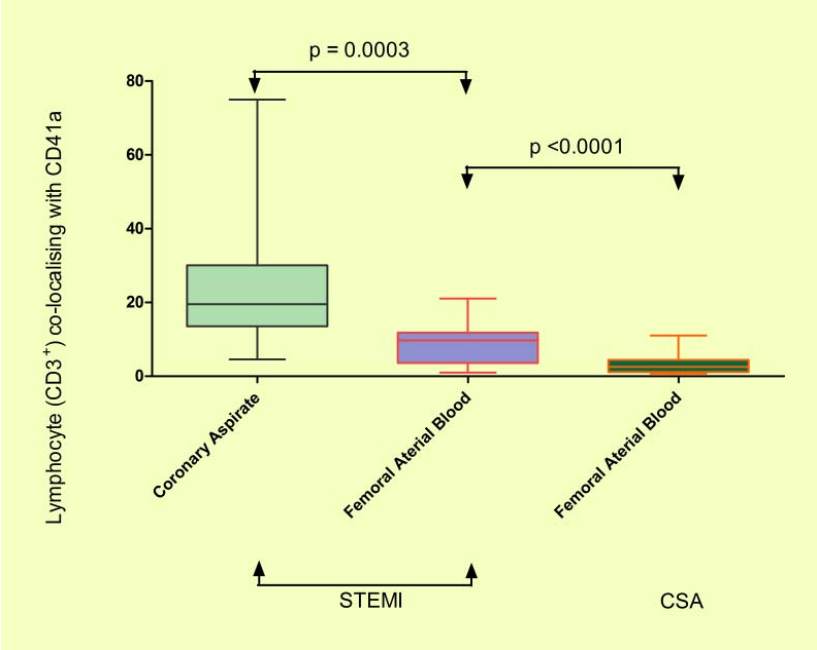


Figure 4.8: Platelet lymphocyte conjugates (Summary)

Since the kinetics of lymphocyte-platelet interaction in vitro is slower than the monocyte–platelet conjugates, a longer duration of interaction would result in higher degree of conjugates formation. To evaluate whether longer duration of coronary occlusion would promote higher lymphocyte-platelet interaction, we correlated the duration between onsets of pain to reperfusion with the percentage of CD3 positive cells co-localizing with CD41a positive platelets. We found that

there was a weak but statistically significant association between the pain to reperfusion times and the lymphocyte-platelet conjugates (r^2 =0.241, p=0.032) (figure 4.9).

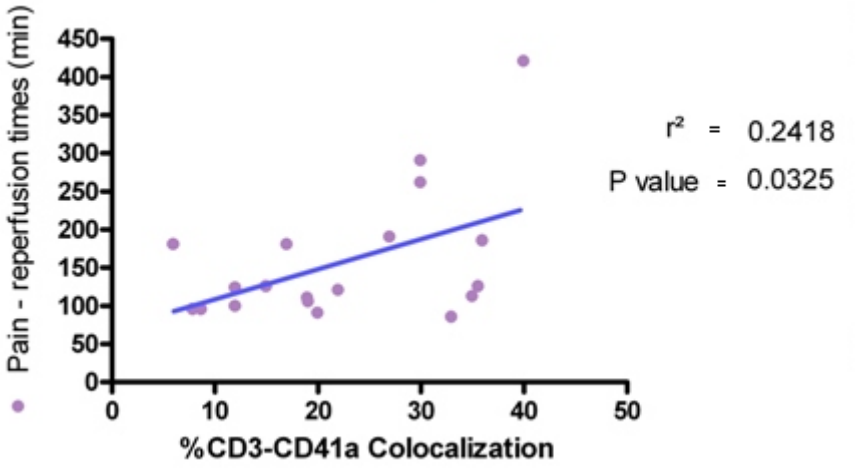


Figure 4.9: Time and lymphocyte platelet conjugates

4.3.5.2. Chronic stable angina (platelet lymphocyte)

In femoral arterial blood of patients with chronic stable angina, 2.0 (1.1, 2.6) of the lymphocytes expressing CD3 were co-localized with platelets expressing CD41a. This level of heterotypic interaction was significantly less when compared to the level in femoral blood of patients with STEMI (p< 0.0001)

4.4. Conclusion

4.4.1 Platelet activation markers

The reduction in CD41a positive events in the coronary aspirate in comparison with the femoral arterial blood sample in patients with STEMI probably reflects consumption of the platelets within the thrombus and their association with the leukocytes to form the conjugates.

Expression of CD62 (P-selectin) at the platelet surface is often used as a general marker of platelet activation.[321] CD62 expression is enhanced in patients with acute coronary syndromes.[322] It was surprising that the degree of expression of CD62P in the platelets obtained from coronary aspirate of patients with STEMI, which conventionally is regarded as marker of platelet activation, was not different from the platelets obtained from femoral artery. However, the expression kinetics of the various inducible markers of the platelets can vary temporally. The P selectin expression is rather transient and probably explains why the CD62P expression in the coronary aspirate is not significantly different than the peripheral circulation. Conversely, CD40L has longer expression kinetics[323] and may explain the differential expression between the coronary aspirate and the femoral artery. Ultimately however the magnitude of the differences was small.

4.4.2 Platelet conjugates

It is perhaps not surprising that the total platelet leukocyte, platelet-monocyte, platelet-granulocyte and platelet lymphocyte conjugates in the coronary thromboaspirate were elevated, and our findings confirm observations made by other groups.[324] However, further research is required to elucidate whether these complexes have any pathogenic role in either the physical generation of occlusive coronary thrombus or subsequent microvascular and or myocardial damage occurring during the reperfusion process.

CHAPTER 5: LIQUID PHASE: ACTIVATED, PROINFLAMMATORY AND REGULATORY T LYMPHOCYTES

5.1. Background

In addition to the initiation and progression of atherosclerosis, accumulating evidence suggest that T cells may also exert a coronary plaque destabilizing effect which is important in the pathogenesis of acute coronary syndromes. Although these acute phenomenona are attributable to the instability of an index culprit plaque, studies have reported synchronous diffuse destabilisation of atherosclerotic plaques in patients with acute myocardial infarction, begetting the concept of "pan-coronaritis".[325,326,327,328] The underlying putative mechanism driving this systemic inflammation may included exogenous microbial components as discussed earlier or autologous proteins for instance, B-2 GP-1, oxidised LDL (oxLDL), and stress or heat shock proteins (HSP). These trigger factors may lead to activation of immune cells perpetuating and propagating the inflammatory process. A transient burst of T-cell activation can be detected systemically in patients with acute coronary syndromes.[329]

Regarding specifically proinflammatory T cells, acute coronary syndromes are accompanied by oligoclonal expansion of a repertoire of $CD4^+$ T cells in the peripheral circulation that have lost the surface expression of co-stimulatory molecule CD28. Although the pathogenic potential of this subpopulation of CD4 T cell is well elucidated in vitro, there is paucity of knowledge pertaining to their phenotypic characteristics and their relative distribution between the peripheral circulation and target site of inflammation. Whilst augmentation of the pro-inflammatory T cells may be important in orchestrating the atherosclerotic

process, the attenuation of immune modulating cells like T_{reg}s may also exert permissive effect in progression of the atherosclerosis. In Humans T_{reg}s has been demonstrated in peripheral blood of subjects with normal coronary arteries[330] and are decreased in the peripheral blood of patients with acute coronary syndromes.[331,332] Furthermore, the frequency of Tregs in atherosclerotic lesion was found to be attenuated and constituted only 0.5–5% of the total number of CD3 $^{+}$ T cells.[333] T_{reg}s are subset of $CD25^{+}$ $CD4^{+}$ T cells and express lineage specific marker FOXP3 (Forkhead Box Protein P3), a member of the forkhead winged helix protein family of transcription factors.[334,335]

Simultaneous sampling of lymphocytes in the liquid phase of the aterothrombotic aspirate at the target site of inflammation (the culprit atherosclerotic lesion) and peripheral circulation of patients with acute myocardial infarction may provide further mechanistic information with regard to the involvement of lymphocytes in acute coronary syndromes. In this series of experiments we aim to evaluate the extent of T cell activation, the degree of expansion of the proinflammatory $CD4^{+}$ $CD28^{null}$ T cells including memory phenotypes and the regulatory T cells expressing the immunoregulatory $FOXP3^{+}$.

5.2. Method

5.2.1. Activated intracoronary and peripheral T lymphocytes.

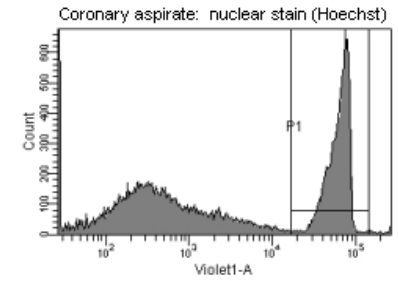
Paired intra-coronary liquid phase aspirate and femoral arterial samples (n=20) from patients with STEMI were obtained during PPCI and femoral arterial blood (n=20) from patients with chronic stable angina undergoing coronary angiography. The samples were stained as whole blood preparation as detailed above using a panel of monoclonal antibodies and matching isotype control in parallel:

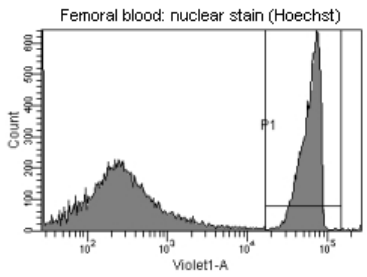
Test	Isotype
CD3-FITC	IgG2a-FITC
CD40L-PC5	IgG1-PC5
HLA-DR-PerCP	IgG2a-PerCP
CD14-FITC	IgG1-FITC
CD11b-PC5	IgG1-PC5

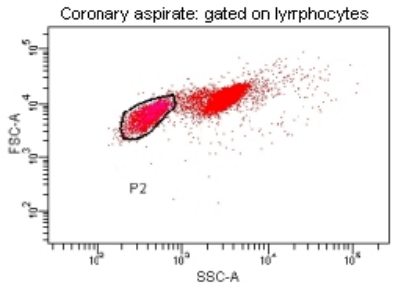
The leukocyte population was gated and 5000 events recorded after appropriate electronic compensation.

