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## The role of bone marrow in thrombus resolution

Wadoodi, Ashar

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# **THE ROLE OF BONE MARROW IN THROMBUS RESOLUTION**

**ASHAR WADOODI**

**April 2012**

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## **ABREVIATIONS**

<b>Ad</b>	<b>Adenovirus</b>
<b>Ang</b>	<b>Angiopoietin</b>
<b>BSA</b>	<b>Bovine serum albumin</b>
<b>BM</b>	<b>Bone marrow</b>
<b>BMMNC</b>	<b>Bone marrow mononuclear cell</b>
<b>CDVT</b>	<b>Catheter directed venous thrombolysis</b>
<b>CXCR4</b>	<b>SDF-1<math>\alpha</math> chemokines receptor</b>
<b>DAPI</b>	<b>4',6-diamidino-2-phenylindole</b>
<b>DC101</b>	<b>VEGFR2 antibody clone</b>
<b>Dil-Ac-LDL</b>	<b>Diioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated LDL</b>
<b>DPBS</b>	<b>Dulbecco's phosphate buffered saline</b>
<b>DMEM</b>	<b>Dulbecco's modified eagle medium</b>
<b>DMSO</b>	<b>Dimethylsulfoxide</b>
<b>DPBS</b>	<b>Dulbeccos phosphate buffered saline</b>
<b>DVT</b>	<b>Deep vein thrombosis</b>
<b>ELISA</b>	<b>Enzyme linked immunosorbent assay</b>
<b>EPC</b>	<b>Endothelial progenitor cell</b>
<b>EPO</b>	<b>Erythropoietin</b>
<b>FACS</b>	<b>Fluorescene-activated cell sorting</b>
<b>FITC</b>	<b>Fluorescein-isothiocyanate</b>
<b>Fsc</b>	<b>Forward scatter</b>

<b>GCSF</b>	<b>Granulocyte colony stimulating factor</b>
<b>GMCSF</b>	<b>Granulocyte macrophage colony stimulating factor</b>
<b>GFP</b>	<b>Green fluorescent protein</b>
<b>H&amp;E</b>	<b>Haematoxylin and eosin</b>
<b>HEK</b>	<b>Human embryonic kidney</b>
<b>HIF</b>	<b>Hypoxia inducible factor</b>
<b>HIT</b>	<b>heparin induced thrombocytopenia</b>
<b>IL-1</b>	<b>Interleukin 1</b>
<b>IVC</b>	<b>Inferior vena cava</b>
<b>LMWH</b>	<b>low molecular weight heparin</b>
<b>MCP-1</b>	<b>Monocyte chemotactic factor 1</b>
<b>MMPs</b>	<b>Matrix metalloproteinases</b>
<b>MOI</b>	<b>Multiplicity of infection</b>
<b>NaCl</b>	<b>Sodium Chloride</b>
<b>NFDM</b>	<b>non-fat dry milk</b>
<b>PAI</b>	<b>Plasminogen activator inhibitor</b>
<b>PAR</b>	<b>Protease activated inhibitor</b>
<b>PC</b>	<b>Progenitor cell</b>
<b>PBMNC</b>	<b>Peripheral blood mononuclear cells</b>
<b>PBS</b>	<b>Phosphate buffered saline</b>
<b>PBST</b>	<b>Phosphate buffered saline tween</b>
<b>PE</b>	<b>Phycoerythrin</b>
<b>PerCP</b>	<b>Peridinin-chlorophyll-protein complex</b>

<b>P-sel</b>	<b>P-selectin</b>
<b>PLGF</b>	<b>Placental growth factor</b>
<b>PMN</b>	<b>Polymorphonuclear cells</b>
<b>PMT</b>	<b>Percutaneous mechanical thrombectomy</b>
<b>Scc</b>	<b>Side scatter</b>
<b>SDF</b>	<b>Stromal derived growth factor</b>
<b>T</b>	<b>Thrombus</b>
<b>TF</b>	<b>Tissue factor</b>
<b>TFPI</b>	<b>Tissue factor pathway inhibitor</b>
<b>tPA</b>	<b>Tissue plasminogen activator</b>
<b>uPA</b>	<b>Urokinase-type plasminogen activator</b>
<b>UEA</b>	<b>Ulex Europaeus Agglutinin</b>
<b>VEGF</b>	<b>Vascular endothelial growth factor</b>
<b>VEGFR</b>	<b>Vascular growth factor receptor</b>
<b>VT</b>	<b>Venous Thrombosis</b>
<b>VTE</b>	<b>Venous thromboembolism</b>
<b>vWF</b>	<b>Von Willebrand factor</b>

## **DEDICATION**

I dedicate this work to my mother without whose commitment to education, I would have not found the courage to pursue a career in academia, my sister, Professor Mona Siddiqui who continues to inspire me in her pursuit of excellence and without whom many a homework assignment would have failed to make the grade. Finally I am indebted to my wife who has encouraged me throughout the many drafts of my final dissertation.

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## ABSTRACT

Deep vein thrombosis (DVT) is a common condition affecting 1-2% of the population. It can be further complicated by serious sequelae such as life threatening pulmonary embolus and the chronically debilitating post-thrombotic syndrome. The main treatment modalities available for DVT are only able to limit disease progression, with resolution occurring physiologically. Natural resolution of DVT occurs through a process of thrombus retraction and recanalisation which is comparable to progenitor mediated neovascularisation. The focus of this project was to examine the circulating progenitor cell response to venous thrombosis and to profile the underlying cytokine response. This data was ultimately used, to manipulate the number of circulating progenitor cells and expression of cytokines to enhance the recanalisation process.

The presence of venous thrombus produced a bimodal circulating haematopoietic progenitor cell response whilst in the bone marrow compartment progenitor cells were simultaneously depleted. GCSF, GMCSF, VEGF, PIGF and SDF1 were expressed differentially in thrombus, vein wall and plasma, with the laparotomy wound mirroring the cytokine profile of the resolving thrombus. The expression pattern of PIGF in the resolving thrombus was however, unusually specific and not seen in the healing laparotomy wound.

In order to enhance recanalisation two approaches were used. The first was peripheral upregulation of bone marrow mononuclear cells which localised to the thrombus vein wall interface and enhanced venous recanalisation 2-fold. Secondly adenoviral constructs were used to upregulate PlGF and SDF1 protein directly within the thrombus. Upregulation of both proteins enhanced thrombus recanalisation by almost 3-fold but only PlGF significantly increased the number of circulating HPCs.

# **1.1 INTRODUCTION & CLINICAL OVERVIEW OF DEEP VEIN THROMBOSIS**

## **1.1.1 DEFINITION**

Deep vein thrombosis (DVT) is the formation of a semi-solid, structured obstructive lesion which develops in the deep veins of the lower limb (*in vivo*). This is distinct to a clot which is an amorphous structure of coagulated blood outside the vein (*ex vivo*).

## **1.1.2 EPIDEMIOLOGY**

DVT is a common condition which can give rise to fatal pulmonary embolism and debilitating post-thrombotic syndrome (PTS)<sup>1</sup>. The annual incidence of DVT is about 5 per 10,000 as shown by a recent meta-analysis of studies, which were carried out mainly in Sweden and the USA<sup>2</sup>. This analysis found that 2 per 10,000 were idiopathic and 1-2 per 10,000 were complicated by a pulmonary embolus. More recently a UK primary care based study estimated the incidence to be 74.5 per 100,000 patient years<sup>3</sup>. The risk of DVT appears to rise exponentially with age, occurring rarely before the age of twenty, but rising significantly there after<sup>4</sup>.

## **1.1.3 CLINICAL PRESENTATION**

DVT presents with calf pain and swelling, which is commonly unilateral. The differential diagnosis includes cellulitis, Baker's cyst, muscular injury, tumour, infection, arterial aneurysm, and Achilles tendon inflammation or rupture<sup>5:6</sup>.

If thrombus is located in the proximal femoral or iliac veins then the leg swelling can be much more pronounced and may lead to one or both of the following:

**Phlegmasia Alba Dolens        (PAD)**

**Phlegmasia Caerulea Dolens   (PCD)**

In PAD, thrombosis involves the major deep venous channels of the extremity only, sparing collateral veins and therefore allowing some venous flow. In PCD there is complete obstruction to venous flow from the limb which can result in *venous gangrene* if the increased pressure within the limb exceeds the local arterial pressure<sup>7</sup>.

#### **1.1.4 RISK FACTORS**

Various factors predispose to DVT and they can be divided into acute (transitory) and chronic (**Table 1.1.1**). These factors are important for risk stratification when assessing patients for prophylaxis and treatment<sup>8-10</sup>.

**Table 1.1.1 Risk factors for deep vein thrombosis**

(adapted from Rawat<sup>11</sup>)

<b>ACUTE</b>	<b>CHRONIC</b>
<b>Trauma</b>	<b>Age</b>
<b>Immobilisation</b>	<b>Obesity</b>
<b>Surgery</b>	<b>Malignancy</b>
<b>Long distance travel (8hrs)</b>	<b>Chemotherapy</b>
<b>Intravascular device (i.e. central line)</b>	<b>Oestrogen therapy</b>
<b>Pregnancy</b>	<b>Inherited or acquired thrombophilia (Table 1.1.2)</b>

### **Thrombophilia**

Abnormalities of clotting factors can also predispose to DVT and they are known collectively as thrombophilias (**Table 1.1.2**). They include the Factor V Leiden gene mutation that confers a seven-fold increase in the incidence of DVT in heterozygotes and an 80-fold increase in homozygotes. The mutation is present in about 5-8% of the general population<sup>12</sup>. Others mutations include the prothrombin gene mutation (G20210A), anti-thrombin III deficiency, protein C and S deficiency, hyperhomocysteinuria and increments in certain clotting factors i.e. VIII, IX and XI<sup>13</sup>.

**Table 1.1.2 Inherited Thrombophilias<sup>14</sup>**

Thrombophilia	Prevalence (European population) %
Factor V Leiden gene mutation (heterozygote)	5-8
Protein C deficiency	0.2-0.33
Protein S deficiency	unknown
prothrombin (G20210A) gene mutation	2
Anti-thrombin deficiency	0.25-0.55
Lupus anticoagulant	2
Homocysteinaemia	11
Dysfibrinogenemia	0.8
Plasminogen deficiency	2-4

### 1.1.5 DIAGNOSIS

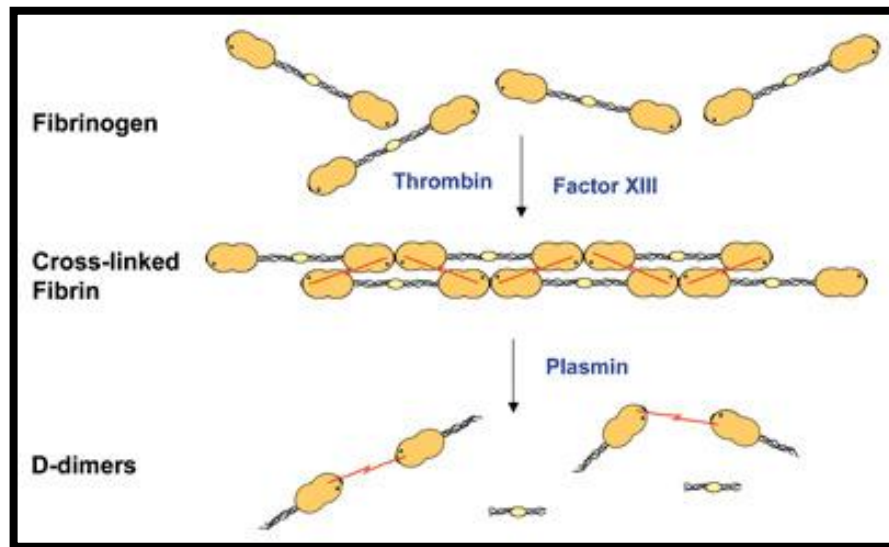
Clinical diagnosis of DVT is difficult, as calf pain and swelling are non-specific symptoms. Scoring systems can help to filter out those unlikely to have a DVT and avoid unnecessary imaging. Although several scoring systems have been developed<sup>6</sup>, the *Well's Criteria* is the only one that has been widely accepted<sup>15</sup>. Below (**Table 1.1.3**) is an example of a modified Well's Scoring Chart<sup>16</sup>, Well's scoring has only been validated in the hospital setting and is not necessarily appropriate for use in primary care<sup>17-19</sup>.

**Table 1.1.3 Modified Well's scoring chart for DVT**

<b>Clinical Feature</b>	<b>Score</b>
Active cancer treatment within the last 6 months or palliative	1
Paralysis, paresis or plaster immobilisation of lower extremities	1
Bedridden for <3 days or major surgery within 12 weeks requiring general/regional anaesthesia	1
Localized tenderness along distribution of the deep venous system	1
Swelling of entire leg	1
Calf swelling >3 cm than asymptomatic leg at 10cm below tibial tuberosity	1
Pitting oedema confined to the symptomatic leg	1
Collateral superficial veins (non-varicose)	1
Previously documented DVT	1
Alternative diagnosis at least as likely as DVT	-2
<i>A score <math>\geq 2</math> indicates that the probability of DVT is likely and a score of <math>&lt; 2</math> indicates that it is unlikely.</i>	

### **D-dimer**

During the 1990's, the addition of the D-dimer blood test increased the negative predictive value of the Well's scoring system<sup>20</sup>. D-dimers are the breakdown products of the fibrin mesh (**Fig 1.1.1**) within the thrombus<sup>21</sup>.



**Fig 1.1.1 Cross-linked fibrin broken down by plasmin to produce D-dimers<sup>22</sup>**

It is a highly sensitive (but non-specific) blood test that is used to exclude the diagnosis of DVT<sup>23</sup>. The test does however have certain limitations e.g. D-dimer levels increase during surgery, pregnancy, trauma, malignancy as well certain inflammatory conditions and are therefore of limited use in these settings<sup>24;25</sup>. It is often used in conjunction with the Well's score; a low pre-test probability with a negative D-dimer test, allows safe discharge without the need for ultrasound. In those with a high pre-test probability duplex ultrasonography is indicated regardless of the D-dimer score<sup>26</sup>. More recently the PROLONG and PROLONG II trials have demonstrated that the D-dimer test can be used to predict the risk of DVT recurrence and may also be useful in predicting treatment duration<sup>27;28</sup>.



### **1.1.6 IMAGING MODALITIES**

Several imaging modalities can be used to visualise DVT in the lower limb. Although duplex ultrasonography is regarded by most as the gold standard, the advantages and disadvantages of alternate modalities are discussed below:

#### **Duplex**

In the UK DVT is largely diagnosed on the basis of duplex scanning; a combination of ultrasound and Doppler which measures flow velocity<sup>29</sup>. Duplex ultrasonography has the advantage of being non-invasive with a sensitivity and specificity that exceeds 95% and 98% respectively for symptomatic proximal DVT i.e. above knee, this falls to 11-100% and 90-100% below the knee<sup>30</sup>. The disadvantage of ultrasound (US) is that it does not visualise the pelvic veins and is of limited value in those who are obese or in plaster cast immobilisation<sup>10</sup>.

#### **Venography**

Venography is still considered by a few to be the gold standard for calf vein (below knee) thrombosis<sup>31</sup>. Intravenous contrast is injected into the foot after which x-ray images are taken of the affected limb. It has been largely superseded by duplex, because it is an invasive and expensive procedure that is not widely available<sup>26</sup>. Computerised Tomographic (CT) venography is a variation on this same format and is 100% sensitive and 96% specific<sup>32</sup>. Its greatest advantage over US scanning is that the patient's body habitus is not a limitation and therefore the pelvic veins can also be visualised<sup>10</sup>.

Indirect CT venography is currently the diagnostic modality used for pulmonary embolism, its use in DVT however appears to be no more sensitive than duplex<sup>33-35</sup>.

### **Magnetic Resonance Direct Thrombus Imaging (MRDTI)**

MRDTI uses the paramagnetic properties of methaemoglobin within thrombus to create a signal which is directly proportional to the amount of methaemoglobin present<sup>36;37</sup>. MRDTI does not require any contrast enhancement and has the added benefit that the peripheral and pulmonary veins within the thorax can be imaged simultaneously<sup>32</sup>. Its sensitivity and specificity for below knee DVT is 92% and 94%<sup>36</sup>. A recent meta-analysis did not demonstrate superiority over duplex ultrasonography<sup>38</sup> but there may be a role for evaluating pelvic veins, particularly in circumstances such as pregnancy where CT would be contraindicated<sup>39</sup>.

### **1.1.7 MEDICAL MANAGEMENT**

Once a DVT has been diagnosed, management consists of limiting thrombus extension, recurrence, PE and long-term complications using a combination of anticoagulation, lower limb compression and in certain circumstances interventional procedures<sup>40-42</sup>. Over the last fifty years, anticoagulation has been achieved by the use of low molecular weight heparin and ultimately warfarin in the majority of patients<sup>43</sup>. Unfortunately both the unfractionated form of heparin and warfarin are limited by their unpredictable pharmacokinetics and interactions<sup>44</sup>.

## **THE ORIGINAL ANTICOAGULANTS:**

### **Unfractionated Heparin (UFH)**

Heparin was originally used only in its unfractionated form, its anticoagulant effect occurs by inactivating thrombin and activated factor X (factor Xa) through an antithrombin (AT)-dependent mechanism<sup>45</sup>. Patients have to remain hospitalised for heparin treatment because it is given intravenously and because of its narrow therapeutic window, which has to be regularly monitored using the activated thromboplastin time (APTT)<sup>46</sup>.

### **Low Molecular Weight Heparin (LMWH)**

The development of Low Molecular Weight Heparins (LMWH) such as *Enoxaparin* have dramatically simplified the management of DVT. LMWHs can be administered subcutaneously allowing out-patient use and do not require monitoring<sup>47</sup>. Several studies comparing LMWH with UFH have shown that LMWHs are just as efficacious as their predecessor UFH<sup>46;48-50</sup>.

### **Warfarin**

Warfarin is a synthetic derivative of coumarin; a chemical found naturally in certain plants<sup>45</sup>. Most famously known as a rodenticide, it wasn't until the 1940's and 1950's that it was first used clinically<sup>45</sup>. Warfarin reduces blood coagulability by preventing the activation of vitamin K dependent clotting factors II, VII, IX and X. It is monitored using the International Normalised Ratio (INR) which is calculated based on the patients prothrombin time (PT)<sup>51</sup>.

The difficulties encountered in administering heparin and warfarin have lead to the development of several new therapies based upon the characteristics of the ideal anticoagulant (**Table 1.1.4**).

**Table 1.1.4 The ideal anticoagulant**

(adapted from Bounameux<sup>52</sup>)

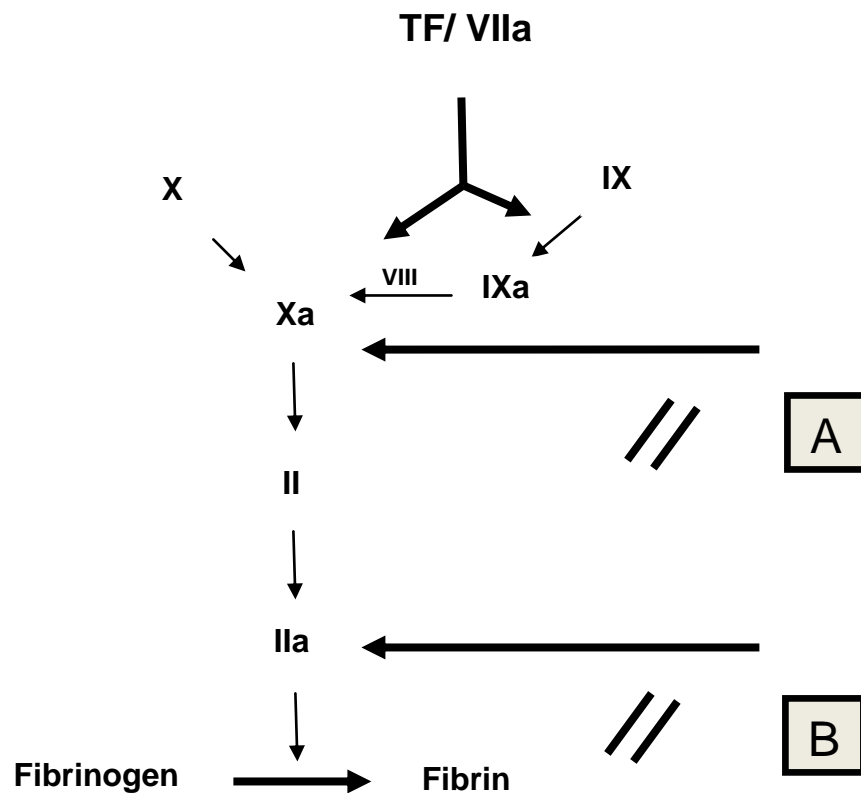
<b>CHARACTERISTICS OF THE IDEAL ANTICOAGULANT</b>
<b>Administered orally, one tablet daily</b>
<b>Highly effective in reducing thromboembolic events</b>
<b>Predictable dose response and kinetics</b>
<b>Low rate of bleeding events</b>
<b>Wide therapeutic window</b>
<b>No dose adjustment required</b>
<b>Little interaction with food or other drugs</b>
<b>Low, nonspecific plasma protein binding</b>
<b>Inhibition of both free and clot-bound activated coagulation factors</b>

### **Emerging therapies**

The new drugs are much more target specific and can be subdivided into classes according to the factors which they affect (**Fig 1.1.2**):

(A) **Specific inhibitors of factor Xa** (Fondaparinux, Rivaroxaban)

(B) **Direct thrombin inhibitors** (Ximelgatran, Dabigatran etexilate)



**Fig 1.1.2 Coagulation steps inhibited by developing anticoagulants**

(Adapted from Bounameaux<sup>52</sup>)

### **Inhibitors of Factor Xa**

#### ***Fondaparinux***

Fondaparinux is a selective inhibitor of Factor Xa. It is a subcutaneously administered pentasaccharide which binds to antithrombin and has a half life of 17hrs<sup>53</sup>. It works by increasing the affinity of antithrombin for factor Xa thereby enhancing its inhibition of the clotting cascade<sup>54</sup>. It appears to be as safe and

effective as LMWH, with the added advantage of not causing heparin induced thrombocytopenia, its use is currently limited by expense<sup>55</sup>.

### ***Rivaroxaban***

Rivaroxaban was the first orally administered factor Xa inhibitor with an 80% bioavailability 3hrs after administration. The RECORD 1-4 trials compared it to enoxaparin in the prevention of DVT and showed that Rivaroxaban was significantly superior to enoxaparin in relation to the primary outcome i.e. total number of VTE with non-significant difference in major haemorrhagic complications<sup>56;57</sup>. NICE has since recommended Rivaroxaban for the prophylaxis of DVT in major orthopaedic surgery. Early literature evaluating Rivaroxaban in the treatment of DVT is also now emerging although it has yet to qualify as an alternative to low molecular weight heparin and warfarin<sup>56</sup>.

### **Direct Thrombin inhibitors**

#### ***Ximelagatran***

Ximelagatran is a prodrug of Melagatran which directly interacts with thrombin's active site. It showed promise in several trials such as EXULT (North American VTE Prevention Trial)<sup>58</sup>, THRIVE (Trial Evaluated Ximelagatran for acute VTE)<sup>59</sup> and the SPORTIF (Non-inferiority trial against dose adjusted warfarin)<sup>60</sup>. The Federal Drug Agency in 2004 however, did not approve Ximelagatran for general use, owing to 6% rate of hepatotoxicity<sup>61</sup>.

### ***Dabigatran etexilate***

Dabigatran is a highly selective, reversible, and potent thrombin inhibitor and is orally available as the prodrug, Dabigatran etexilate. It is the current favourite as a suitable replacement for warfarin with a half life of approximately 15hrs making it ideal for once daily dosing. It is renally metabolised and so far no evidence of hepatotoxicity has been documented. Trials such as RE-MODEL<sup>62</sup>, RE-NOVATE<sup>63</sup> and RE-MOBILIZE<sup>64</sup> have all demonstrated non-inferiority to enoxaparin and further trials looking at secondary prevention of DVT are underway<sup>54</sup>.

### **Non-pharmaceutical adjuncts**

Physical compression is used both in the prevention of DVT in hospitalised patients and also in the treatment for people who have had a DVT<sup>65</sup>. The two commonest methods used, are graded elastic compression stockings and intermittent pneumatic compression.

### **Graduated compression stockings**

Graduated stockings produce a higher pressure at the level of the ankle than at points above the ankle. In this way blood flow is encouraged from back up the leg into the central circulation. Several mechanisms underpin stocking function: prevention of stasis, enhancing the calf muscle pump, improving venous valve function and potentially altering the clotting profile of blood<sup>66;67</sup>.

## **Pneumatic Compression**

Intermittent pneumatic compression (IPC) devices consist of an inflatable sleeve for the leg and an electrical pneumatic pump that fills it with compressed air. The sleeve cycles between inflation and deflation increasing blood flow through venous system by up to 200%, the shear effect of the increase in blood flow increases fibrinolytic activity<sup>68</sup> as well as a rise in Tissue factor pathway inhibitor protein (TFPI) and a reduction of FVIIa<sup>67</sup>. Although it is accepted that compression stockings and pneumatic compression reduce the incidence of DVT, no clear difference between the two technologies has been identified<sup>69</sup>.

### **1.1.8 INVASIVE THERAPIES FOR REMOVING THROMBUS**

Invasive management of DVT involves removing the thrombus either mechanically or chemically. Its indications are still limited to a select number of patients, usually those with occlusive iliofemoral DVT<sup>41</sup>.

#### ***Percutaneous mechanical thrombectomy***

Percutaneous mechanical thrombectomy (PMT) has advanced considerably since its inception over fifty years ago, this has been facilitated by the embolectomy catheter developed by Fogarty<sup>70</sup>. A catheter with an inflatable balloon is passed down the vein through the thrombus at which point the balloon is inflated and the catheter retrieved, bringing with it the thrombus. Maceration and fragmentation of the thrombus can occur and there is also the theoretical risk of valvular damage. It has been shown that long-term vein patency is improved by thrombectomy compared with anti-coagulation alone and leads to reduced venous



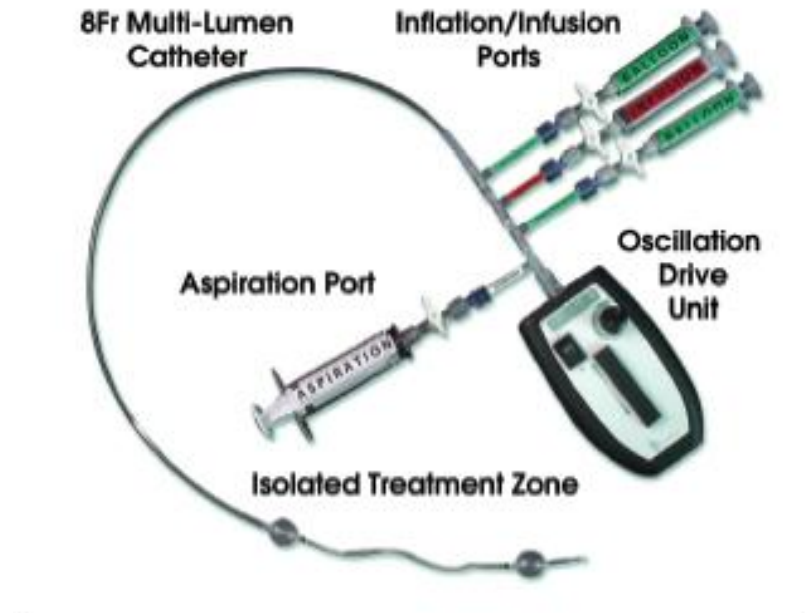
insufficiency<sup>71;72</sup>. Since its development there have been trials examining its effect in combination fibrinolytic agents which have proven superior to PMT in isolation<sup>73</sup>.

### **Pharmaco-mechanical thrombolysis**

Mechanical thrombectomy has now largely been superseded by pharmacological catheter directed venous thrombolysis (CDVT) which has improved patency rates compared with anti-coagulation alone<sup>74</sup>. This technique remains interventional in that a catheter has to be passed down into the thrombus using image guidance to allow localised delivery of the chemical agent. There are several types of commercially available catheter, each with its own potential advantages:

#### ***Trellis 8***

The Trellis 8 incorporates both pharmacological and mechanical thrombolysis<sup>75</sup>. It uses two inflatable balloons to enclose the thrombus, the enclosed segment contains a wire which rotates at high speed to mechanically fragment the thrombus mass. This mechanical process is combined with thrombolytic drug infusion (**Fig 1.1.3**)<sup>75</sup>.



**Fig 1.1.3 The Trellis 8<sup>75</sup>**

### ***Angiojet Power Pulse***

The Angiojet Power Pulse™ uses pressurised high velocity saline stream for thrombus dissolution at the catheter tip. Once in the pathway of the jets, thrombus is broken up into small particles, the jets also provide the driving force for evacuation of the thrombus debris through the catheter and associated tubing. As it does not use thrombolytic agents it is particularly useful when chemical thrombolysis is contraindicated<sup>76</sup>.

### ***Ekos Endowave***

The Ekos™ Endowave uses ultrasound (2MHz) in a process known as acoustic streaming to alter fibrin structure and increase the number of plasminogen receptor sites available for thrombolytic agents<sup>77;78</sup>. In trials of CDVT for iliofemoral DVT, the Ekos Endowave has achieved success rates of approximately 80% in reducing thrombus load to at least 50% of pre-treatment size<sup>79;80</sup>. Complications included significant bleeding in up to 10% of patients, intracranial haemorrhage however, occurred in less than 1% of patients<sup>79;80</sup>.

#### **1.1.9 POST THROMBOTIC SYNDROME: THE CASE FOR ENHANCING THROMBUS RESOLUTION**

Post thrombotic syndrome (PTS) encompasses a wide spectrum of signs and symptoms which develop in chronic manner following DVT (**Table 1.1.5**)<sup>81</sup>.

**Table 1.1.5 Features of PTS**

(adapted from Kahn et al.<sup>82</sup>)

<b>Symptoms</b>	<b>Signs</b>
<b>Heaviness</b>	<b>Oedema</b>
<b>Pain</b>	<b>Telangiectasia</b>
<b>Swelling</b>	<b>Venous Ectasia</b>
<b>Itching</b>	<b>Hyperpigmentation</b>
<b>Cramps</b>	<b>Dependent cyanosis</b>
<b>Paraesthesia</b>	<b>Lipodermatosclerosis</b>
<b>Bursting pain</b>	<b>Ulceration</b>

## **Epidemiology of PTS**

The incidence of PTS has been reported to be as high as 50%, with significant morbidity occurring in 5-10% of patients<sup>82-84</sup>. In most cases PTS develops within 2yrs of the primary DVT<sup>85</sup>. The condition remains underreported, because of a lack of consistency in how it is defined<sup>86</sup>. It is estimated that more than one quarter of the 170000 new cases of venous stasis syndrome per year represent PTS<sup>87</sup>. The socioeconomic cost of this has been estimated by a Swedish study to be £3000 per incident, or approximately 75% the cost of treating the primary DVT<sup>88</sup>.

## **Pathophysiology of PTS**

It is thought that DVT results in ambulatory venous hypertension through a combination of venous obstruction and valvular dysfunction. The development of PTS appears to be multi-factorial, but some clinical studies have suggested that persistent and more proximal venous obstruction results in distal valvular incompetence and more severe PTS<sup>89-92</sup>. In canine models thrombolysis resulting in early resolution preserves both endothelial function and valve integrity compared with placebo<sup>93</sup>. It follows therefore that early removal of obstructing thrombus using interventional means may reduce long-term morbidity. Several prospective cohort studies have shown CDVT to be a safe and effective method of removing thrombus burden<sup>93;94</sup>. The CaVenT and ATTRACT studies are international multicenter studies comparing CDVT with anticoagulation<sup>95;96</sup>. Initial results have shown increased early vein patency with CDVT, it is not known however, if this will translate into long-term benefits<sup>95-97</sup>.

## Management of PTS

Compression therapy is currently the main treatment strategy for managing PTS. Stockings have proved beneficial in reducing the impact of PTS<sup>98</sup> and at the seventh American College of Chest Physicians conference, it was suggested that stockings should be used for at least 2 years following a DVT (2004)<sup>99</sup>. Compression strength and stocking length have not been clarified, although the below knee stocking appears far more acceptable to patients than the above knee. The evidence supporting pneumatic compression pumps is limited, however their use is restricted by impracticality for the wearer<sup>100</sup>. The Venowave (a light weight calf compression device) has been tested in a trial in patients with severe PTS based on the Villalta scale<sup>101</sup> and has shown some promise in reducing PTS scores at the end of an eight week trial period when compared with compression stockings<sup>102</sup>.

There is also emerging evidence that *venoactive* therapies such as aescin and rutosides give some symptomatic relief<sup>103</sup>. Horse chestnut seed extract (aescin) was shown in a Cochrane review to be effective for treating PTS symptoms with minimal adverse effects reported<sup>104</sup>. Therapies such as this require more robust assessment before they can be recommended for general use.

## 1.2 MECHANISM OF VENOUS THROMBUS FORMATION

### 1.2.1 HISTORICAL OVERVIEW

The first documented report of DVT came from Wiseman<sup>105</sup> in 1676 when he noted that following a difficult labour, his patient developed a swollen leg, he was the first to consider that the underlying cause was an abnormality in the circulation. In 1846, Hewson<sup>106</sup> hypothesised that venous thrombosis was caused by obstruction of veins by blood clots, with venous pooling playing a major role in this process. Interestingly Hewson<sup>106</sup> also believed that thrombus formation was triggered by lymph within the plasma; this substance was later renamed fibrinogen.

The most noteworthy understanding of DVT came from Virchow<sup>107</sup> and Rokitansky<sup>108</sup> although the latter is less well known. The triad of **vessel wall (endothelial injury), venous stasis and hypercoagulability** still remain fundamental to the understanding of venous thrombogenesis. It was noticed initially in conditions which leant themselves to elements of the triad i.e. in the later stages of pregnancy venous stasis was caused by immobility often in the form of bed rest. In addition pelvic vein trauma, caused by manipulation of the uterus, put patients at the highest risk of DVT immediately following delivery<sup>109</sup>. An increasingly elderly population has changed the demographics of those affected by DVT. In patients undergoing hip replacement without any form of prophylaxis, the incidence of DVT has been reported as high as 50%<sup>110</sup>, this in turn has renewed orthopaedic interest in surgical prophylaxis<sup>111</sup>.

## **Virchow's Triad Explored**

A venous thrombus consists of a laminar structure formed from layers of platelets, leucocytes and fibrin<sup>112</sup>. The laminar thrombus is structurally very different from what we consider a 'clot' which forms *ex vivo* or on circulatory arrest<sup>112</sup>. Virchow's triad categorises the different elements contributing to the formation of DVT as listed below: <sup>113;114</sup>

**Vessel wall**

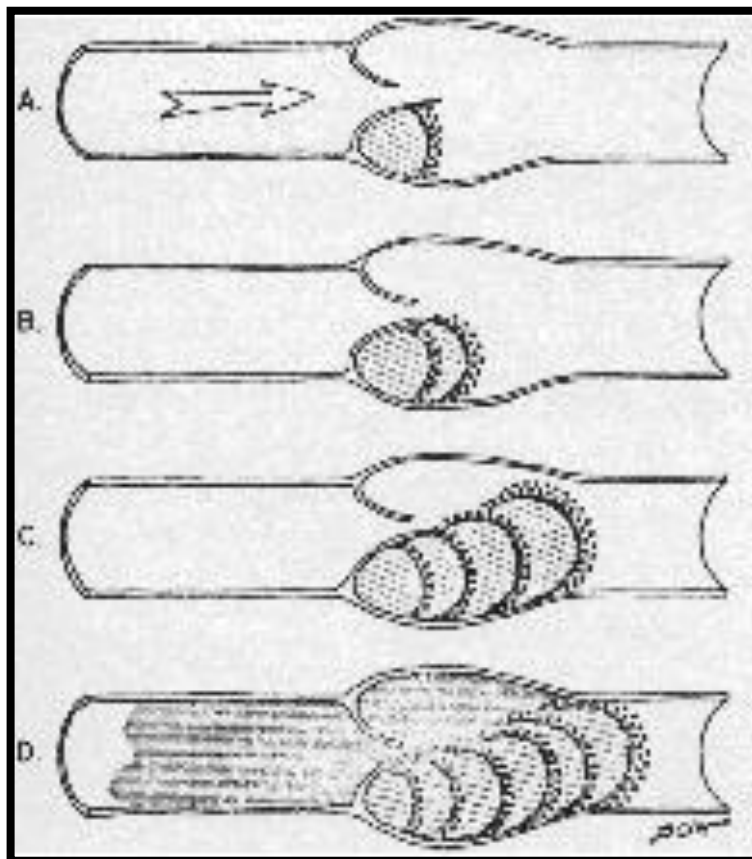
**Blood flow**

**Blood constituents**

The role of each of these elements has been explored through a combination of work in humans and animal models<sup>112;115-121</sup>.

### **1.2.2 VESSEL WALL / ENDOTHELIUM**

Sevitt's post-mortem work suggested that venous thrombi arise in both the valve pockets and dilated sinuses of the lower limbs<sup>115;122-124</sup>.



**Fig 1.2.1 The propagation of deep vein thrombi from a nidus in a valve pocket (A-C) and the deposition of successive layers of fibrin, platelets etc in a valve pocket, (D) retrograde thrombus propagation occurs when there is proximal venous obstruction (Reproduced from Sevitt<sup>123</sup>)**

The exact mechanism behind Sevitt's theory was unknown, but he hypothesised that a nidus consisting of red and white cells was held together by platelet strands<sup>124</sup>. The role of endothelial injury was also unclear as the majority of thrombi studied by Sevitt, were lying over macroscopically intact endothelium<sup>123;124</sup>. More recently examination of autopsy specimens of iliofemoral veins in patients who succumbed to VTE, revealed that Von Williebrand Factor (vWF); a haemostatic



glycoprotein was consistently present, in the same study anti-vWF antibody resulted in a reduction in thrombus burden in a rabbit model<sup>125</sup>. VWFs function in venous thrombosis appears to be distinct to its role in platelet adhesion and aggregation<sup>126</sup>, a reduction in platelet number or inhibition of aggregation does not appear to influence thrombus formation<sup>127-129</sup>. This substantiates the fact that venous thrombi are relatively platelet independent in contrast to platelet rich arterial thrombi<sup>116</sup>.