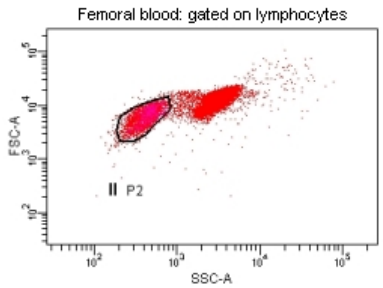
5.2.2. Proinflammatory $CD4^+$ $CD28^{null}$ T lymphocytes

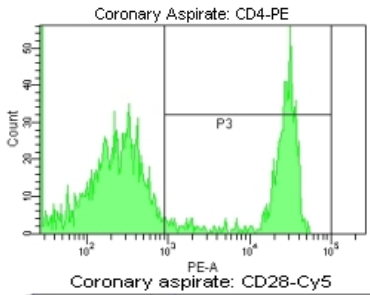
Paired coronary aspirate and femoral arterial blood from patients with STEMI (n=22) femoral arterial blood from chronic stable angina (n=20), and peripheral venous blood from healthy volunteers (n=10) were stained as a whole blood preparation using following antibodies: CD4-PE, CD62L-APC, CD45RO-CY7, CCR7-FITC, and CD28-PC5. The lymphocyte population was identified by side and forward scatter and were gated to exclude debris and aggregates. The lymphocyte population expressing the CD4 was gated to evaluate for the expression of CD28 molecule. The $CD4^+CD28^+$ and $CD4^+CD28^{null}$ T cell population were both dichotomised on the basis of expression of phosphatase CD45R0 in to naïve and memory $CD4^+CD28^{null}$ T cells. These cell populations were then interrogated for expression of adhesion molecules CD62L and CCR7 to classify them into TEM and TCM. (Figure 5.1)

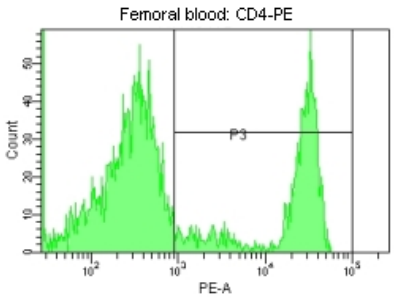

Coronary aspirate: nuclear stain (Hoechst)
Count
P1
Violet1-A

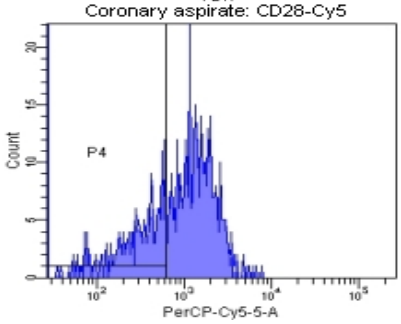

Femoral blood: nuclear stain (Hoechst)
Count
P1
Violet1-A

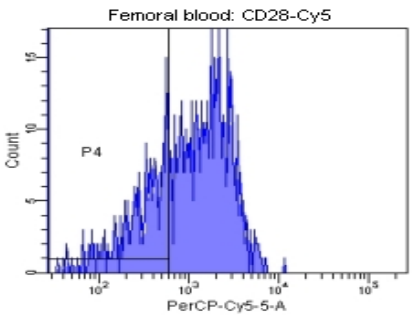

Coronary aspirate: gated on lyrrphocytes
FSC-A
P2
SSC-A

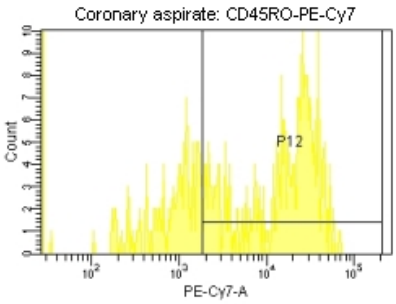

Femoral blood: gated on lymphocytes
FSC-A
P2
SSC-A

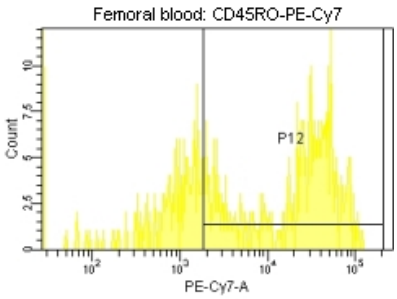

Coronary Aspirate: CD4-PE
Count
P3
PE-A

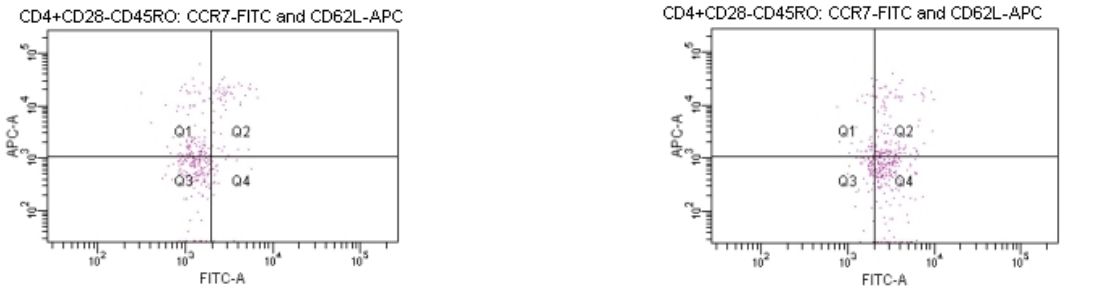

Femoral blood: CD4-PE
Count
P3
PE-A


Coronary aspirate: CD28-Cy5
Count
P4
PerCP-Cy5-5-A


Femoral blood: CD28-Cy5
Count
P4
PerCP-Cy5-5-A


Coronary aspirate: CD45RO-PE-Cy7
Count
P12
PE-Cy7-A


Femoral blood: CD45RO-PE-Cy7
Count
P12
PE-Cy7-A



A B

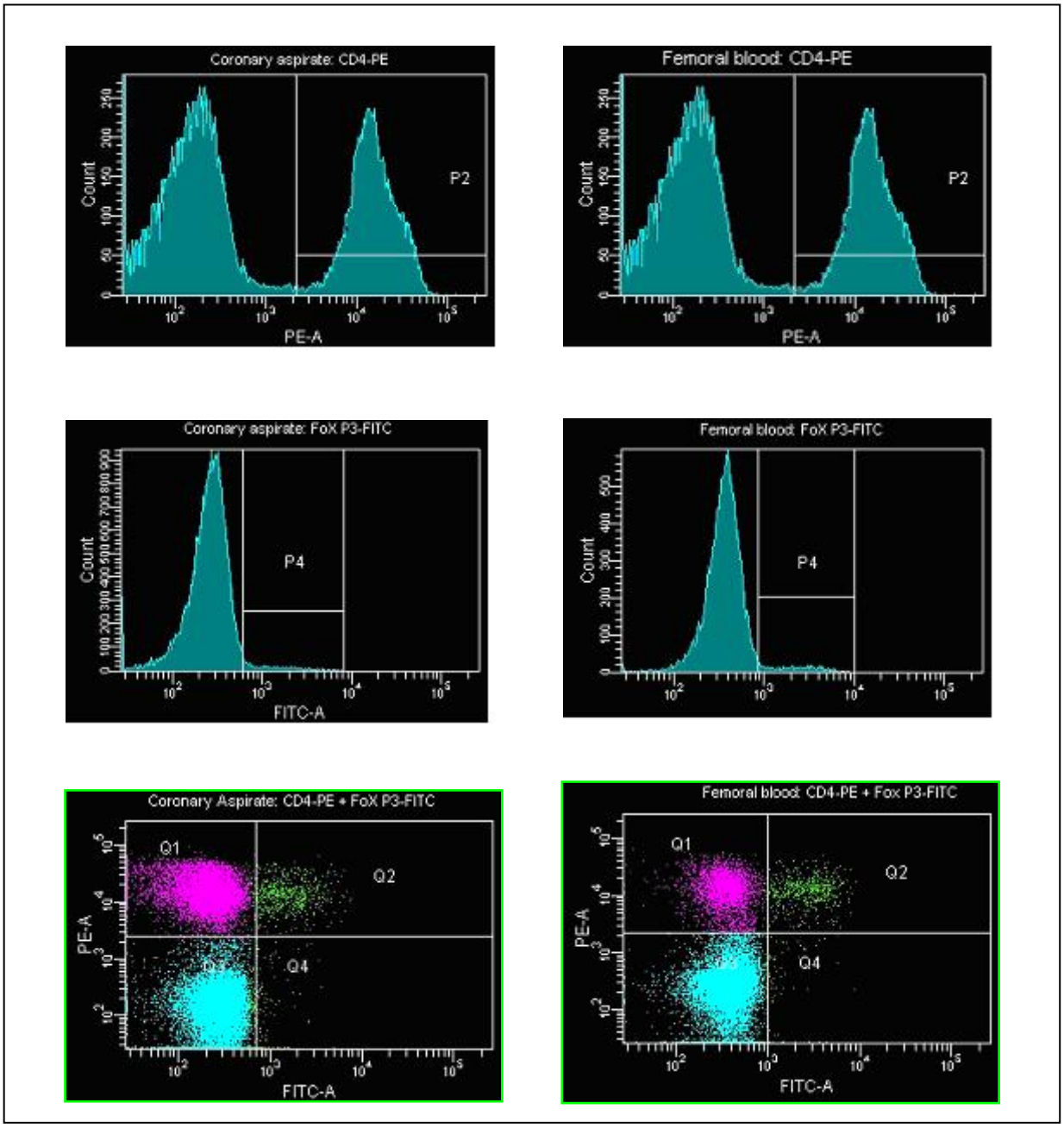
Figure 5.1: Multichannel multicolour flow cytometry for $CD4^+$ $CD28^{null}$ T lymphocytes

***A**: intracoronary Aspirate, **B**: Femoral Artery. **P1**: Total Leukocytes **P2**: Total Lymphocytes **P3**: Total CD4 T Lymphocytes **P4**: $CD4^+$ $CD28^{null}$ T Lymphocytes **P12**: Memory CD4 T Lymphocytes P4:$CD4^+$ $CD28^{null}$ T Lymphocytes, **Q2** and **Q4**: Central Memory CD4 T Lymphocytes P4:$CD4^+$ $CD28^{null}$ T Lymphocytes, **Q1** and **Q3**: Effector Memory CD4 T Lymphocytes P4:$CD4^+$ $CD28^{null}$ T Lymphocytes.*

5.2.3. Intracytoplasmic flow cytometry for FoxP3 expression.

Mononuclear cells were isolated from the coronary filtrate and the femoral arterial blood by gradient centrifugation as described earlier. $0.5\text{-}1\times10^6$ of the mononuclear cells at concentration of $1X10^6$/ml was taken in to paired falcon tubes. 2ml of flow Cytometry Staining Buffer (PBS) was added to the tube, centrifuged for 6 minutes at 1500 rpm, and the supernatant discarded. The cells were stained with 20 μL of antihuman CD4- PE (BD Bioscience, San Jose CA, USA) and IgG1-PE isotype (Beckman coulter) and incubated at room temperature for 20 minutes. The cells were washed in PBS and centrifuged again at 1500 rpm for 5 minutes. The pellet was re-suspended in 750 μL of fixation/permeabilization working solution (eBioscience), incubated at 4 °C for 45 minutes and subsequently washed twice with 2ml of 1x permeabilization Buffer (eBioscience). The cells were

blocked with 2μl normal rat serum at 4°C for 15 minutes. Without washing after blocking step, 10 μl of anti-FOXP3-FITC antibody (eBioscience) was added and incubated at 4°C for 30 minutes. The cells were washed again with 2ml of 1× permeabilization Buffer, Centrifuged and supernatant decanted. To label leukocytes, Hoechst 33342 (Molecular Probes, Eugene, OR, USA), diluted in PBS (1 μg/L final concentration), was added and analysed in FACS after 30 min.



A **B**

Figure 5.2: Intracytoplasmic flow cytometry

***A**: intracoronary Aspirate, **B**: Femoral artery. **P4**: Total $CD4^+$ T lymphocytes, **Q2**: $CD4^+$ $FOXP3^+$ T lymphocytes*

5.3. Results

Clinical characteristics:

	STEMI (n=22)	CSA (n=20)	Healthy Volunteer (n=10)
Age: Mean years.	59 (15)	54 (12)	30 (4)
Sex (% Male)	70	80	50
Pain to reperfusion time: Minutes(SD)	165 (174)	N/A	N/A
Peak Creatinine Kinase U/l(SD)	1216 (1038)	N/A	N/A
Peak Troponin I ug/l (SD)	80 (229)	N/A	N/A
Total Cholesterol mmol/l (SD)	4.9 (1.1)	4.5 (1.4)	N/A
Random Glucose mmol/l (SD)	10 (5)	7 (2)	N/A
Hypertension %	54	60	0
Hypercholesterolemia %	31	40	0
Diabetes %	27	30	0
Current Smoking %	33	25	0
Family history of premature CAD %	22.7	25	0
Prior Beta Blocker use %	22.7	70	0
Prior ACE inhibitor use %	25	65	0
Prior Statin Use %	31.9	90	0
Prior Clopidogrel use %	0.9	25	0

5.3.1.1. Activated intracoronary T- lymphocyte in STEMI

There was no difference in number of mononuclear cells expressing the pan T cell marker CD3 between the coronary aspirate and femoral arterial blood , 10.9 (9, 16.8) versus 11.5 (7.25, 15.75), p=0.638. However, 28.55 (16, 48) of CD3 positive cells in the coronary aspirate and 17.85 (8, 229) percentage in the femoral blood (p=0.0001) expressed CD40L. Similarly, 36.55 (19, 48) and 17 (12, 21) percentage of the CD3 T cells respectively expressed MHC class 2 antigen HLA-DR (p=0.0004) (Figure 5.3). In contrast, CD14 positive monocytes (6.6% versus 6.5%, p=0.65) and co-expression with Mac-1 (85.3% versus 88.6% p=0.60) and HLA-DR (90.3% versus 88.6%, p=0.45) did not differ significantly in coronary aspirate and femoral blood. Figure 5.3

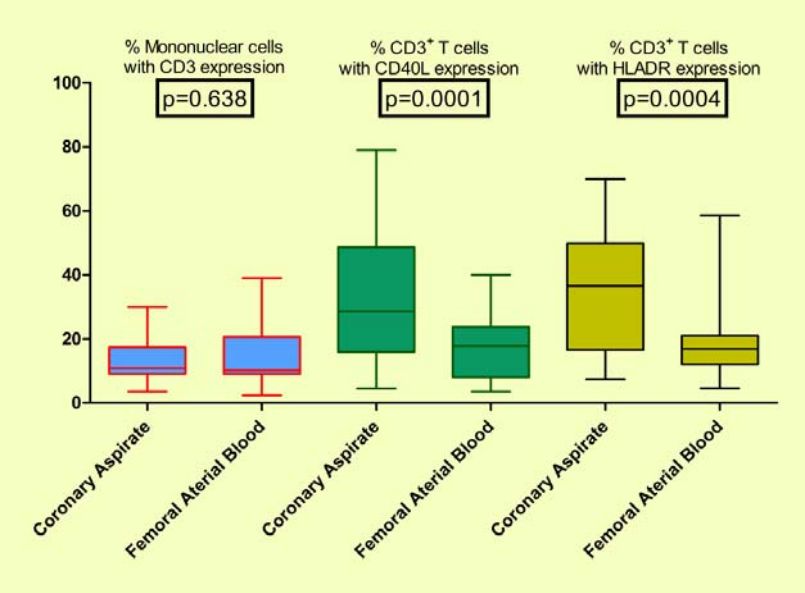


Figure 5.3: Activated T-Lymphocytes in STEMI

5.3.1.2. Activated peripheral T lymphocyte in Chronic Stable Angina

In the femoral artery of patients with chronic stable angina, 13% of the mononuclear cells expressed CD3. Median of 6.5 (8.5, 16) and 16 (9, 23) of the CD3 positive cells expressed CD40L and HLA-DR respectively.

5.3.2. Proinflammatory $CD4^+$ $CD28^{null}$ T cells across the spectrum of Coronary Artery Disease

5.3.2.1. ST Elevation Myocardial Infarction ($CD4^+$ $CD28^{null}$)

In patients with STEMI, 33 (29, 39) and 35 (28, 42) of lymphocytes, identified by their forward and side scatter characteristics, expressed $CD4^+$ in coronary aspirate and femoral arterial blood respectively (p= 0.352). Of the $CD4^+$ T cells, 48% (26, 68) in coronary aspirate and 31% (13, 52) in femoral blood were negative for surface expression of CD28 molecule (p=0.0005) (Figure 5.4).

5.3.2.2. Chronic Stable Angina ($CD4^+$ $CD28^{null}$)

Although the total percentage of CD4 T cells in femoral arterial blood of age matched patients with chronic stable angina was similar to the patients with STEMI (mean age 54 years versus 59 years); significantly fewer CD4+ T cells demonstrated the loss of surface expression of the CD28 molecule: 12 (10, 18), p = 0.0102 (Figure 5.4).

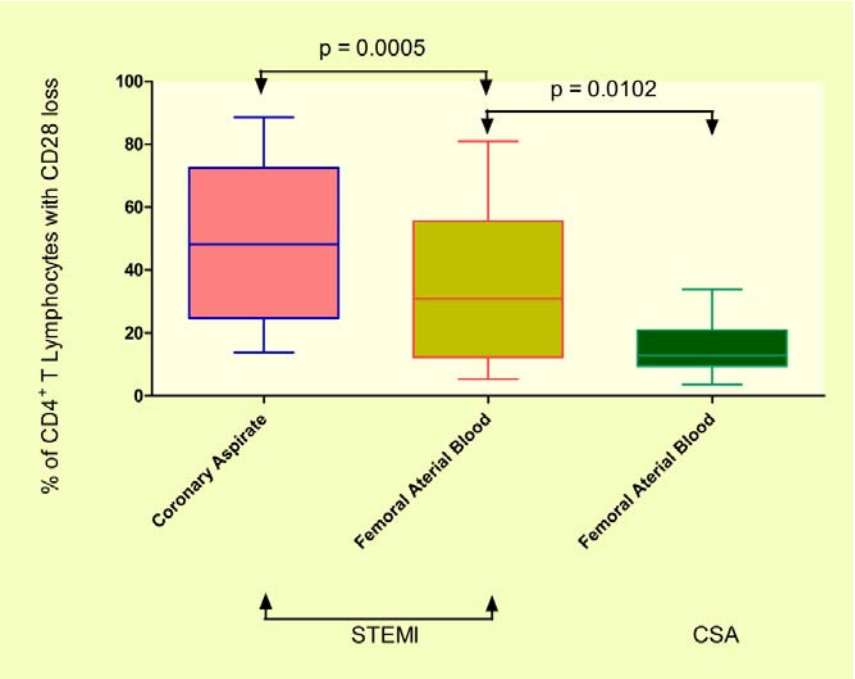


Figure 5.4: Proinflammatory (CD4+ CD 28 null) Lymphocytes

In STEMI and stable coronary disease

5.3.2.3. Healthy Volunteers ($CD4^+$ $CD28^{null}$)

Only 3 (2, 5) of the $CD4^+$ T cells in young healthy volunteers (mean age 32) were CD4+ and $CD28^{null}$ T cells.

5.3.3.1 Naïve versus memory $CD4^+$ $CD28^{null}$ T cells

In patients with STEMI, 44 (19, 62) of the $CD4^+$ $CD28^{null}$ cells in femoral artery expressed CD45RO indicating a memory phenotype in contrast to 29 (15, 50) in coronary aspirate (p=0.0083) (Figure 5.5).

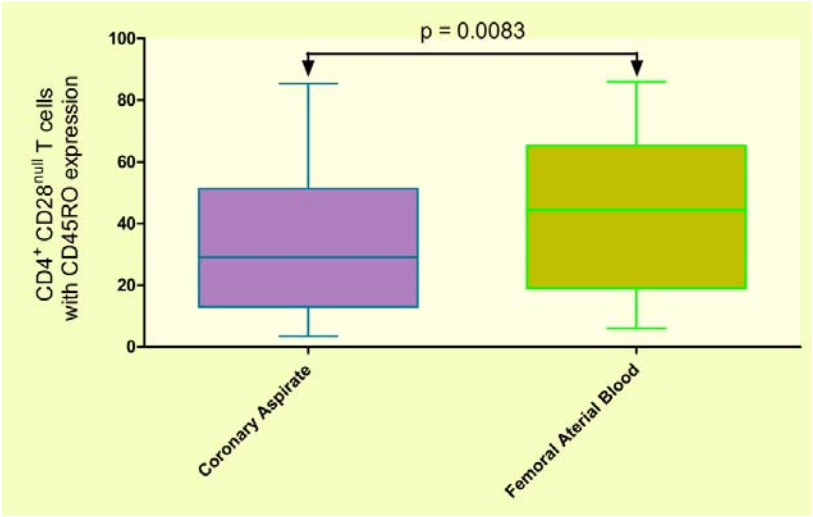


Figure 5.5: CD4+ CD28null cells expressing memory T cell marker CD45RO.

5.3.3.2 Memory subsets of proinflammatory T cells ($CD4^+$ $CD28^{null}$)

Central memory cells: Similarly, 74 (50, 86) of the $CD4^+$ $CD28^{null}$ memory T cells ($CD45RO^+$) in the femoral artery and 43 (29,70) of the $CD4^+$ $CD28^{null}$ memory T cells in the coronary aspirate expressed the phenotypic marker of *central memory* T cells (CCR7+) (p=0.0017).