The endothelial lining of the vessel wall has several roles but most crucially, it maintains the balance between blood flow, fluidity and thrombus formation. **Table 1.2.1** lists both the anti-thrombogenic and pro-thrombotic factors associated with the endothelium<sup>130;131</sup>. Procoagulant factors are released during periods of endothelial disturbance initiating the inflammatory phase of thrombogenesis<sup>130;131</sup>. Animal models have been used to shed further light on the role of endothelium in thrombogenesis<sup>132-134</sup>. In a rabbit model of jugular vein thrombosis, isolated venous injury did not result in thrombus formation, however both platelets and leukocytes adhered to the site of injury, with leukocyte migration occurring through the vessel wall when a short period of stasis was also induced<sup>135</sup>. Endothelial injury combined with prolonged stasis however resulted in thrombus formation in 90% of test animals<sup>136</sup> suggesting that thrombus formation requires more than just vessel wall injury and probably a combination of at least two elements of Virchow's triad<sup>137-139</sup>.

**Table 1.2.1 Anti-thrombogenic and Procoagulant factors in the vein wall**  
(adapted from Becker<sup>130</sup>)

<b>Anti-thrombogenic factors</b>	<b>Procoagulant factors</b>
<b>Thrombomodulin</b>	<b>Platelet activating factor (PAF)</b>
<b>Heparin, dermatan sulfate</b>	<b>Endothelin-1</b>
<b>tPA and uPA</b>	<b>vWF (Von Willie Brand Factor)</b>
<b>Nitric oxide</b>	<b>Tissue factor</b>
<b>Prostacyclin</b>	<b>Plasminogen activator inhibitor (PAI)-I</b>
<b>Interleukin 10</b>	<b>Factor V</b>
<b>-</b>	<b>Adhesion molecules: P-selectin, E-selectin</b>

tPA-tissue plasminogen activator, uPA urokinase plasminogen activator

### **1.2.3 BLOOD FLOW**

Analysis of flow patterns behind venous valves in canine long saphenous veins has shown that under physiological conditions, cells continually entered venous valve pockets and created vortices<sup>140</sup>. Red cell aggregates formed within these vortices subsequently dissolving as the vortex blood re-entered luminal flow<sup>140</sup>. The partial pressure of oxygen rapidly decreased within valve pockets, causing a relative hypoxia during low flow states, that occur during periods of immobility<sup>141</sup>. Once flow was made pulsatile however (mimicking normal calf pump activity), oxygen levels within the valve pockets returned to that of the lumen<sup>141</sup>. Thrombus formation has been found in valve cusps as early as 2hrs after non-pulsatile flow<sup>141</sup>. This is consistent with data from gamma imaging studies which used radioactively

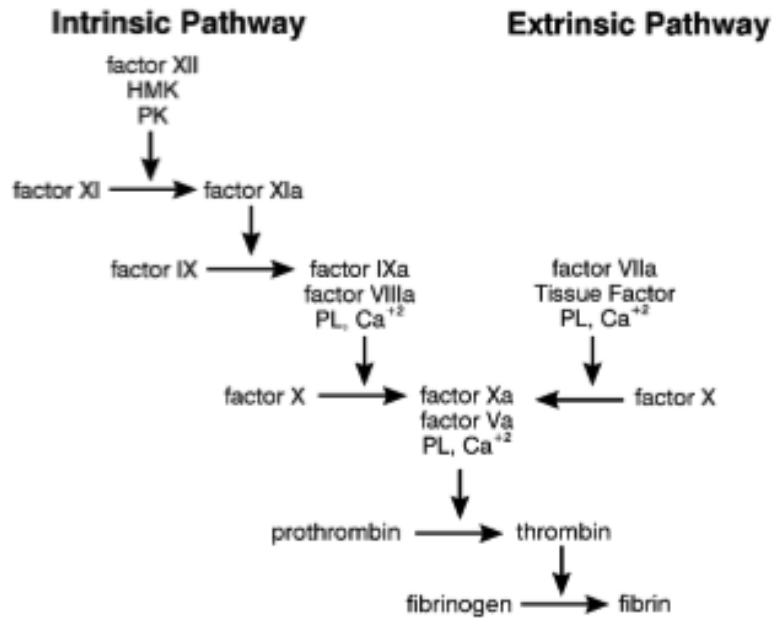
labelled fibrinogen to show that thrombus formation in limb veins occurred 2-4hrs after the beginning of surgery<sup>142</sup>. This has resulted in the use of intraoperative Flowtron™ stockings, which encourage lower limb venous return through pneumatic compression in bed bound surgical patients to reduce thrombotic events<sup>143</sup>. Pneumatic compression was thought to be effective by increasing lower limb blood flow but work by Giddings *et al.*<sup>144</sup> has shown that it also positively affects systemic haemostasis by increasing tissue factor pathway inhibitor (TFPI) and urokinase-type plasminogen activator (uPA) activity reducing the likelihood of thrombus formation<sup>144</sup>. TFPI is the only known serine protease inhibitor of the tissue factor (TF) protein a key element of the extrinsic pathway<sup>145</sup>. Upregulation of TFPI attenuates thrombosis in animal models<sup>146;147</sup>.

Hypoxia within valve pockets may stimulate thrombus formation by increasing the expression of the adhesion molecule P-selectin on the surface of endothelial cells<sup>148</sup>. P-Selectin and vWF are stored in secretory vesicles known as *Weibel-palade* bodies within endothelial cells<sup>149</sup>, their translocation to the cell surface leads to platelet and leucocyte adhesion to the endothelium<sup>150;151</sup>. P-selectin inhibition in a primate model of thrombosis resulted in diminished thrombus formation<sup>152;153</sup>. Its receptor P-selectin glycoprotein ligand 1 (PSGL-1) is found both on leucocytes and platelets as well as their respective microparticles<sup>154</sup>. Microparticles (MPs) are small phospholipid vesicles that are released from the surface of certain cells (platelets, leucocytes and endothelial cells). Once thought to occur only in disease states, they are now believed to occur physiologically<sup>154</sup>.

Thrombin and hypoxia amongst other mediators are thought to trigger their release, the evidence for this however, remains sparse<sup>155</sup>. MPs containing TF have been shown to accumulate at sites of venous stasis and thrombus formation in a P-selectin dependent fashion<sup>156</sup>. The role of MPs in thrombosis is likely to be mechanism dependent, i.e. if there is significant vascular trauma exposing vein wall TF then thrombosis here is likely to be MP independent, at least in the first instance<sup>157</sup>.

#### **1.2.4 BLOOD CONSTITUENTS**

Haemostasis was historically described as a cascade or a waterfall model involving various clotting factors as part of the extrinsic or intrinsic pathways<sup>158</sup>. This model highlights the interactions of the pro-coagulant proteins as a series of lytic reactions culminating in a common final pathway which results in the formation of fibrin (**Fig 1.2.2**)<sup>158</sup>. It also identified that the anionic phospholipid surface provided by platelets or the injured vessel wall was essential for optimal function of the cascade. The artificial separation into separate pathways and the absence of cellular elements from the cascade has limited its application to *in vivo* haemostasis and thrombosis.



**Fig 1.2.2 Cascade model of coagulation**

(adapted from Hoffman<sup>158</sup>)

In the last decade the **cell based model** (Fig 1.2.3) of coagulation has been accepted as a much more comprehensive view of intravascular haemostasis. It can be divided into three overlapping phases:

### Initiation

TF was previously known as the initiator of the extrinsic pathway in the clotting cascade but it is now generally accepted that TF initiates *in vivo* coagulation. It has both sub-endothelial and circulating isoforms and is expressed on several cell types, including stromal fibroblasts, endothelial cells, platelets and leukocytes<sup>147</sup>. Recently evidence has also been found of the existence of TF in MPs as

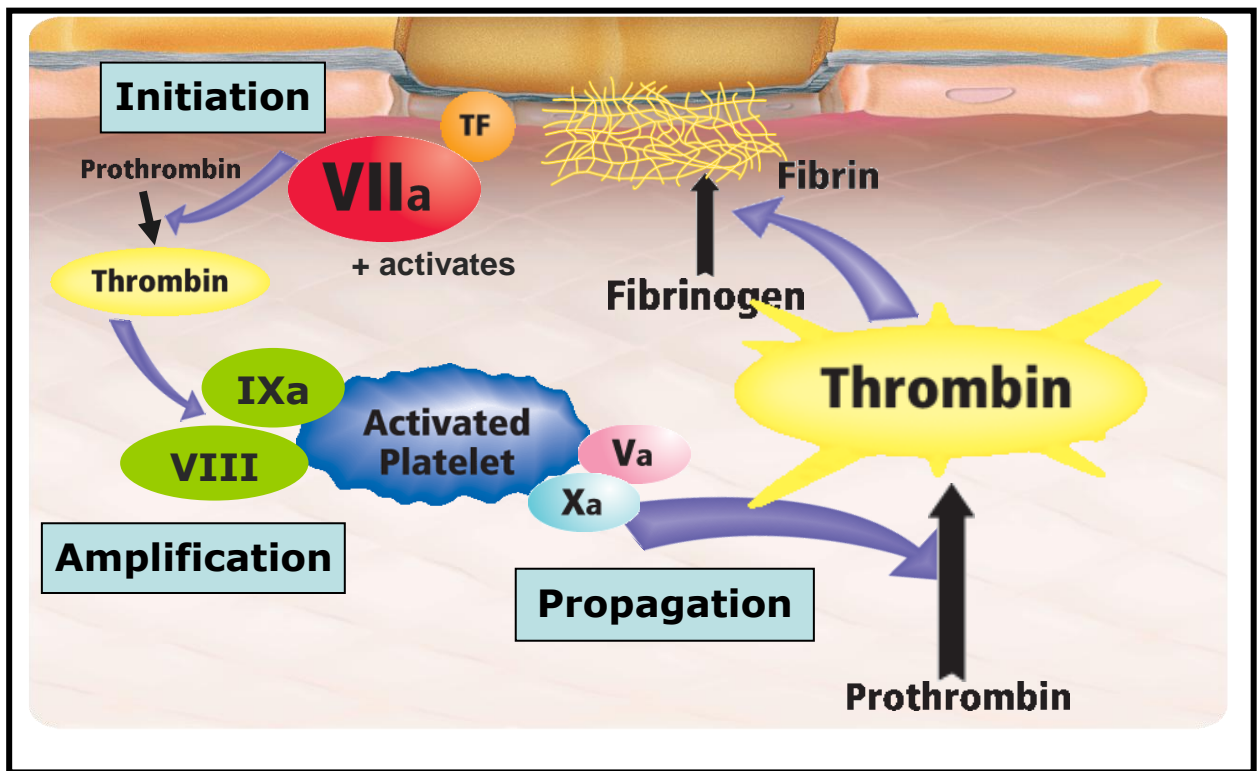
mentioned above<sup>159</sup>. Exposed TF (vein wall and MP derived) activates **FVII**, the **TF/FVII** complex then activates **FX** and **FIX**, **FXa** then activates **FV**<sup>160</sup>. Any cell surface bound **FXa** and **FV** convert a small amount of the zymogen; prothrombin to thrombin.

### **Amplification**

The small amount of thrombin generated on TF exposed cells then fully activate platelets through protease-activated receptors (PARs): PAR1 and PAR4 and also factors V, VIII and XI<sup>161</sup>.

### **Propagation**

This occurs at the platelet surface which coordinates the assembly of the tenase complex (**FVIIIa/ FIXa**), this activates **FX** which then complexes with **FV** to form the prothrombinase complex producing a burst of thrombin which converts soluble fibrinogen to fibrin<sup>160</sup>.

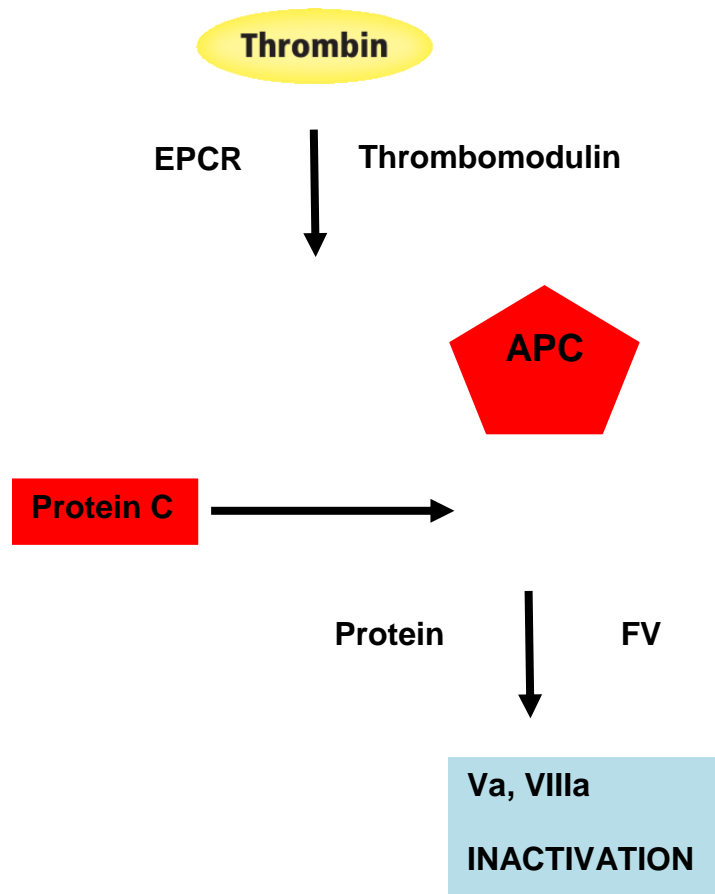


**Fig 1.2.3 Cellular model of the clotting cascade**

(adapted from Hoffman<sup>162</sup>)

### Termination of thrombosis

Activated protein C (APC) terminates the haemostatic cascade by proteolytically cleaving and inactivating **Va** and **VIIIa**. This pathway is activated by proteolytic cleavage of protein C by thrombin. This reaction is catalysed by both thrombomodulin (TM) and the endothelial protein C receptor (EPCR) (**Fig 1.2.4**). The primary objective of this system is to confine thrombin formation to the area of injury. A safety mechanism which prevents widespread thrombogenesis includes the protease inhibitors ATIII and TFPI<sup>160</sup>.



**Fig 1.2.4 Termination of haemostatic mechanism**

(adapted from Bertina <sup>160</sup>) EPCR (endothelial protein C receptor), APC (Activated protein C)

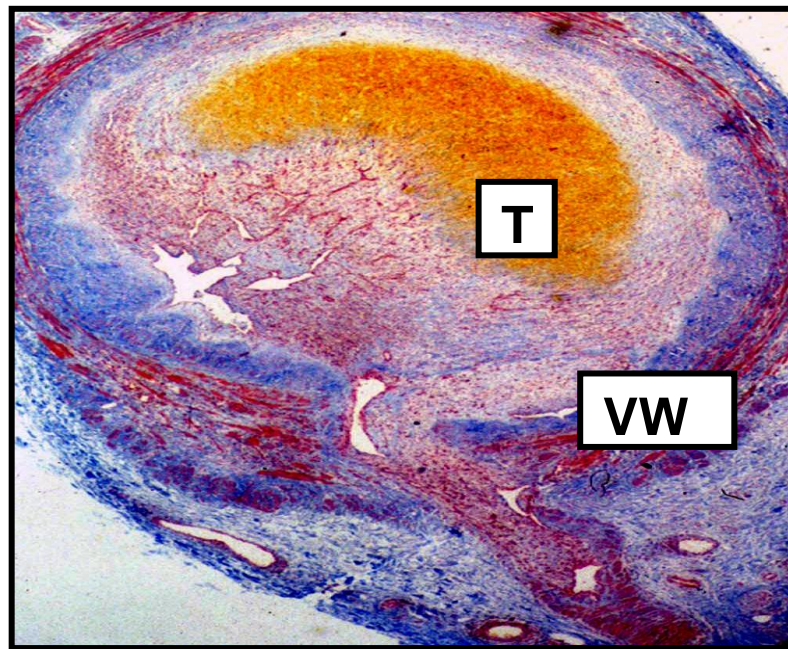


## 1.3 VENOUS THROMBUS RESOLUTION

### 1.3.1 ORGANISATION

Natural thrombus resolution occurs through a process of organisation that is accompanied by the recruitment of a variety of cells, into the thrombus (**Fig 1.3.1**).

This includes neutrophils, monocytes, myofibroblasts, endothelial cells and their progenitors, from the vessel wall and the circulation<sup>120;163-167</sup>. This influx of cells facilitates the organisation and resolution of the thrombus in a process reminiscent of that seen during normal wound healing<sup>120;163;168;169,170</sup>.



**Fig 1.3.1 Histological section of a resolving human thrombus**

Dense cell infiltrate (dark nuclei), deposition of collagen (bright blue) and formation of recanalising channels in the thrombus. T- Thrombus, VW-Vein Wall

## Early inflammatory phase

An acute inflammatory response results in early thrombus amplification and organisation<sup>157</sup>. The early phase of thrombus resolution is associated with a neutrophil influx, neutrophils are polymorphonuclear (PMN) cells sometimes referred to as granulocytic because they contain small packages of preformed proteins<sup>171;172</sup>. They have a fairly short half life of 7-12hrs which is modulated by the presence of infection or inflammation<sup>172</sup>. Mature neutrophils are terminally differentiated cells that develop from CD34+ bone marrow precursors under the influence of various cytokines: interleukin-3 (IL-3), granulocyte-CSF (G-CSF) and granulocyte macrophage-CSF (GM-CSF)<sup>173</sup>. Many of these cytokines continue to influence neutrophil activity outside the bone marrow<sup>174</sup>.

Previously an artificial model of thrombosis using stasis (The Chandler Loop) showed that PMN dependent uPA was responsible for resolution<sup>175</sup>. Subsequently in a rat model of thrombosis neutropenia resulted in a 60% increase in overall thrombus size, increased vein wall stiffness and fibrotic changes<sup>176</sup>. There was also a neutrophil dependent reduction in uPA which was two-fold lower in neutropenic animals<sup>177</sup>. This was associated with changes in local matrix metalloproteinases (MMPs), most noticeably a reduction in thrombus MMP9 levels and a concomitant increase in MMP2 levels in the vein wall, 2 days following thrombosis<sup>177</sup>.

## **Late inflammatory phase**

Monocytes are a large heterogeneous population of circulating leucocytes of myeloid lineage. They were once thought to be merely a precursor cell to macrophages, but over the last decade it has come to light that they in themselves have both inflammatory and anti-inflammatory roles<sup>178</sup>. Monocytes express a large variety of inflammatory mediators and growth factors that are known to orchestrate tissue remodeling<sup>165;179</sup>. They are recruited into the thrombus with a characteristic temporal and spatial pattern, peaking at day 8 after thrombosis<sup>119;180</sup>. Monocyte chemoattractant protein 1 (MCP-1) is the primary orchestrator of monocyte chemotaxis and activation, its upregulation enhances thrombus resolution<sup>179</sup>. Thrombus resolution is impaired in CCR-2 (MCP-1 receptor) knockout models, but not in MCP-1 knockout animals. This suggests that there are likely to be other MCP subtypes<sup>181</sup>. MCP-1 also appears to effect thrombus resolution independent of its effect on monocytes<sup>179</sup>.

### **1.3.2 FIBRINOLYSIS**

Several proteins are involved in controlling the ultimate dissolution of the thrombus. Plasmin is a key protein in fibrinolysis, plasminogen (its non-active form) binds to lysine and arginine residues on fibrin through secondary structural motifs known as kringles<sup>182</sup>. Plasminogen activators, uPA and tPA (serine proteases) activate plasminogen and are therefore important mediators of fibrinolysis and cell migration<sup>183;184</sup>. Plasminogen activator inhibitor 1 (PAI -1) secreted from both liver and endothelial cells is their primary inhibitor<sup>185</sup>. PAI-1 deficiency in mice makes them highly resistant to thrombosis<sup>186</sup>.

This has made the inhibition of PAI-1 increasingly attractive as an alternate therapeutic option to treat DVT<sup>186</sup>. Plasminogen activators are well recognised for their effectiveness in the lysis of coronary artery thrombi<sup>187;188</sup>. Work in mouse knockout models has shown that thrombus resolution is diminished in uPA deficient but not tPA deficient animals<sup>189</sup>. Rescuing the uPA<sup>-/-</sup> phenotype with wild-type bone marrow transplant rescues normal thrombus resolution<sup>189</sup>. This is in keeping with the theory that bone marrow (BM) plays an important role in thrombus resolution.

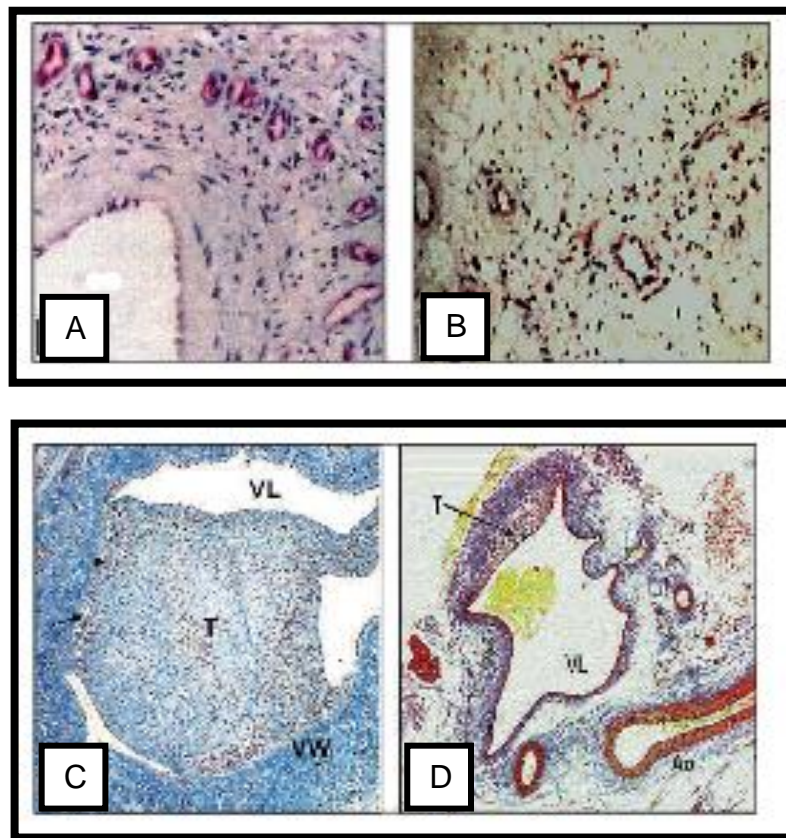
### **1.3.3 VEIN WALL REMODELLING**

Vein wall remodeling occurs during the inflammatory response that ensues after thrombus formation<sup>186;190</sup>. The early invasion by PMNs is essential for fibrinolysis and collagenolysis<sup>157</sup>, and crucially they must be present early during thrombus resolution<sup>176</sup>. The occurrence of elastinolysis within the vein wall, results in wall stiffening and correlates with elevated levels of MMP2 and MMP9 as well as well as collagen I and III gene expression<sup>157</sup>. The thrombosis related vein wall injury results in elevated levels of profibrotic mediators such transforming growth factor  $\beta$ , IL-3 and MCP-1<sup>191</sup>. Although MCP-1 is known to mediate resolution, it also appears to correlate with vein wall stiffness<sup>192</sup>. The inflammatory changes within the vein wall respond to treatment with LMWH or oral P-selectin, reducing both vein wall stiffness and thickness<sup>192</sup>. MMP9 levels are raised following venous thrombosis, gene deletion studies have however, shown that it is not essential for thrombus resolution but may play a more significant role in vein wall remodeling<sup>193</sup>.

## 1.4 THE ROLE OF PROGENITOR CELLS IN THROMBUS RESOLUTION

### 1.4.1 POST-NATAL PROGENITOR CELLS

The concept that as fully developed adults we can produce cells capable of endothelial regeneration has been a revolution in the world of vascular biology<sup>194-201</sup>. Early evidence for post-natal vasculogenesis came from patients who underwent bone marrow or peripheral stem cell transplantation and subsequently developed a chimeric endothelial lining to their vasculature<sup>202</sup>. This pointed to the post-natal existence of circulating PCs. Indirect evidence has come from studies correlating a reduction in putative PCs with increased atherosclerotic morbidity and cardiovascular risk<sup>203;204</sup>. There has been a large amount of work looking at the characterisation, function and role of these cells in the neovascularisation within the context of tumorigenesis, coronary artery disease and peripheral vascular disease<sup>200;205-214</sup>. It has been suggested that by manipulating PCs, it may be possible to accelerate wound healing, tissue regeneration and new vessel formation<sup>215</sup>. Thrombus resolution is an active and organised process which involves clot retraction and formation of endothelial lined bypass channels within the body of the thrombus. In human and rat thrombi, vascular channels form early in the resolution process and stain with the endothelial cell markers CD31 and vascular adhesion molecule VCAM-1(**Fig 1.4.1**)<sup>120</sup>.

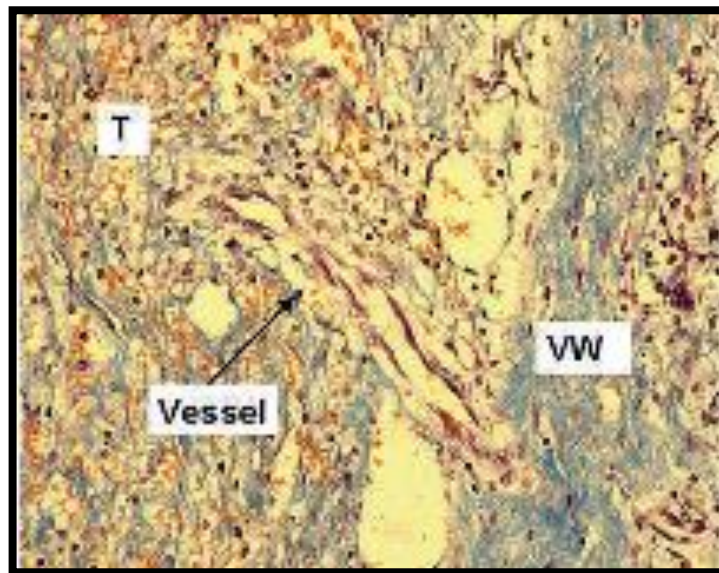


**Fig 1.4.1 Recanalisation in human and murine thrombus**

(A) VCAM-1 staining (B) CD31 staining channels formed within thrombi (C) H&E stain of rat thrombus at 2 weeks (D) MSB stain of 4 week thrombus showing residual vein wall thickening (adapted from Moderai<sup>120</sup>)

In an elegant study *Moderai et al.*<sup>209</sup> demonstrated a temporal pattern in the influx of bone marrow derived progenitor cells into the resolving mouse thrombus<sup>209</sup>. They subsequently went onto to demonstrate that adenoviral upregulation of intra-thrombus vascular endothelial growth factor (VEGF) enhanced thrombus resolution and upregulated Sca-1+ hematopoietic progenitor cells (HPCs) in the circulation<sup>216</sup>.

Human PCs peripherally injected into nude rats following thrombus formation has demonstrated enhanced recanalisation and increased blood flow when compared with controls animals<sup>217</sup>. Much like thrombus formation, resolution appears to be a highly organized process, involving both clot retraction and new vessel formation (**Fig 1.4.2**)<sup>120;218</sup>.



**Fig 1.4.2 New vessel formation in rat thrombus**

Cross section through rat thrombus showing a vessel  
Growing into the (T) thrombus mass from the (V) vein wall  
(adapted from Modera<sup>120</sup>)

New vessel formation occurs through two processes: **angiogenesis** and **vasculogenesis**, which may be PC dependent.

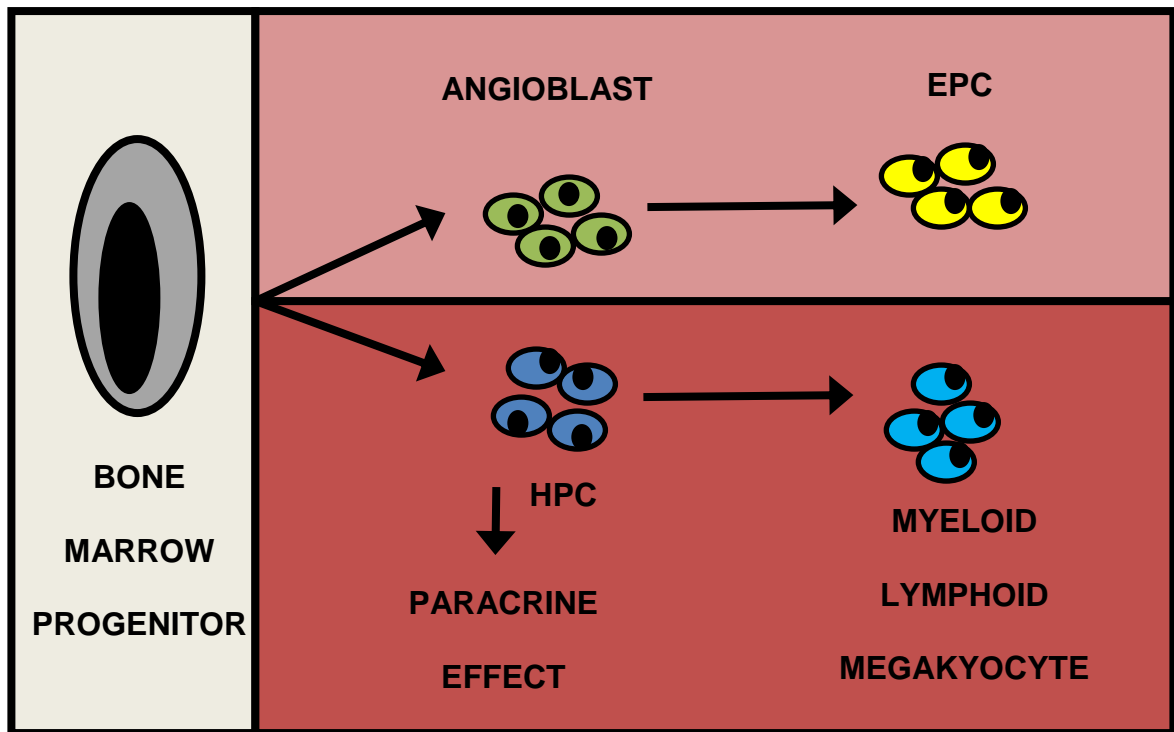
**Angiogenesis** is the sprouting of blood vessels from pre-existing vascular structures and occurs during normal physiological and pathological processes<sup>219</sup>.

**Vasculogenesis** is the formation of blood vessels *de novo* from precursor or progenitor cells<sup>194</sup>. This process was thought only to occur in the prenatal period until *Asahara et al.*<sup>220</sup> first demonstrated that PCs persisted into adult life.

#### 1.4.2 HAEMATOPOETIC PROGENITOR CELL CHARACTERISATION

Although there is no agreement as to what objectively defines a PC in terms of its biology<sup>221</sup>, theoretically these cells are likely to reside within the bone marrow (BM) and are capable of self-renewal and regeneration<sup>222</sup>. The BM is a source of both mesenchymal and HPCs, (**Fig 1.4.3**). HPCs are known to give rise to red cells, leucocytes, endothelial cells and platelets<sup>223</sup>. HPCs are capable of reconstituting the haematopoietic system of lethally irradiated mice after transplantation of even a single HPC<sup>224</sup>. Current evidence suggests that HPCs indirectly promote neovascularisation but are unlikely to be the precursors of post-natal endothelial cells i.e. endothelial progenitor cells (EPCs)<sup>225</sup>.





**Fig 1.4.3 HPC development from bone marrow progenitor cells**

The practical characterisation of PCs particularly HPCs remains controversial partly because of the array of techniques used for their identification<sup>206;226</sup>. Cell culture techniques and flow cytometric analysis of peripheral blood have formed the basis of a number of PC related papers<sup>195;198;202;209;214</sup>. In order to better comprehend what we know about PCs, it is first necessary to understand how both of these techniques have been developed over the last ten years.

### **Cell culture and colony forming units (CFUs)**

Culture of peripheral blood mononuclear cells has produced spindle like cells with an endothelial morphology<sup>227</sup>. These cells can be labelled with VEGFR2 (vascular endothelial growth factor receptor 2), Tie-2 (tyrosine kinase receptor in vascular

endothelium) and VE-Cadherin (endothelial specific cadherin)<sup>198</sup>. They also positively stain with Lectin Europeaus Agglutinin-1 (UEA-1) and acetylated Low Density Lipoprotein (AcLDL). Under certain culture conditions these cells form colonies i.e. clusters of cells with rounder central cells and spindle like cells at their periphery, also known as colony forming units (CFUs) which have been used as a surrogate marker of PC number and function<sup>228-230</sup>.

Several methods have since been used to grow CFUs from peripheral blood<sup>231</sup>. Characterisation of the cultured cells has revealed that only the method used by Lin<sup>232</sup> gives rise to colonies containing cells with a high proliferative capacity. These cells are known as **late colonies**, because of their latent formation when compared to **early colonies** generated by the Hill<sup>231</sup> or Ito<sup>228</sup> methods (**Table 1.4.1**). The method used by Hill *et al.*<sup>231</sup> gained popularity because of its inverse correlation to the Framingham cardiovascular risk score, giving it greater notoriety<sup>229</sup>.

**Table 1.4.1 Differences in Early and Late Colony Forming Units**

ALDH-aldehyde dehydrogenase, UEA-1 Ulex erupeaus agglutinin-1, acLDL-acetylated low density lipoprotein, eNOS-endothelial nitric oxide synthase  
(adapted from Yoder<sup>233</sup>)

	CFU Hill	CFU Lin
<b>Clonal Proliferation</b>	No	Yes
<b>Passage ability</b>	No	Yes
<b>In vitro tube formation</b>	Partial	Yes
<b>De novo vessel formation</b>	no	Yes
<b>Ischaemic homing</b>	Yes	Yes
<b>Paracrine augmentation of angiogenesis</b>	Yes	Yes
<b>Phagocytosis of bacteria</b>	Yes	No
<b>Non-specific esterase expression</b>	Yes	Yes
<b>Phenotypic characterisation</b>	CD34+/-	CD34+
	CD133+	CD133-
	VEGFR2+	VEGFR2+
	CD45+/-	CD45-
	CD14+/-	CD14-
	CD115+	CD115-
	CD31+	CD31+
	ALDH <sup>bright</sup>	ALDH <sup>bright/lo</sup>
	acLDL uptake	acLDL uptake
	UEA-1 lectin binding	UEA-1 lectin binding
	eNOS+	eNOS+
	Von williebrand +	Von williebrand +

The validity of CFU staining with UEA-1 and AcLDL has recently been questioned as UEA-1 recognises the L-fucosylated molecules on the cell surface of endothelial cells, and this molecule has now been found on epithelial cells and platelets<sup>234;235</sup>. It has been suggested that CFU cultures are being contaminated by platelets which form microparticles that fuse with and contribute to the cell surface markers of CFUs, allowing them to bind UEA-1<sup>236</sup>. Analysis of CFU mRNA also corroborates this finding as it shows that these cells do not contain the necessary mRNA for the proteins expressed on their cell surface<sup>236</sup>. Secondly AcLDL is taken up by monocytes as well as endothelial cells, proteomic comparison of monocytes and putative EPCs has shown an almost 80% congruency and although this means they are not identical it suggests that differentiating between them using UEA-1 and AcLDL is likely to be flawed<sup>237;238</sup>.

### **Flow cytometry**

Flow cytometry is used to identify cells based on the expression of cell surface and intracellular markers. Its strength rests in the ability to analyse large numbers of cells rapidly and efficiently. Using two or three cell markers to adequately identify PCs is a limited method for identifying a cell with a complex phenotype, but has been the preferred method for analysing these cells in fresh blood samples<sup>239</sup>. There are numerous antigens which have been used to identify PCs both by their presence or absence (**Table 1.4.2**).

**Table 1.4.2 Endothelial progenitor cell markers used to identify EPC**

HSC-Haematopoietic stem cell, MSC-Mesenchymal stem cell, EPC-Endothelial progenitor cell

Marker	Expressed
CD133 (AC133) <sup>240</sup>	HSCs, EPCs , neural and glial stem cells
Sca-1 (Stem cell antigen 1) <sup>241</sup>	Early stem cell
CD34 <sup>220</sup>	MSCs, HSCs, EPCs
VEGFR2 (Vascular endothelial growth factor receptor 2) <sup>220</sup>	EPCs, mature endothelial cells
CD31 (PECAM-1: Platelet/endothelial cell adhesion molecule 1) <sup>242</sup>	Platelets, EPCs, macrophages
Von Williebrand factor <sup>243</sup>	Endothelial cells, Weibel Palade bodies, megakaryocytes
CD45 <sup>225</sup> (used as negative marker)	Lymphocytes, monocytes, granulocytes

The original combination of CD34 and VEGFR2 used by Asahara<sup>220</sup> to characterise the putative *endothelial progenitor cell* initially gained popularity as these cells were incorporated into the vasculature of ischaemic rabbit hind limbs in a manner consistent with them becoming ECs<sup>220</sup>. It is now known however that mature ECs also express CD34 and VEGFR2<sup>244</sup> and in order to exclude them a third antigen denoting a more immature cell type i.e. CD133 (human), Promonin-1(mice) or Sca-1(mice), which are not found on mature endothelial cells have been used in combination with CD34 and VEGFR2<sup>245</sup>. Despite the extensive literature using

only two of CD34, CD133 or VEGFR2<sup>221;246-249</sup>, triple positive CD133+CD34+VEGFR2+ cells are still considered by many to represent the gold standard progenitor cell with an endothelial phenotype<sup>227</sup>. These cells however do not form tubular structures in either *in vitro* studies nor do they participate in the formation vasculature channels *in vivo* making them more likely to be HPCs<sup>244;250</sup>. This has lead to the conclusion that these cells are effecting change through the paracrine production of cytokines and not by lining newly formed vascular channels<sup>251</sup>. A lack of consensus regarding what defines EPCs and HPCs has made the study of this area increasingly difficult.

#### **1.4.3 REGULATION OF BONE MARROW MOBILISATION AND RECRUITMENT**

Since the discovery of circulating progenitors by Asahara<sup>252</sup>, a large number of chemokines have been implicated in progenitor mobilisation and recruitment **(Table 1.4.3)**.

**Table 1.4.3 Factors responsible for progenitor mobilisation**

<b>Factors influencing progenitor mobilization</b>	<b>Receptors</b>	<b>Role</b>
<b>VEGF</b> Vascular endothelial growth factor	VEGFR-1, VEGFR-2, VEGFR-3	Physiological and pathological angiogenesis <sup>195;253</sup>
<b>PLGF</b> Placental derived growth factor	VEGFR-1 MMP-9 mediated	Pathological angiogenesis <sup>195;253</sup>
<b>SDF-1</b> Stromal derived growth factor	CXCR4	Stem cell mobilisation from bone marrow niche <sup>254</sup>
<b>GCSF</b> Granulocyte stimulating factor	GCSF receptor MMP-9 mediated	Reduction of SDF-1 in bone marrow and increased CXCR4 <sup>255</sup>
<b>GMCSF</b> Granulocyte-Macrophage Colony Stimulating Factor	GMCSF receptor + others	Stem cell mobilisation into circulation + homing to sites of ischaemia <sup>256</sup>
<b>Epo</b> Erythropoietin	Epo –R	stimulates proliferation of early erythroid precursors <sup>257</sup>
<b>Ang-1</b> Angiopoietin-1	Tie-2 , alpha(5)beta(1), alpha(v)beta(5) integrins	Embryonic vascular development and tumor angiogenesis <sup>258</sup>
<b>SCF</b> Stem cell factor	C-kit	Stem cell mobilisation <sup>259</sup>
<b>MCP-1</b> Monocyte chemoattractant protein	CCR-2	Tumor angiogenesis <sup>260</sup>
<b>MMP-2, MMP-9</b>	Metalloproteinase receptor (TIMP)	Increase vascular permeability for stem cell migration <sup>261</sup>
<b>Statins</b>	? VEGFR-2	VEGF mediated stem cell mobilisation <sup>262</sup>
<b>Exercise</b>	Unknown mechanism	Increases circulating EPCs via ischaemic conditioning <sup>263</sup>

It is almost certainly the intricate interaction of several factors which results in PC mobilisation into the circulation and recruitment to areas of injury and hypoxia<sup>249;264;265</sup>. The cytokines measured during thrombus resolution in this study are reviewed in greater detail below:

### **Granulocyte colony stimulating factor (GCSF)**

GCSF is a 25 kDa glycoprotein secreted by monocytes, macrophages, and neutrophils after cellular activation. It is also produced by stromal cells, fibroblasts and endothelial cells<sup>266</sup>. The GCSF receptor CD114 is expressed on all cells of granulocytic lineage as well as being expressed on placental, endothelial, cardiac myocytes and some tumor cell lines<sup>267</sup>. It has been used successfully in the clinical setting as Filgrastim to treat neutropenia, as well as prior to bone marrow transplantation to induce the release of bone marrow stem cells (BMSCs) into the circulation<sup>267</sup>. The realisation that it was produced by infarcted myocardium<sup>268</sup> resulted in animal studies in which GCSF administration resulted in mobilisation of BMSCs to the infarcted heart in parallel with improved cardiac function<sup>269-271</sup>. This led to several randomised control trials which sadly weren't able to produce the desired clinical response (**Table 1.4.4**).



**Table 1.4.4 Trials assessing benefit of GCSF after myocardial infarction**  
(adapted from Kurdi<sup>272</sup>)

Trial	Outcome
Stem Cells in Myocardial Infarction (STEMI) <sup>273</sup>	No improvement, no obvious adverse effects
Reinfusion of Enriched Progenitor Cells And Infarct Remodelling in Acute Myocardial Infarction (REPAIR-AMI) <sup>274</sup>	Moderate improvement in ejection fraction
Front-Integrated Revascularisation and Stem Cell Liberation In Evolving Acute Myocardial Infarction by Use of GCSF (FIRSTLINE-AMI) <sup>275</sup>	Improved LV wall thickening and wall motion in infarction zone with dobutamine stress
Regenerate Vital Myocardium by Vigorous Activation of Bone Marrow Stem Cells (REVIVAL -2) <sup>276</sup>	No difference in LV function

We now know that GCSF also has stem cell independent cardio-protective effects<sup>268</sup>. GCSF also appears to mobilise bone marrow CXCR4 through upregulation of stromal derived factor 1 (SDF-1) and matrix metalloproteinase 1 (MMP-1)<sup>277</sup>. In a mouse model of thrombosis, the expression of circulating CCR2 mRNA (the MCP1 receptor) increased following subcutaneous injection of recombinant GCSF<sup>278</sup>. GCSF injection resulted in greater macrophage ingress into the thrombus and enhanced resolution and organisation characteristics compared with the sham control<sup>278</sup>. The mechanisms by which GCSF produces its effects still require further investigation before we embark on further clinical trials. Studies with

animal models use otherwise healthy young animals and do not always translate clinically as trial patients are generally from an aged population resulting in a varied progenitor cell response to GCSF<sup>272;279</sup>. A study in rabbits examined this age related phenomenon and found that GCSF lost its efficacy in infarction remodeling in older animals<sup>280</sup>. Whilst we are continually advancing our knowledge of GCSF, it is not yet clear as to whether it will be useful as a mobilising agent for HPCs<sup>272</sup>.

### **Granulocyte macrophage colony stimulating factor (GMCSF)**

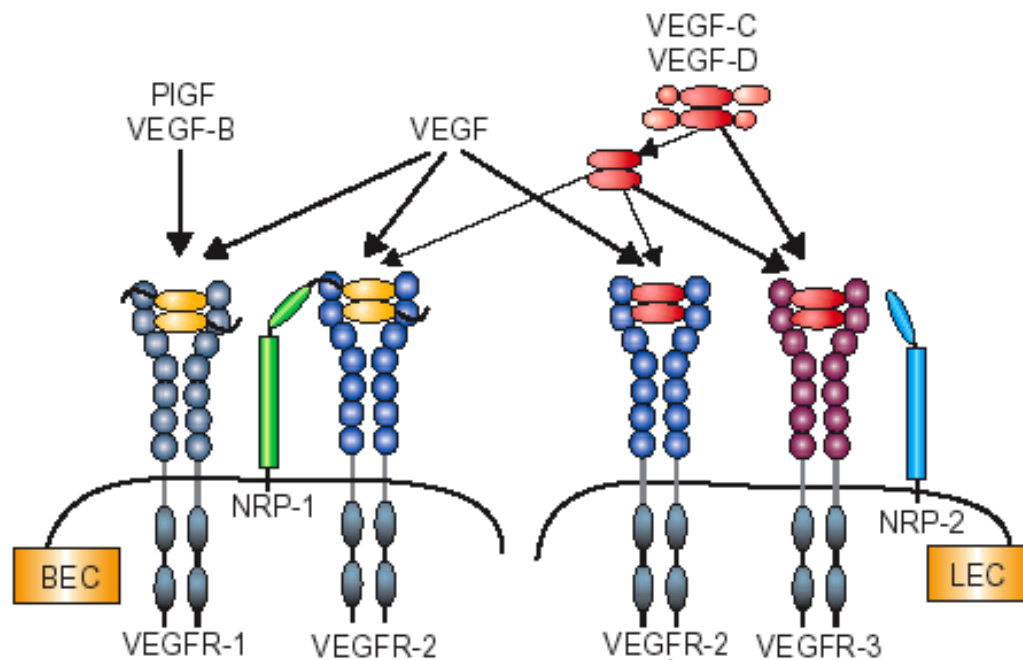
GMSCF is a 25 kda protein in its glycosylated form and like GCSF it is best known for its use in bone marrow transplantation<sup>256</sup>. Under the influence of various interleukins and tumor necrosis factor (TNF) it can be secreted from several cell types including fibroblasts, chondrocytes, smooth muscle, endothelial cells and inflammatory cells<sup>281</sup>. GMCSF mobilises neutrophils as well as cells of a macrophage lineage via the CD116 receptor<sup>256</sup>. Systemic administration of GMCSF mobilises progenitors from the BM compartment into ischemic hind limbs<sup>282</sup>. Both GCSF and GMCSF also appear to accelerate the healing process of damaged endothelium in animal models<sup>283;284</sup>. Studies in patients with coronary artery disease have shown that GMCSF improves collateral circulation<sup>285</sup>. Although there is no definitive evidence that GMCSF results in the mobilisation of CD34+ progenitor cells<sup>283;284</sup>, it does appear however to enhance the mobilisation of PCs when combined with other chemokines such as SDF-1<sup>286</sup> and GCSF<sup>287</sup>

### **Stromal derived factor 1 (SDF1)**

SDF1 also known as CXCL12 is an 8.5 kDa protein which is part of the C-X-C chemokine family. It is produced by multiple bone marrow stromal cell types as well as epithelial cells<sup>288</sup>. It has only one known receptor, CXCR4 which is expressed by a number of cell types including hematopoietic, immature endothelial, stromal and neuronal cells<sup>289</sup>. It is implicated in the migration, proliferation, differentiation of both murine and human hematopoietic stem cells<sup>290;291</sup> and is involved in homing of putative PCs to sites of vascular and cardiac ischemia<sup>292;293</sup>. SDF1/CXCR4 murine knockout embryos develop multiple defects which are often fatal<sup>294</sup>. The SDF1 gradients established between the circulation and BM compartments are thought to control the direction of progenitor mobilisation and migration<sup>295;296</sup>. Platelets also express CXCR4 and respond to SDF-1 gradients<sup>295</sup>. The binding of platelet MPs to immature CD34 progenitors via P-Selectin and Mac-1 integrin receptors may be the mechanism by which CD34+ progenitors home to sites of thrombosis<sup>293</sup>.

### **Vascular Endothelial Growth Factor (VEGF)**

It was initially designated Vascular Permeability Factor (VPF) by Senger *et al.*<sup>297</sup> because of its histamine like ability to increase capillary permeability. It was renamed VEGF when it became evident that it also possessed some mitogenic activity particularly in relation to EC replication<sup>298</sup>. It is probably the most potent angiogenic factor in man and other mammals, with 5 major subtypes (A-D and PlGF (Placental Growth Factor)) which have varying affinities for the 6 receptor subtypes (**Fig 1.4.4**)<sup>299</sup>.



**Fig 1.4.4 VEGF subtypes and their receptor affinities**

(NRP-neuropilin receptor) <sup>299,300</sup>.

VEGF (**A**) is the main circulating form in mammals and plays an important role in regulating vascular development, it has several distinct isoforms, with equivalent mouse isoforms being a single amino acid shorter (**Table 1.4.5**)<sup>301</sup>. It is thought that the VEGF<sub>121</sub> isoform is the most diffusible whereas VEGF<sub>165</sub> (the main circulating form in man and VEGF<sub>164</sub> in mice) have both matrix bound and diffusible components<sup>302</sup>. Mouse knockout models of VEGF<sub>164</sub> are fatal either at birth or within two weeks, whereas VEGF<sub>120</sub><sup>-/-</sup> results in live birth with deficient endothelial distribution (**Table 1.4.6**)<sup>303;304</sup>.

**Table 1.4.5 VEGF, human and mouse isoforms**

<b>Human</b>	<b>Mouse</b>
<b>VEGF 121</b>	<b>VEGF 120</b>
<b>VEGF 145</b>	<b>VEGF 144</b>
<b>VEGF 165</b>	<b>VEGF 164</b>
<b>VEGF 189</b>	<b>VEGF 188</b>
<b>VEGF 206</b>	<b>VEGF 205</b>

**Table 1.4.6 VEGF & VEGFR knockouts**

<b>Gene</b>	<b>+/-</b>	<b>-/-</b>	<b>Phenotype</b>
<b>VEGFA</b>	lethal	lethal	absence of vasculature
<b>VEGFB</b>	viable	viable	↓ heart size, ↓ recovery from MI,
<b>VEGFC</b>	?	lethal	no lymphatic development
<b>VEGFD</b>	?	?	?
<b>PLGF</b>	viable	viable	↓ angiogenesis & arteriogenesis
<b>VEGFR1</b>	viable	viable	↓ EC formation, abnormal vessels
<b>VEGFR2</b>	viable	lethal	↓ Haematopoietic precursors, no blood vessels
<b>VEGFR3</b>	lethal	lethal	?

Its place in prenatal vasculogenesis came to light through *in utero* gene targeting studies; VEGF is crucial during early prenatal development, important in controlling vascular permeability, as well as the maintenance and protection of ECs<sup>305;306</sup>. There is now considerable evidence that VEGF plays a vital role in vasculogenesis<sup>253</sup>. Firstly, over expression of VEGF in rodent muscle results in the formation of newly formed blood vessels and is accompanied by infiltration of the same area with cells which carry markers of both bone marrow lineage (Sca1) and endothelial lineage (CD34 and VEGFR2)<sup>307</sup>. Secondly, systemic administration of VEGF results in upregulation of circulating progenitor cells and enhanced blood flow in cardiac and hind limb ischaemia models<sup>274;308</sup>.

The mechanisms controlling VEGF release are no less complex. Hypoxia appears to be the most potent factor in increasing both *in vivo* and *in vitro* VEGF expression<sup>309;310</sup>. VEGF is regulated at the transcriptional, posttranscriptional and translational levels<sup>311</sup>. Although there are numerous transcription factors which have the potential to upregulate VEGF; HIF-1 $\alpha$  and signal transducer and activator factor transcription-3 (STAT3) facilitate hypoxia related VEGF expression<sup>312</sup>. A variety of cytokines control the expression of VEGF, including inflammatory cytokines such as Interleukin-1 $\alpha$  and Interleukin-1 $\beta$  as well as factors which down regulate VEGF; IL-4 and IL-10<sup>312</sup> (**Table 1.4.7**).

**Table 1.4.7 Cytokines affecting VEGF expression**

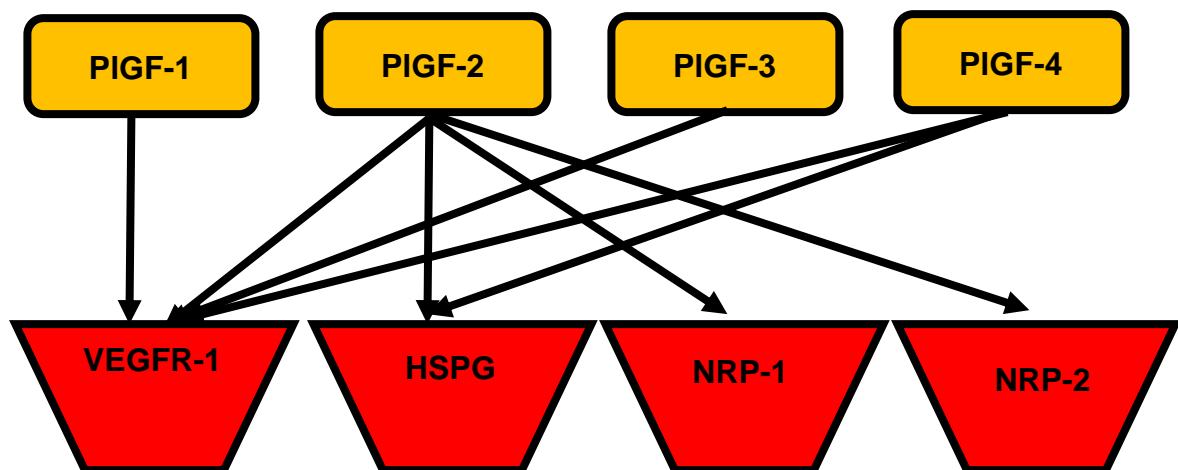
(adapted from Birk<sup>312</sup>)

<b>Cytokines upregulating VEGF</b>	<b>Cytokines down regulating VEGF</b>
IL-1 $\alpha$ (interleukin)	IL-4
IL-1 $\beta$	IL-10
IL-3	IFN- $\alpha$ (interferon)
IL-15	IFN- $\beta$
IL-18	<p>There are some factors which can cause both upregulation and down regulation of VEGF:</p> <p>IFN-<math>\gamma</math></p> <p>IL-12</p> <p>IL-13</p>
TPO (thrombopoietin)	
bFGF (basic fibroblast growth factor)	
EGF (endothelial growth factor)	
HGF (hepatocyte growth factor)	
KGF (keratinocyte growth factor)	
IGF-1 ((insulin like growth factor)	
Oncostatin-M	
PDGF (platelet-derived growth factor)	
TGF (transforming growth factor)	
TNF (tumor necrosis factor)	
MIF (migration inhibitory factor)	

In the mouse model of venous thrombosis there is a temporal expression of VEGF during resolution and increasing VEGF expression in the thrombus using plasmid and adenoviral upregulation enhances thrombus resolution<sup>121;167;313</sup>. There is also evidence that this is related to migration of BM cells into the thrombus, although the role of these cells remains unclear<sup>313</sup>.

### Placental growth factor (PIGF)

PIGF is also a member of the VEGF family<sup>301</sup>. The structure of PIGF exhibits remarkable similarities to the structure of VEGF-A, although PIGF and VEGF-A have only a 42% amino acid sequence homology<sup>314</sup>. PIGF is a 46 KDa protein with 4 isoforms. PIGF1 and PIGF3 isoforms bind to VEGFR1 only but PIGF2 also binds to the heparin sulphate proteoglycan (HSPG) receptor and NRP-1(neuropilin) and NRP-2, and PIGF4 to HSPG (**Fig 1.4.5**)<sup>315</sup>.



**Fig 1.4.5 PIGF isoforms and their receptors**

(adapted from Ribatti<sup>315</sup>) NRP-neuropilin, HSPG-heparin sulphate proteoglycan



Complete PlGF gene knock out is not fatal as with VEGF<sup>316</sup>, but when PlGF<sup>-/-</sup> are exposed to ischaemic conditions i.e. myocardial infarction or following ligation of the hind limb artery, these mice produce a diminished angiogenic response<sup>317</sup>. In PlGF<sup>-/-</sup> mice, 7–14 days after ligation of the left coronary artery, there is reduced neovascularisation at the border of infarcted area and a reduced number of macrophages, suggesting that PlGF may be important in the regulation of angiogenesis following a pathological insult<sup>318</sup>. Administration of recombinant human PlGF is able to reverse the revascularisation defect and increases macrophage accumulation within the infarcted area. PlGF may contribute to angiogenesis via several mechanisms<sup>317;319;320</sup>. Studies have shown that PlGF enhances pathological angiogenesis by the initiation of signaling between VEGFR-1 and VEGFR-2<sup>319</sup>. Murine PlGF amplifies VEGF signaling in endothelial cells and although VEGF and PlGF both bind VEGFR-1, only PlGF stimulates the phosphorylation of specific VEGFR-1 tyrosine residues and the expression of distinct downstream target genes<sup>317</sup>. This data indicates that PlGF binding to VEGFR-1 can somehow sensitise the VEGFR-2 receptor to VEGF<sup>321</sup>. PlGF can however, also directly stimulate angiogenesis via pro-angiogenic genes<sup>322</sup>. Delivery of recombinant PlGF stimulates angiogenesis in skin, revascularisation of ischaemic heart muscle and limbs, as well as the healing of diabetic wounds<sup>323</sup>.

#### **1.4.4 CLINICAL APPLICATION OF HAEMATOPOIETIC PROGENITOR CELLS**

Since the discovery of circulating adult progenitor cells in 1997 by Asahara<sup>252</sup> there has been a rush towards developing the clinical applications of stem cell therapy<sup>324</sup>.

A large number of trials in autoimmune disease, acute and chronic myocardial ischaemia and stroke have shown promise although this has been hampered by several trials showing equivocal results<sup>324-326</sup>.

### **Studies in myocardial ischaemia**

Early interventional studies using BMMNCs were small and non-randomised, studies such as the one by Strauer *et al.*<sup>327</sup> showed that intracoronary BMMNC injection reduced infarct size at 3 months. The first randomised trial of progenitor cell therapy was BOOST<sup>328</sup> (Bone Marrow Transfer to Enhance ST-elevation Infarct Regeneration) trial; this showed improved left ventricular function using MRI 6 months post intervention although the 5 year results suggest that this effect is not longterm<sup>329</sup>. The REPAIR-AMI<sup>330</sup> (Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction) assessed the effect of intracoronary delivery of progenitor cells after angioplasty compared with placebo. At 4 months LV function was improved when measured by angiography, there was also a trend to suggest that progenitor cell injection modified end-points such as repeat MI or death<sup>330</sup>. The MAGIC<sup>331</sup> (Myocardial Regeneration and Angiogenesis in Myocardial Infarction with GCSF and Intracoronary Stem Cell Infusion) trial combined peripheral blood stem cells with GCSF infusion against GCSF infusion alone or no treatment as a control. Interestingly this demonstrated that although stem cells with GCSF improved LV function, GCSF alone produced an increased tendency towards coronary artery restenosis, illustrating the deficiencies in the understanding of basic stem cell biology<sup>331</sup>.

Although there is evidence from animal studies that transplantation of progenitor cells improves neovascularisation in ischaemic heart muscle as well as ischaemic hind limbs<sup>282;332-334</sup>, clinical studies of myocardial ischaemia have been outcome centred and measured LV function using imaging techniques such as MRI and ECHO, and less focused on neovascularisation<sup>335</sup>. Using cardiac scintigraphy there is some evidence that cell therapy can improve tissue perfusion<sup>336</sup>. In the TOPCARE-AMI study, there was improved coronary blood flow in the infarct zone following intracoronary injection of progenitor cells<sup>337</sup>. These studies have shown moderate improvement in cardiac perfusion, their biggest limitation being that the majority used unselected BMMNCs and not specific PC phenotypes<sup>335</sup> (**Table 1.4.8**).

**Table 1.4.8 Cardiac trials based on bone marrow or mononuclear cell therapy using mononuclear** (adapted from Jujo<sup>335</sup>). IHF-Ischaemic heart failure, OMI-Old myocardial infarction, AMI-Acute myocardial infarction RMI-Recent myocardial infarction, PB-Peripheral blood, BM-MNC-Bone marrow mononuclear cells, R-Randomised, RC- Randomised control, MC-Multicentre, NA-Not applicable

Study	Disease	Cells	Design	Follow up (months)	Outcome
Assmus et al. <sup>338</sup>	OMI	BM-MNCs	RC	3	Effective
ASTAMI <sup>339</sup>	AMI	BM-MNCs	R	12	Ineffective
Beeres et al. <sup>340</sup>	IHF	BM-MNCs	NR	3-6	NA
BOOST <sup>328</sup>	AMI	BMCs	R	18	Ineffective
Chen et al. <sup>341</sup>	AMI	BMCs	R	6	Ineffective
Fernandez-Aviles et al. <sup>342</sup>	AMI	BM-MNCs	NR	6	Effective
HEBE <sup>343</sup>	AMI	BM-MNCs	Pilot	4	NA
Henrikx et al. <sup>344</sup> (183)	IHF	BM-MNCs	R	4	Ineffective
Janssens et al. <sup>345</sup>	AMI	BM-MNCs	RC	4	Ineffective
Kueth <sup>346</sup>	AMI	BM-MNCs	NR	12	NA
MAGIC <sup>347</sup>	AMI	PB-MNCs (+GCSF)	R	6	Effective
Meluzin et al. <sup>348</sup>	AMI	BM-MNCs	R	12	Effective
Mocini et al. <sup>349</sup>	RMI	BM-MNCs	NR	12	Effective
Perin et al. <sup>350</sup>	IHF	BM-MNCs	NR	12	Effective
REPAIR-AMI <sup>351</sup>	AMI	BM-MNCs	R/MC	12	Effective
Ruan et al. <sup>352</sup>	AMI	BMCs	R	6	Effective
Strauer et al. <sup>353</sup>	AMI	BM-MNCs	R	4	Effective
Strauer et al. <sup>354</sup>	OMI	BM-MNCs	R	3	Effective
Tatsumi et al. <sup>355</sup>	AMI	PB-MNCs	Cohorts	6	Effective
TCT-STAMI <sup>356</sup>	AMI	BM-MNCs	R/MC	6	Effective
TOPCARE-AMI <sup>357</sup>	AMI	PB/BM MNCs	R	12	Effective
Tse et al. <sup>358</sup>	IHF	BM-MNCs	NR	3	NA

## Studies in peripheral vascular disease

Studies in PVD have focused on using BM cells and their derivative PCs to improve perfusion of ischaemic lower limbs in order to alleviate pain and allow wound healing<sup>359-361</sup>. Animal work in rabbits, rats and mice shows that hind limb ischaemia results in local PC incorporation<sup>362</sup>. A limitation of these models is that they represent acute limb ischaemia in otherwise healthy animals as opposed to chronic limb ischaemia in clinical studies and as with cardiac studies BMMNCs have been used rather than progenitor subsets<sup>363</sup>. The Therapeutic Angiogenesis using Cell Transplantation (TACT)<sup>364</sup> study was the first large clinical study using direct injection of BMMNCs into ischaemic limbs. Rest pain improved with BMMNC injection and in objective measures both ankle brachial pressure indices (ABPI) and transcutaneous oxygen pressure (TcPO<sub>2</sub>) also improved. Several subsequent studies have corroborated these results (**Table 1.4.9**)

**Table 1.4.9 Mononuclear cell therapy for lower limb ischaemia**

(adapted from Alaiti<sup>363</sup>) CLI-chronic limb ischaemia

Study	Disease	Cells	Design	Follow up (months)	Outcome/ABPI
TACT <sup>364</sup>	PVD	BMMNC	RCT	6	Effective
Huang et al. <sup>365</sup>	Lower limb arteriosclerosis obliterans	PBMNC (GCSF)	RCT	3	Effective
Higashi et al. <sup>361</sup>	PVD	BMMNC	Series	6	Effective
Amann et al. <sup>360</sup>	CLI	BMMNC	CT	6	Effective
TAM-PAD <sup>359</sup>	PVD	BMMNC	CT	13	Effective

The vast literature examining the impact of progenitor cell therapy in both animal and human models suggest that it is safe and may have a beneficial effect, the mixed trial results however suggest that more work needs to be done at a basic science level if progenitor cell therapy is to prove advantageous<sup>366</sup>.

## **1.5 HYPOTHESIS & AIMS**

### **1.5.1 PROJECT OVERVIEW**

The aim of this project was to examine the progenitor cell response to thrombus resolution using a mouse model of venous thrombosis. Flow cytometry was used to examine changes in circulating and bone marrow resident progenitor cell response to venous thrombosis. The expression of cytokines which are known to recruit and mobilise progenitor cells were then measured in the thrombus, vein wall, plasma, and laparotomy wound tissues. The final stage of this project assessed whether venous recanalisation could be enhanced by upregulating circulating progenitor cell numbers or increasing the amount of specific progenitor cell mobilising cytokines within the thrombus using adenoviral technology.

### **1.5.2 HYPOTHESIS**

The mobilisation and recruitment of pluripotent bone marrow derived progenitor cells is an important mechanism in venous thrombus resolution.

### **1.5.3 AIMS**

The aims of this study were:

1. to delineate the changes in the circulating and bone marrow resident progenitor phenotypes following thrombus formation.
2. to determine whether there is a pattern in the local and circulating expression of growth factors and cytokines that are known to regulate the mobilisation and recruitment of bone marrow progenitor cells.
3. to determine whether enriching the thrombus or circulation with bone marrow cells (with an endothelial phenotype) has a differential effect on resolution.
4. to determine the effect of upregulating cytokines within the thrombus that are known to effect recruitment of bone marrow progenitor cells.



## 2.0 GENERAL METHODS

### 2.1 ANIMAL MODEL

#### 2.1.1 OVERVIEW

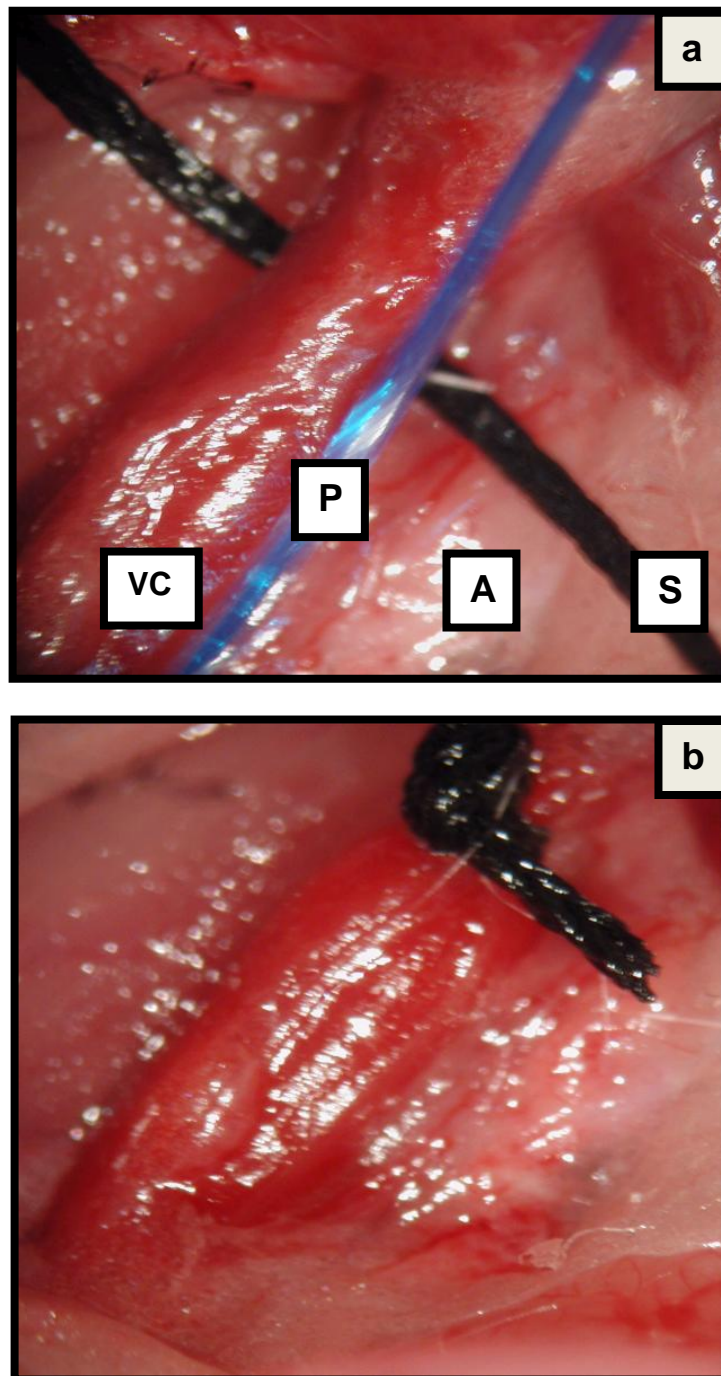
The study of venous thrombosis (VT) can be difficult in the clinical setting because thrombus tissue samples are not readily available from afflicted patients. Accurately ageing the thrombus radiologically is also not yet possible, although ultrasound studies examining elasticity in the thrombus have shown some initial promise<sup>367;368</sup>. In addition attempting to clinically study DVT in isolation is difficult, as most patients have underlying health problems such as malignancy which can complicate any biochemical analysis<sup>369-372</sup>. A reproducible *in vivo* model is therefore advantageous in both accurately ageing the thrombus as well as supplying tissue samples. Several animal models have been used but rodent models have become popular as work in primates is now limited, both by finance and ethical ambiguity<sup>136;373;374</sup>. The main advantage of using mice over rats in this project was the availability of antibodies for progenitor antigens<sup>136</sup>. The model used here employs a combination of reduced blood flow and vessel wall damage to create thrombus in the infra-renal vena cava<sup>136</sup>. The architecture of thrombus formed in the mouse vena cava is similar to thrombus that is formed in humans<sup>119</sup>. Thrombi consist mainly of red cells and are typically laminated with platelets, fibrin, and leukocytes<sup>136</sup>. The vena cava however has no valves and is therefore anatomically different from the human lower limb vessels where DVT commonly occurs<sup>140;141</sup>. The mouse model has revealed that inducing a single component of

Virchow's triad<sup>113;114</sup> in isolation does not result in thrombosis and that it requires at least two components from vessel wall injury, stasis or coagulopathy<sup>136</sup>. Ligation of the cava alone does not produce a thrombus as consistently as when it is used in combination with vessel wall injury, which results in a 90% success rate<sup>136</sup>. This corroborates the work of others which has shown that using either vein wall damage or stasis alone did not result in thrombus formation, in a rabbit model<sup>375</sup>.

### 2.1.2 MOUSE MODEL

All animal work was carried out in accordance with the Animals scientific procedures act 1986. Male Balb/c mice were anaesthetised using isoflurane (Abott, UK) anaesthetic and laparotomised. The bowel was externalised and kept moist in damp gauze, in order to visualise the vena cava. The renal veins were identified and the aorta was dissected away from the vena cava using DeBakey fine tissue forceps (Ethicon, UK). A 4.0 silk suture (Ethicon, UK) was placed around the infra-renal vena cava, care was taken to avoid damaging the lumbar vessels. A silk suture was tied over a 5.0 prolene (Ethicon, UK) (**Fig 2.1.1a**) which was then removed to allow minimal blood flow or a '**reduced flow**' model (**Fig 2.1.1b**). A crocodile clip (Braun Medical, UK) was then placed on the vena cava to cause vessel wall injury. This was repeated twice at different points on the vein, for 45 second intervals. The animal was rehydrated with approximately 1ml of intra-peritoneal 0.9% normal saline. Intraperitoneal Buprenorphine (Veteregesic, UK) was used for analgesia 50µl (0.3µg/µl). The peritoneum and skin were closed with 4.0 PDS (Polydioxanone, Ethicon, UK) in two layers with continuous running

suture. The **sham-control operation** was identical except that a loose tie was placed around the vena cava and endothelial injury was not created.



**Fig 2.1.1 Mouse model of venous thrombosis**

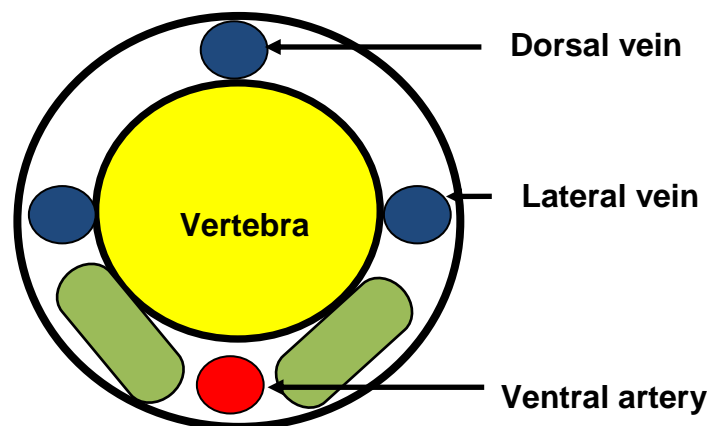
**(a)** Placement of silk suture (S) around the vena cava (VC) and the prolene (P), aorta (A), **(b)** Tied silk ligature with prolene removed

### 2.1.3 ADMINISTRATION OF TREATMENTS

In this model two interventional techniques were employed for delivery of interventions:

#### Peripheral tail vein injection of substances into the circulation

Peripheral tail vein injection has certain advantages over direct thrombus injection; it is less traumatic to the animal and allows for greater volumes of substances to be delivered (250µl v 10µl directly into the thrombus). At 24hrs following thrombus formation animals were re-anesthetised for tail vein injection (**Sec 2.1.2**). The tail was cleaned using alcohol and a 1ml, 25G insulin syringe (BD, UK) used to inject a volume of up to 250µl of solution into either the dorsal or lateral tail vein (**Fig 2.1.2**). After removing the needle, direct pressure was used to prevent further bleeding and the animal placed in a warming chamber at 24°C.



**Fig 2.1.2 Cross section of mouse tail vein**

## **Direct thrombus injection**

Direct thrombus injection was chosen for adenoviral upregulation to concentrate chemokine upregulation and prevent hepatic sequestration of the virus<sup>376</sup>. Under isoflurane anaesthetic, the primary laparotomy incision was re-opened and the small bowel placed in moist gauze as described above. The thrombus was injected using 33G needle (Hamilton, Switzerland) and 50µl syringe (Hamilton, Switzerland), where it appeared most solid to reduce the risk of post injection haemorrhage. Once the injection was complete any haemorrhage was controlled with direct pressure using a cotton bud (Johnson & Johnson™). Analgesia, hydration and wound closure were as previously described (**Sec 2.1.2**).

### **2.1.4 TISSUE PROCESSING FOR FLOW CYTOMETRY AND ELISA**

Animals were anaesthetised using isoflurane anaesthetic, the primary laparotomy wound was reopened and the heart punctured with a 25G needle (BD, UK). Blood was collected in a 2.5ml syringe (BD, UK) and placed immediately in an EDTA tube (BD, UK). After exsanguination, thrombus, vein wall and wound tissues were harvested, snap frozen and stored at -80°C. Femur bones were also harvested with sharp dissection and stored at 4°C in sterile saline until BM extraction was carried out for flow cytometry (**Sec 3.2.5**).

Frozen tissues were homogenized for 2-3mins on ice using mortar and pestle (Eppendorf, UK), wound, vein wall and thrombus samples were all homogenised in PBS with a cocktail protease inhibitor III (0.5ml/mg) (Calbiochem, UK) added to prevent protein degradation.

Samples were placed in Eppendorf vials and centrifuged at 20,000 X g for 10mins at -4°C and the supernatants were collected for both ELISA and protein analysis. The protein content of aliquoted samples was calculated using a Bradford Assay (Appendix1A).