Effector memory cells: Conversely, 26 (13, 45) and 56 (30, 71) of the $CD4^+$ $CD28^{null}$ memory T cells expressed the phenotypic marker of *effector memory* T cells ($CCR7^-$) in the femoral and coronary aspirate respectively (p=0.0034). Further, phenotypic characterization of the $CD4^+$ $CD28^{null}$ memory T cells and comparison with $CD4^+$ $CD28^+$ memory T cells is depicted in the following table (Table 5.1). The phenotypic subanalysis of the CD 4 28 null cells is depicted in figure 5.6

	Femoral Blood	Coronary Aspirate	P
CD4+CD28-CD45RO+ CCR7+ CD62L+	36.4 (18, 46)	25 (15, 35)	0.01
CD4+CD28+CD45RO+ CCR7+ CD62L-	33.3 (18, 50)	12 (8, 28)	0.05
CD4+CD28-CD45RO+ CCR7- CD62L+	8 (3, 13)	19 (7, 34)	0.11
CD4+CD28-CD45RO+ CCR7- CD62L-	17 (3, 22)	24 (11, 50)	0.002
CD4+CD28+CD45RO+ CCR7+ CD62L+	57 (50, 70)	54 (40, 66)	0.141
CD4+CD28+CD45RO+ CCR7+ CD62L-	22 (13, 40)	19 (12, 33)	0.441
CD4+CD28+CD45RO+ CCR7- CD62L+	4 (2, 17)	7 (4, 15)	0.802
CD4+CD28+CD45RO+ CCR7- CD62L-	3 (2, 7)	8 (2, 13)	0.091

Phenotypic subanalysis of the $CD4^{+}$ $CD28^{null}$ memory T ($CD45RO^{+}$) lymphocytes

Based on surface of expression of the homing molecules CD62L and CCR7

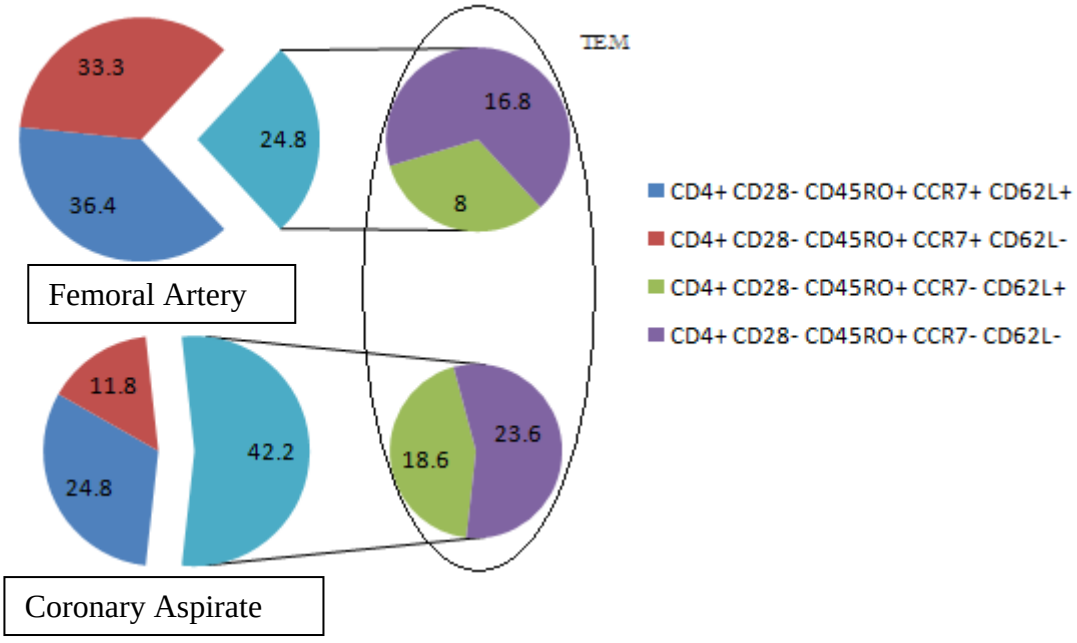


Figure 5.6: Phenotypic subanalysis of the $CD4^{+}$ $CD28^{null}$ memory T ($CD45RO^{+}$) lymphocytes

Based on surface of expression of the homing molecules CD62L and CCR7. TCM: Central memory Tcells, TEM: Effector memory T cells

5.3.4. Immune modulatory FOXP3$^+$ T_{reg}s in STEMI

Intracytoplasmic flow cytometry was performed in mononuclear cells present in 11 paired coronary aspirates and femoral arterial blood. The mean proportion of the mononuclear cells expressing CD4 did not differ between the coronary aspirate and the femoral artery. Of $CD4^+$ T cells, a median of 10 (7, 12)% in the femoral blood expressed FOXP3 in contrary to 6 (3,6) of the $CD4^+$ T cells in the coronary artery (p=0.0036) (Figure 5.7).

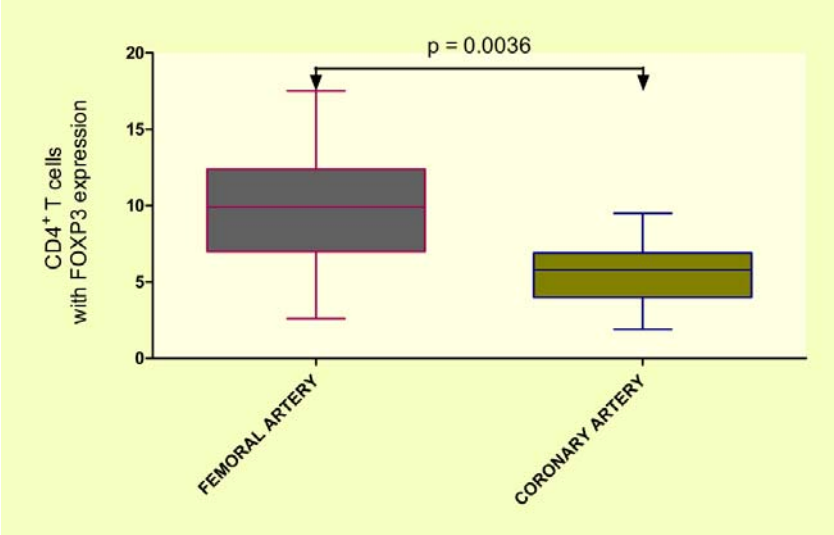


Figure 5.7: Immunoregulatory FOXP3 expression

5.4. Conclusion

These experiments demonstrated relatively higher expression of CD40L, a marker of antigen specific T cell activation and HLA-DR, a marker of non-specific T cell activation, at the culprit site of an infarct related artery than in the femoral artery of patients with STEMI. One of the most interesting observations was that there were increased numbers of CD4+ CD28null T lymphocytes in the liquid phase coronary

aspirate when compared with femoral blood. There were relatively fewer FOXP3+ regulatory T cells at the index culprit site of plaque rupture compared to the peripheral circulation in patients with ST elevation myocardial infarction.

These findings suggest that augmented proinflammatory and attenuated immunomodulatory T lymphocytes may be associated with atherosclerotic plaque instability.

The circulating $CD4^{+}CD28^{null}$ cell population is amplified in acute coronary syndromes and constitutes up to 30-50 percent of total $CD4^{+}$ T cell population,[336] and their frequency predicts the risk of recurrence of acute coronary events.[337] To our knowledge this is the first time the intracoronary $CD4^{+}CD28^{null}$ cell population had been studied in this manner. The CD4+ CD28null T lymphocytes have been shown previously to have cytolytic potential against endothelial cells[338] and their release downstream into the myocardium during primary angioplasty may help to explain the phenomenon of distal microvascular and myocardial damage during reperfusion in STEMI. Regarding memory cell types, the differences observed were modest, however whilst there were relatively fewer memory type CD4+CD28null cells in the coronary than femoral artery, phenotypic sub analysis of CD4+CD28null memory (CD45RO) cells indicated a greater proportion of effector than central memory cells in the coronary than femoral artery. Since

One of the mechanism of genesis of the effector memory cell is exposure to its cognate antigen and has homed to target site of inflammation thus losing their homing receptor CCR7, these cells if found at higher concentration at site of inflammation may indicate that they have tropism for that particular site. In our experiment we clearly demonstrated a clear gradient in the number of the effector memory phenotype CD4+28null cells between coronary and femoral artery

samples, thus indicating that they have been released from the ruptured atherosclerotic plaque as they have already homed to their cognate site of inflammation, rather than generated locally in the arterial lumen during the thrombotic process, or ‘enmeshed’ in the clot whilst circulating. Thus coronary thromboaspirate could potentially yield cells from an unstable plaque potentially facilitating the study of factors associated with plaque instability.

CHAPTER 6: LIQUID PHASE- REACTIVE ANTIBODIES

6.1. Background

Many organ specific autoimmune diseases are associated with accelerated atherosclerosis and increased cardiovascular mortality.[339,340] The complement system also plays a pivotal role in initiation and propagation of inflammation and evidence suggests that atherosclerosis is no exception. Complement activation is known to occur during acute myocardial infarction.[341] This activation inflicts inflammatory damage,[342,343] is associated with larger infarct size,[344] and predicts poor clinical outcomes.[345] The accumulation of terminal complement complexes in ischemic myocardium early during the reperfusion suggests its involvement in reperfusion myocardial damage.[346]

Although there is substantial research in to the role of cell-mediated immunity in atherosclerosis and there is evidence of its alteration during acute coronary syndromes, there is relatively less data regarding the involvement of humoral immunity during acute coronary syndromes. We aimed to evaluate the presence of autoantibodies in the liquid phase coronary thromboaspirate of patients with acute myocardial infarction using complement mediated cytotoxicity and apoptosis assays.

6.2. Method

6.2.1. Complement Dependent Cyototoxicity (CDC)

6.2.1.1. Methodological principle:

All sera were screened for auto-reactive antibodies by a modified microcytotoxicity technique.[347] A panel of viable HLA-typed lymphocytes were incubated with patient's sera so that antibodies present in the serum bind with the target antigen expressed on the surface of the lymphocytes. When rabbit complement is added, the antibody attached to the cell surface activates the classical complement pathway, leading to the production of the membrane attack complex of the complement (C5-9), which ultimately causes cell lysis. The cell lysis was assessed by viability staining using combination of fluorescent stains ethidium bromide and acridine orange. CDC assays are able detect both IgG and IgM by using dithiothreitol (DDT). DDT cleaves disulphide bonds, and because IgM contains significantly more than IgG, the IgM reactivity is preferentially degraded. In addition, IgM non-HLA auto-reactive antibodies can be detected on this assay. A patient was considered to be antibody positive if panel reactivity was greater than 5% and the strength of reaction was greater than 10% above background levels.

6.2.1.2. Procedures

Twenty six paired coronary and femoral sera were tested using an HLA Class I (A, B, C) and Class II (DR and DQ) typed panel of peripheral blood mononuclear cells (PBMC) from 10 healthy volunteers.1 μl of the paired coronary and femoral sera with 0.5% phenol red was coated in duplicates into the terasaki plates. 1μl of AB serum, IgG control and IgM control respectively were introduced in to the wells of row 1 as shown below (Figure 6.1)

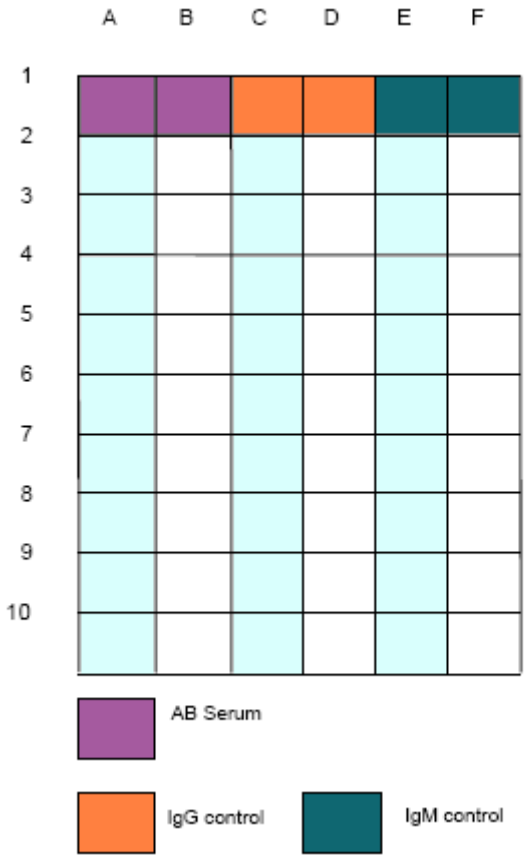


Figure 6.1: Terasaki plate

1µl of DTT and PBS were added to the alternate column. 1µl of the PBMC cell suspension were added in to the wells and incubated at 22°C for 60 minutes. 5µl of the lyophilised rabbit complement (Cedarlane Laboratories, Canada) dissolved in PBS/cystine was added to each well using an HLA microsyringe multiple dispenser and incubated for a further 120mins.