## **2.2 CHEMOKINE ANALYSIS**

### **2.2.1 INTRODUCTION**

A large number of chemokines have been implicated in the mobilisation of bone marrow progenitor cells. VEGF, PLGF and SDF-1, GCSF and GMCSF discussed in the introductory chapter to this thesis all appear to play a role<sup>377;378</sup>. The aim of this section of work was to examine the temporal expression pattern of these chemokinetic proteins during thrombus resolution. Thrombus, vein wall, plasma and laparotomy wound (control) protein extracts were all analysed using ELISA (enzyme linked Immunosorbent assay).

Several techniques can be used for protein analysis; immunohistochemistry for protein localisation, Western blotting and ELISA for protein quantification. ELISA has however, proven to be an efficient and accurate method for protein quantification when suitable antibodies are available.

### **2.2.2 ELISA**

ELISA is a technique used to detect a specific protein in a given solution. The limited amount of mouse tissues available makes it difficult to perform multiple

standard sandwich ELISAs. The amount of plasma extracted from mouse blood is limited to a total volume of roughly 300µl which if undiluted is only sufficient for a single ELISA. Combining both multiplex and standard ELISA systems allowed analysis of a larger number of chemokines and limited animal usage. Although the Sandwich ELISA technique has become one of the most popular methods of protein quantification, there are several other types of ELISA:

**1) Direct**

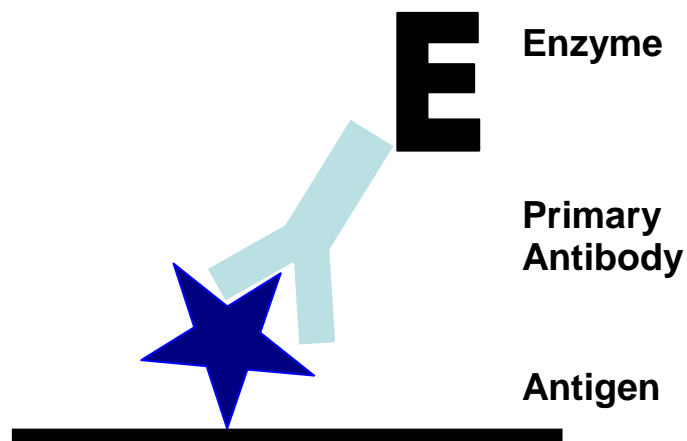
**2) Indirect**

**3) Multiplex**

**Direct**

ELISA techniques are all based on the interaction of an antigen with an antibody. In a single antibody system the primary antibody is linked to an enzymatic reaction which results in a colour change in a specific substrate. This colour change is then quantified using an absorbance plate reader. The greater the colour change the greater the amount of protein being measured.





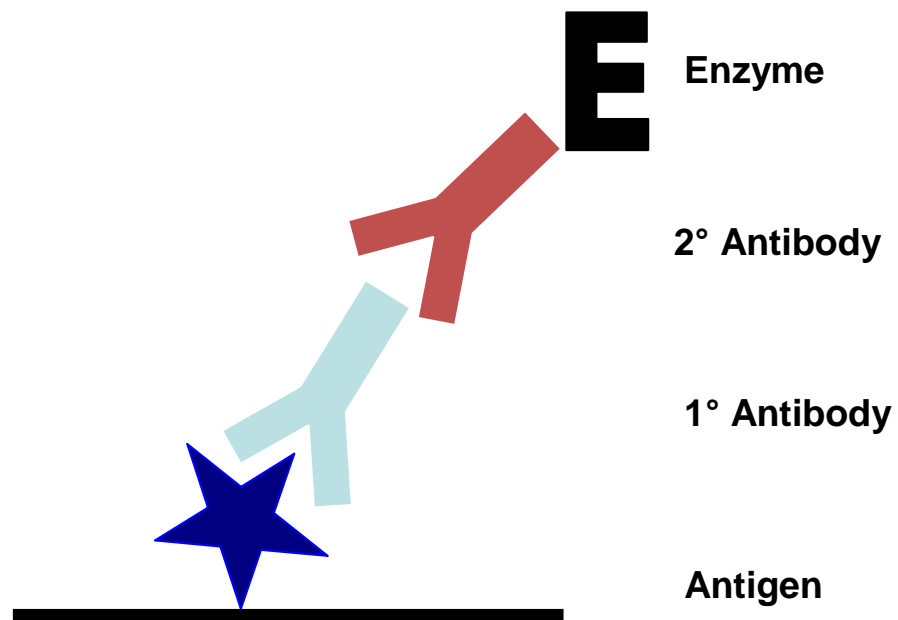
**Fig 2.2.1 Direct ELISA**

The direct ELISA (**Fig 2.2.1**) method uses a single primary labelled antibody to detect the antigen. It has the advantage of being a single step process, conjugation of the primary antibody to the chromogenic enzyme however, may affect its antigen binding ability. Signal amplification is also limited with a single antibody system making it less efficient in detecting small protein concentrations.

### **Indirect ELISA**

In this system there is a secondary antibody which is conjugated to the chromogenic enzyme. Its advantages are: a single conjugated secondary antibody can be used in multiple ELISAs; if the secondary antibody binds to multiple epitopes on the primary antibody then signal strength can be amplified.

Its drawback is that it is a two stage process with the potential risk of cross reactivity between the secondary antibody and the antigen resulting in a non-specific signal (**Fig 2.2.2**).



**Fig 2.2.2 Indirect ELISA**

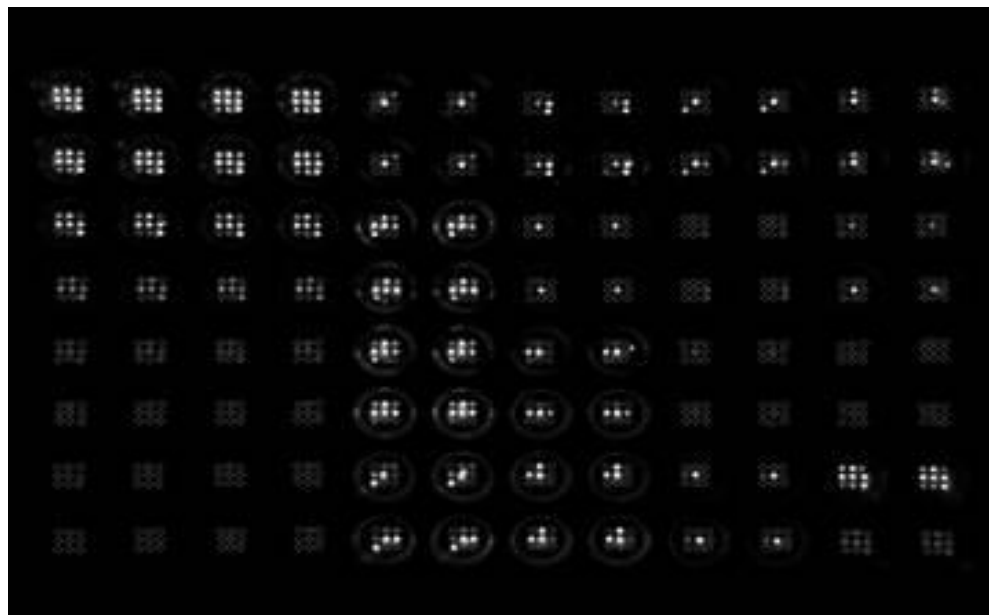
### **Sandwich ELISA**

The Sandwich ELISA system is a type of indirect ELISA also using a secondary enzyme linked antibody. It differs from the indirect system in that there is also capture antibody which holds the antigen on the ELISA plate thereby reducing the interaction from non-specific proteins. Adding in a third antibody however raises the complexity of the overall process as well as increasing the likelihood of cross reactivity.

## Multiplex ELISA

Multi-Plex technology enables the quantification of multiple proteins from a single sample. It is essentially a sandwich ELISA, but rather than only one capture antibody and one detection antibody it uses multiple antibodies. The luminescent reaction created is measured at different spots within the same well for different analytes. Below is a photograph of the luminescence created by reactions occurring within the same well (**Fig 2.2.3**); the luminescence for each spot within the well is photographed using a *charged coupling device* (CCD) camera. In the Perbio© system luminescence is created using the SuperSignal ELISA Femto Maximum Sensitivity Substrate, and the plate is placed under the CCD camera in a light-tight environment.

The CCD chip is exposed to the image over a specific exposure time and a digitised image is acquired and displayed on a monitor. The Perbio© CCD image analysis system calculates exact values (pg/ml) for unknowns from standard curves based on the position of the spots.



**Fig 2.2.3 Multitplex ELISA**

### **2.2.3 STANDARD SANDWICH ELISA PROTOCOL**

The protocol for the PIGF ELISA is given below, it is derived from the R&D Duo kit manual and follows the same set of steps as the SDF1, GMCSF, GCSF and VEGF ELISA, individual ELISA kit components and working dilutions are described in **Table 2.2.1.**

#### **PIGF Protocol**

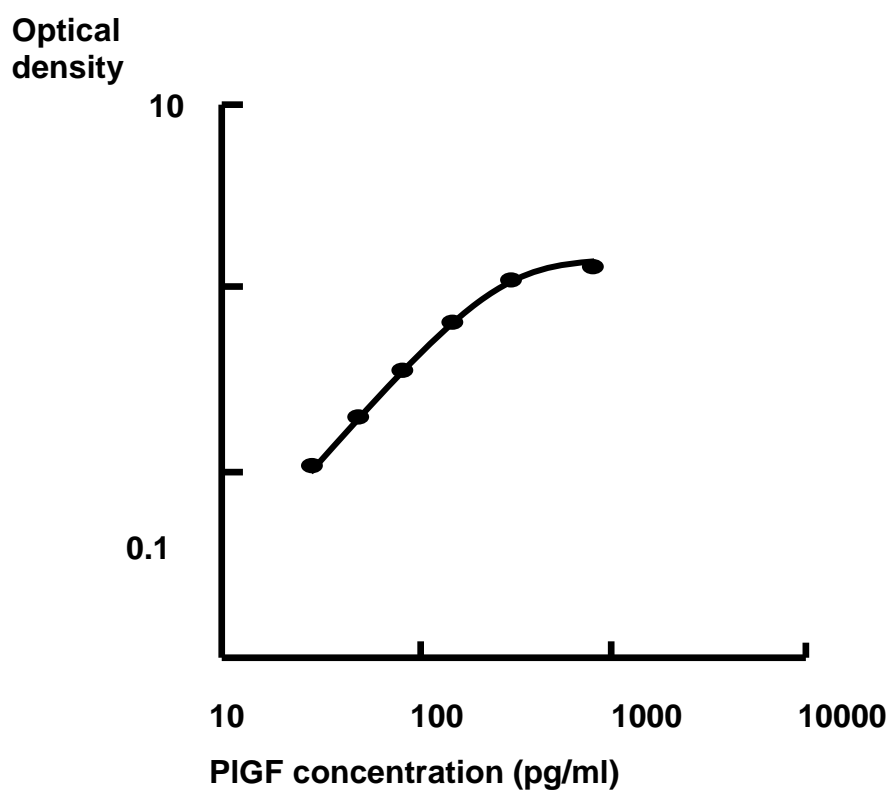
A 96 well plate (NUNC, UK) was coated with 100 $\mu$ l of the appropriate dilution (2 $\mu$ g/ml) of capture antibody (rat anti-mouse PIGF) and the plate was incubated overnight.

The plate was washed using a plate washer with 400µl of wash buffer (0.05% Tween© 20 in PBS, pH 7.2-7.4 (R&D, UK)) per well X 3. Then 400µl of reagent diluent (1% BSA in PBS, pH 7.2-7.4, 0.2µm filtered) was added to each well and incubated for 1hr. The wash step was repeated and 100µl of PIGF protein sample standard was added in triplicate to the left hand wells (serially diluted from a top standard of 100pg/ml down to 15pg/ml). 100µl protein samples were then added in duplicate and incubated for 2hrs. The wash step was repeated and 100µl (200ng/ml) of detection antibody (goat anti-mouse PIGF) was added to each well and incubated for 2hrs. The wash step was repeated and 100µl of streptavidin HRP (1/200, from kit) working dilution was added and incubated for 20mins. The wash step was repeated and 100µl of TMB substrate solution (Thermoscientific, UK) (1:1 mixture of colour reagent A (H<sub>2</sub>O<sub>2</sub>) and colour reagent B (Tetramethylbenzidine)) was then added to each well and incubated for 20mins. Finally 50µl of 1M H<sub>2</sub>SO<sub>4</sub> was added to each well to stop any further reaction. The plate was analysed using a spectrophotometer at 450nm. Below is an example of the standard curve generated from the PIGF standards (**Fig 2.2.4**).

***All steps were carried out at (18-20°C.***

**Table 2.2.1 ELISA kit solutions and working concentrations**

ELISA	GCSF	VEGF	SDF1	GMCSF
<b>capture</b>	Rat anti-mouse 2.0µg/ml	Goat-anti mouse 0.4µg/ml	Mouse anti- mouse 360 µg/ml (2.0µg/ml)	Rat anti-mouse 2.0µg/ml
<b>Detection</b>	Biotinylated goat anti-mouse 200ng/ml	Biotinylated goat anti-mouse 100ng/ml	Biotinylated goat anti-mouse 400ng/ml	Biotinylated goat anti- mouse 50ng/ml
<b>Standard</b>	Recombinant mouse GCSF high standard 2000 pg/ml	Recombinant mouse VEGF high standard 1000pg/ml	Recombinant mouse SDF1 high standard 3000pg/ml	Recombinant mouse GMCSF high standard 500pg/ml
<b>Secondary anibody</b>	Streptavidin HRP (1/200 working dilution)	Streptavidin HRP (1/200 working dilution)	Streptavidin HRP (1/200 working dilution)	Streptavidin HRP (1/200 working dilution)
<b>Substrate solution</b>	TMB	TMB	TMB	TMB



**Fig 2.2.4 PIGF standard curve**

## **2.3 HISTOLOGY**

### **2.3.1 FROZEN SECTION**

IVC and thrombus were excised together just below the left renal vein to the bifurcation of the common iliac veins. Vein wall and thrombus *en mass* and spleens were mounted using O.C.T. (optimal cutting temperature) compound (Tissue-Tek, UK) and frozen in liquid nitrogen. A microtome was used to cut 10µm tissue sections at 300µm intervals throughout the length of the vein or spleen and placed on slides coated with Vectabond (Vector Labs, UK) which were air dried for 1hr (18-20°C). Coverslips were mounted using 1-2 drops of Vectashield mounting medium with DAPI (Vector Labs, UK) and images were captured using a microscope-mounted colour digital camera (CoolSnap Pro CF) and motorised stage (ProScan, Datacell, UK).

### **2.3.2 PARAFFIN SECTION**

IVC and thrombus were excised together as above (**Sec 2.3.1**). Tissue was fixed in 10% formalin, dehydrated through graded alcohols and processed automatically for paraffin embedding (Tissue-Tek, UK). Using a microtome 5µm sections were cut at 300µm intervals along the entire length of the vein. Images were captured using a microscope-mounted colour digital camera (CoolSnap Pro CF) and motorised stage (ProScan, Datacell, UK).

### **2.3.3 HAEMATOXYLIN & EOSIN STAINING**

Tissue sections were warmed for 20mins at 40°C and deparaffinised in xylene for 2mins after which they were rehydrated by processing through graded alcohols to water. They were stained with Mayer's haematoxylin dye (Sigma, UK, which stains nuclei blue) for 5mins (18-20°C) and washed in distilled water for 5mins. Differentiation was carried out by immersion in acid alcohol (1% HCl in 70% ethanol, BDH) for 3-5 seconds. The sections were washed again as above and stained with eosin dye which stains cytoplasm red (Sigma, UK) for 2mins (18-20°C). Sections were dehydrated through graded alcohols to xylene and mounted on glass slides (BDH, UK) using DPX resin. Sections were visualised using a light microscope (Leitz LaborLux S, Leica, UK) and images were captured using a microscope mounted camera (Coolpix 990, Nikon).

### **2.3.4 IMAGE ANALYSIS**

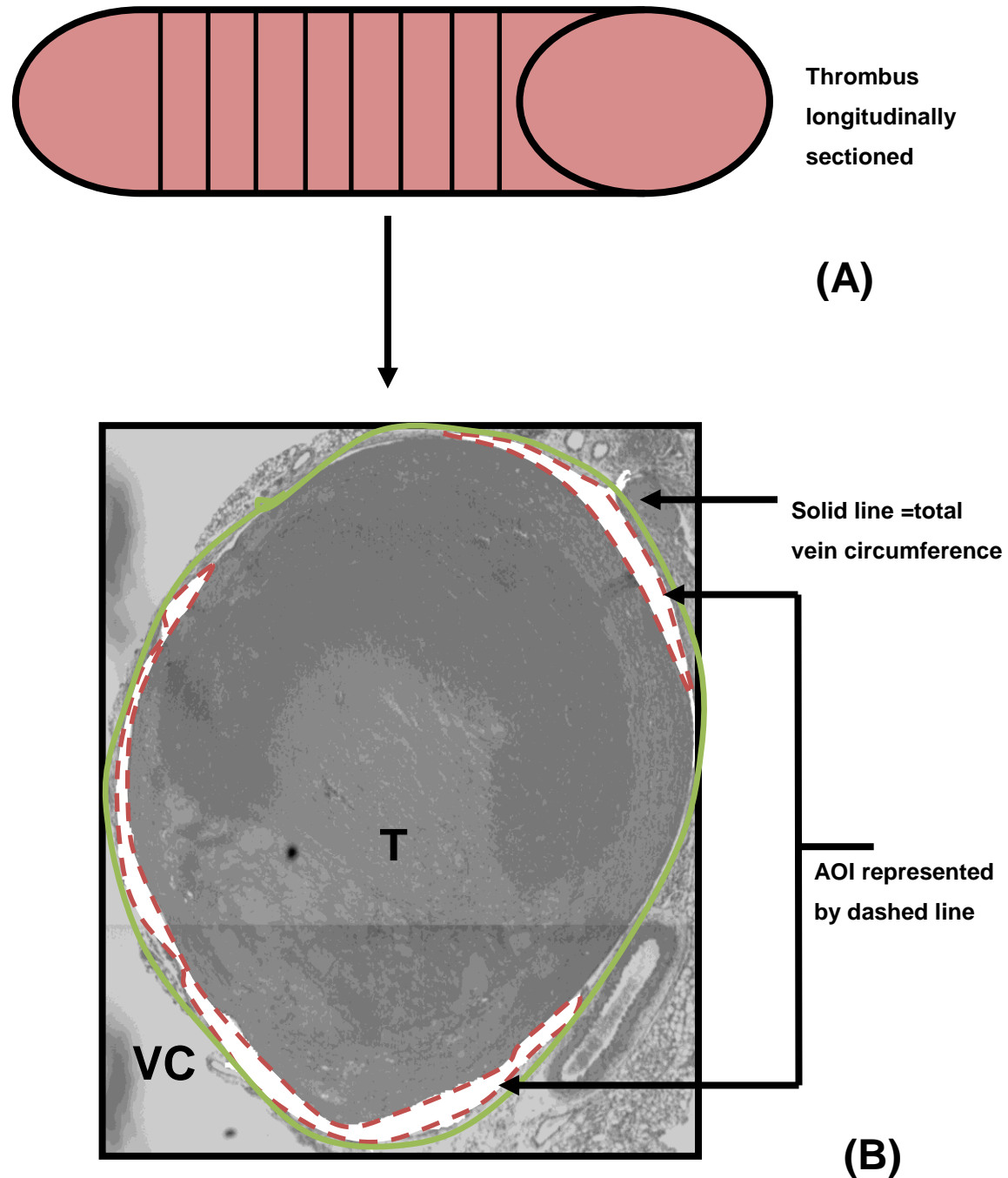
All sections were analysed using a Leica (Leitz DMRB) microscope at X5 magnification. Images were then captured using CoolSnap Pro CF colour digital camera. Images were typically captured whole or tiled to create a single composite. Image analysis was then carried out using the Image Pro Plus package™ (Media Cybernetics, USA) with specific macros to allow efficient analysis of up to ten images per thrombus (**Sec 2.3.5**).

### **2.3.5 ANALYSIS OF THROMBUS SIZE AND RECANALISATION**

The Image Pro Plus package was calibrated using a 1mm graticule. Recanalisation was assumed of all areas not containing thrombus lying within the confines of the



vein wall. Using a highlighting tool these areas were drawn around and identified as areas of interest (AOI). After this the vein wall itself was traced around and the AOIs were subtracted from it as a function of the macro (**Fig 2.3.1**). This would generate two numbers; the area of thrombus recanalisation per section and also the area occupied by the thrombus. This calculation was performed for approximately 10 sections per thrombus from which the mean percentage recanalisation was calculated.



**Fig 2.3.1 Cross section of vena caval wall with thrombus**

**(A)** schematic of a thrombus in its long axis, cross-sectioned **(B)** sample cross section: dashed line represents areas of interest (AOI) which can be calculated as a percentage of the total vein circumference. T (thrombus), VC (vena cava)

## 2.4. PROGENITOR CELL TRACKING

The ability to track the distribution and migration of biologically active cells *in vivo* can be invaluable in determining the mechanisms underlying cell based therapies. Although monocyte migration into the resolving mouse thrombus has been previously demonstrated, it is unknown if progenitor cells follow a similar course<sup>119</sup>. Cell tracking can be performed through both direct and indirect labelling techniques. Direct labelling, as used here has the advantage of being a relatively straightforward process that is not limited by cell modification. Its major disadvantage is that the label can be lost or diluted during subsequent cell division. Indirect labelling is a more complex process that requires a reporter gene, which if it remains stable can be indefinitely measured in the labelled cells progeny. The PKH26 system used here allows the labelling of the lipid membrane of the cell with a fluorescent dye which was used here to distinguish the native progenitors from the transplanted bone marrow mononuclear cells.

### 2.4.1 PKH26 CELL LINKER KIT METHOD

*(The following is adapted from the PKH 26 red fluorescent linker kit (Sigma, UK) instruction manual, **all steps were performed at 18-20°C**)*

Approximately  $2 \times 10^7$  BMMNCs were placed in a 15ml conical tube (Falcon BD, UK) and washed once in Dulbecco's modified eagles medium (DMEM). Cells were centrifuged at (400 X g) for 5mins into a loose pellet (18-20°C).The supernatant was then aspirated leaving no more than 25µl of supernatant on the pellet.

1ml of diluent C (from kit) was added and the cells were resuspended, pipetting to insure complete dispersion (do not vortex). Immediately prior to staining,  $4 \times 10^{-6}$  molar PKH26 dye was prepared in a 15ml falcon conical tube using diluent C. 1ml of 2X cells was then added to 1ml of 2X dye, pipetting to mix. Cells were incubated at 25°C in a water bath for 2-5mins. Periodically, the tube was inverted gently to assure mixing during the staining period. The staining reaction was stopped by adding an equal volume of DMEM with 1% bovine serum albumin (BSA, R&D UK). The cells were then centrifuged (400 X g, 10mins at 25 °C) to remove them from the staining solution. The supernatant was aspirated and the cells washed as above a further 3 times with DMEM. Cells were then suspended in DMEM at a concentration of approximately 4 million cells/ ml (1million cells/ 250µl).

## **2.5 CELL CULTURE**

Cell culture was used to grow and characterise the adenoviruses used for the interventional studies. Human Embryonic Kidney (HEK) 293 cells have been transformed by the introduction of approximately 4.5 kb of viral genome into the human chromosome 19 of the HEK cell line. It is a stable cell line which produces the adenovirus E1 gene in *trans* allowing the replication of E1-deleted adenoviruses such AdPIGF and AdSDF1.

### **2.5.1 HEK293 CELL CULTURE**

Frozen HEK 293 cells (Stratagene, UK) were thawed by brief immersion in a 37°C water bath (e.g. 2–3mins with constant agitation).

The contents of the vial were diluted in supplemented growth media (DMEM (Lonza, UK) +10% FBS (Gibco, UK) and transferred into a T25 flask (Falcon BD, UK). Cells were placed in a 37°C, 5% CO<sub>2</sub>, humidified incubator. Cell morphology and viability were verified under a microscope after 24hrs (healthy cells display a flat morphology and adhered well to the plate, **Fig 6.2.1A**). Old growth medium was aspirated and replaced with fresh, pre-warmed (37°) growth medium at 24-48hr intervals. Cell cultures were then expanded as required. Cells in culture were passaged (**Sec 2.5.2**) every 2–4 days, when they reached 70–80% confluency.

### **2.5.2 HEK 293 CELL PASSAGE**

Old growth medium was aspirated and the cells were washed once with pre-warmed sterile PBS (containing no Ca<sup>2+</sup> or Mg<sup>2+</sup>). 1–2mls of trypsin-EDTA solution (Sigma, UK) was added for 1–2mins, until cells detached. Trypsinisation was terminated by the addition 5–10mls of growth medium. Cells were then transferred to the desired number of culture flasks containing medium and the incubation process was restarted.

## **3.0 OPTIMISATION OF FLOW CYTOMETRY FOR HAEMATOPOIETIC PROGENITOR CELLS**

### **3.1 FLOW CYTOMETRY**

#### **3.1.1 OVERVIEW**

The aim of this section of work was to determine whether venous thrombosis has an effect on the number and phenotype of circulating and bone marrow resident progenitor cells. Flow cytometry was used to enumerate HPCs in the blood and BM by using antibodies against cell surface markers to Sca-1, CD34 and VEGFR2 (DC101).

#### **3.1.2 THE FLOW CYTOMETER**

Flow cytometry is a technique that is ideally suited to identifying and counting populations of cells in solution. Single cells in a continuous fluid stream are passed through one or more laser beams which cause light to scatter and fluorescent dyes to emit light at various frequencies. Photomultiplier tubes (PMT) convert light into electrical signals and the data is collected on a computer. Three types of data can be derived from the analysis: cell size, cell complexity (i.e. granularity) and lastly cell populations can be quantified by tagging them with a variety of fluorescently labelled antibodies<sup>208</sup>. Cytometers have gone through some major advances over the last few years<sup>379</sup> but the basic components of the machine have not changed.

For this project we used a FACSCalibur®, single laser cytometer (BD, UK, **Fig**

**3.1.1**). The flow cytometer consists of three main components:

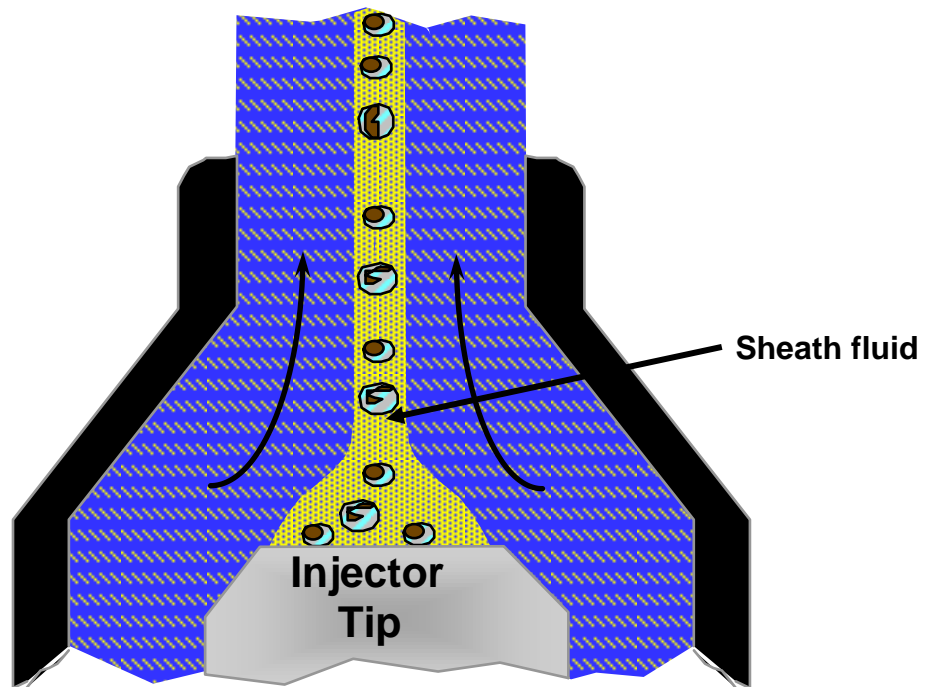
(1) **Flow Cell:** This is a *fluidic system* by which cells are drawn up into the machine through an external port. The purpose of this is to keep the cells at the centre of a fluid stream, in single file; so that only one cell at a time is targeted by the laser. This process is known as “**Hydrodynamic Focusing**” (**Fig 3.1.2**)<sup>380</sup>.

(2) **Optical System:** Basic machines are fitted with a single laser e.g. argon, which delivers approximately 15mW at 488nm as a light source. As the single cells filter into the cuvet they are hit by the laser. Light is then refracted off the cells and the degree of refraction, which is dependent on the size and granularity of the cells, is detected by silicon photodiodes or photomultiplier tubes that convert light into electrical impulses. The refracted light from the cells is interpreted as forward scatter (Fsc: cell size) and side scatter (Scc: cell granularity). An example of a peripheral blood forward and side scatter dot-plot is shown below (**Fig 3.1.3**).

(3) **Analogue-Digital Convertor:** Converts the light signals as detected by the photodiodes into digital signals which can be processed by a computer.



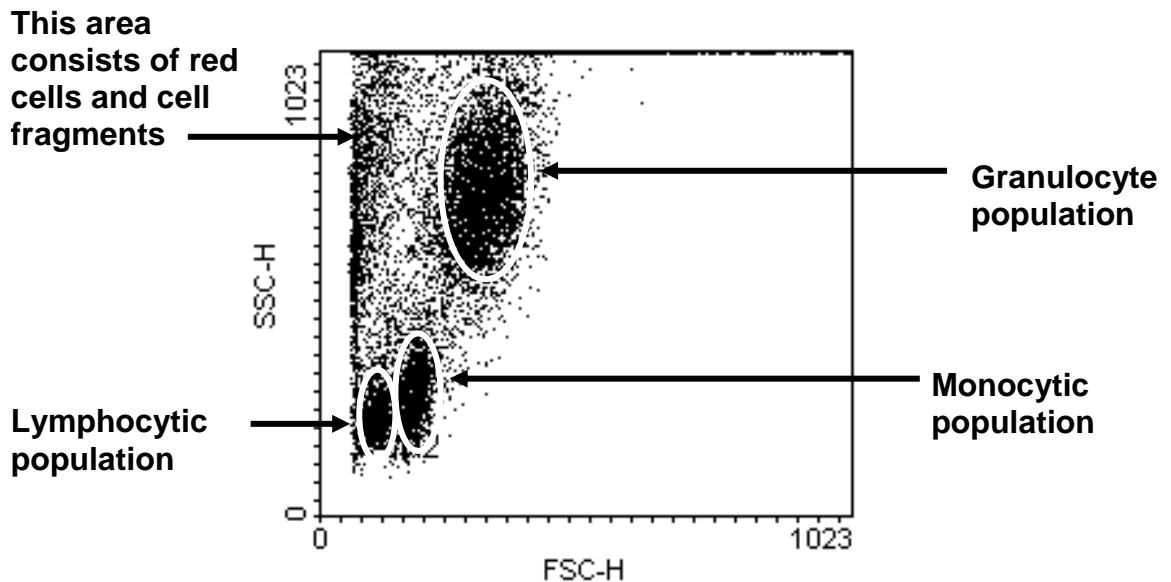
**Fig 3.1.1 Flow Cytometer**



**Fig 3.1.2 Flow cytometer tip**

The sheath fluid (Phosphate buffer) draws the cells into a central chamber by creating a single cell stream, 'hydrodynamic focusing'.





**Fig 3.1.3 Scatter plot of mouse whole blood after red cell lysis (white cells are displayed in three distinct groups)**

### **3.1.3 MEASUREMENT OF CELL POPULATIONS**

Measurement of specific cell populations within the scatter plots is performed using fluorophore tagged antibodies. A **fluorophore** is a molecule that when excited by a laser, emits fluorescence. Fluorescence detectors within the cytometer are fitted with filters that only perceive light, different in colour to that of the laser. Argon produces a blue laser so typical filters are red, orange and green, this means that any light detected by these receptors is only generated by antibody labelled cells which have crossed the path of the lasers. Some commonly used fluorophores are shown in (**Table 3.1.1**)

**Table 3.1.1 Commonly used fluorophores**

<b>Dye or fluorophore</b>	<b>Laser excitation (nm)</b>	<b>Maximal Emission (nm)</b>	<b>Colour</b>
<b>FITC</b>	488	525	Green
<b>PerCP</b>	488	675	Red
<b>Pe</b>	488	578	Yellow
<b>Alexa Fluor</b>	405	421	Turquoise

**FITC**            **Fluorescein isothiocyanate**

**PE**             **Phycoerythrin**

**PerCP**        **Peridinin-chlorophyll-protein complex**

An antibody bound to a cell will emit the typical fluorescence for that fluorophore e.g. FITC will produce a green emission. To adjust for non-specific antibody signalling, a non-specific IgG antibody conjugated to the same fluorophore should be used as a control. A single laser machine can detect up to three fluorescently labelled antibodies per sample.

#### **3.1.4 FLOW CYTOMETRIC DATA ANALYSIS**

Data was analysed using the flow cytometry-specific software package, *Win MDI*, which is a freely downloadable software package available from the internet (<http://flowcyt.salk.edu/software.html>). The software can be used to produce two dimensional histograms known as dot plots for data analysis. This allows cell populations to be selected and quantified for the presence or absence of specific antigens. Although any combination of parameters can be used, a commonly used

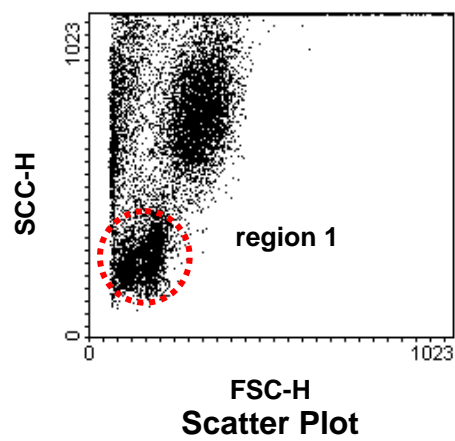
system is to plot Fsc versus (v) Scc (see **Fig 3.1.4 (i)**), fluorophore, channel 1 (FL1) against fluorophore channel 2 (FL2), and FL1 against channel 3 (FL3) (**Fig 3.1.4 (iv) & (v)**). Each fluorophore should produce a signal in its respective channel (**Table 3.1.2**).

**Table 3.1.2 Channel, antibody and fluorophore optimised**

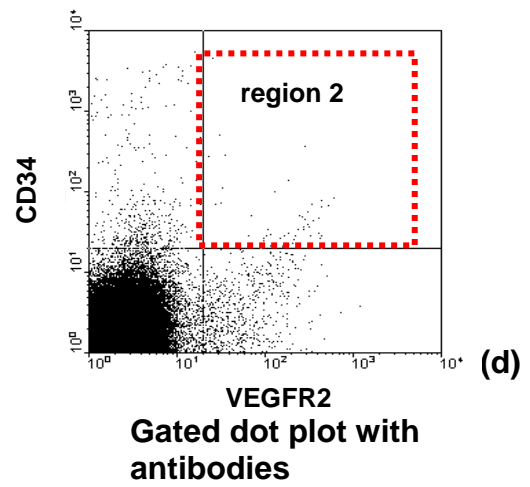
CHANNEL	ANTIBODY	FLUOROPHORE
FL1	VEGFR2 (DC101)	FITC
FL2	CD34	Pe
FL3	Sca1	PerCP

Using the Fsc v Scc plot, populations of interest can be selected and analysed for the presence or absence of fluorescently labelled antibodies bound to the antigens of interest. The selected area of interest is known as a 'region', a region can then be used to either exclude or include data from any further analysis. This allows analysis of specific cell phenotypes and reduces the impact of non-specific signalling on data interpretation. **Fig 3.1.4 (a)** is a typical Fsc v Scc plot, the mononuclear region has been selected from this plot. **Fig 3.1.4 (b)** is a dot plot of a VEGFR2 versus CD34 for the same data without the application of gating (region selection or exclusion). This results in a large amount of non-specific labelling, making data interpretation difficult. **Fig 3.1.4 (d)** has been 'gated', which means the plot only display data from the region selected in **Fig. 3.1.4 (a)**.

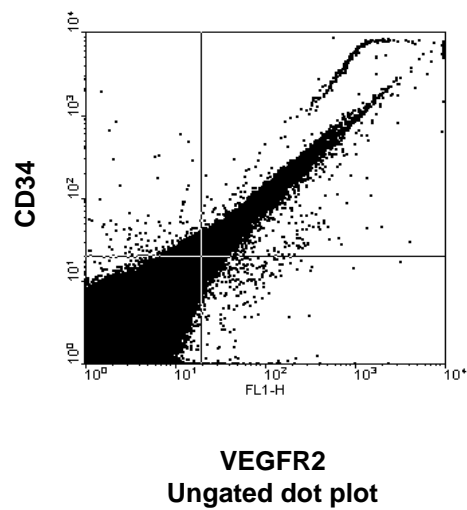
To account for auto-fluorescence and non-specific binding, a control sample containing fluorescently labelled non-specific IgG of the same subgroup is used to set the quadrants to confine background staining to the bottom left quadrant (**Fig 3.1.4 (c)**). **Fig. 3.1.4 (d)** shows positive cells in both the VEGFR2 channel and the CD34 channel, cells in the larger upper right quadrant are positive for both VEGFR2 and CD34 and are known as **double positive**. Gating also enables elucidation of triple positive cells. **Fig 3.1.4 (e)** is a dot plot which has been gated for region 1 and region 2, this data has then been plotted in the VEGFR2 v Sca1 dot plot. Region 1 is selected for mononuclear cells and region 2 has been selected for the population of mononuclear cells positive for both VEGFR2 and CD34. Plotting the same data in the VEGFR2 v Sca1 plot selects for triple positive Sca1+CD34+VEGFR2+ cells in the upper right quadrant.



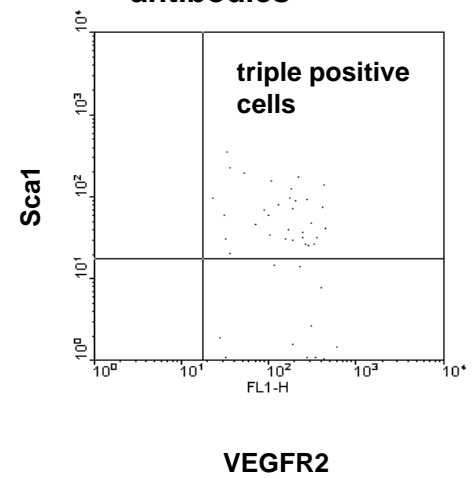
a)



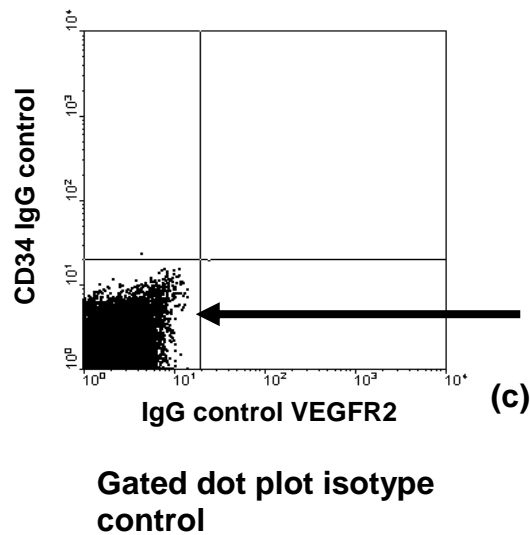
(d)



(b)



(e)



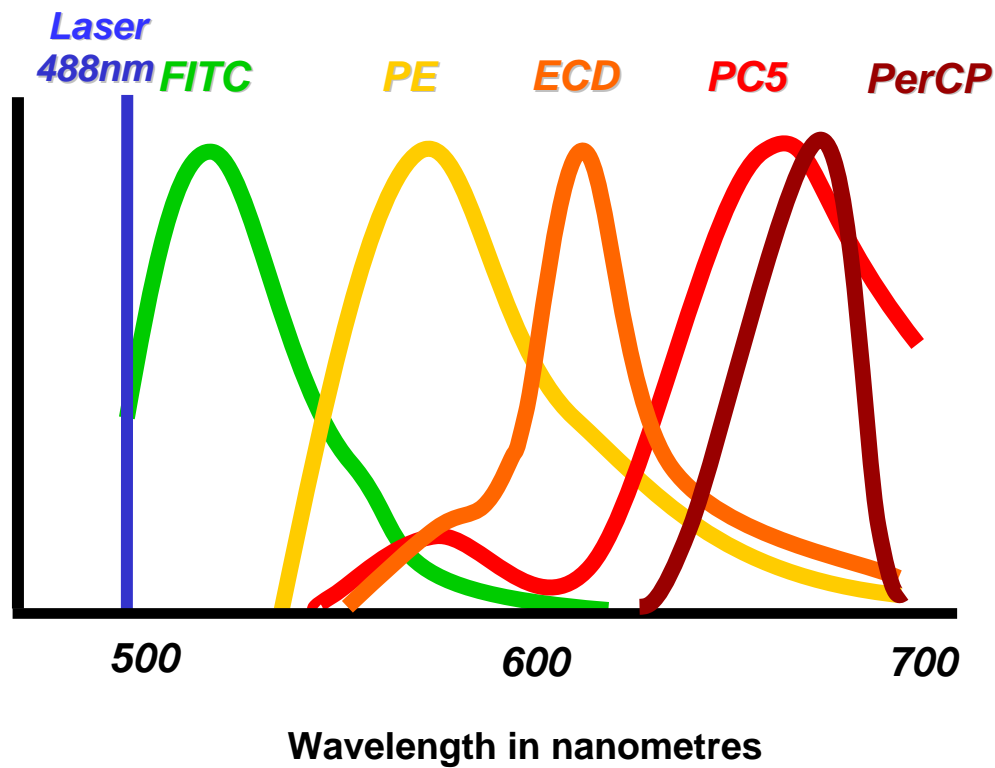
(c)

**Fig 3.1.4 Dot plots using regions and gates**

### 3.1.5 VOLTAGE SETTING

Optimising the voltage settings on the flow cytometer is performed during the set-up stage, before data collection can begin. The voltage is set according to the size and the complexity of the cells being analysed. This reduces the impact of non-specific staining on data collection. Using a sample containing only control antibodies the voltage is either increased or reduced to place all non-specific staining into the bottom left quadrant or the first decade of the log plot (**Fig 3.1.4 (c)**).

Secondly, fluorescence emitted from one fluorophore can overlap with the emission from that of another fluorophore being used in the analysis of the same blood or tissue sample i.e. the fluorescent signal from FITC overlaps with signal from PE (**Fig 3.1.5**). This is a potential problem when using more than one antibody and can be overcome in two ways: (1) is to use antibodies which have markedly different emission spectra (2) to perform a compensation experiment in which spectral overlap is compensated for by manipulating the voltage settings. For cells present in only small quantities the use of compensation beads (Compbeads, Invitrogen UK) is an alternative to direct compensation using cells.



**Fig 3.1.5 Overlapping emission spectra of commonly used fluorophores**  
 FITC-Fluorescein isothiocyanate, ECD-Energy Coupled Dye, PE-Phycoerythrin,  
 PC5- CyChrome Tricolor, PerCP- Peridinin chlorophyll protein

### 3.1.6 THRESHOLD

Setting threshold allows exclusion of cells or cellular debris of a certain size and granularity from the analysis. This reduces the signal to noise ratio, any cells excluded from analysis using the threshold function are not recorded as data points.

## 3.2 OPTIMISATION OF COMPENSATION SETTINGS

### 3.2.1 INTRODUCTION

In order to run experiments with multiple fluorescently labelled antibodies, the voltage settings are manipulated to reduce the impact of spectral overlap from the any fluorophore labelled antibodies used (**Fig 3.1.5**).

### 3.2.2 BASIC SETUP AND COMPENSATION USING LYSED WHOLE BLOOD

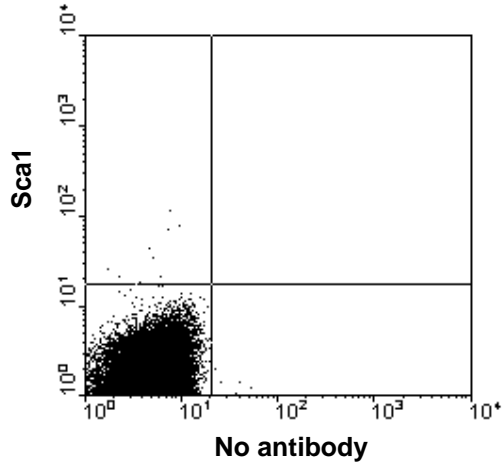
#### Method

Five samples of mouse blood were required to set up a compensation experiment for three antibodies; one containing all three non-specific IgG isotype control antibodies, one containing the three antibodies of interest and three further samples each containing one antibody of interest only.

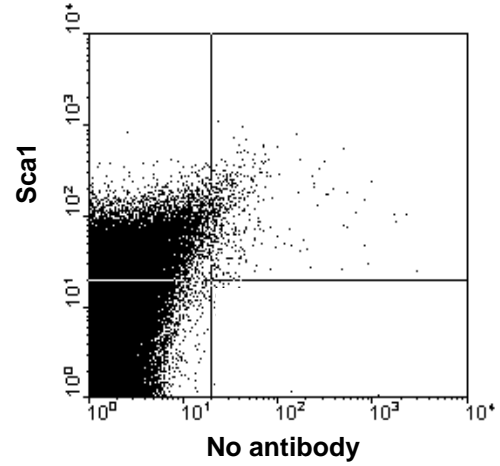
First blood containing all three IgG isotypes was analysed, the voltage settings were adjusted to place all non-specific signal generated in the bottom left quadrant (**Fig 3.2.1(a)**). Antibodies of interest were then tested individually to look for spectral overlap in their fluorescence, i.e. individual antibodies should only produce positive signals in one channel. The voltage settings were then altered and fixed to compensate for all overlapping spectral signals.

***Once these settings were created they were not altered during any subsequent experiments***

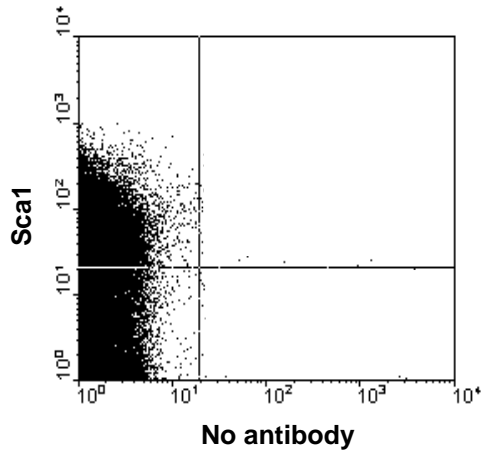




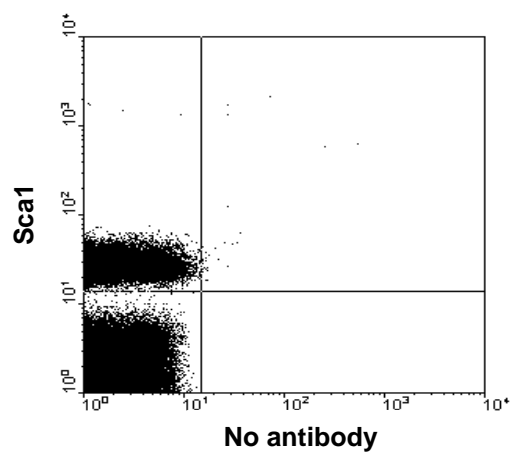
**(a) Isotype control dot plot**



**(b) Sca1 PeCy7 fluorochrome prior to compensation**



**(c) Sca1 PeCy7 fluorochrome after to compensation**



**(d) Strong Sca1 PeCy7 signal using compensation beads**

**Fig 3.2.1 Compensation setting plot for Sca-1 antibody in the FL3 channel**

### 3.2.3 SETTING COMPENSATION SETTING USING BEADS

#### Methods

The described method is based on the manufacturer's instructions, ***all steps were carried out at 18-20°C unless otherwise stated:***

Adjustment of the voltage settings was performed as above to exclude non-specific IgG signalling. The beads were then used only to compensate for spectral overlap. 200µl of stain buffer was added to three 15ml conical tubes (Falcon BD, UK) and approx. 120µl (2 drops) of antibody positive and 120µl of antibody negative beads were added to each tube (CompBeads, Invitrogen, UK). The antibody requiring compensation e.g. Sca1 was then added to each tube, the concentration of antibody used was according to manufacturer's instruction. 2mls of stain buffer was added to each sample of beads and centrifuged (600 X g for 5mins). The supernatant was discarded and samples resuspended in 1.5mls of stain buffer. The voltage settings were then adjusted to compensate for spectral overlap.

#### Results

Illustrated in **Fig 3.2.1** are the dot plots generated during the Sca-1 compensation study. **Fig 3.2.1 (a)** accounts for fluorescence generated from the non-specific binding of the isotype control antibodies. It is filtered into the lower left quadrant by adjusting the voltage settings. The fluorescence generated by the Sca-1 antibody produced some spectral overlap into FL2 (**Fig 3.2.1 (b)**). Adequate compensation is demonstrated in **Fig 3.2.1 (c)**.

CD34 and VEGFR2 antibodies were similarly compensated for background fluorescence and spectral overlap. In **(Fig. 3.2.1 (d))** the Sca-1 antibody compensation using beads produces a distinct dot plot.

### **Conclusion**

After testing both methods, compensation beads were used as the signal from VEGFR2 /DC101 was insufficient to adequately compensate for its spectral overlap.

### **3.2.4 WHOLE BLOOD PREPARATION FOR FLOW CYTOMETRY**

Approximately 800µl -1000µl of blood was harvested per animal and split into two replicates of 400µl. One replicate was used for the antibodies of interest and one for IgG isotype controls.

### **FACS protocol for mouse blood**

*All steps were carried out at room temperature (18°-20°), centrifugation was always 400 X g and incubations were performed out of direct light*

400µl of whole blood was aliquoted into 15ml conical tubes (Falcon BD, UK) 4µl (1µg/ul) Fc receptor block (BD, UK) was added to each replicate and incubated for 15mins. Titrated concentrations of the antibodies were then added and incubated for 30mins (**Sec 3.3.1**). Equivalent concentrations of IgG isotypes were added to the second replicate, to serve as a control. 2mls of stain buffer\* (PBS (Phosphate Buffer Solution), 2% (v/v) heat inactivated fetal calf serum and sodium azide 0.9%

(w/v)) was added to each sample and centrifuged for 5mins, the wash step was repeated. After centrifugation the supernatant was removed and 8mls of lysis buffer (Pharmolyse®, BD Biosciences, UK) was added. This was incubated for 10mins (added at a ratio of 2mls per 100µl of blood volume). The sample was centrifuged for 5mins and the supernatant removed. Samples were resuspended in 1-2mls of stain buffer for FACS analysis.

### **3.2.5 BONE MARROW PREPARATION FOR FLOW CYTOMETRY**

***All the following steps were carried out at room temperature (18°-20°) and centrifugation was always 400 X g.***

The ends of a mouse femur were excised using a scalpel blade, this allowed access to the femur lumen containing BM. BM was flushed out using a 30G (BD, UK) needle into a sterile petri dish with 10% stain buffer (phosphate buffer with 10% (v/v) heat inactivated foetal calf serum and 0.09% sodium azide); a 1-2mls suspension was centrifuged for 5mins. The supernatant was discarded and the red cells in the pellet were lysed for 30secs Pharmolyse®. A further 2mls of modified stain buffer was added to the sample and it was centrifuged for 5mins, the supernatant was again discarded. Cells were resuspended in 2mls of modified stain buffer and filtered through a 70µm filter (BD). A haemocytometer was used to count the cells, and each sample was diluted to contain approximately 1-2million cells per 500µl. 4µl of Fc blocker (1µg/µl) was added to each aliquot and it was

incubated for 30mins. Titrated antibody concentrations (**Sec 3.3.1**) were added to the bone marrow cells and a 30mins incubation performed. Samples were washed with 2mls of modified stain buffer and centrifuged for 5mins; the supernatant was discarded and cells were resuspended in 1-2mls of modified stain buffer for FACS analysis.

### **3.3 OPTIMISATION OF FLOW CYTOMETRY FOR MOUSE HPCS**

#### **3.3.1 INTRODUCTION**

The definition of progenitor cells has continued to develop over the last decade<sup>221</sup> but in this project the HPC population was defined as Sca-1+CD34+VEGFR2+ HPCs<sup>194;208;221;225</sup>. In order to examine the circulatory and BM resident HPC response to venous thrombosis, the cytometer settings were first optimised for the antibodies of interest. This was initially attempted using only the commercially available mouse antibodies.

#### **3.3.2 DETECTION OF SCA-1+CD34+VEGFR2+CELLS**

##### **Methods**

Mouse blood was collected (**Sec 2.1.4**) and stained as described with general FACS protocol (**Sec 3.2.4**). Sca1 (BD, UK) CD34 (Serotec, UK) and VEGFR2 (BD, UK) were used at the optimal titrated dilutions (**Table 3.3.1**). Duplicate samples were run using the (BD) FACSCalibur®, approximately  $5 \times 10^5$  cells were counted per sample. Data was recorded and analysed using Win MDI (2.9).

The specificity of the antibody was calculated by excluding the number cells labelled with non-specific IgG as a percentage of the number of cells labelled with the antibody of interest.

**Table 3.3.1 Flow cytometry antibodies**

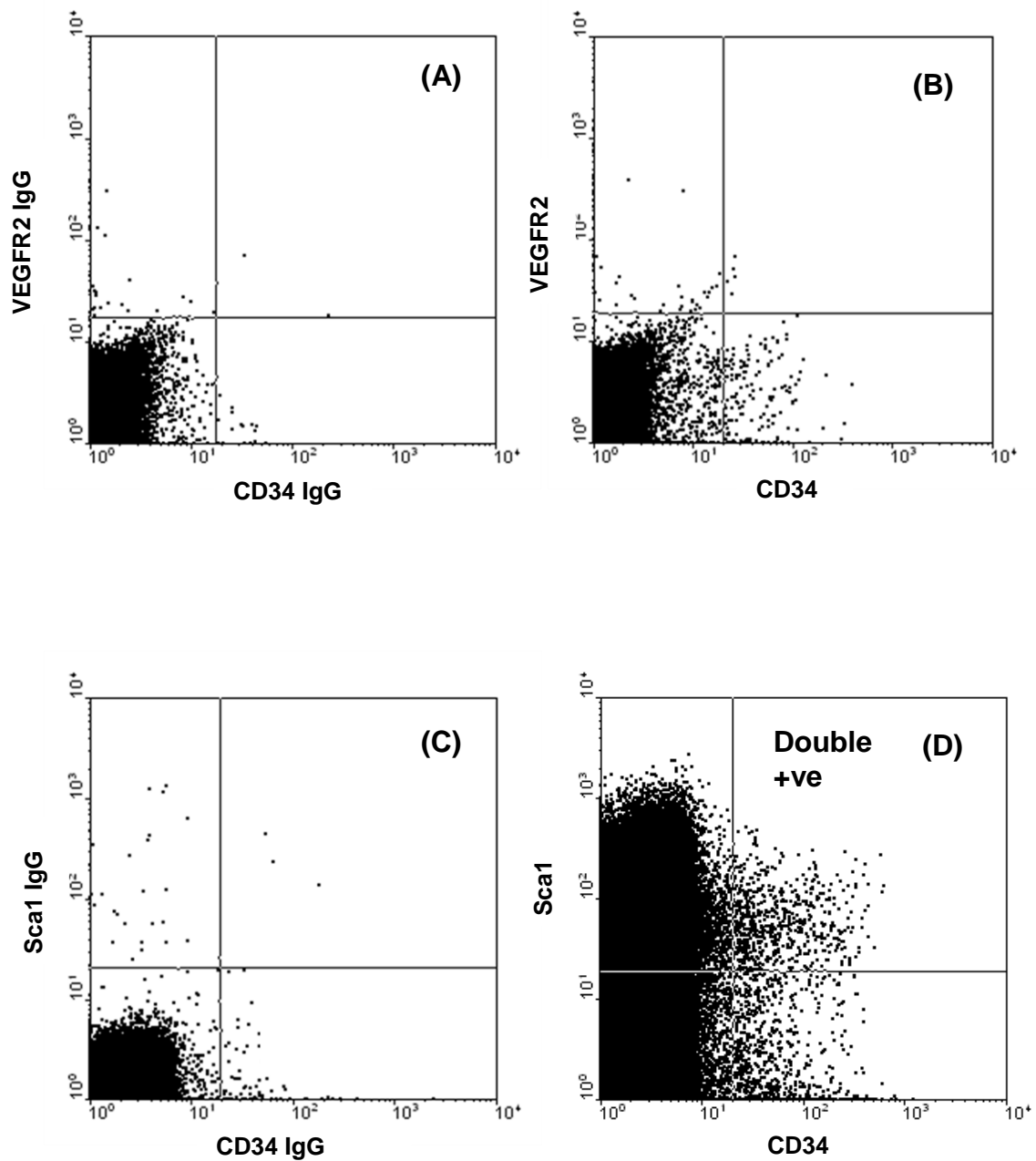
Antibody	Concentration (mg/ml)	Concentration used (mg/ml)	Fluorophore
Sca1	0.2	0.04	Pe-Cy7
CD34	0.5	0.05	PE
VEGFR2	0.2	0.04	Fluorescein

## **Results**

**Table 3.3.2** shows the numerical signal generated from antibodies and IgG controls on the same blood sample. The Sca1 antibody had a greater than 99% specificity and the CD34 antibody a greater 90% specificity, VEGFR2 was however less than 40% specific. **Fig 3.3.1** compares the fluorescent signal generated from the Sca1, CD34 and VEGFR2 antibodies with their IgG isotypes. Clear differences were seen between the isotypes and the antibody samples for Sca1 and CD34, but for the VEGFR2 antibody and its respective IgG control, the fluorescence generated was highly comparable.

**Table 3.3.2 Sca-1, CD34, VEGFR2 positive cell populations**

Sample		% of positively labelled mononuclear cells		
		Sca-1	CD34	VEGFR2
<b>1</b>	<b>Isotype</b>	0.0034	0.06	0.0021
	<b>Antibody</b>	8.01	0.73	0.003
<b>2</b>	<b>Isotype</b>	0.0013	.049	0.001
	<b>Antibody</b>	7.34	0.68	0.002
<b>% False positives</b>		<b>&lt;1%</b>	<b>&lt;10%</b>	<b>&gt;60%</b>



**Fig 3.3.1 Dot plots of mouse blood using fluorescent antibodies**

(A) and (B) compare both CD34 and VEGFR2 antibodies against their IgG controls, (C) and (D) compares Sca1 and CD34 against their isotypes. Double positive cells can be seen clearly in (D)



### 3.3.3 FURTHER ASSESSMENT OF ANTI-VEGFR2 FOR FLOW CYTOMETRY

#### Method

VEGFR2 was assessed in isolation using a new antibody and isotype control purchased to exclude contamination. The FACS protocol (**Sec 3.2.4**) was used with two different concentrations of VEGFR2: 0.025mg/ml and 0.1mg/ml. Specificity was calculated as described in section **3.3.2**.

#### Results

Reducing the antibody concentration did not reduce the level of non-specific binding and it remained greater than 60% (**Table 3.3.3**).

**Table 3.3.3 Effect of altering VEGFR2 antibody concentration**

Sample		Concentration of VEGFR2 antibody	
		2µg/µl	4µg/µl
1	Isotypes	0.13	0.21
	Antibodies	0.25	0.32
2	Isotypes	0.16	0.19
	Antibodies	0.22	0.29
Percentage false positive		>60%	>60%

## **Conclusion**

The commercially available VEGFR2 antibody was found to be unsuitable for flow cytometry and the alternative antibody DC101 clone acquired from Imclone™ systems was conjugated and tested.

### **3.3.4 CONJUGATION OF DC101 (VEGFR2) FOR FLOW CYTOMETRY**

#### **Method**

The DC101 antibody clone was conjugated to fluorescein isothiocyanate (FITC) using a commercially available conjugation kit (Invitrogen, UK). Conjugation was performed as per the data sheet, the main steps of the procedure are listed below:

200µl of DC101 was added to the vial (**A**, kit component) which contained the stir bar (small metal bar already in place for stirring). 20µl of 1M Bicarbonate pH~8.4 was then added to vial (**A**). 50µl of Dimethylsulfoxide (DMSO) was added to a vial of fluorescein (**B**, kit component), the amount of dye required for conjugation was based on the following calculation:

$$\text{mg/ml protein} = \frac{\text{X 0.2 ml X 590 X 100 X MR}}{\text{MW of protein}}$$

0.2 ml - volume of protein (antibody solution)

590 – MW (molecular weight) of the reactive dye

100 -unit conversion factor

The MW weight of most IgG is 145,000

MR (molar ratio) of dye to protein in the reaction mixture, which is related to the concentration of the antibody; for an antibody concentration of 3mg/ml it should be 20-25.

$$\frac{2 \times 0.2 \times 590 \times 100 \times 22}{145,000} = 3.6 \mu\text{l}$$

3.6µl of reactive dye solution was then added to vial (**A**) and it was stirred for 1 hour at (18-20°) out of direct light. The contents of vial (**A**) was placed drop wise into the filtration column (kit component, separating bound and unbound fluorescein). The filtration column and attached collection tube (kit component) were then centrifuged (1100 X g for 5mins, 18-20°). The collection tube ultimately collected the labelled 200-250µl protein i.e. VEGF2 antibody. The new clone of

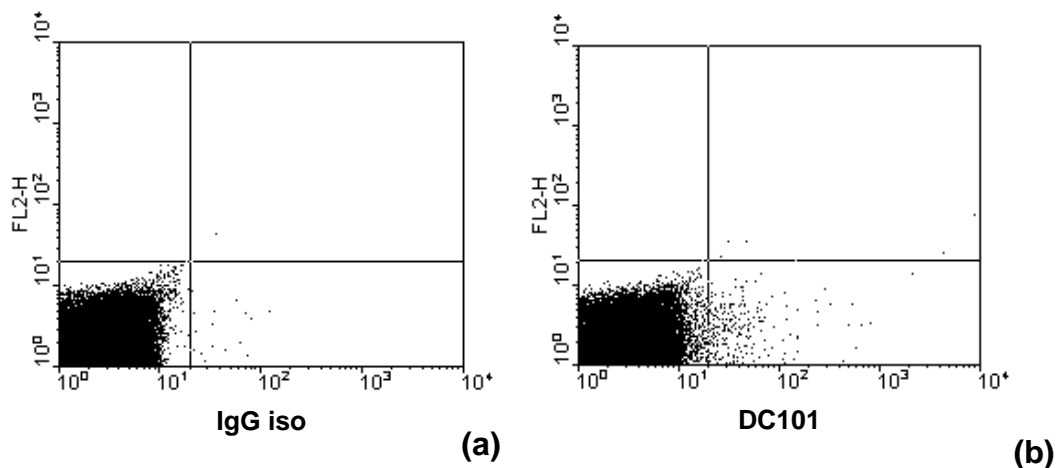
VEGFR2 (DC101) was then assessed using an appropriate isotype control at three different concentrations: 5µg/ml, 10µg/ml and 20µg/ml (**Sec 3.2.4**)

## **Results**

10µg/ml of VEGFR2 (DC101) demonstrated only 6% of false positive staining (**Table 3.3.4**), lowering the concentration of DC101 further resulted in significant loss of signal. The signal strength at 10µg/ml of DC101 is also shown in dot plot form (**Fig 3.3.2**).

**Table 3.3.4 Evaluation of VEGFR2 (DC101) antibody**

		Amount of isotype or antibody (µg/ml)		
		5	10	20
% binding	IgG Isotype	0.04	0.06	0.56
	VEGFR2 (DC101)	0.062	0.87	1.8
% false positive binding		Over diluted, weak signal	6.8 %	31 %



**Fig 3.3.2 Dot plots for (a) IgG isotype signal, (b) DC101 signal 10µg/ml**

### 3.3.5 OPTIMISATION FOR SCA-1+CD34+DC101+ CELLS

Sca1 (BD, UK) CD34 (Serotec, UK) and DC101 (Imclone™, USA) were used at the titrated dilutions (**Table 3.3.1, Sec 3.3.4**). Two sets of samples were run with all three antibodies and two samples were run with all three isotypes. Triple positive cells were calculated using the WinMDI 2.9 region and gating function (**Sec 3.1.4**). Approximately 500,000 cells were counted per sample and the specificity of the antibodies was calculated as described above.

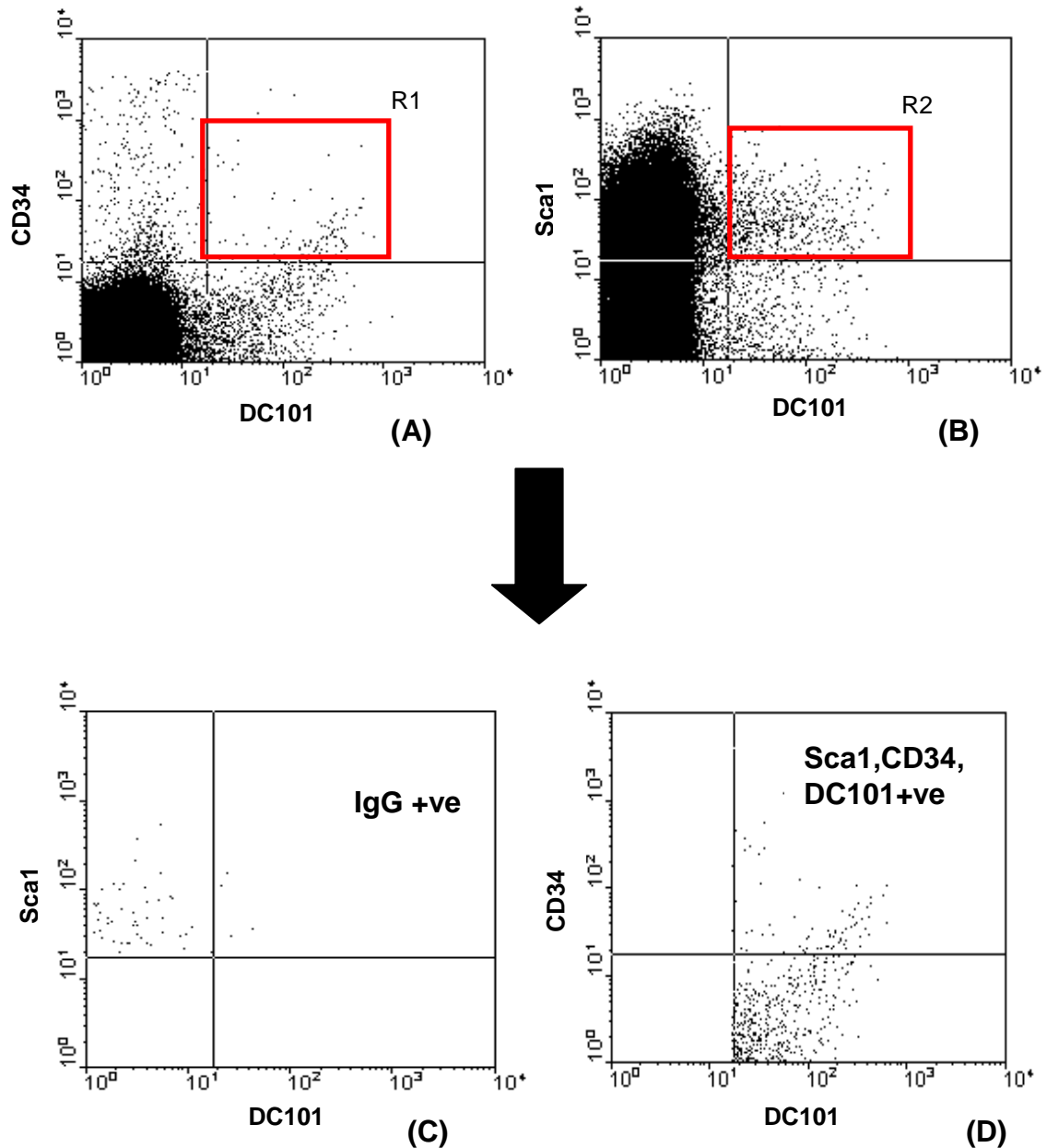
## **Results**

Using all three antibodies the specificity of the antibodies was increased with only approx 5% false positive staining (**Table 3.3.5**). Both **Fig 3.3.3 (A)** and **(B)** show double positive CD34+VEGFR2+ cells in **region 1** and Sca1+ VEGFR2+ cells in **region 2**. Using the Win MDI software package these two regions can be

reanalysed for cells positive for all three antibodies shown in **Fig 3.4.3 (D)**, for comparison triple isotype positive cells are also shown in **Fig 3.4.3 (C)**. Comparing **(C)** and **(D)** it can be seen that the triple positive antibodies are highly specific and there is minimal background fluorescence from the isotype antibodies.

**Table 3.3.5 Specificity of Sca1+CD34+VEGFR2+ antibodies**

		Sample	
		1	2
% binding	IgG Isotype controls	0.001	0.002
	Sca1+CD34+DC101+	0.021	0.034
% false positive binding		4.7	5.9



**Fig 3.3.3 Dot plots using regions and gates to show triple positive cells**  
**(A)&(B) show double positive cells in regions 1 + 2,(C) is an example of the**  
**number of triple positive isotype control cells, with (D) showing the actual**  
**triple positive Sca1+CD34+DC101+ cells.**

## **Conclusion**

The above studies demonstrated that Sca-1 and CD34 binding was specific but that the commercially available mouse VEGFR2 demonstrated a high level of non-specific binding. Antibody contamination and dilution were considered as possible reasons for this non-specificity but excluded through further assessment; because of this the VEGFR2 antibody (BD-555308) from BD Biosciences was concluded to be unsuitable for use in flow cytometry. A novel form of the VEGFR2 named DC101 from Imclone™ Systems was used as an alternative to the commercially available VEGFR2 (BD, UK)<sup>381</sup>. A small quantity of this antibody was donated to this project by the manufacturers. DC101 (VEGFR2) was conjugated with FITC and then optimised for use with Sca1 and CD34 antibodies. In combination these antibodies were found to be highly specific.



## 4.0 THE PROGENITOR RESPONSE TO VENOUS THROMBOSIS

### 4.1.1 INTRODUCTION

Progenitor cells have for over a decade been implicated in post-natal vascularisation<sup>194;366;382</sup>. In venous thrombosis, bone marrow Tie-2 positive cells are recruited into the resolving thrombus in a temporal pattern. These cells were once thought to be EPCs but are now believed to be a subset of pro-angiogenic monocytes<sup>209</sup>. Furthermore the direct upregulation of VEGF in the murine thrombus results in greater numbers of circulating Sca1+ (BM marker) cells and enhanced thrombus resolution<sup>313</sup>. In an effort to further classify the progenitor response to venous thrombosis, this study examined the temporal changes in circulating and BM resident Sca1+CD34+VEGFR2+ HPCs following induction of thrombus, using flow cytometry. The growth factor or cytokine VEGF has previously implicated in thrombus resolution in the rat model of venous thrombosis<sup>120;121;167;313</sup>. Here we aimed to elucidate the temporal changes in a wider range of cytokines in the thrombus, vein wall, circulation and laparotomy wound associated with HPC mobilisation and recruitment. Cytokines were measured using a combination of ELISA and multiplex ELISA.

#### **4.1.2 SCA1+CD34+VEGFR2+ (DC101) HPCS AT 12HRS**

##### **Method**

Circulating and BM resident HPCs were measured in three groups of wild type Balb/C mice. Ten animals had a thrombus forming procedure, ten had a sham operation and 6 animals had no intervention (**Sec 2.1.2**). Approximately 12hrs following thrombus induction all animals were sacrificed under isoflurane anaesthetic by exsanguination, blood, thrombus, vein wall, wounds and femur bones were collected (**Sec 2.1.4**). Blood and BM were then processed for flow cytometry (**Sec 3.2.4 & 3.2.5**). HPCs were measured in the mononuclear fraction of lysed whole blood and BM and calculated as a percentage of the total number of gated mononuclear cells. Any IgG isotype background signal detected was subtracted and the three groups were compared in pairs using the Mann Whitney U test.

##### **Results**

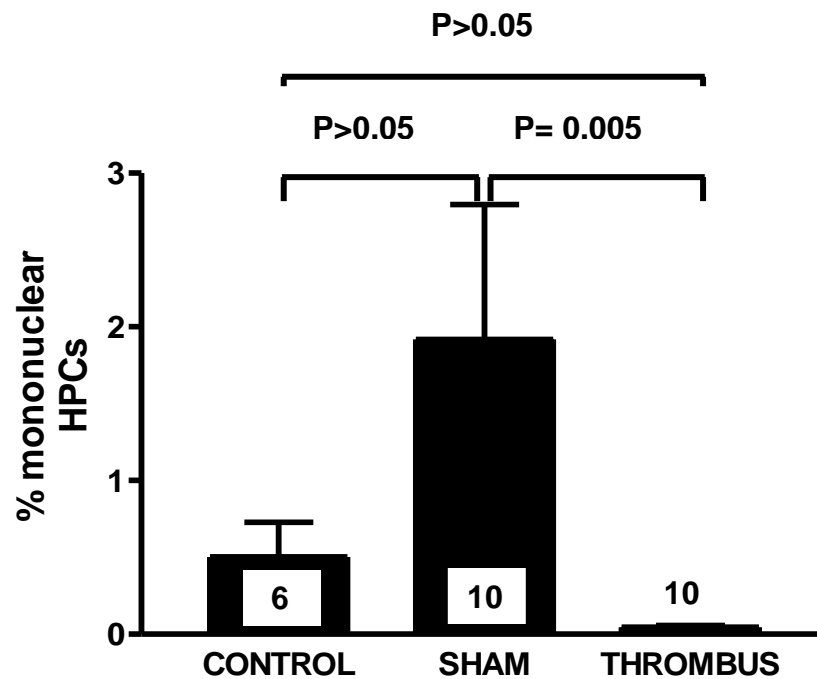
12hrs post thrombus induction, there was a depletion of circulating HPCs in thrombus group mean 0.04% (CI: 0.004-0.09) compared to the sham control group mean of 1.92%, (CI: 0.07-3.9, **P=0.004, Table 4.1.1, Fig 4.1.1**).

The BM resident HPCs also fell from a mean 6.1% (CI:3.2-8.9) to a mean of 1.7% (CI:0.75-2.6) in the thrombus group and a mean of 0.95% (CI:0.6-1.3) in the sham operated group (**Table 4.1.2, Fig 4.1.2**).

There was a statistical difference between both operated groups and the non-interventional group ( $P<0.05$ ), however there was no difference between the sham operated and the thrombus group.