Cell viability was assessed by the addition of 5µl of Fluoroquench in to the wells and observation under a fluorescent microscope in which viable cells fluoresce green and dead cells orange/red. The viability of cells in each well was assessed using the following scale: (figure 6.2a-c)

0 = Negative

1 = 10-20% cell death - doubtful positive

2 = 21-40% cell death- weak positive

4 = 41-60% cell death- weak positive

6 = 61-80% cell death- strong positive

8 = 81-100% cell death- strong positive

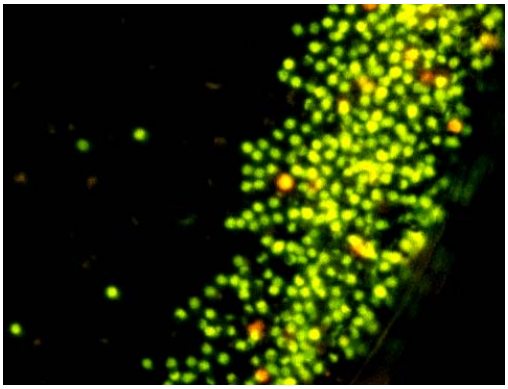


Figure 6.2a Negative control: Viable cells

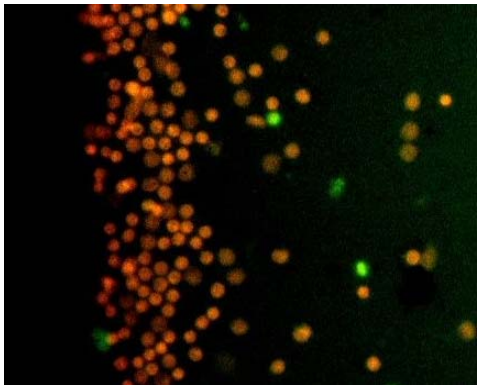


Figure 6.2b Positive control: Dead cells

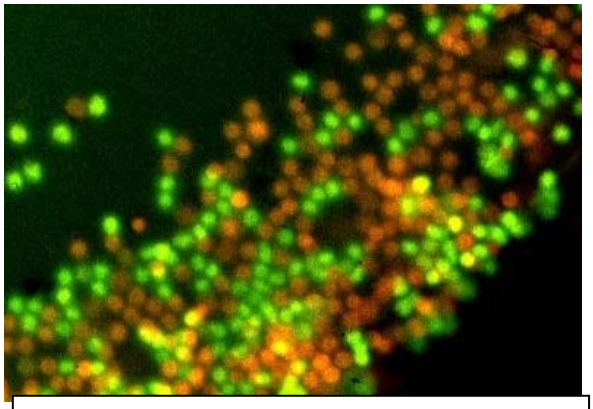


Figure 6.2c Test: Weak positive

Figure 6.2 Cell viability

6.2.2. Apoptosis assay

6.2.2.1. Principles

The complement dependent cytotoxic assay dichotomises target leukocytes into binary viable or dead cell populations, and this assay is not sensitive enough to

identify those cell populations that are undergoing apoptosis or are partially dead. Furthermore, reactive antibodies against target cell populations other than lymphocytes are not assessed in the CDC assay. We therefore incubated the paired coronary and femoral sera in presence of rabbit complement to induce complement dependent cell injury as a more sensitive assay of cell damage. The induction of apoptosis and cell death was assessed by flow cytometry after double staining with annexin V and propidium iodide.

6.2.2.2. Procedures

Buffy coat preparation for leukocytes from blood of healthy volunteers was prepared as detailed in chapter 2.6.2. Approximately, 10^6 cells dissolved in 50 μl of RPMI were taken in falcon tubes and incubated with:

Positive control: 50 μl of positive antibody

Positive control2: 50 μl 10% formaldehyde (Sigma)

Negative control: 50 μl of AB serum

Test 1: 50 μl of intracoronary plasma

Test 2: 50 μl of femoral arterial plasma

The samples were incubated at 37 °C for 60 minutes. Subsequently of 50 μl lyophilised rabbit complement (Cedarlane Laboratories, Canada) was added in to each tube and they were further incubated at 22 °C for 60 minutes.

The cells were then suspended in ice cold RPMI and centrifuged at 500 X g for 5 minutes at 4°C. The supernatant were discarded and the cells suspended in 100 μl of 1X Binding Buffer (Beckman Coulter, Marseille, France).

1 μl of annexin V-FITC (Beckman Coulter) and 5 μl of propidium iodide (Beckman Coulter, 250 μl dissolved in 1ml of 1X Binding Buffer) were added in the cell suspension and incubated at dark 4 °C for 15 minutes. The cells were further diluted in 400 μl of 1X binding buffer and analysed in flow cytometer (Beckman coulter) within 30 minutes.

6.3. Results

Clinical Characteristics:

	STEMI (n=26)
Age: Mean years.	62 (15)
Sex (% Male)	69.9
Pain to reperfusion time: Minutes(SD)	168 (180)
Peak Creatinine Kinase U/l(SD)	1500(1013)
Peak Troponin I ug/l (SD)	78 (283)
Total Cholesterol mmol/l (SD)	5.9 (1.3)
Random Glucose mmol/l (SD)	8 (4)
Hypertension %	50
Hypercholesterolemia %	31
Diabetes %	27
Current Smoking %	33
Family history of premature CAD %	31.8
Prior Beta Blocker use %	31.8
Prior ACE inhibitor use %	19
Prior Statin Use %	31
Prior Clopidogrel use %	7.7

6.3.1. CDC microassay

Of the 26 pairs of coronary and femoral samples analysed, 11.1% of the coronary aspirate (2/26 weakly positive and 1/26 strongly positive) and 3.5% of the femoral (1/26 weakly positive) induced significant PBMCs death indicative of the presence of IgM non-HLA antibody.

6.3.2. Apoptosis assay

Thirteen paired coronary and femoral sera were incubated with the buffy coat leukocytes in the presence of rabbit complement. After 2 hours of incubation, a mean of 9.5 (10.5, 7.5) of leukocytes incubated with coronary sera and 5.2 (2.5, 9) leukocytes incubated with femoral sera were positive for the apoptosis marker annexin V ($p<0.001$). This suggests the presence of more reactive antibodies in the coronary than femoral sera, which bind to the leukocyte cell surface and activate the complement system (Figure 6.3a).

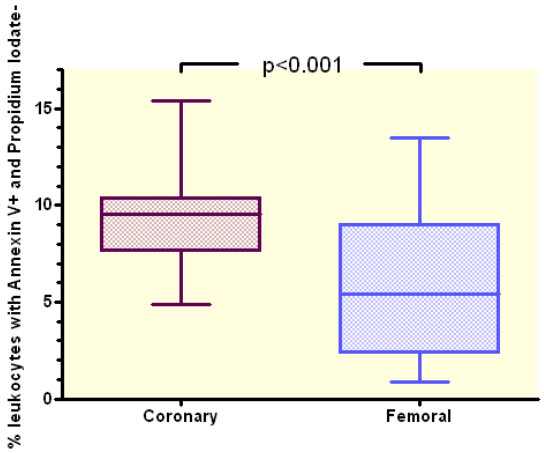


Figure 6.3a: Apoptotic cells

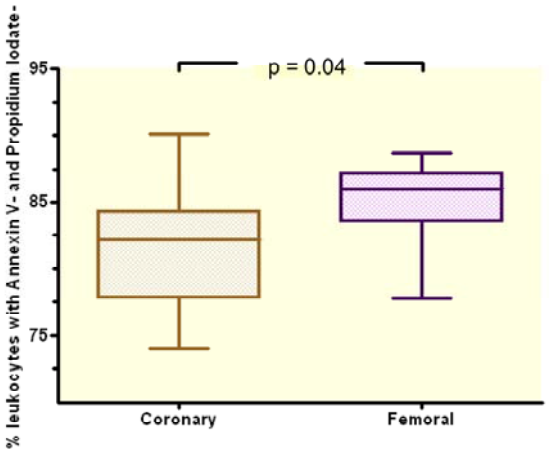


Figure 6.3b: Viable cells

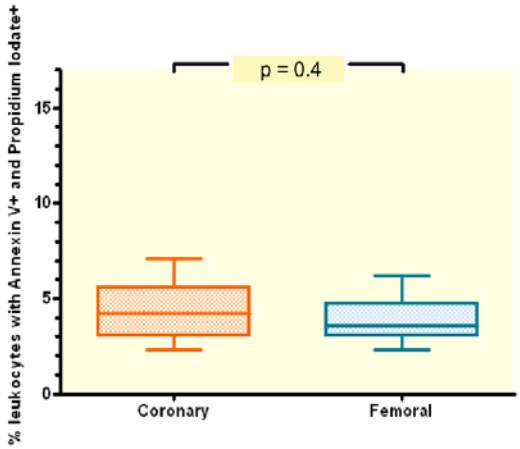


Figure 6.3c: Dead cells

The median magnitude of leukocytes that were viable (annexin V negative and propidium iodide negative) after incubation with coronary sera compared to that following incubation with femoral sera was respectively 82 (76, 84) and 86 (84, 87), p=0.04 (Figure 6.3b). There was no difference in the cell death (annexin V positive and propidium iodide positive) induced by the coronary versus femoral sera 4. 2 (3, 6) versus 3.8 (3, 5) respectively, p=0.4) (Figure 6.3c).

To ascertain whether the apoptosis was induced by the effect of the rabbit complement alone, the leukocytes were incubated in absence of the patient's serum but with serial concentration of the rabbit complement (1 RPMI: 1 rabbit complement to 1 RPMI: ¼ rabbit complement). The median of the leukocytes

showing apoptosis (positive for annexin v and negative for propidium iodate) was 0.8% in contrast to 9.5% and 5.2% showing apoptosis when incubated with coronary and femoral sera respectively (Figure 6.4).