**Table 4.1.1 Percentage of circulating mononuclear HPCs at 12hrs**

Animal	% SCA-1+CD34+ VEGFR2+ mononuclear cells		
	Control (no intervention)	Sham	Thrombus
Mean	0.503	1.91	0.086
Standard Error	0.25	0.88	0.043



**Fig 4.1.1 Circulating HPCs at 12hrs (Mann Whitney U)**

Table 4.1.2 Percentage of BM mononuclear HPCs at 12hrs

Animal	% SCA-1+CD34+ VEGFR2+ mononuclear cells		
	Control (no intervention)	Sham	Thrombus
Mean	6.1	0.95	1.7
Standard Error	1.1	0.14	0.36

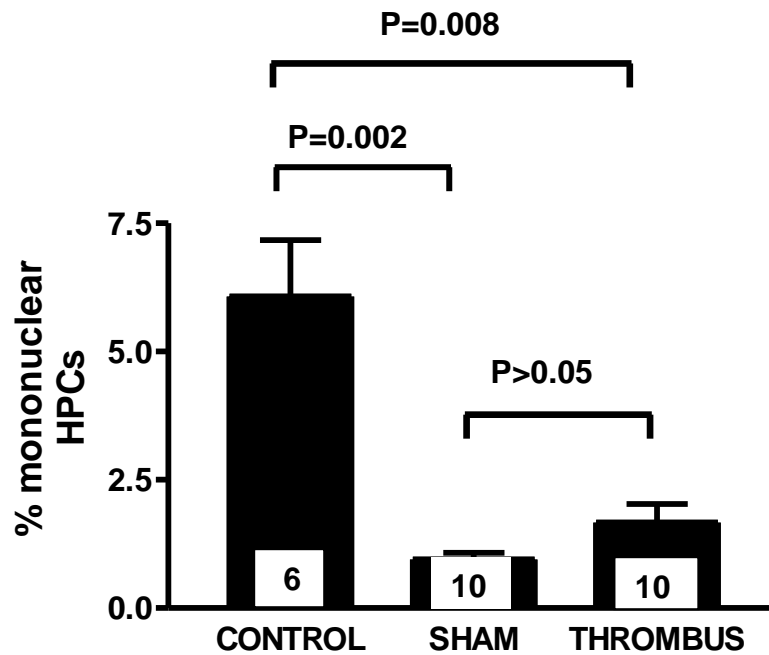


Fig 4.1.2 BM resident HPCs at 12hrs (Mann Whitney U)

### **4.1.3 CIRCULATING AND BM RESIDENT HPCS FROM 24HRS TO DAY14 FOLLOWING THROMBOGENESIS**

#### **Method**

Circulating and BM resident HPCs were examined in animals at 24hrs, 48hrs, 7 days and 14 days. Thrombus was formed in 6 animals at each time point, with an equivalent number of sham operated animals (**Sec 2.1.2**). Blood and BM were prepared for flow cytometry (**Sec 3.2.4, 3.2.5**). Groups were compared in pairs at each time point using Mann Whitney U and temporal profiles were compared using two-way ANOVA.

#### **Results**

##### **Circulating HPCs**

There was a difference in the temporal circulating profile of HPCs of animals in which thrombus had been induced when compared with sham control animals ( $P < 0.006$ , ANOVA). At 24hrs there was a 25-fold increase in circulating SCA-1+, CD34+, VEGFR2+ cells above sham controls: 0.378% (CI:0.12-0.76) vs 0.015% (0.01-0.13),  $P = 0.004$ , with a lesser sustained increase between day 7 ( $P > 0.05$ ) and 14 ( $P = 0.007$ , **Table 4.1.3, Fig 4.1.3**).

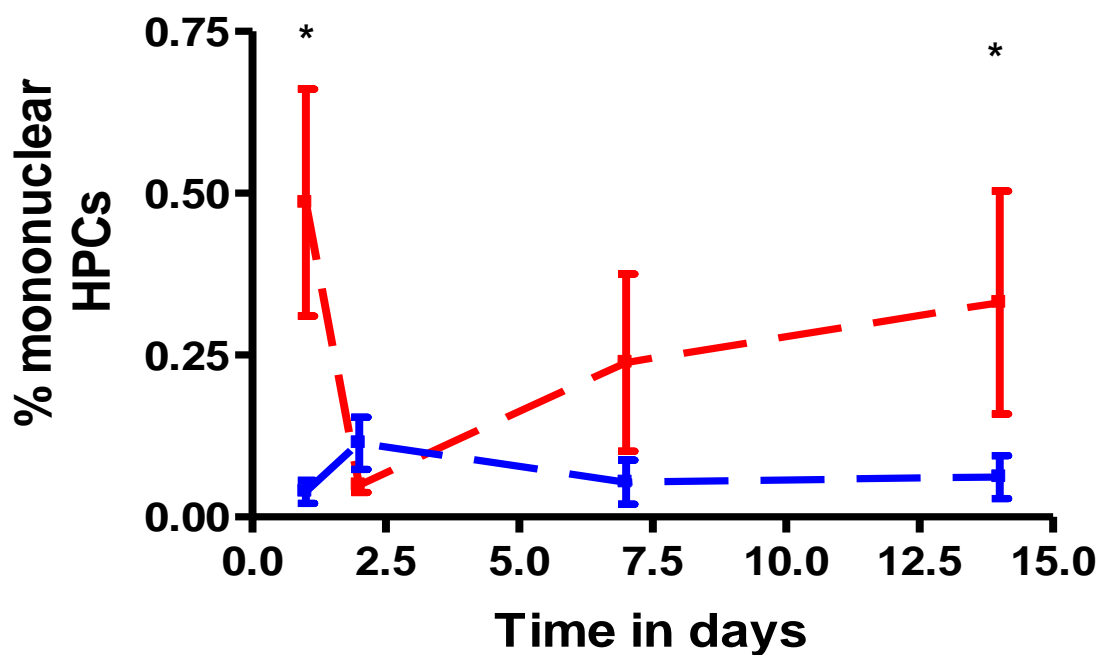
##### **BM resident HPCs**

There was no difference in the BM resident HPC profiles between those animal in which thrombus had been induced and sham controls ( $P > 0.05$ ). BM resident HPCs were depleted over the 48hr period in both the sham and thrombus groups (0.05

%, CI: 0.03-0.12) when compared with non-operated controls (6.69%, CI: 1.6-9.5, P=0.002, **Table 4.1.4, Fig 4.1.4**).

**Table 4.1.3 Circulating HPCs day 1-14**

Time in days	Animal no.	Sham mean	SEM	Thrombus Mean	SEM	P value
1	10	0.039	0.033	0.485	0.17	0.006
2	10	0.114	0.040	0.049	0.011	>0.05
7	6	0.054	0.034	0.24	0.137	>0.05
14	6	0.061	0.018	0.33	0.172	0.007



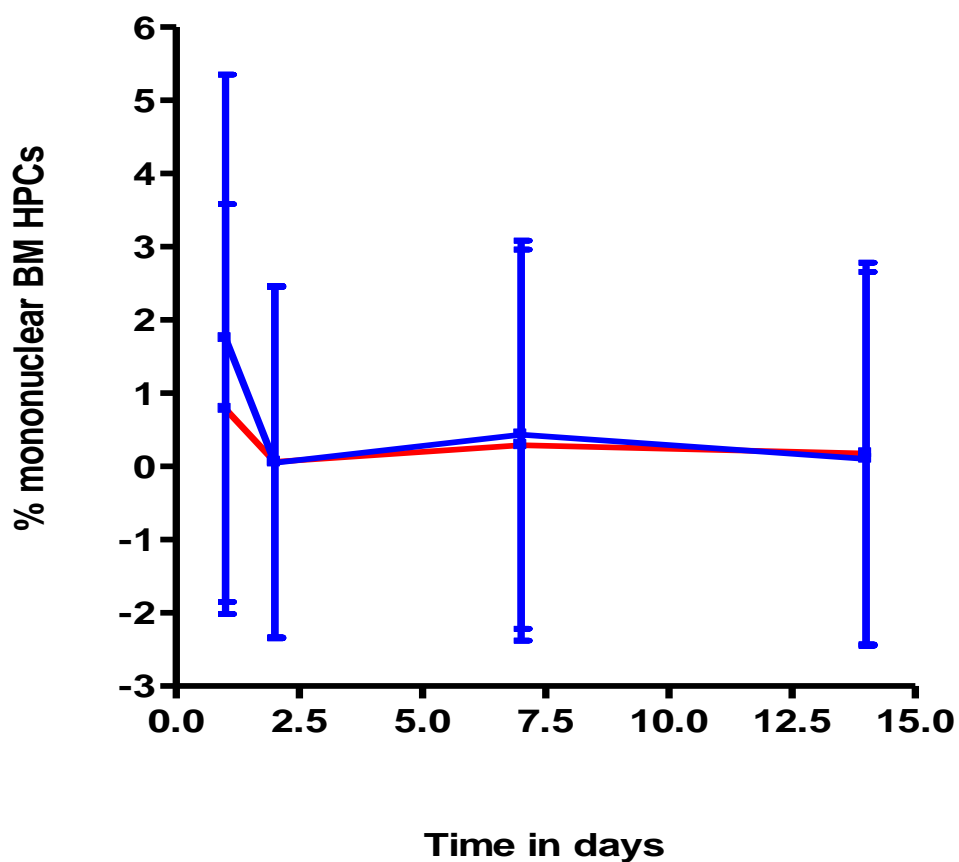
**Fig 4.1.3 Expression of circulating HPCs day 1-14**

Thrombus v. sham operated animals (■ sham, ■ thrombus)

P<0.006 by ANOVA, \*P<0.05 Mann Whi

**Table 4.1.4 BM resident HPCs day 1-14**

Time in days	Animal no.	Sham mean	SEM	Thrombus Mean	SEM	P value
1	10	1.75	3.60	0.79	2.80	>0.05
2	10	0.05	2.40	0.07	2.40	>0.05
7	6	0.43	2.65	0.29	2.67	>0.05
14	6	0.10	2.55	0.18	2.60	>0.05



**Fig 4.1.4 Expression of BM resident HPCs day 1-14**

Thrombus v. sham operated animals (■ Sham, ■ Thrombus),  
P>0.05 ANOVA



## **4.2 CYTOKINE RESPONSE TO VENOUS THROMBOSIS**

### **4.2.1 AIMS**

A combination of multiplex and sandwich ELISA were used to measure the cytokine changes which occurred between 12hrs and day 14 following induction of thrombus. GCSF, GMCSF, VEGF, PIGF and SDF1 were measured in mouse thrombus, vein wall, plasma and laparotomy wound tissue.

### **Measurement of cytokines using ELISA**

#### **Method**

All snap frozen tissue specimens underwent protein extraction (**Sec 2.1.4**). Individual sample protein concentrations were then measured using a Bradford Assay (**Appendix 1**). Vein wall and thrombus were aliquoted into 110µl replicates for single sandwich ELISA and 200µl replicates which were sent to Perbio® laboratories for Multiplex ELISA. GMCSF, VEGF and SDF1 in thrombus, vein wall and plasma were all evaluated using the Multiplex system. PIGF and GCSF were analysed as separated sandwich ELISAs. All wound tissue samples were also analysed using sandwich ELISA. All results were normalised for protein concentration and compared using ANOVA with Bonferroni post-testing. Actual protein concentration has not been commented on except for when both thrombus and wound protein have been measured by the same ELISA method.

## **Results**

### **4.2.2 GCSF EXPRESSION**

Thrombus expression of GCSF was found to be significantly elevated 12hrs after thrombus formation when compared to all other time points (1-way ANOVA,  $P=0.0002$ ) (**Table 4.2.1, Fig 4.2.1**). Wound GCSF similarly peaked at 12hrs (1-way ANOVA,  $P=0.0001$ ), however wound GCSF protein concentration was 10-fold greater than that in the thrombus (**Table 4.2.2, Fig 4.2.2**). Vein wall expression (**Table 4.2.3, Fig 4.2.3**) of GCSF was significantly greater than sham vein levels of the protein at 12hrs but fell to sham vein levels by day 7 ( $P=0.0047$ , 2-way ANOVA). Data for plasma GCSF was not available owing to limited plasma available from mouse blood.

### **4.2.3 GMCSF EXPRESSION**

Thrombus expression of GMCSF (**Table 4.2.4, Fig 4.2.4**) rose significantly by day 14 ( $P=0.002$ , 1-way ANOVA). Comparatively wound GMCSF (**Table 4.2.5, Fig 4.2.5**) also peaked at day 14 ( $P=0.0047$ , 1-way ANOVA). Vein wall GMCSF (**Table 4.2.6, Fig 4.2.6**) was significantly higher than sham levels at 12 and 24hrs post thrombus induction falling to sham levels after this point ( $P=0.004$ , 2-way ANOVA). Plasma GMCSF (**Table 4.2.7, Fig 4.2.7**) expression in thrombus animals was only elevated above sham levels at 12hrs ( $P<0.01$ , Mann Whitney U).

#### 4.2.4 VEGF EXPRESSION

Thrombus VEGF expression (**Table 4.2.8, Fig 4.2.8**) was maximal 48hrs after thrombus formation, producing a bell shaped curve ( $P=0.016$ , 1-way ANOVA). Wound tissue produced a similar expression pattern ( $P=0.014$ , 1-way ANOVA) (**Table 4.2.9, Fig 4.2.9**). Vein wall expression (**Table 4.2.10, Fig 4.2.10**) were statistically higher than the sham expression at 12 and 24hrs, ( $P=0.0001$ , 2-way ANOVA). Plasma VEGF expression (**Table 4.2.11, Fig 4.2.11**) was elevated above sham levels at only 12hrs ( $P < 0.05$ , Mann Whitney U).

#### 4.2.5 PLGF EXPRESSION

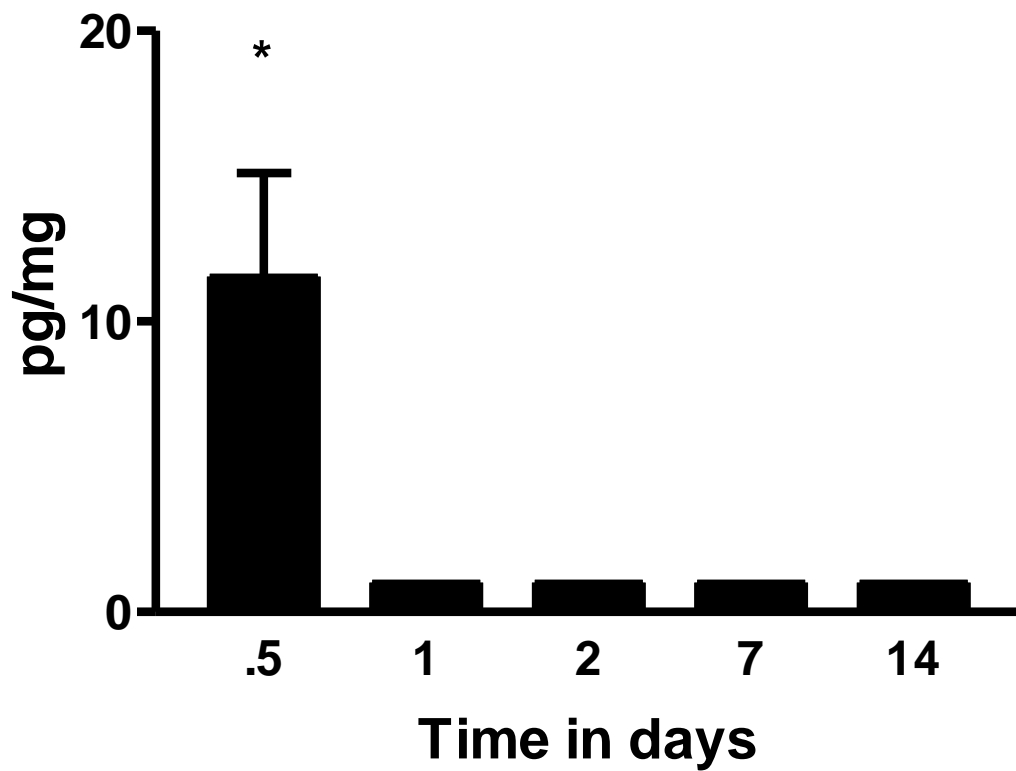
Thrombus PLGF expression (**Table 4.2.12, Fig 4.2.12**) peaked at day 7 and was falling by day 14, ( $P=0.0001$ , 1-way ANOVA). Wound PLGF expression (**Table 4.2.13, Fig 4.5.13**) did not produce any peaks or troughs like its expression in thrombus but produced a constant level of expression which was higher than that of thrombus levels until they peaked at day 7. There was bimodal expression of PLGF in both the sham and thrombus vein (**Table 4.2.14, Fig 4.2.14**), the greatest expression was at 24hrs, falling by 48hrs and rising again between day 7 and 14. Vein wall PLGF expression after thrombus induction remained continuously greater than that found in sham vein wall ( $P=0.0001$ , 2-way ANOVA). Plasma PLGF expression (**Table 4.2.15, Fig 4.2.15**) was elevated above sham levels at 12 and 24hrs ( $P=0.004$ , 2-way ANOVA).

#### **4.2.6 SDF-1 EXPRESSION**

SDF-1 expression in the thrombus (**Table 4.2.16, Fig 4.2.16**) was significantly higher by day 14 than at earlier time points ( $P=0.0005$ , 1-way ANOVA). A similar pattern was seen in wound tissue  $P=0.035$  (**Table 4.2.17, Fig 4.2.17**). In the vein wall (**Table 4.2.18, Fig 4.2.18**) there was a bimodal expression pattern which was highest at 12hrs in both sham and thrombus operated animals, falling by 24hrs but then climbing again only in the thrombus group and remaining elevated above sham levels until day 14 ( $P=0.0036$ , 2-way ANOVA). There was no statistically significant difference between sham or thrombus plasma levels of SDF-1 (**Table 4.2.19, Fig 4.2.19**).

**Table 4.2.1 Thrombus GCSF expression**

TIME	12 hrs	24 hrs	48 hrs	7 days	14 days
MEAN pg/mg	11.5	1.0	1.0	1.0	1.0
SEM	3.6	0.0	0.0	0.0	0.0

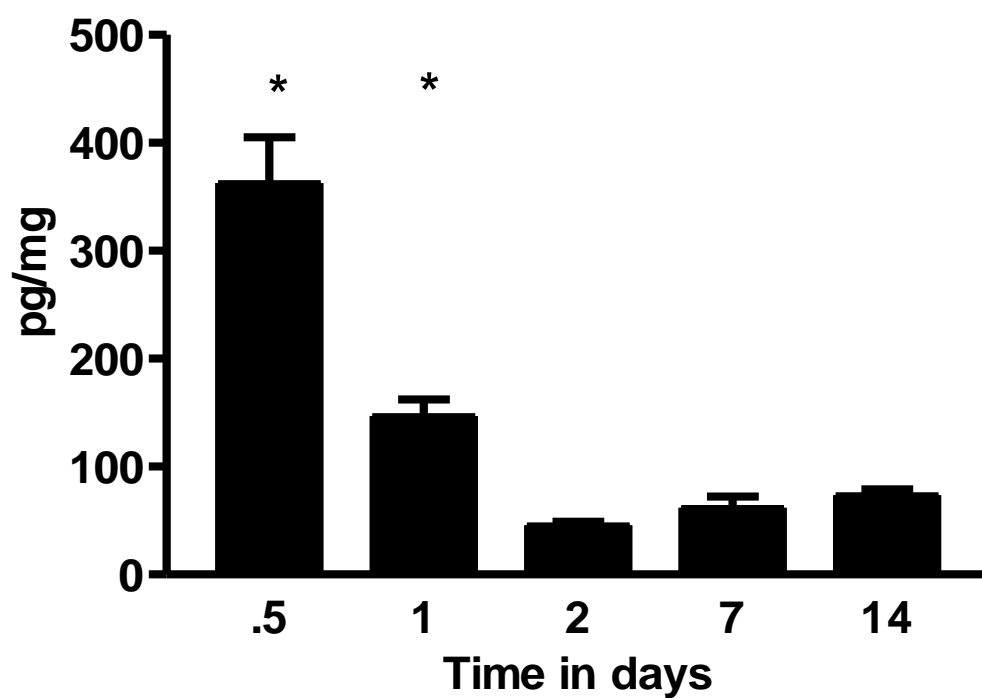


**Fig 4.2.1 Thrombus GCSF expression**

P=0.0002 (1-way ANOVA) \*Bonferroni post test P<0.05

**Table 4.2.2 Wound GCSF expression**

TIME	12 hrs	24 hrs	48 hrs	7 days	14 days
MEAN pg/mg	362.8	146.7	45.8	61.9	73.7
SEM	42.5	15.5	3.1	10.6	5.2



**Fig 4.2.2 Wound GCSF expression**

P=0.0001 (1-way ANOVA) \* Bonferroni post test P<0.05

Table 4.2.3 Vein wall GCSF expression

TIME		12 hrs	24 hrs	48 hrs	7 days	14 days
SHAM VEIN pg/ml	MEAN	638.0	383.0	189.0	111.0	101.0
	SEM	129.0	98.0	47.0	42.0	27.0
THROMBUS pg/ml	MEAN	1148.0	632.0	374.0	60.0	144.0
	SEM	116.0	144.0	118.0	12.0	144.0

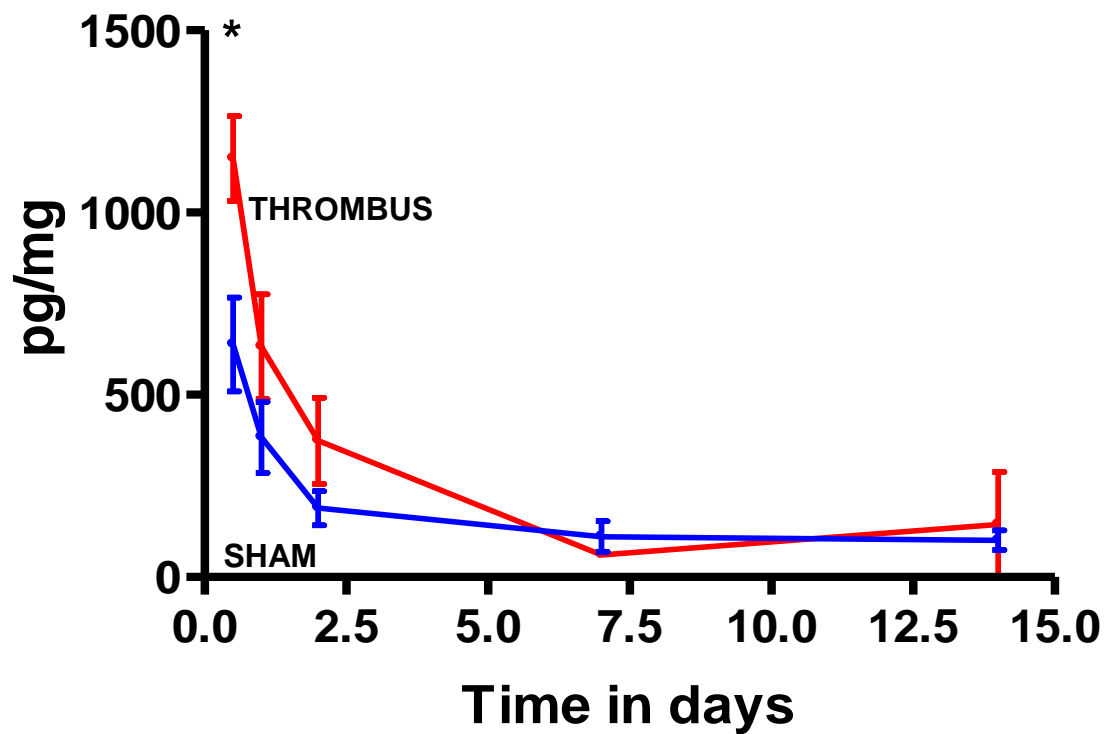
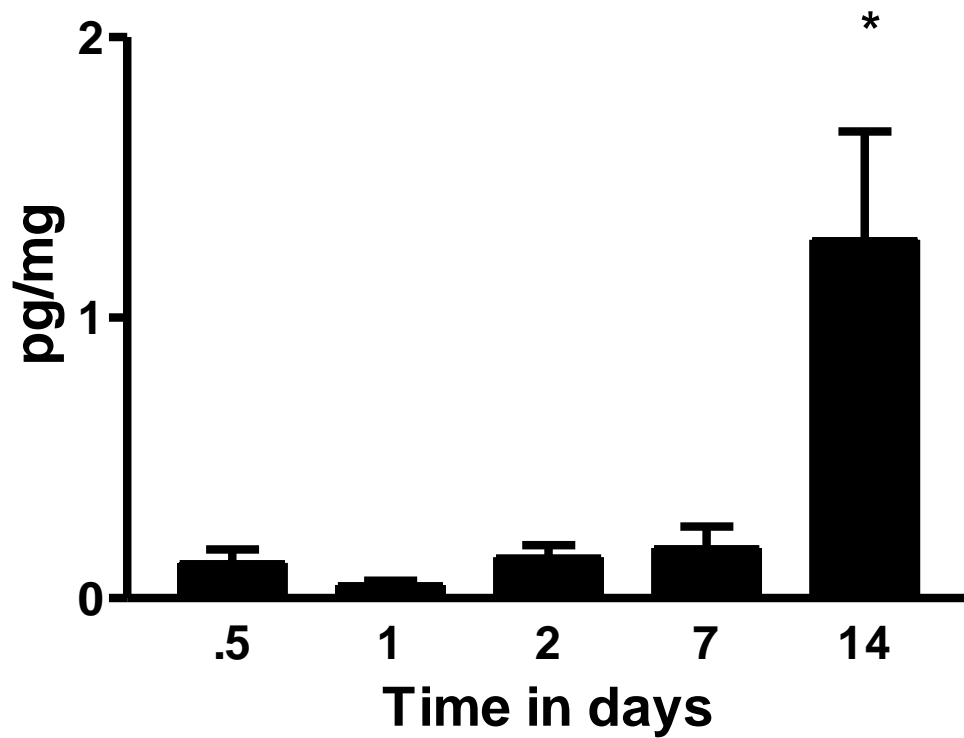


Fig 4.2.3 Vein wall GCSF expression

P=0.0047 (2-way ANOVA) \*Bonferroni post test P<0.05

**Table 4.2.4 Thrombus GMCSF expression**

TIME	12 hrs	24 hrs	48 hrs	7 days	14 days
MEAN pg/mg	0.12	0.047	0.15	0.18	1.3
SEM	0.05	0.02	0.04	0.08	0.39



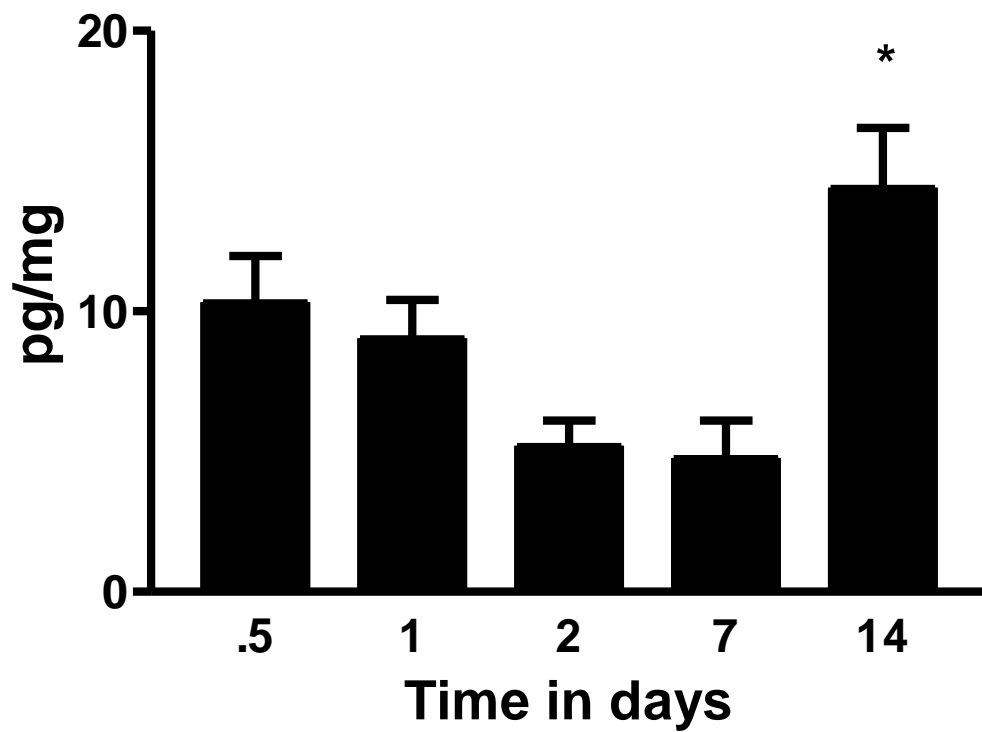
**Fig 4.2.4 Thrombus GMCSF expression**

P=0.002 (1-way ANOVA) \* Bonferroni post test P<0.05



**Table 4.2.5 Wound GMCSF expression**

TIME	12 hrs	24 hrs	48 hrs	7 days	14 days
MEAN pg/mg	10.3	9.0	5.2	4.8	14.2
SEM	4.2	3.7	2.1	1.95	5.9



**Fig 4.2.5 Wound GMCSF expression**

P=0.0047 (1-way ANOVA) \* Bonferroni post test P<0.05

Table 4.2.6 Vein wall GMCSF expression

TIME		12 hrs	24 hrs	48 hrs	7 days	14 days
SHAM VEIN pg/ml	MEAN	2.2	1.93	1.30	2.35	1.83
	SEM	0.42	0.32	0.39	0.63	0.60
THROMBUS pg/ml	MEAN	4.8	4.78	3.27	0.83	2.1
	SEM	0.98	0.64	0.78	0.16	0.93

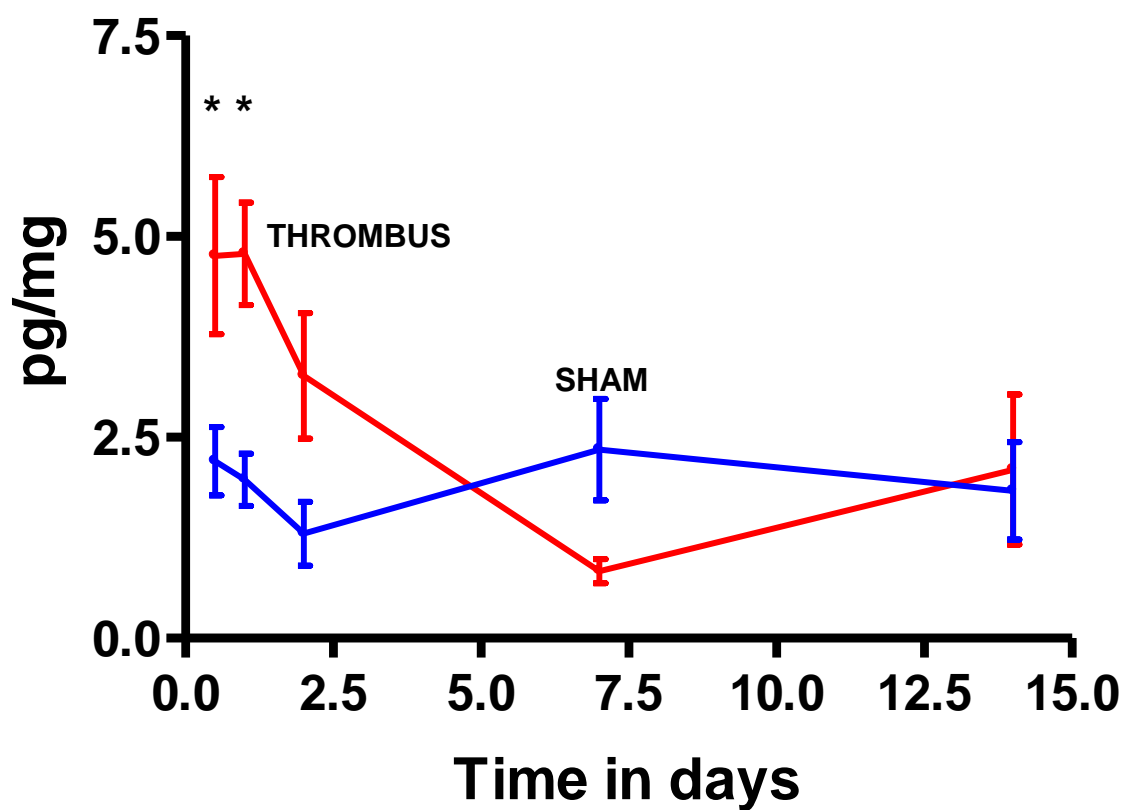
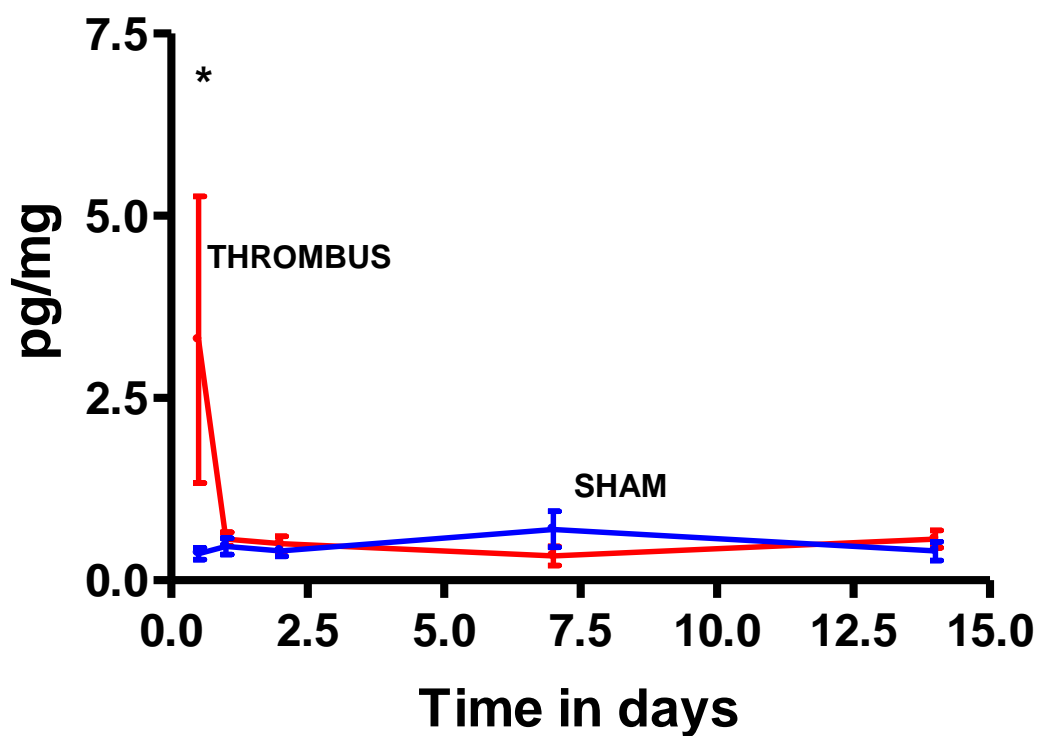


Fig 4.2.6 Vein wall GMCSF expression

P=0.004 (2-way ANOVA) \* Bonferroni post test P<0.05

**Table 4.2.7 Plasma GMCSF expression**

TIME		12 hrs	24 hrs	48 hrs	7 days	14 days
SHAM VEIN pg/ml	MEAN	0.37	0.47	0.40	0.70	0.40
	SEM	0.08	0.1	0.07	0.26	0.13
THROMBUS pg/ml	MEAN	3.30	0.57	0.50	0.33	0.57
	SEM	2.0	0.1	0.1	0.1	0.1

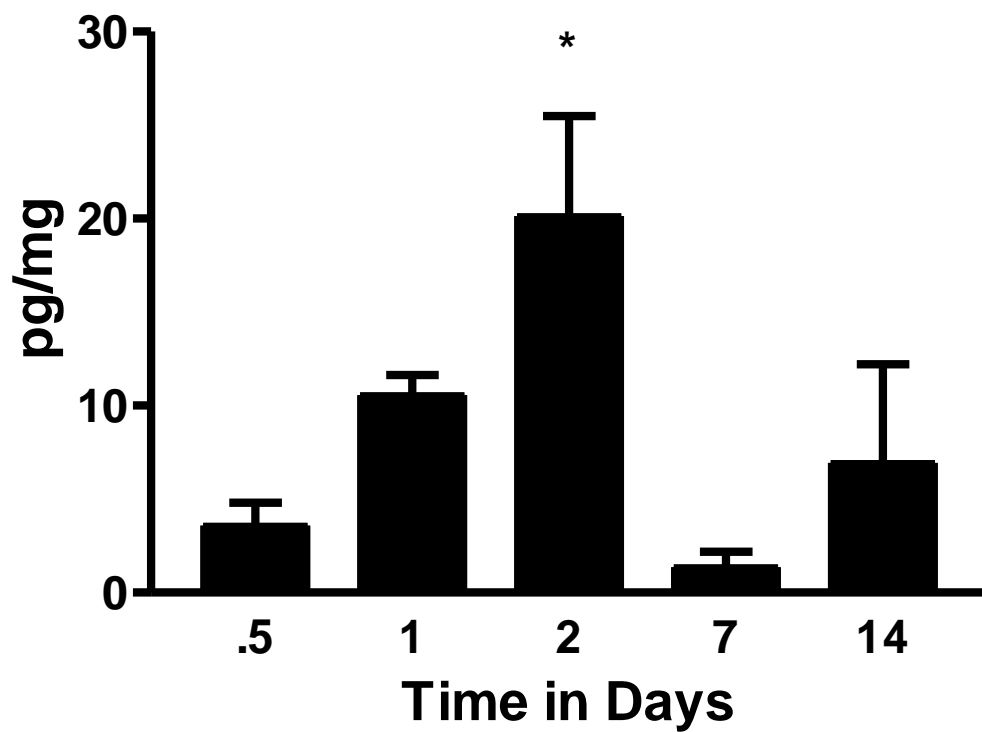


**Fig 4.2.7 Plasma GMCSF expression**

\* Mann Whitney P<0.01

**Table 4.2.8 Thrombus VEGF expression**

<b>TIME</b>	<b>12 hrs</b>	<b>24 hrs</b>	<b>48 hrs</b>	<b>7 days</b>	<b>14 days</b>
<b>MEAN pg/mg</b>	3.6	10.6	20.1	1.4	6.9
<b>SEM</b>	1.2	1.1	5.4	0.8	5.3

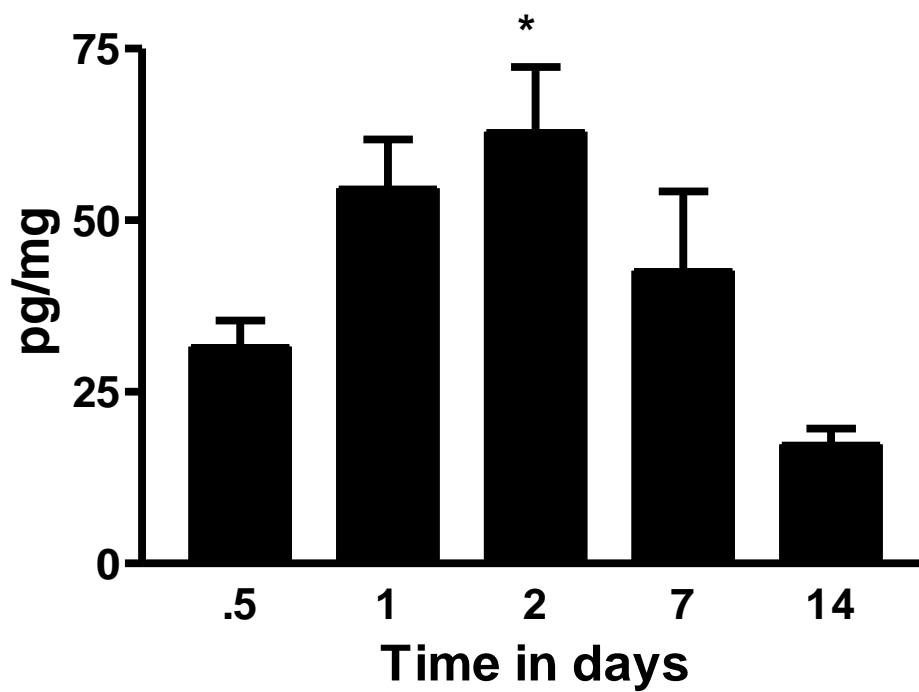


**Fig 4.2.8 Thrombus VEGF expression**

P=0.016 (1-way ANOVA) \* Bonferroni post test P<0.05

**Table 4.2.9 Wound VEGF expression**

TIME	12 hrs	24 hrs	48 hrs	7 days	14 days
MEAN pg/mg	31.6	54.7	62.9	42.6	17.3
SEM	3.8	7.1	9.5	11.6	2.3



**Fig 4.2.9 Wound VEGF expression**

P=0.014 (1-way ANOVA), \* Bonferroni post test P<0.05

Table 4.2.10 Vein wall VEGF expression

TIME		12 hrs	24 hrs	48 hrs	7 days	14 days
SHAM VEIN pg/ml	MEAN	31.9	6.4	5.5	3.8	3.2
	SEM	4.4	0.4	0.9	0.1	0.1
THROMBUS pg/ml	MEAN	52.8	40.6	22.2	2.9	3.8
	SEM	2.8	1.5	1.9	0.11	0.12

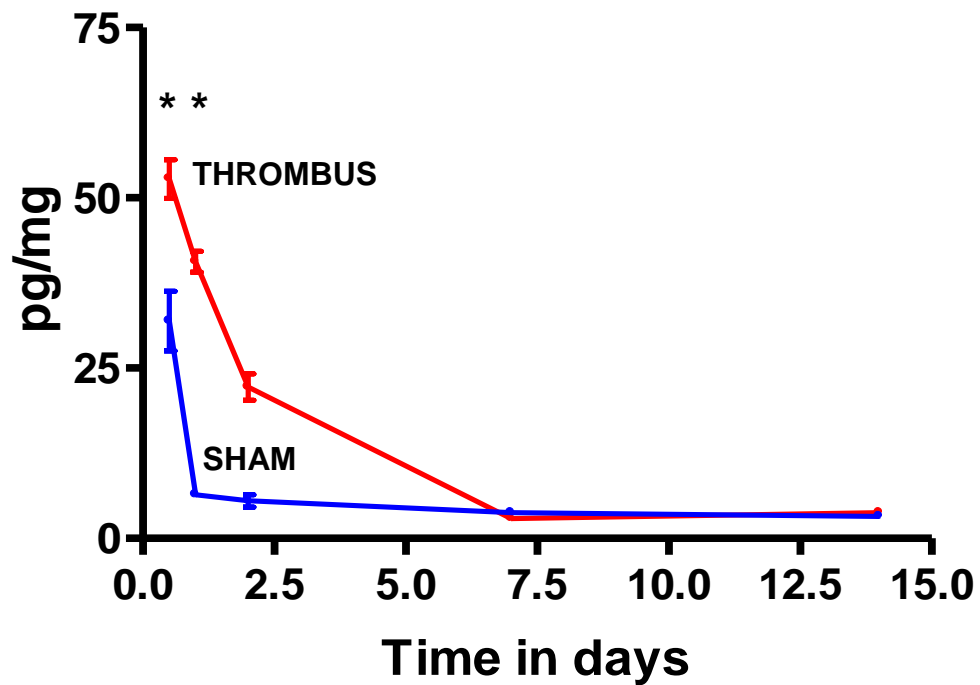
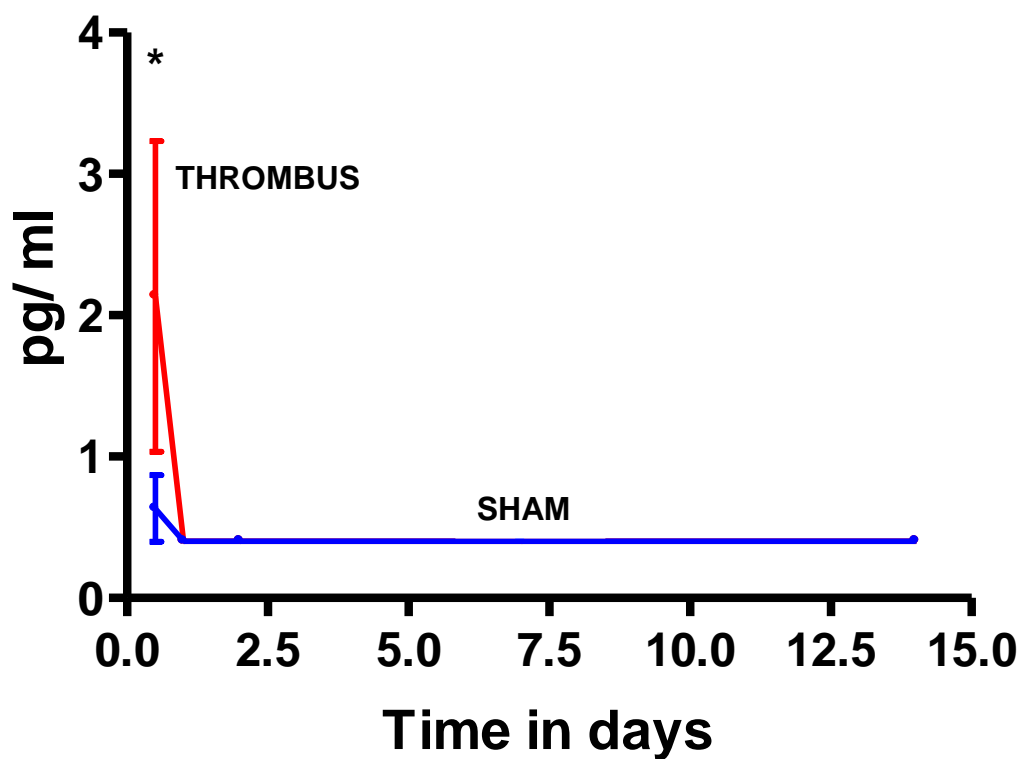


Fig 4.2.10 Vein wall VEGF expression

P=0.0001 (2-way ANOVA), \* Bonferroni post test P<0.05

**Table 4.2.11 Plasma VEGF expression**

TIME		12 hrs	24 hrs	48 hrs	7 days	14 days
SHAM VEIN pg/ml	MEAN	0.63	0.40	0.40	0.40	0.40
	SEM	0.23	0.00001	0.00001	0.00001	0.00001
THROMBUS pg/ml	MEAN	2.13	0.40	0.40	0.40	0.40
	SEM	1.1	0.00001	0.00001	0.00001	0.00001

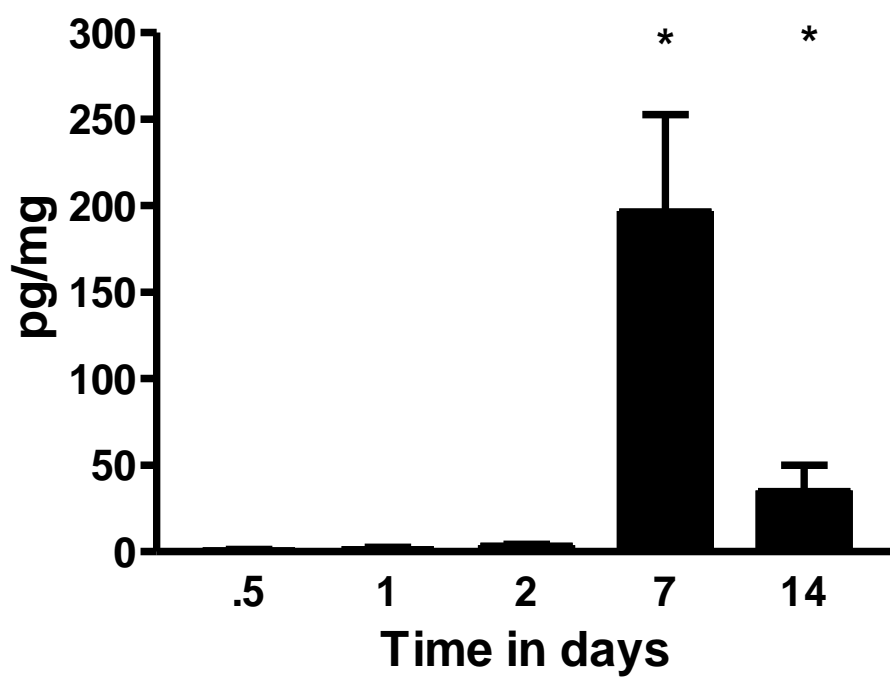


**Fig 4.2.11 Plasma VEGF expression**

P=0.02 (2-way ANOVA), \*Bonferroni post test P<0.05

**Table 4.2.12 Thrombus PIGF expression**

TIME	12 hrs	24 hrs	48 hrs	7 days	14 days
MEAN pg/mg	1.1	1.8	3.8	197.1	35.4
SEM	0.2	0.7	0.5	55.5	14.7



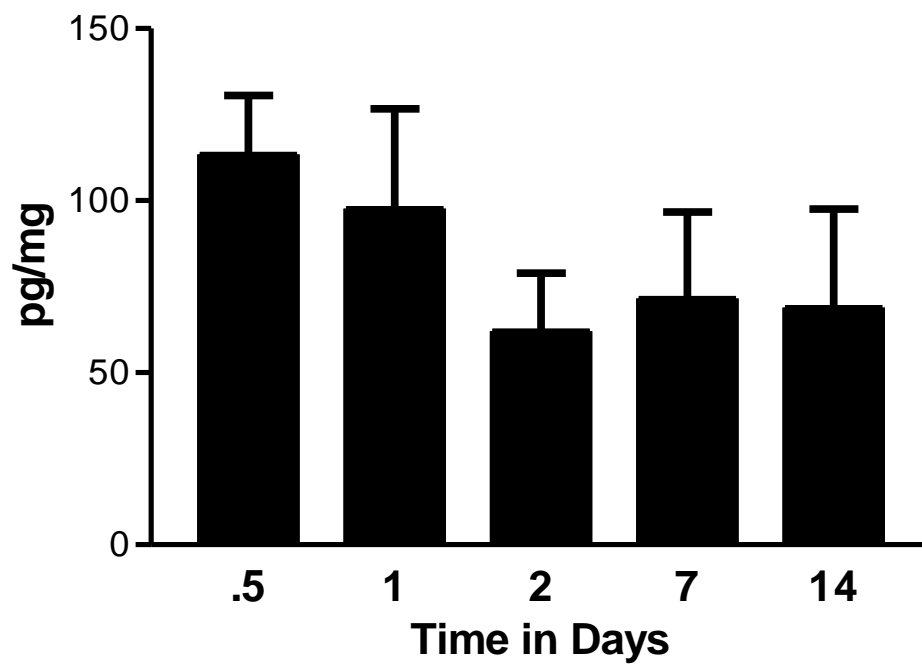
**Fig 4.2.12 Thrombus PIGF expression**

P=0.0001 (1-way ANOVA), \* Bonferroni post test P<0.05



**Table 4.2.13 Wound PIGF expression**

<b>TIME</b>	<b>12 hrs</b>	<b>24 hrs</b>	<b>48 hrs</b>	<b>7 days</b>	<b>14 days</b>
<b>MEAN pg/mg</b>	113.4	98.0	62.1	71.6	69.0
<b>SEM</b>	46.0	39.9	25.3	29.2	28.1



**Fig 4.2.13 Wound PIGF expression**

P>0.05 (2-way ANOVA)

Table 4.2.14 Vein wall PLGF expression

TIME		12 hrs	24 hrs	48 hrs	7 days	14 days
SHAM VEIN pg/ml	MEAN	116.0	108.0	32.0	92.0	81.0
	SEM	24.0	30.0	6.9	18.0	13.0
THROMBUS pg/ml	MEAN	297.0	310.0	119.0	241.0	197.0
	SEM	52.0	33.0	17.0	25.0	26.0

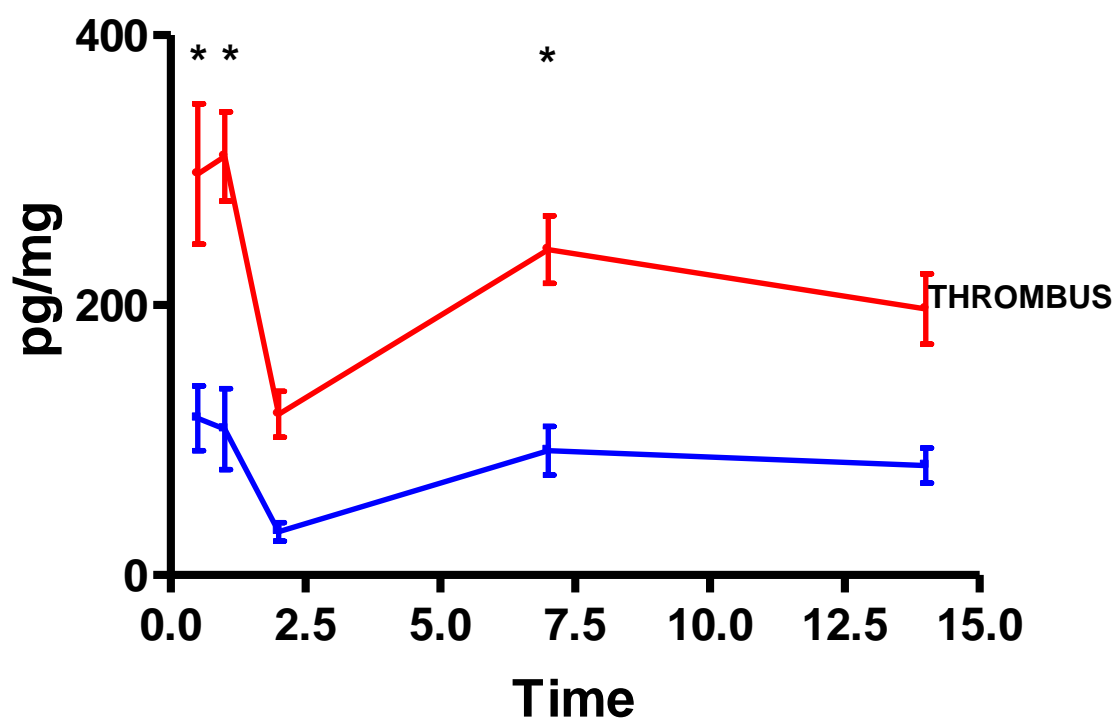


Fig 4.2.14 Vein wall PLGF expression

P= 0.0001(2-way ANOVA),\* Bonferroni post test P<0.05

Table 4.2.15 Plasma PIGF expression

TIME		12 hrs	24 hrs	48 hrs	7 days	14 days
SHAM VEIN pg/ml	MEAN	24.5	10	10	10	10
	SEM	12	0	0	0	0
THROMBUS pg/ml	MEAN	106.5	110	10	10	10
	SEM	19	56	0	0	0

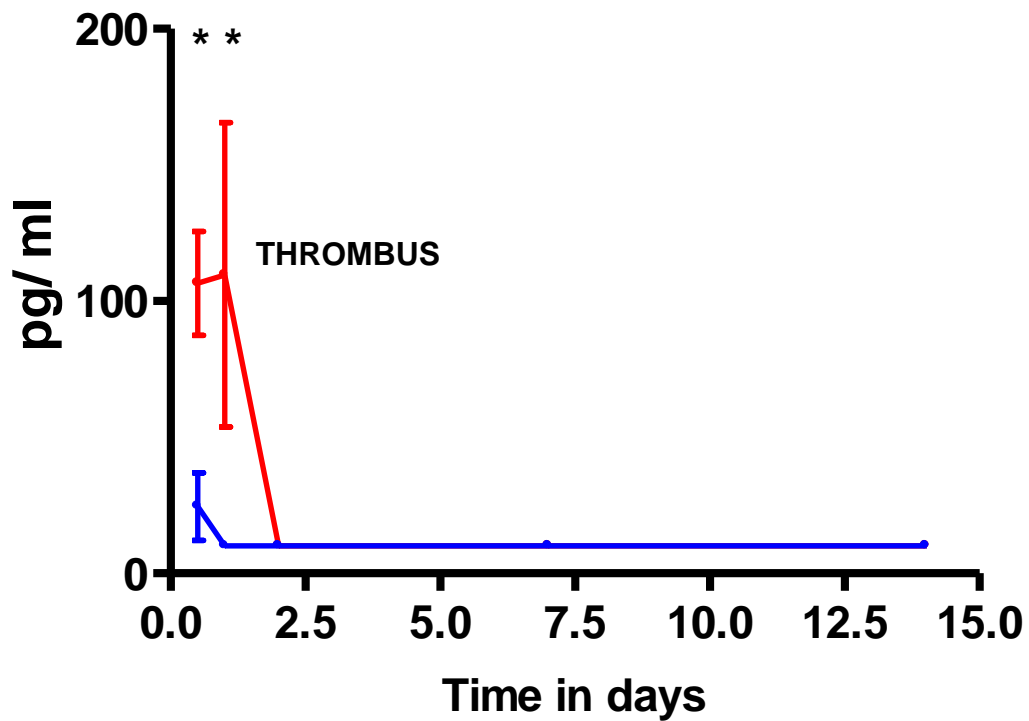
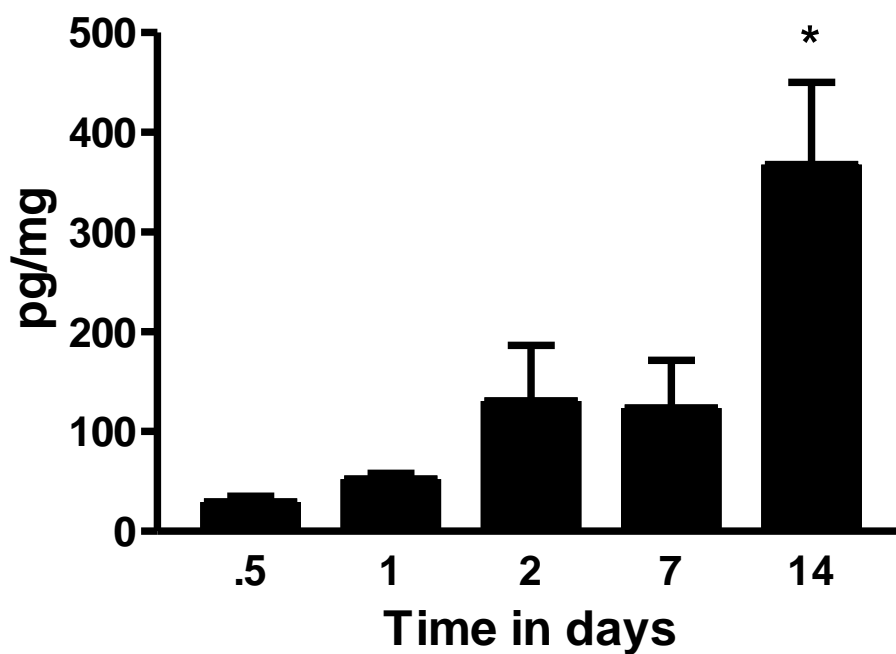


Fig 4.2.15 Plasma PIGF expression

P= 0.004(2-way ANOVA), \* Bonferroni post test P<0.05

**Table 4.2.16 Thrombus SDF-1 expression**

TIME	12 hrs	24 hrs	48 hrs	7 days	14 days
MEAN pg/ml	29.2	52.2	130.5	123.7	367.6
SEM	5.9	5.8	56.1	47.5	82.6

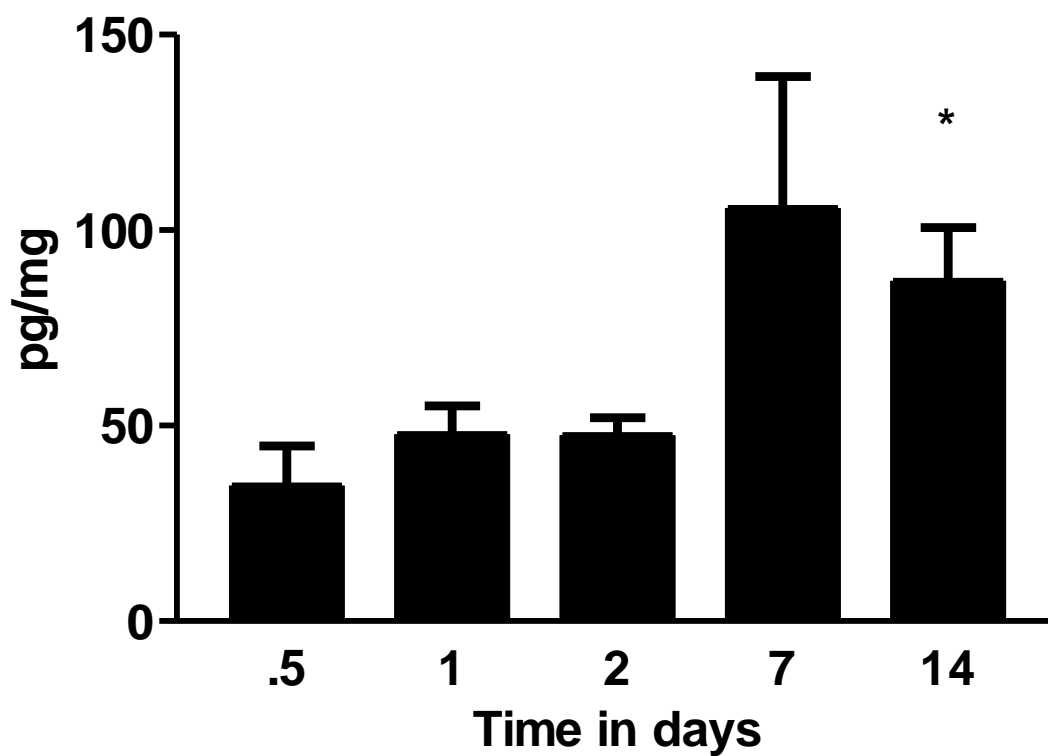


**Fig 4.2.16 Thrombus SDF-1 expression**

P=0.0005 (1-way ANOVA), \*Bonferroni post testing P<0.05

**Table 4.2.17 Wound SDF-1 expression**

TIME	12 hrs	24 hrs	48 hrs	7 days	14 days
MEAN pg/mg	29.8	41.2	41.0	91.6	76.6
SEM	10.6	9.8	8.2	34.3	16.8



**Fig 4.2.17 Wound SDF-1 expression**

P=0.035 (1-way ANOVA), \*Bonferroni post test

Table 4.2.18 Vein wall SDF-1 expression

TIME		12 hrs	24 hrs	48 hrs	7 days	14 days
SHAM	MEAN	648.0	163.0	135.0	218.0	145.0
	SEM	478.0	48.0	19.0	23.0	60.3
THROMBUS	MEAN	631.0	262.0	458.0	357.0	479.0
	SEM	407.0	21.0	110.0	226.0	111.0

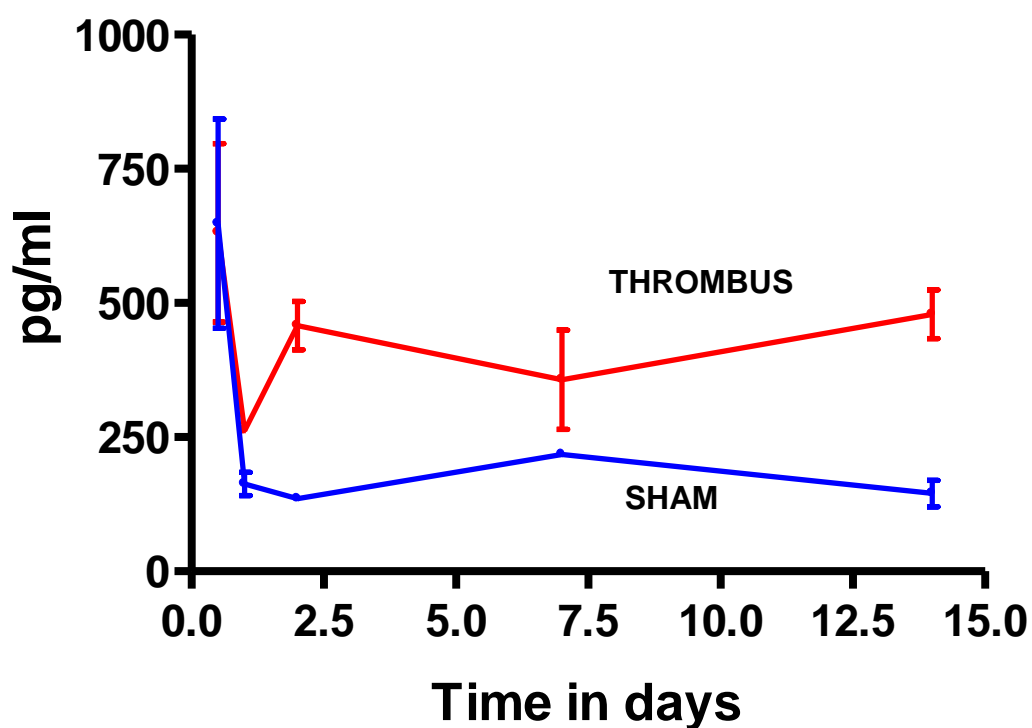


Fig 4.2.18 Vein wall SDF-1 expression

P=0.0036 (2-way ANOVA)

Table 4.2.19 Plasma SDF1 expression

TIME		12 hrs	24 hrs	48 hrs	7 days	14 days
SHAM VEIN pg/ml	MEAN	1408.4	1819.7	1852.0	843.3	1050.2
	SEM	267.6	142.4	262.2	251.1	179.9
THROMBUS pg/ml	MEAN	845.6	1114.9	1152.0	1348.5	1209.5
	SEM	280.2	159.4	100.7	129.8	237.6

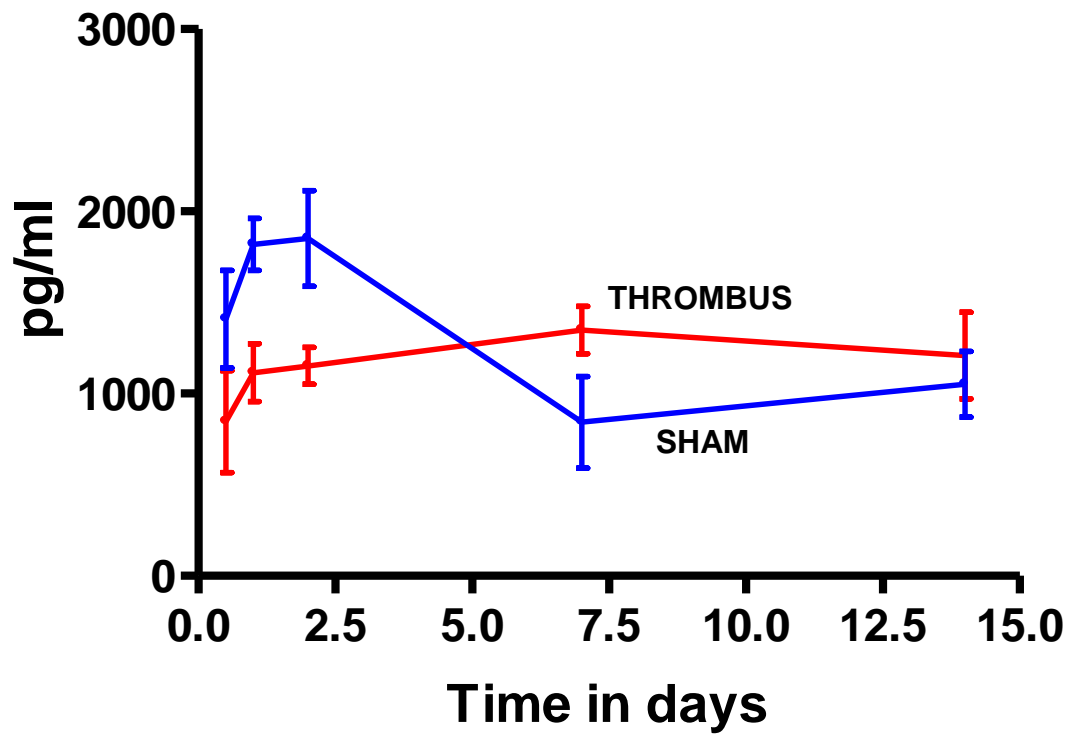


Fig 4.2.19 Plasma SDF-1 expression

P>0.05 (2-way ANOVA)

## **5.0 ENRICHMENT OF CIRCULATING BONE MARROW DERIVED MONONUCLEAR CELLS ON THROMBUS RESOLUTION**

### **5.1.1 INTRODUCTION**

Studies have demonstrated that enriching the circulation with progenitor cells has an effect on a variety of different pathologies such as myocardial infarction and lower limb ischaemia<sup>383-385</sup>. At the time of this study it was suggested that the uptake of 3,3,3',3'-tetramethylindocarbocyanine–labelled acetylated LDL (Dil-Ac-LDL) and binding of *Ulex Europaeus Lectin* in combination isolated progenitor cells with an endothelial phenotype<sup>221;229</sup>. These markers were therefore used for our studies in order to assess the mouse spleen and bone marrow for their potential to generate progenitor cells for use in interventional studies<sup>198;208;209;386;387</sup>.

### **5.1.2 MOUSE SPLEEN PROGENITOR CELL CULTURE**

#### **Aim**

To grow and characterise progenitor cells derived from mouse spleen mononuclear cells.



## **Methods**

Mouse spleens were removed from Balb/C mice immediately after death, under sterile conditions, the spleens were homogenised using a mortar and pestle in PBS (on ice). The suspension was filtered through a sterile 70µm filter (BD Bioscience, UK) and the filtrate centrifuged at 600 X g for 10mins (18-20°C), the supernatant was aspirated and the pellet resuspended in 5mls of DMEM (Invitrogen, UK, 37° C). The mononuclear fraction of the spleen cells was isolated using a Ficoll density gradient (**appendix 2**). The isolated mononuclear cells were washed with DMEM (37° C) and centrifuged at 600 X g for 10mins (18-20°C). The pellet was resuspended in Endothelial Basal Medium 2 (EBM-2: Gibco, UK) supplemented with 5% fetal calf serum (FCS, Cambrex, UK). Concentrations of two, four or eight million cells per ml were placed in 1ml aliquots on a 12 well culture plate (NUNC, UK).

The plate had been pre-coated with fibronectin solution 10µg/ml (Sigma, UK). The plate was incubated at 37°C and the EBM2 media replenished under sterile conditions every 48hrs. Dil-Ac-LDL and Lectin staining was carried out at day 1 and 7.

Cultured cells were washed with sterile warm PBS and incubated in 2µg/ml 3,3',3'-tetramethylindocarbocyanine-labelled acetylated LDL (Dil-Ac-LDL, Sigma UK) for 3hrs at 37°C. Cells were then fixed in 4% formaldehyde and counterstained with

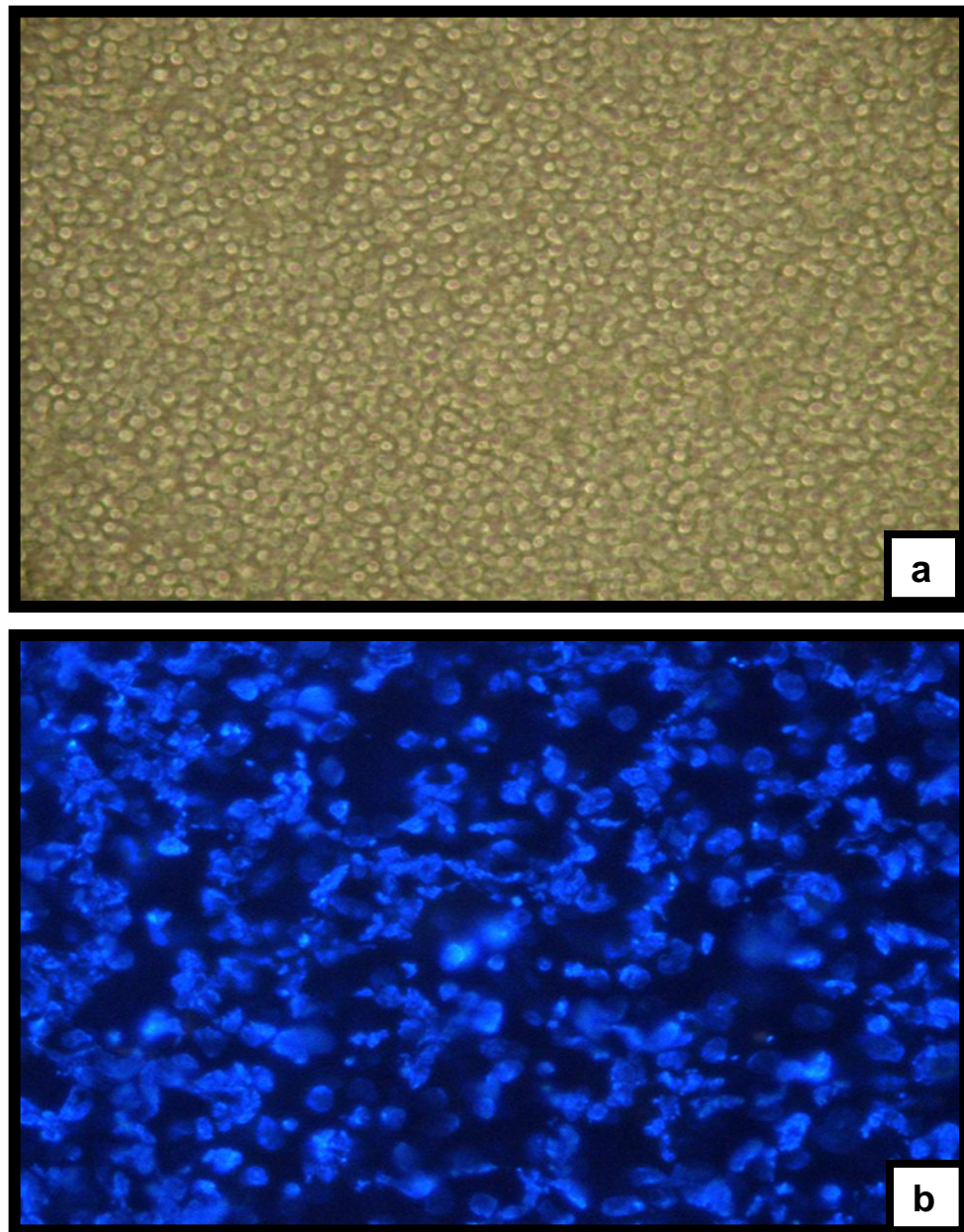
50µg/ml FITC-labelled Lectin from *Ulex Europaeus* (Sigma, UK) for 1 hour at 37°C in the dark. Cells were ultimately incubated in the blue fluorescent nuclear stain, 4',6-diamidino-2-phenylindole (DAPI), and viewed under a fluorescent microscope.

## **Results**

At day 1, spleen cells at all plating densities were phenotypically spherical (**Table 5.1.1, Fig 5.1.1 (a)**), and there was no positive staining with Dil-Ac-LDL or Lectin (**Fig 5.1.1(b)**). At day 7 a significant number of spleen cells changed morphology becoming elongated and spindle shaped (**Table 5.1.1, Fig 5.1.2(a)**), as well as staining positively for Dil-Ac-LDL and Lectin suggesting a progenitor phenotype (**Fig 5.1.2(b)**).