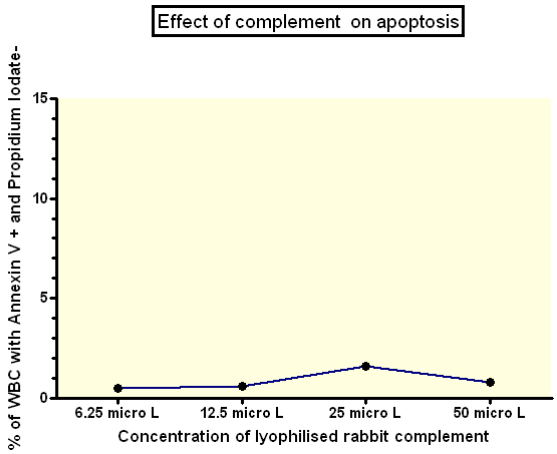


Figure 6.4: Apoptosis with Rabbit complement alone

6.4. 4. Conclusion

The CDC assay suggested the presence of autoreactive non-HLA IgM antibody more frequently in the sera derived from the coronary aspirate of patients with acute myocardial infarction when compared with the sera of peripheral circulating blood. The sensitivity of CDC assay is however limited since it dichotomises the target cell population into binary viable and dead cells. When similar experiments were repeated using the more sensitive assay of annexin V and propidium iodide as markers of complement dependent cell injury the apoptosis inducing effect of the serum was amplified (though there was little change in viability or death). This suggests that there may be a higher concentration of reactive IgM in the liquid phase of the coronary thromboaspirate than in the femoral artery during myocardial infarction.

Complement is known to be deposited on cardiomyocytes after myocardial infarction and IgM can fix complement initiating the classical cascade culminating

in formation of membrane attack complex. Although the positive findings of this series of experiments were limited to apoptosis, liquid phase reactive antibody may have a role in the pathogenesis of myocardial injury following reperfusion in acute myocardial infarction due to downstream release at the restoration of flow.

CHAPTER 7: VIMENTIN AND ANTIVIMENTIN ANTIBODIES

7.1 Background

Vimentin is a highly conserved intermediate filament protein expressed intracellularly in cells of mesenchymal origin including endothelial cells. However, cellular activation[348] or apoptosis can induce surface expression of these proteins.[349,350] Apoptotic cells are an important source of autoantigens.[351,352] Previously, injection of apoptotic cells into normal mice resulted in production of various autoantibodies including antivimentin autoantibodies.[353] Autoantibody against vimentin is an independent predictor of transplant associated coronary artery disease.[354] However this antibody has not been evaluated in atherosclerotic ischemic heart disease.

7.2 Methods: ELISA for Antivimentin antibodies

The antivimentin antibodies were measured at Professor Marlene Rose`s Laboratory at Harefiled Heart science centre. The lab is certified for measuring antivimentin antibody for clinical purpose. The ELISA procedure uses both positive and negative controls to ensure that there is no cross reaction between vimentin and other proteins. 100 μl of vimentin (1mg/ml) was coated overnight into the rows B, D F and H of 96 well ELISA plates. The plates were blocked with 5% non-fat dried milk (Marvel, Ireland) in PBS, 0.1% Tween-20 (200 μl Milk/PBST) at 22 °C for 60 minutes. Subsequently, flicking in to the sink and striking the plate on to the towel for at least five times discarded the solution.

Serial dilutions of the positive vimentin control were prepared by taking 5 X 2ml tubes. To the first, 1000 μl and to the next four, 500 μl of the blocking solution was added. To the first tube 20 μl of positive vimentin control was added and vortexed. 500 μl of the mixture was transferred to the second tube and vortexed. Similarly 500 μl of the mixture was transferred from the second in to the third and so forth. The negative control was prepared by mixing 10 μl of negative serum with 500 μl of blocking solution and similarly 10 μl of the test serum was mixed with 500 μl of the blocking solution. These samples were introduced in the ELISA plate as per the following design (figure 7.1):

A1, A2, B1, B2: 100 μl of negative control

A3, A4, B3, B4: 100 μl of 1/50 dilution positive control

A5, A6, B5, B6: 100 μl of 1/50 dilution positive control and so on

C1, C2…. H12: test sera

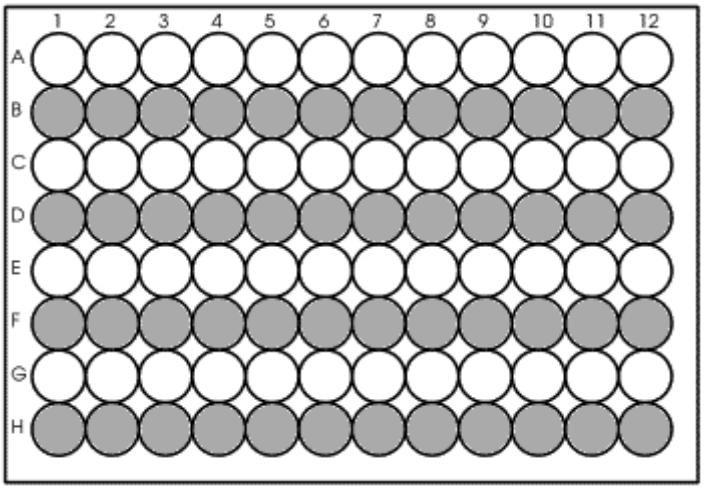


Figure 7.1: ELISA plate

The plate was incubated at 22 °for 60 minutes and the fluid was decanted off. The plate was washed using 200 μl PBS/Tween 1% 5 times. The Horseraddish peroxidase conjugated to rabbit anti-human IgM antibody (Dako Corp, CA) was

diluted to 1/100 and 100 μl was added into each well, incubated for 45 minutes and washed 5 times.

The colorimetric solution was prepared by dissolving a 1 mg tablet of TMB (Tetremethylbenzidine dihydrochloride, Sigma, UK) in to 9ml of distilled water, 5 μl of hydrogen peroxide and 1 ml of 10X phosphate-citrate buffer. 50 μl of the reagent was added to each well and the ensuing chromogenic reaction was allowed to develop for 3 minutes before being terminated by adding 50 μl of sulphuric acid in to each well. The absorbance was read on ELISA reader (Thermo Scientific) at 450 nm. The antibody titres were expressed as titre units.

7.3 Results

Patient Characteristics:

Prior ACE inhibitor use %	19
	STEMI (n=26)
Prior Statin Use %	31
Age: Mean years.	62 (15)
Prior Clopidogrel use %	7.7
Sex (% Male)	69.9
Pain to reperfusion time: Minutes(SD)	168 (180)
Peak Creatinine Kinase U/l(SD)	1500(1013)
Peak Troponin I ug/l (SD)	78 (283)
Total Cholesterol mmol/l (SD)	5.9 (1.3)
Random Glucose mmol/l (SD)	8 (4)
Hypertension %	50
Hypercholesterolemia %	31
Diabetes %	27
Current Smoking %	33
Family history of premature CAD %	31.8
Prior Beta Blocker use %	31.8

Twenty six paired coronary aspirate and femoral arterial sera were assayed for both IgG and IgM antivimentin antibody. The median coronary IgG titre was 55 (38, 65) in contrast to the titre of 70 (61, 77) in the femoral sera ($p < 0.0001$). Similarly the coronary and the femoral titres of IgM antivimentin antibody were 71(47, 85) and 48 (61, 92) respectively, $p= 0.0002$ (fig 7.3).

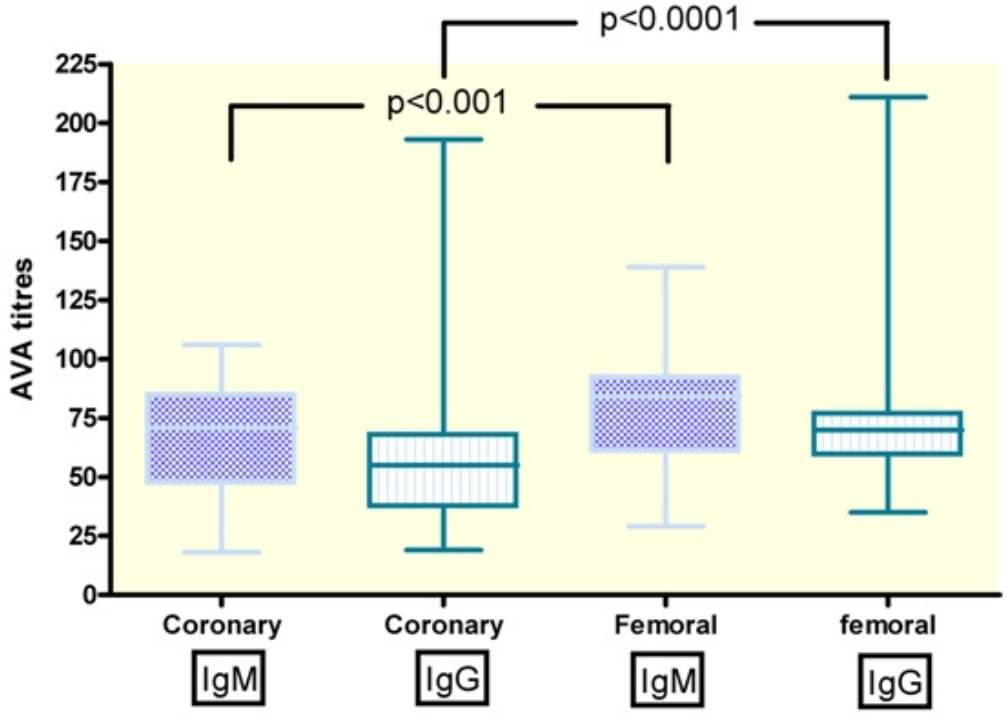


Figure 7.3: Coronary and femoral antivimentin antibody titres

7.4 Conclusions

The coronary aspirate sera demonstrated consistently slightly lower titres of both IgG and IgM antivimentin antibodies.

Although this is interesting, all titres in this study are in the range of those found in the peripheral blood of healthy volunteers (50-70).

As discussed earlier, cells undergoing apoptosis can express vimentin extracellularly. The lower titres of coronary thromboaspirate antivimentin antibody could potentially be caused by sequestration of the antibody by formation of antigen antibody complexes on the surface of the endothelial cells, though this remains speculative and requires further investigation.

CHAPTER 8: LIQUID PHASE- CYTOTOXICITY AGAINST ENDOTHELIAL CELLS

8.1. Background

Derangement in the integrity of endothelial function is a surrogate marker of cardiovascular disease and persistent deterioration is predictive of poor clinical outcomes.[355] Furthermore, among patients presenting with ACS, impaired peripheral endothelial function measured during the first 5 days following an index event, independently identifies patients at an increased risk of recurrent coronary instability.[356,357] We aimed to evaluate the effect of the sera obtained from atherothrombotic aspirate of patients with ST elevation myocardial infarction on healthy endothelial cells (Primary human umbilical vein endothelial cells, HUVECS) as a surrogate for potential cytotoxicity against distal vascular endothelium beyond the site of coronary occlusion during reperfusion.

8.2. Methods

Primary human umbilical vein endothelial cells (HUVECs) were sub-cultured as a monolayer on a 24 well plate for 24 hours and the plating confirmed by optical microscope. The wells were washed with modified Eagle's media (Sigma, UK) and 500 μl of paired sera from coronary aspirate and femoral artery diluted 50 % on media was introduced into each well and incubated at 37 °C for 4 hours. The endothelial cells were harvested by trypsinyzing the wells with 250 microlitres of trypsin (Sigma, UK). The cells were suspended to a concentration of 100,000/ ml, washed, centrifuged at 1500 RPM for 6 minutes, and re-suspended with 100 μl of

Binding Buffer (Beckman Coulter). A positive control was obtained by incubating the cells with 3% formaldehyde for 30 minutes. Both the positive control and the test samples were subjected to annexin V and Propidium Iodide staining as detailed earlier and analysed in the flow cytometer within 30 minutes.

8.3. Results

Patient Characterstics:

	STEMI (n=20)
Age: Mean years.	62 (17)
Sex (% Male)	75
Pain to reperfusion time: Minutes(SD)	168 (180)
Peak Creatinine Kinase U/l(SD)	1500 (1380)
Peak Troponin I ug/l (SD)	98 (239)
Total Cholesterol mmol/l (SD)	5.1 (1.4)
Random Glucose mmol/l (SD)	9 (5)
Hypertension %	60
Hypercholesterolemia %	40
Diabetes %	30
Current Smoking %	30
Family history of premature CAD %	20
Prior Beta Blocker use %	20
Prior ACE inhibitor use %	20
Prior Statin Use %	40
Prior Clopidogrel use %	10

Twenty paired coronary and femoral sera were incubated with the HUVECS: 17 pairs were incubated for 4 hours and the remaining 3 pairs for 24 hours. Within 4

hours 19 (14, 24) of the HUVECS incubated with the coronary sera underwent apoptosis (annexin v positive, propidium iodate negative) in contrast to 8 (17, 4) of the HUVECS incubated with the femoral arterial sera, p<0.001.

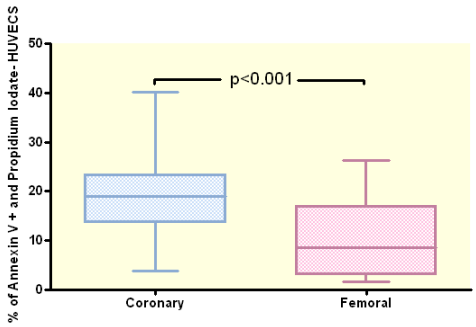


Figure 8.1a Apoptotic cells

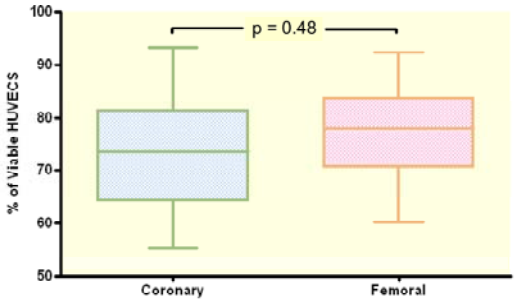


Figure 8.1b viable cells

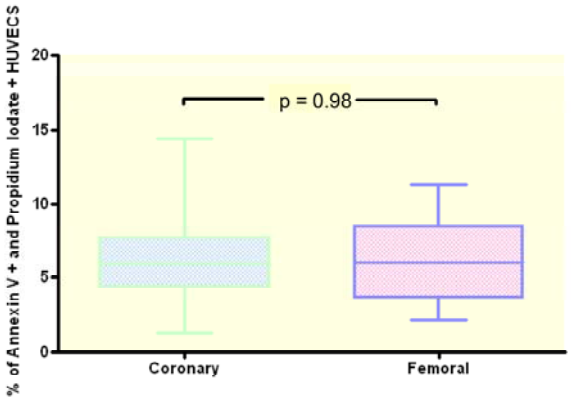
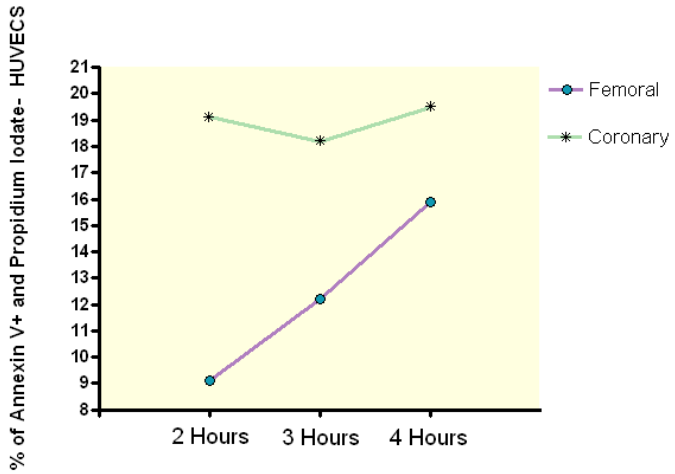


Figure 8.1c dead cells

Figure 8.1 Cytotoxicity against endothelial cells

There was no difference in the magnitude of viable (annexin v and propidium iodate negative; 74(64, 82) versus 77 (70, 84), p=0.48 and dead (doubly positive - annexin v and propidium iodate positive; 6 (4, 7.5) versus 6.2 (3, 8), p=0.98 endothelial cells that were incubated with coronary sera and femoral sera respectively for 4 hours

To delineate the temporal change in the degree of apoptosis, in a separate set of experiments, the HUVECS were incubated with paired coronary and femoral sera for 2 hours, 3 hours, 4 hours and 24 hours consecutively and doubly stained with annexin V and propidium iodate (Figure 8.2). As illustrated below, near maximal apoptosis (19.2) was evident on the HUVECS incubated with the coronary sera. In contrast the HUVECS incubated with the femoral sera demonstrated progressive increment in apoptosis (9, 12.5 and 16 at 2hours, 3 hours and 4 hours respectively. The magnitude of the viable HUVECS did not alter significantly with coronary sera (71, 74, and 67) in contrast to the femoral sera (79, 76, and 70).



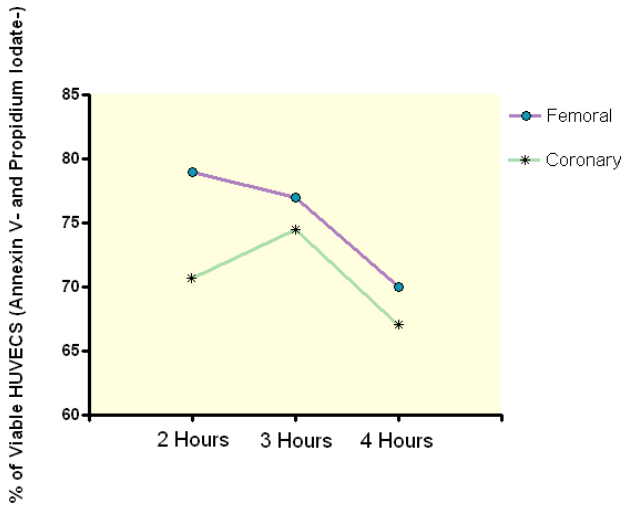


Figure 8.2: Time course of endothelial cell apoptosis

At 24 hours of 12 of the HUVECS incubated with coronary sera were positive for annexin v and negative for propidium iodate, in contrast to 6.5 of HUVECS incubated with femoral sera. More interestingly, the magnitude of viable endothelial cells when incubated with the coronary aspirate was 38 in contrast to 10 in the femoral sera.

Femoral sera @ 24 hours	Coronary sera @ 24 hours

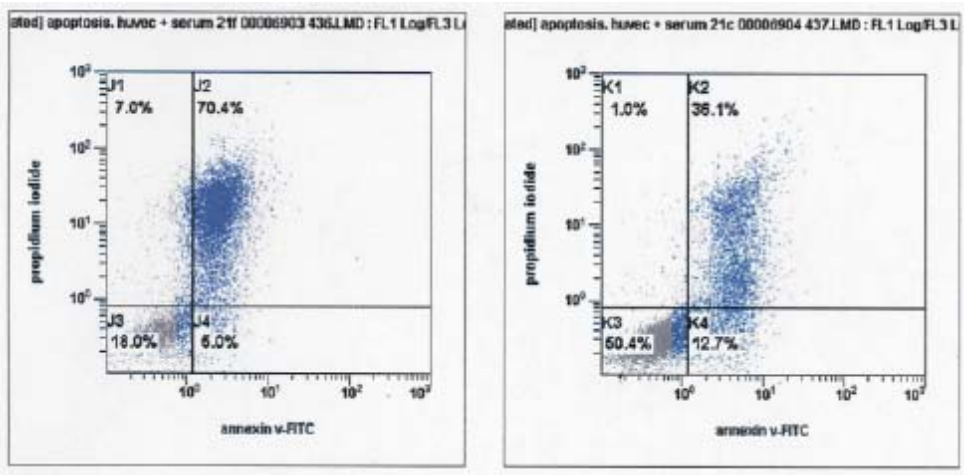


Figure 8.3: Endothelial cell viability at 24 hours

8.4. Conclusion

It appeared from this small study that coronary artery sera induced more apoptosis on endothelial cells than femoral artery sera. Such an effect could be important in the mechanism of myocardial damage following restoration of flow in reperfusion of STEMI. This serum toxicity effect may be antibody related, possibly due to IgM as suggested by the data from chapter 6. However there remains the possibility that these effects are not due to antibody. Further studies need to be undertaken including dose-response curves and removal of immunoglobulin from patients' serum.

CHAPTER 9: CONCLUSIONS

9.1. Rationale and plan of investigation

As set out in chapter 1, the primary objective of this thesis was to define the involvement of immune cells and the soluble auto-reactive antibodies, contained within ex-vivo athero-thrombotic aspirate obtained from the occlusion site of the infarct related coronary artery of patients with acute myocardial infarction undergoing primary percutaneous angioplasty. The reasons for this were both to investigate the pathogenesis of coronary atherothrombosis, and to examine possible mechanisms involved in myocardial damage at the restoration of flow in STEMI.

In order to achieve this aim the coronary athero-thrombotic aspirate was first filtered to segregate the particulate thrombus. The ex-vivo thrombus was sectioned and subjected to immunohistochemistry (Chapter 3). The filtrate of the coronary aspirate, its paired femoral arterial blood and their derivatives (for example the platelet rich plasma) were stained with monoclonal antibodies for multicolour flow cytometry. Detailed T cell subset phenotypic analysis was undertaken (Chapter 5). Similarly the sera from both the sites were assayed for reactive antibodies and assessment of cytoxicity (Chapter 6 + 8). By necessity the investigations in this thesis were global in terms of both thrombus composition and detailed analysis of many cell types in the thromboaspirate. This was necessary because of the paucity of available data. However this work will lay down the basis for more focussed subsequent studies upon the specific findings.

9.2. Specific findings of the investigations

9.2.1. General

Although the adaptive immune system may contribute to the plaque instability and initiation of acute coronary syndrome, T-lymphocytes were not observed in significant amounts within the architecture of intracoronary thrombus, (chapter 3), since immunohistochemistry of the ex-vivo thrombus failed to demonstrate lymphocytes in any significant number, although they comprise about 30% of the circulating blood cells. However, the innate immune cells including monocytes and granulocytes seemed to be an integral part of the thrombus and they expressed ligand (CD11b) for the fibrin polymer. In our preliminary thermoelastography experiments, in which in-vitro clots were generated in the presence of inhibiting monoclonal antibody against CD11b, the antibody reduced the tensile strength of the thrombus (Data not included in the Thesis). Since this thesis planned to address principally the adaptive immune system, and very few lymphocytes were seen in the thrombus, the plan of investigation focussed principally on the resident lymphocytes (chapter 5), Immmunoreactive antibodies (chapters 6 and 7) and the cytotoxic potential of coronary serum (chapter 8) .

9.2.2. Platelet activation

Platelet activation occurs in acute coronary syndromes, however, only approximately 1/5th of the platelets in the coronary aspirate of patients with ST elevation myocardial infarction expressed the conventional activation marker P-selectin on their surface (chapter 4). The occurrence of the index thrombotic event in acute ST elevation myocardial infarction is temporally heterogeneous, with more than 50% patients harbouring old thrombus.[358,359] The expression kinetics of

inducible markers of platelet activation are variable: The P-selectin expression is rather transient and probably explains why the observed CD62P expression in the coronary aspirate is not significantly different than in the peripheral circulation. Conversely, CD40L has longer expression kinetics and this may explain the differential expression between the coronary aspirate and the femoral artery. Similarly PAI-1 and vimentin may also be more reliable markers of platelet activation. Using novel markers of platelet activation with different expression kinetics like CD40L, PAI-1 and vimentin it may be possible to obtain incremental information regarding the degree of platelet activation. Due to the absence of robust and validated assays to determine the degree of in-vivo activation of platelets, patients with acute coronary syndromes are empirically treated with multiple antiplatelet and antithrombotic therapies. Although this approach has improved the prognosis, it has also significantly augmented haemorrhagic risks. It needs to be evaluated whether these novel platelets activation markers can be used as predictors of risk and assist in tailoring antiplatelet therapies.

9.2.3. Platelet-Leukocyte conjugates

Activation of platelets enhances the heterotypic interaction between platelet-monocytes and platelet-granulocyte. Therefore, platelet-monocytes and platelet-granulocyte conjugates can be considered a surrogate marker of platelet activation. As anticipated platelet-monocyte and platelet-granulocyte conjugates were greatly enhanced in the coronary aspirate compared to femoral blood in patients with acute myocardial infarction (Chapter 4). Since platelets facilitate the transmigration of leukocytes across the endothelium, platelet leukocyte co-aggregation at the site of coronary occlusion could potentially enhance the

delivery of leukocytes to the infarcted myocardium. Whether this effect is pathogenic and contributes to ischemia reperfusion injury is unknown.

We also observed an increase in lymphocyte platelet conjugates in the coronary aspirate (chapter 4). The kinetics of heterotypic interaction between lymphocyte and platelet is slower than that of monocyte and granulocyte-platelet interaction, thus the degree of lymphocyte-platelet co-aggregation may also depend on the duration for which coronary artery had remained occluded. A correlation between the pain to reperfusion time and the percent of lymphocyte-platelet conjugates showed the presence of a weak but statistically significant association. Since pain to reperfusion times in one of the major determinants of outcome after acute myocardial infarction, the lymphocyte-platelet conjugate may also predict outcomes. However, a large outcome based clinical research would be required to clarify this.

Previous studies have suggested that activation of lymphocytes and not platelets per se increases in vitro conjugate formation between lymphocyte and platelet. Thus, the enhanced lymphocyte-platelet conjugates in the coronary aspirate may be indicative of increased lymphocyte activation on the coronary aspirate. We corroborated this hypothesis by assessing the activation of T lymphocytes using CD40L as marker of antigen specific T cell activation and HLA-DR as marker of non-specific T cell activation (chapter 5) we found that the lymphocytes from coronary aspirate had enhanced expression of the activation markers when compared to the circulating T lymphocytes from the femoral artery.

9.2.4. T-Lymphocytes

$CD4^+$ T lymphocytes are the dominant proatherogenic cell population amongst the T lymphocytes, of which $CD4^+CD28^{null}$ T cells are of special interest due to their proinflammatory cytotoxic potential despite having a helper T cell phenotype. We evaluated the degree of loss of co-stimulatory molecule CD28 on the CD4 T cell population across the spectrum of the coronary disease (chapter 5) and observed that in ST elevation myocardial infarction there were more $CD4^+$ T cells of $CD28^{null}$ phenotype in the coronary aspirate when compared to the circulating $CD4^+$ T cells in the femoral artery.

In vitro studies have suggested these cell populations exert a cytolytic effect against endothelial cells. However, cloned and activated cell lines of the $CD4^+CD28^{null}$ cells were used in the experiments. In vitro cloning and activation could have altered the properties of the cells and the results may not be directly applicable to in vi-vivo situation. So, based upon our findings, further experiments assessing the cytoxicity against endothelial cells by primary $CD4^+CD28^{null}$ cells isolated from the coronary artery of patients with STEMI, may provide more relevant mechanistic insight.

We observed relative concentration of $CD4^+CD28^{null}$ cells at the culprit site of the occluded coronary artery of patients with STEMI, and these cells may be released downstream in to the distal microvascular bed during reperfusion. It is possible that they may contribute to reperfusion injury.

Chronic antigenic stimulation of the $CD4^+$ T cells is one of the proposed mechanisms of generation of the $CD4^+CD28^{null}$ T cells. Thus one would expect the $CD4^+CD28^{null}$ T cells to have memory T cell phenotype ($CD45RO^+$). Interestingly, $CD4^+CD28^{null}$ T cells in the coronary aspirate less frequently expressed the

CD45RO than the circulating $CD4^+CD28^{null}$ T cells in the femoral artery. This suggests that whilst exposure to antigen frequently contributes to loss of CD28 in the $CD4^+$ T cells in the peripheral circulation, a different mechanism may exist at the site of coronary occlusion. Research in the cytokine and mRNA profile of $CD4^+CD28^{null}$ T cells obtained from the coronary aspirate and femoral artery may clarify this.

Of the memory $CD4^+CD28^{null}$ T cells, the cells from the coronary aspirate more frequently expressed the effector memory T cells phenotype ($CCR7^-$) in contrast to the central memory phenotype ($CCR7^+$) of the peripheral blood memory $CD4^+CD28^{null}$ T cells. This may indicate that the $CD4^+CD28^{null}$ T cells obtained from the coronary aspirate originate from the ruptured plaque.

Although amplification of the pro-atherogenic cells may be important in the progression of atherosclerotic diseases, the attenuation of immune modulating cells may also be important. Indeed, in patients with ST elevation myocardial infarction, the immune regulatory $CD4^+$ T cells (Tregs) that expressed the nuclear transcription factor FoxP3 were present in reduced number on the coronary aspirate when compared to the peripheral circulation (chapter 5). Profiling the titres of the cytokines IL-10 and TGF-β on the coronary aspirate and the femoral artery may increase the robustness of this finding.

9.2.5. Immunoreactive IgM non-HLA antibodies

Significantly more auto-reactive IgM non-HLA antibodies were present in the coronary aspirate sera compared to the femoral sera (chapter 6). This could potentially induce higher degree of cellular apoptosis of the leukocytes in presence of complement. Pentameric IgM antibodies can avidly fix and activate

the classical pathway of complement system to produce the membrane attack complex. Since complement is known to be deposited on the surface of blood vessels and myocardium after myocardial infarction, the presence of IgM could be mechanistically important in the pathogenesis of acute coronary syndromes, and the aetiology of myocyte damage following the restoration of flow in STEMI.

9.2.6. Antivimentin antibodies

Our group has a special interest in vimentin, and since it was not practical to study vimentin directly, we sought antivimentin antibody evidence. Interestingly sera from the coronary aspirate contained less of both IgG and IgM antivimentin antibodies (chapter 7). This was an unexpected finding. Although vimentin is an intracellular intermediate filament, it is known to be expressed extracellularly in response to activation, stress and apoptosis. Since the endothelial cells of the infarct related artery are stressed and apoptotic they may have extracellular expression of vimentin. This may sequestrate the antivimentin antibody thus reducing the titre of the antivimentin antibody in the coronary sera.

9.2.7. Serum cytotoxicity

The coronary sera induced more apoptosis than the femoral artery sera when incubated with the HUVECS. Whilst the identifying the specific humoral factor or factors responsible for this was beyond the scope of the work of this thesis, this ability to induce apoptosis may be important in the aetiology of myocyte damage following the restoration of flow in STEMI.

9.3. Study Limitations

9.3.1. Sample size:

Although 62 patients with ST elevation myocardial infarction undergoing primary angioplasty were recruited in to this study, most of the experiments were performed in twenty patients. This magnitude of sample size is underpowered for assessment of clinical outcomes, so no clinical conclusions are drawn from this mechanistic series of studies.

9.3.2. Dilutional effect:

During aspiration of the coronary athero-thrombotic material some blood upstream of the occlusion site is often aspirated, though the intention of the operator in the therapeutic procedure is to remove primarily the thromboaspirate form the site of the occlusion. Since the blood proximal to the occlusion site is likely similar to the femoral artery this would reduce the gradient of difference between coronary aspirate and femoral blood, suggesting that the differences we observed may be under- rather than over-estimates.

9.3.3. Aspirin and heparin effect:

Patients admitted to our hospital with suspected ST elevation myocardial infarction invariably received aspirin therapy during their transport to the hospital. Furthermore the coronary aspiration thrombectomy was performed after anticoagulation with heparin. The changes we observed were therefore in the presence of aspirin and heparin.

9.3.4. Sampling Issues:

Due to the basic methodology of aspiration thrombectomy there is loss of spatial orientation and fragmentation of the aspirated thrombus limiting any 'polarity' inferences from the clot architecture such as the 'head and 'tail'.

9.3.5 Cytotoxicity assays

Studies investigating cytotoxicity were limited for a number of reasons in these exploratory assays:

1. In all cases small amounts of cytotoxicity were detected (maximum 20%). These studies did not use patient derived target cells which may be more sensitive. Further studies should include cytokine treatments of target cells to measure their sensitivity.

2. Full dose dependency assays were not performed.

3. Immunoglobulin should be removed from sera to confirm the toxic effects are immunoglobulin induced.

4. The study group was small.

9.4. Future directions

The exploratory work of this thesis explored a number of new areas and raises a number of key questions surrounding the involvement of the adaptive immune system in acute coronary syndromes at the site of coronary occlusion. Two key areas that the group is taking forward at present are:

9.4.1 CD24+28null cell clonality

Further investigation into CD24+28null cell typing may be valuable from the perspective of gaining insight into atherogenesis: It is possible that the effector

memory phenotype cells we observed at higher concentration in the coronary than the femoral artery may have originated from the ruptured atherosclerotic plaque. We aim therefore to define the clonality of these T-cells defined by receptor mRNA. If they are clonal, then a search for the common antigen concerned may help understand the pathogenesis of plaque development.

9.4.2 Coronary serum proteomics

Understanding which components of the coronary filtrate serum are causing cytotoxicity may be therapeutically important. Reperfusion injury on restoring flow to an occluded infarct related coronary artery is a well recognised clinical problem with significant morbidity and mortality, and based upon our findings, it is possible that humoral factors released downstream during reperfusion therapy may be important in this regard. We plan to perform a detailed proteomic analysis on coronary thromboaspirate filtrate serum to search for candidate proteins. If we can demonstrate high concentrations of proteins potentially implicated in the reperfusion injury phenomenon then this may give us the opportunity to work towards the development of potential strategies to reduce the clinical burden of this problem.

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