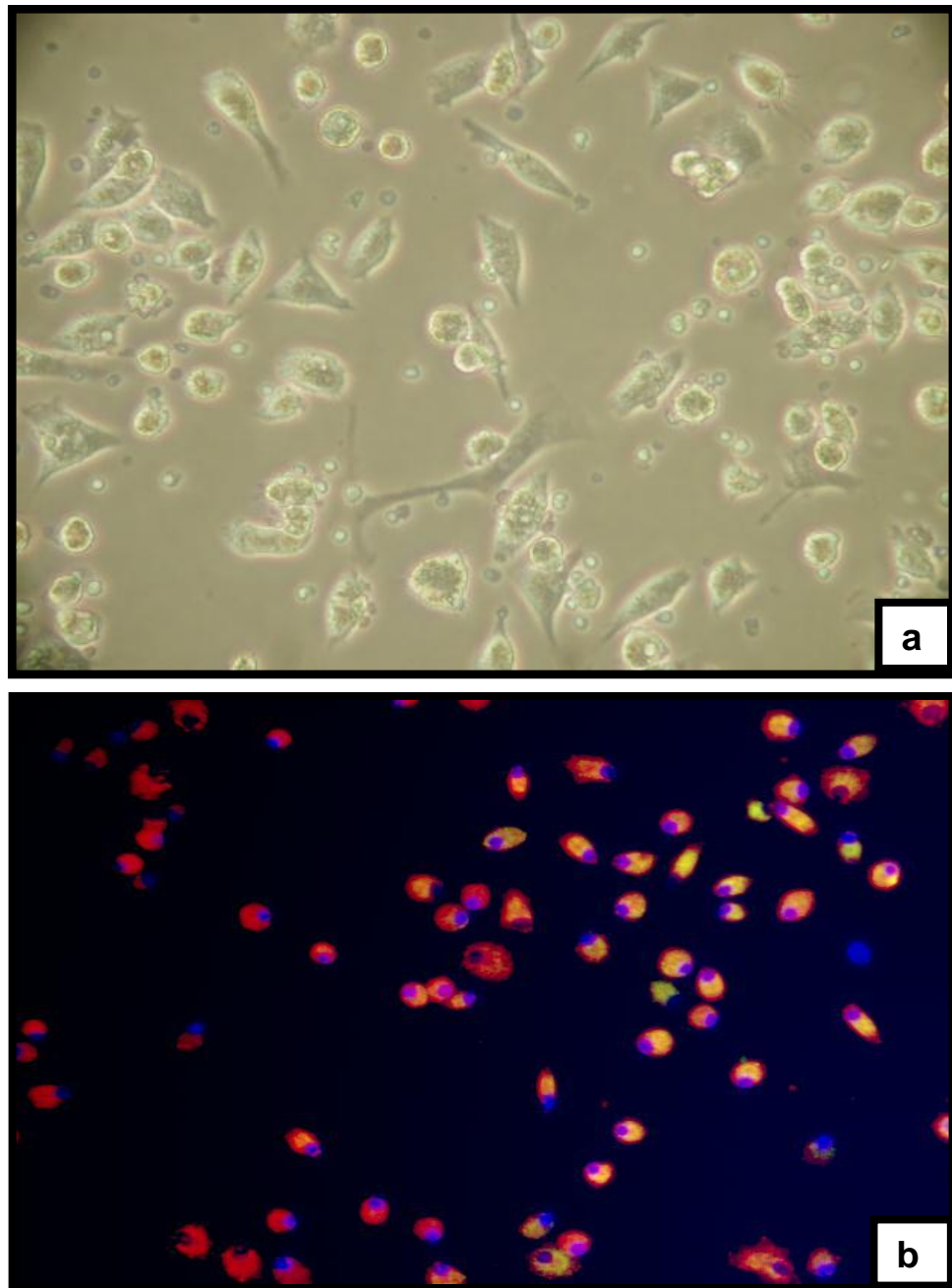
**Table 5.1.1 Spleen mononuclear cell generation of progenitor cells**

Time (days)	Sample	Dil-Ac-LDL and Lectin staining progenitor cells		
		2 million cells/ml	4 million cells/ml	8million cells/ ml
Day 1	1	-ve	-ve	-ve
	2	-ve	-ve	-ve
Day 7	1	+ve	+ve	+ve
	2	Infection	+ve	+ve



**Fig 5.1.1 Spleen cells in culture on fibronectin**

(a) Spherical mononuclear spleen cells in culture (b) Cells stained with DAPI but no evidence of DIL-Ac-LDL (red) or lectin (green) staining, X20 magnification



**Fig 5.1.2 Endothelial lineage cells**

(a) Phenotypically spindle shaped cells in culture between day 7, (b) cells staining positively for Dil-Ac-LDL (red) and Lectin (green), X20 magnification

### 5.1.3 MOUSE BONE MARROW PROGENITOR CELL CULTURE

#### Aim

To grow and characterise progenitor cells derived from the mononuclear fraction of mouse bone marrow

#### Methods

*All steps were carried out a room temperature (18-20°C) unless otherwise stated*

The femurs of BALB/C mice were removed immediately after death and under sterile conditions the BM was flushed from the femur with DMEM (RTP) using a 30G needle (BD, UK). BM effluent was suspended in 10mls of DMEM solution, pre-warmed to 37° C. The suspension was filtered through a sterile 70µm filter (BD, UK). The filtrate was centrifuged at 600 X g for 10mins and the pellet resuspended in 5mls of DMEM (37° C). The mononuclear fraction of the BM cells were isolated using a Ficoll density gradient (**appendix 2**) and the cells washed with DMEM (37° C) and centrifuged at 600 X g for 10mins. The pellet was resuspended in EBM2 supplemented with 5% FCS at a concentration of 2,4 or 8 million cells per ml. Cells were plated and cultured and stained as described (**Sec 5.1.2**).

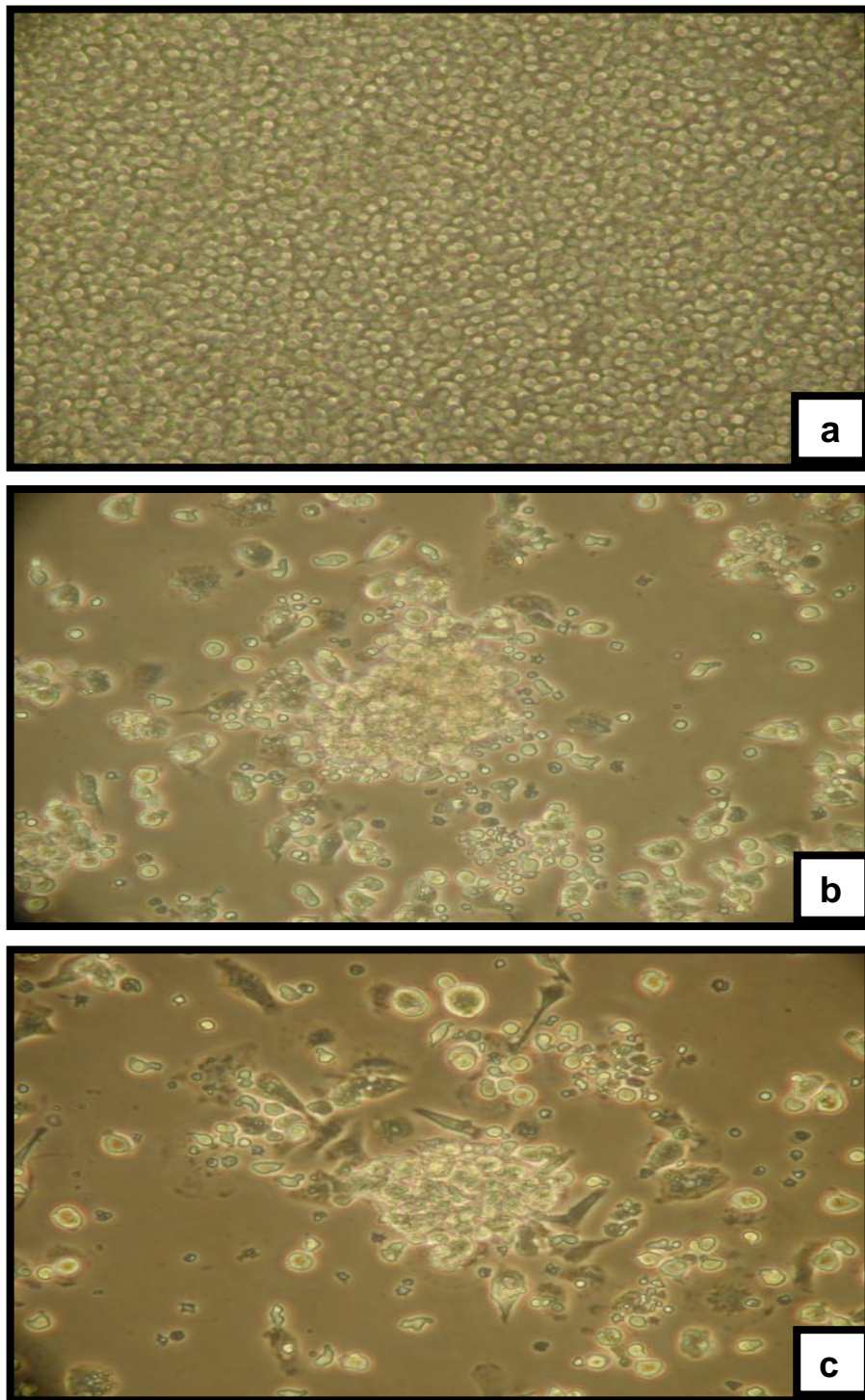
## **Results**

At day 1 there were no spindle shaped cells or CFUs seen at any plating density (**Table 5.1.2, Fig 5.1.3(a)**) but by day 7 at the lower plating densities of 2 and 4 million cells/ml, CFUs were apparent (**Table 5.1.2, Fig 5.1.3 (b) & (c)**). CFUs also stained positively with the Dil-Ac-LDL and Lectin stains (**Fig 5.1.4 (a), (b) & (c)**). At the higher plating density some spindle shaped cells also co-stained with Dil Ac LDL and Lectin.

**Table 5.1.2 BMMNC generation of progenitor cells (CFUs)**

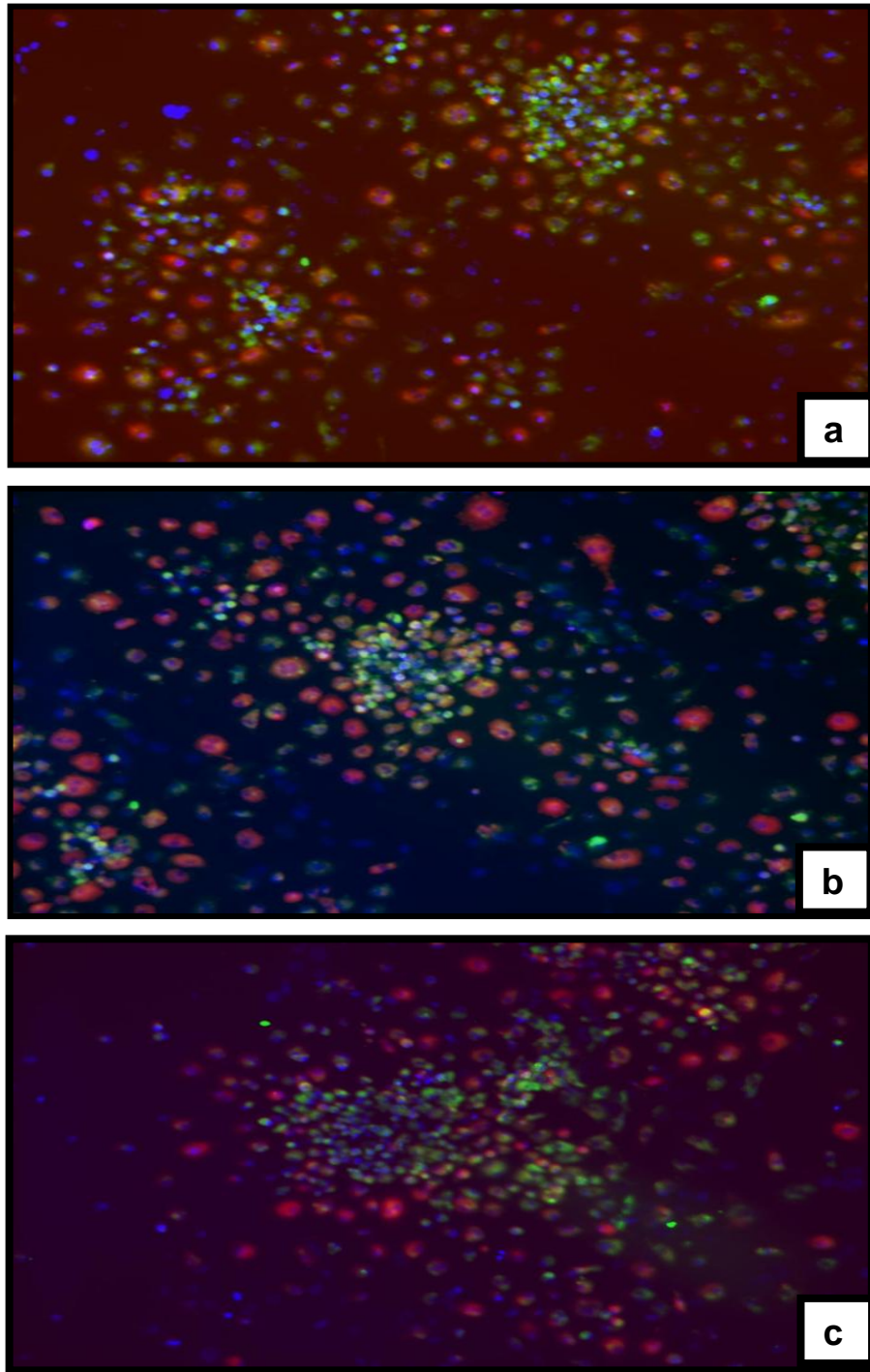
<b>Time (days)</b>	<b>Sample</b>	<b>Dil-Ac-LDL and Lectin staining colony forming units</b>		
		<b>2 million cells/ml</b>	<b>4 million cells/ml</b>	<b>8 million cells/ ml</b>
<b>Day 1</b>	<b>1</b>	<b>-ve</b>	<b>-ve</b>	<b>-ve</b>
	<b>2</b>	<b>-ve</b>	<b>-ve</b>	<b>-ve</b>
<b>Day 7</b>	<b>1</b>	<b>+ve</b>	<b>+ve</b>	<b>-ve</b>
	<b>2</b>	<b>+ve</b>	<b>+ve</b>	<b>-ve</b>





**Fig 5.1.3 Bone marrow cells in culture**

(a) Day 1 BM cells, (b) &(c) CFUs, X20 magnification



**Fig 5.1.4 CFUs derived from mouse BM**

(a-c) stained with DAPI, Dil-Ac-LDL and Lectin, X20 magnification



#### 5.1.4 EXPANSION OF CFUS FOR USE IN INTERVENTIONAL STUDIES

##### **Aim**

To assess whether BM derived CFUs could undergo passage and expansion *in vitro*, for subsequent interventional studies.

##### **Methods**

CFUs were cultured as described (**Sec 5.1.2**). At day 7 cells were washed X3 with 0.9% saline solution and then incubated in one of the following agents: trypsin (Serotec, UK), Accutase (Invitrogen, UK) or 1% lignocaine (Pfizer, UK) at the recommended concentrations. Incubation in these agents was performed until at least 50% of cells had detached from the culture plate. Passaging agents were neutralised using 10mls of media (DMEM +5% FCS). Cells were then centrifuged at 400 X g for 10mins (18-20°C). All live cells were put back into fibronectin culture.

##### **Results**

Trypsin EDTA produced a cell harvest which was several fold better than the other agents tested, however none of the agents used produced CFUs from BMMNCs after passage (**Table 5.1.3**).

**Table 5.1.3 Passage of BM CFUs at using different reagents**

Compound	Cells plated Million cells/ml	Original CFU no. MEAN (SEM)	Cell harvest MEAN (SEM)	Passaged CFU no.
<b>Trypsin + EDTA</b>	2	44 (1.5)	58,000 (10,000)	0
<b>Trypsin</b>	2	34 (8.1)	11,000 (3600)	0
<b>Acutase</b>	2	35 (5)	25,000 (4500)	0
<b>Lignocaine</b>	2	37 (3.5)	13,000 (3600)	0

### **Conclusion**

It was therefore decided that interventional studies would proceed with BMMNCs rich in HPCs rather than developed CFU cells which could only be generated in small numbers.

### 5.1.5 LOCALISATION AND IMPACT OF PERIPHERALLY INJECTED BMMNCs FOLLOWING THROMBUS FORMATION

#### Aim

To evaluate the effect of upregulating circulating BMMNCs on thrombus resolution.

#### Methods

##### Localisation of BMMNCs after tail vein injection

Thrombus was formed in two groups of four Balb/C mice. At 24hrs following thrombus formation, 4 animals underwent tail vein injection of PKH 26 (Invitrogen, UK) labelled red fluorescent BMMNCs (**Sec 2.4**). A further 4 animals were injected with carrier solution (DMEM). All injections were carried out with animals under anesthetic. At day 2 and day 7 spleen, BM, blood, thrombus and cava were harvested. Blood and BM were analysed for the presence of PKH26 red fluorescent cells using flow cytometry. Spleen and thrombus were analysed in frozen section using fluorescence microscopy (**Sec 2.3.3**).

##### Impact of BMMNCs on thrombus resolution

Thrombus was formed in two groups of 8 Balb/c mice (**Sec 2.1.2**). After 24 hrs, mice were peripherally injected with 250µl of either carrier solution (DMEM) or 1 million BMMNCs (**Sec 2.1.3**). At day 7, all animals were culled, thrombus and vein wall were removed *en mass* for paraffin section (**Sec 2.3.4**).

Image analysis (**Sec 2.3.6**) was used to compare vena caval recanalisation in control vs. BMMNC injected animals (Mann Whitney U).

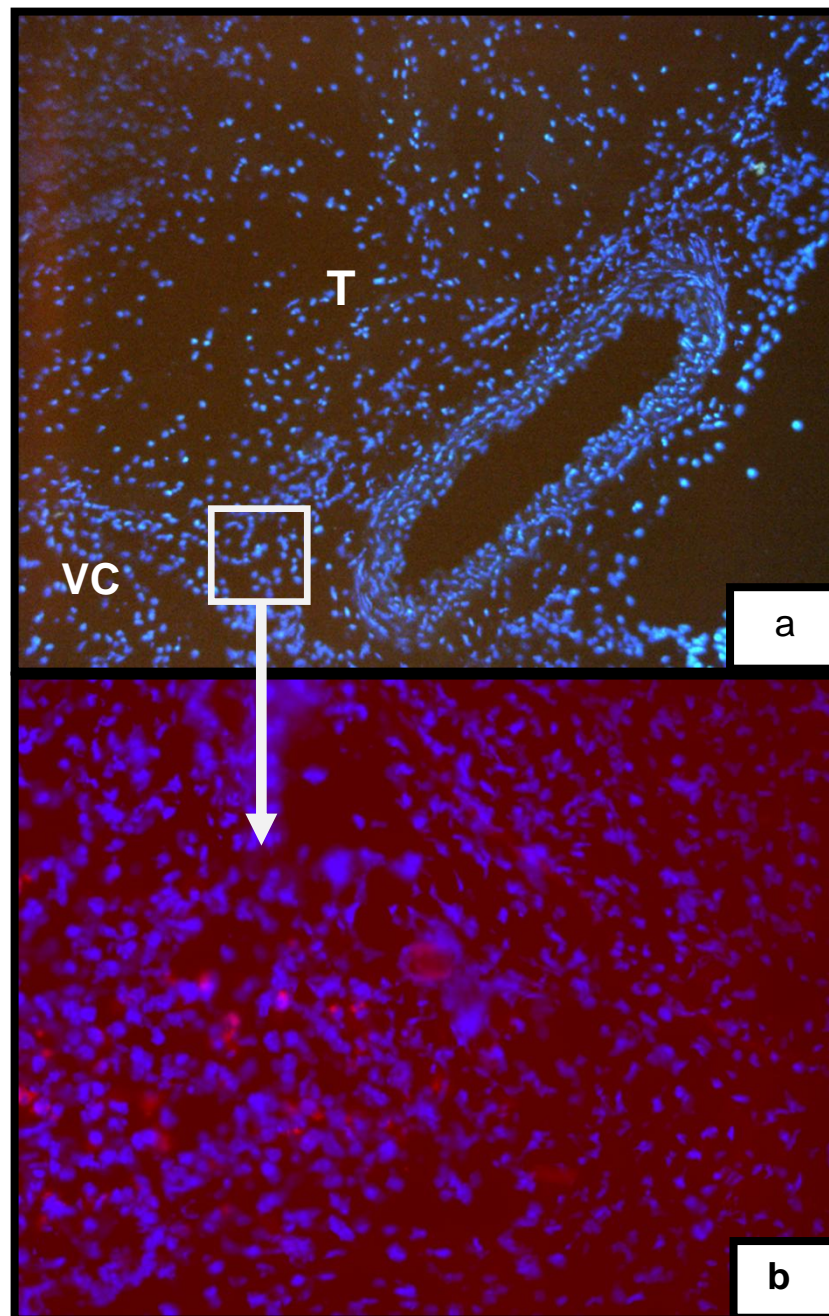
## **Results**

### **Day 2**

No labelled BMMNCs were identified in the spleen or thrombus.

### **Day 7**

No labelled BMMNCs were identified in the spleen, a small number of red fluorescent cells were seen in the vein wall thrombus interface (**Fig 5.1.4**).



**Fig 5.1.4 PKH 26 labelled BMMNCs at thrombus-vein wall interface**

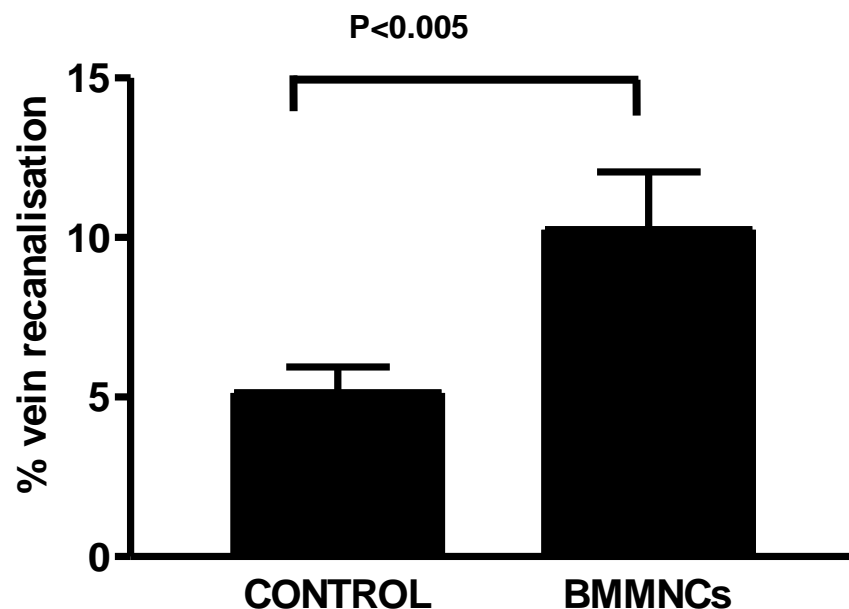
Panel (a) is a DAPI stained cross section of thrombus at X20 magnification, (b) X40 magnification of outlined box from (a) showing red fluorescent cell labelling

### Impact of BMMNCs on thrombus resolution

Injection of the BMMNCs resulted in a doubling of recanalisation, this was a mean increase from 5% to 10% ( $P=0.005$ , **Table 5.1.4**). A selection of cross-sections taken from control animals and those from animals injected with BMMNCs (**Fig 5.1.6**) shows 3 examples of significantly larger recanalisation channels at the periphery of the thrombus in those animals injected with BMMNCs.

**Table 5.1.4 Percentage caval recanalisation after injection of BMMNCs**

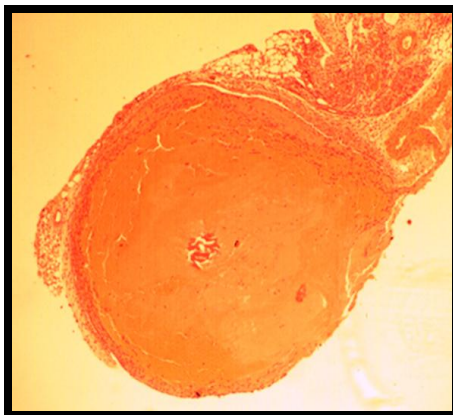
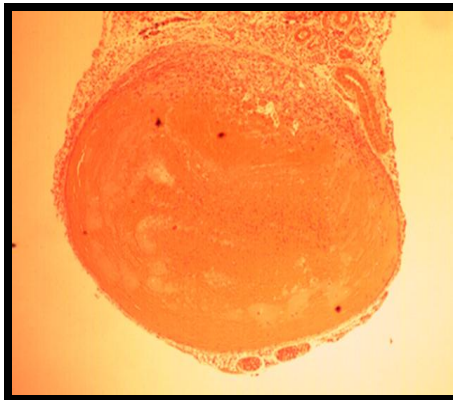
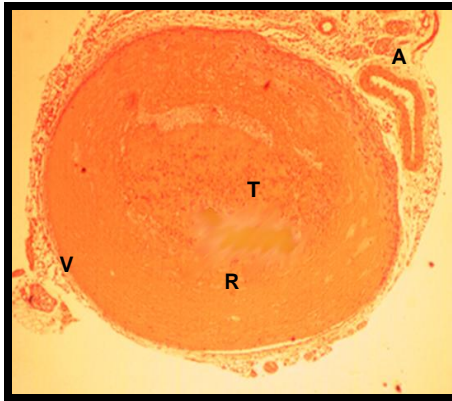
% Mean Recanalisation (SEM)	GROUP	
	CONTROL	BMMNC
	5.2 (0.1)	10 (0.1)



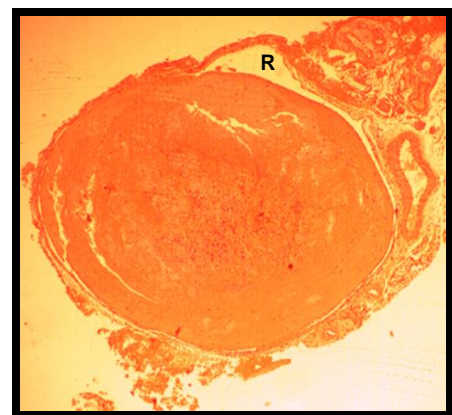
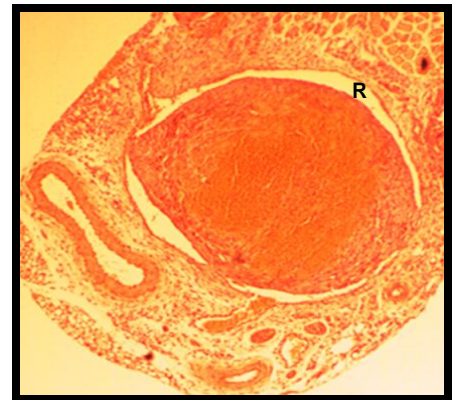
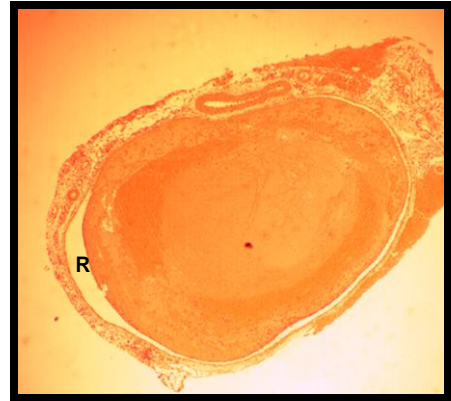
**Fig 5.1.5 Percentage caval recanalisation**

5% increase in recanalisation following BMMNC injection,  
Mann Whitney U

## CONTROL



## BMMNC



**Fig 5.1.6 Cross section of thrombi treated with DMEM or BMMNCs**  
(X5 magnification), A (aorta), VC (vena cava), R (recanalisation), T  
(thrombus)

## 6.0 ADENOVIRAL CYTOKINE UPREGULATION

### 6.1 ADENOVIRAL TECHNOLOGY

#### 6.1.1 INTRODUCTION

Analysis of the cytokine expression in thrombus, vein wall and wound tissues suggested that both PIGF and SDF1 may be pivotal to thrombus resolution. PIGF produced a unique expression pattern within the thrombus not seen in wound tissue and also a bimodal expression pattern within the vein wall. SDF1 also produced a bimodal expression in the vein wall and was able to migrate BM derived CFU cells *in vitro*. The expression patterns of these two cytokines within the vein wall, mirrored to some extent the expression of the circulating progenitor cells. In order to further evaluate this hypothesis the effect of upregulating these proteins within the thrombus was assessed using adenovirus technology. Viruses have been extensively studied as a method of gene delivery in cancer and coronary artery disease<sup>388-392</sup>. Several types of virus have been used both in the laboratory and in clinically based studies, including retroviruses, lentiviruses, adenoviruses and adeno-associated viruses<sup>391-394</sup>. Retroviruses use reverse transcriptase to integrate into the host genome, the viruses potential to integrate efficiently however requires actively dividing cells, which may not always be present<sup>395</sup>. Adenoviruses such as the ones used here have several features which make them ideal for gene therapy studies; they are able to rapidly infect a variety of cell types, they can house large segments of DNA and have a low pathogenicity

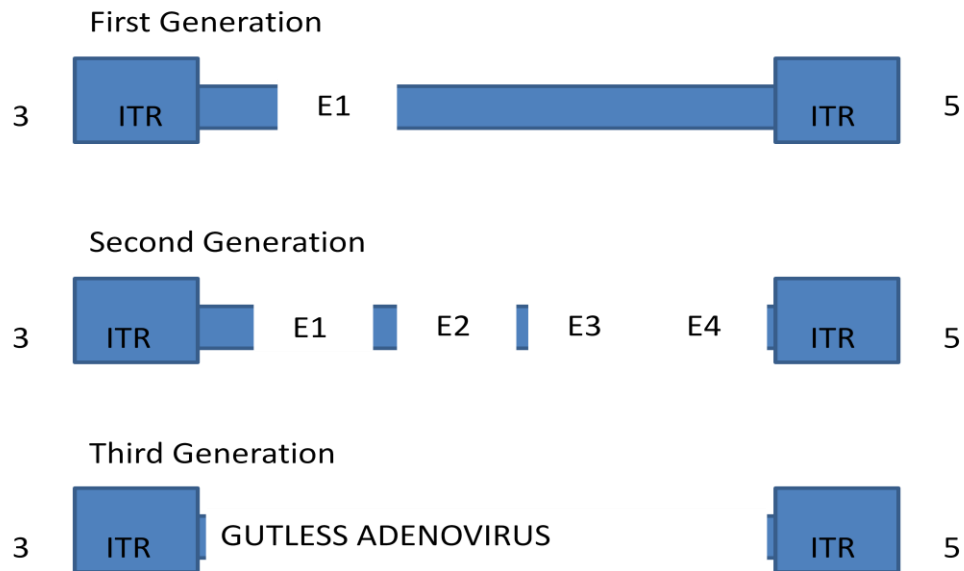


in humans<sup>396</sup>. VEGF has been successfully used to promote thrombus resolution in a rat model of thrombosis using an adenovirus<sup>313</sup>, adenoviral constructs have also previously been used to upregulate angiogenic proteins such as SDF-1 and PIGF in other models<sup>295;321;397</sup>.

**Adenoviruses** are double stranded DNA icosahedral viruses which will normally result in mild coryzal symptoms in the immunocompetent host. The normal pathophysiology of this virus results in receptor mediated endocytosis after which the virus invades the nucleus, where viral transcription and replication begin<sup>398</sup>.

Certain key gene deletions have enhanced the use of adenoviruses both in basic and clinical science. Replication defective viruses, known as **1<sup>st</sup> generation adenoviruses** have an E1 region deletion and are therefore only able to replicate in specifically engineered cells containing the E1 transgene e.g. Human Embryonic Kidney (HEK) 293 cells<sup>399</sup>. Cells infected with replication deficient adenovirus, will only produce the experimental transgene for the life span of the virus. The E3 region encodes for proteins which enable the virus to evade the hosts defence system. This deletion also allows the adenovirus to accommodate up to 8kb of foreign DNA. Second generation adenoviruses have had further gene deletions allowing up to 14kb of foreign DNA to be introduced into the host cell. At the cutting edge of adenoviral technology are the third generation or “gutless” viruses which are devoid of all viral coding regions<sup>400</sup>. They are an attractive option for long-term transgene expression because of a dampened immune response generated from the host and the ability to hold up to 36 kb of DNA<sup>400</sup>. Furthermore the adenoviral

genome does not integrate into the host cell, making it ideal for transient gene expression<sup>401</sup>.



**Fig 6.1.1 Gene deletions in adenoviruses** (adapted from Lindeman<sup>393;402;403</sup>)

## 6.2 MATERIALS & METHODS

### 6.2.1 GROWTH, PURIFICATION AND CHARACTERIZATION OF ADENOVIRUS CONSTRUCTS

The aim of this section of work was to grow and characterise the adenoviral construct containing the genes for SDF1; a small quantity of which had been donated to the project courtesy of Professor Ron Crystal, Cornell University, New York, USA<sup>295</sup>. The AdPIGF and AdEGFP viruses were acquired from Professor Desire Collen's group at the Department of Molecular and Cellular Medicine, University of Leuven. The latter were kindly donated in sufficient quantities for all our studies, however titre and function were confirmed prior to use<sup>318;319</sup>.

### 6.2.2 ADSDF1 GROWTH AND CHARACTERISATION

AdSDF1 is an Ad5-derived E1a-, E3-deficient (E1a-E3-E4-) vector with an expression cassette in the E1a region containing the human SDF1 complementary DNA (cDNA) and is driven by the cytomegalovirus (CMV) major immediate/early promoter/enhancer<sup>295</sup>. The cassette for the EGFP and the PIGF viruses were identical.

### 6.2.3 ADSDF GROWTH

The initial AdSDF1 stock was grown and purified using the **Adeno-X Maxi purification kit** (Clontech, UK). The methodology described below was taken from the manufacturer's instructions:

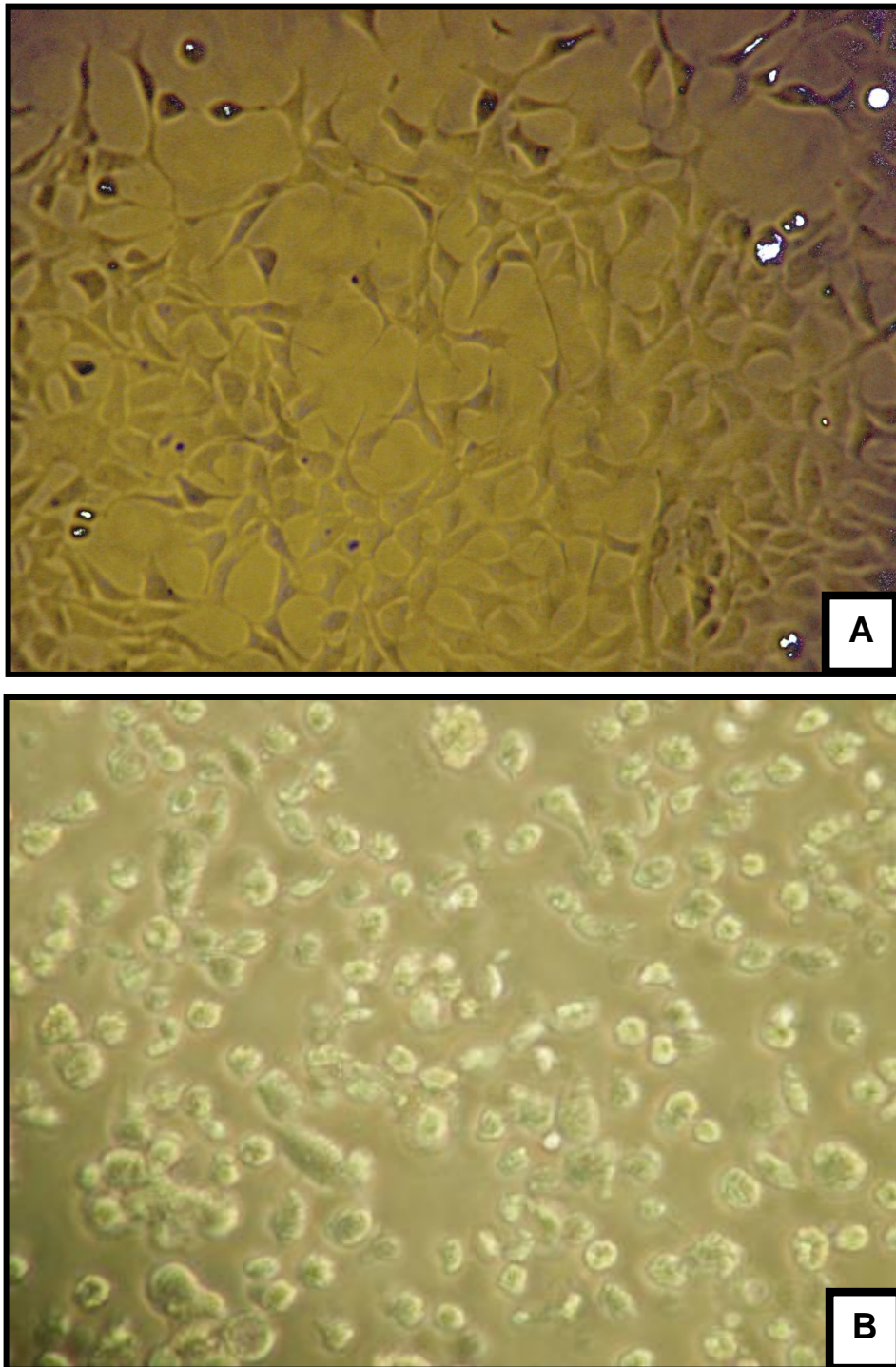
### **Stage I Growth**

HEK 293 cells were grown to 70% confluence in six 175cm<sup>2</sup> cell culture flasks (Falcon BD, UK, **Sec 2.5, Fig 6.2.1 (A)**). The amount of virus added to each flask was based on the viral titre. The AdSDF1 was provided at a titre of  $1 \times 10^9$  pfu/ml, therefore 5µl of AdSDF1 was added per 175cm<sup>2</sup> of HEK cells, aiming for a multiplicity of infection (M.O.I) of 1-2 (if the viral titre is unknown this can be calculated using a titre testing experiment, **appendix 3**). Cells were incubated at 37°C until the optimal cytopathic effect (CPE) was reached i.e. when 80-90% of cells had lifted off the bottom of the plate (**Fig 6.2.1 (B)**). Cells were placed in a sterile 15ml conical tube and pelleted by centrifugation at 1250 x g for 5mins (18-20°C). The supernatant was discarded and the pellet was resuspended in 5mls of DMEM. This suspension was freeze thawed three times in liquid nitrogen and the lysate was centrifuged at 1250 x g for 5mins (18-20°C). The supernatant was collected and placed in a sterile 15ml conical tube. 5µl of benzonase nuclease (kit component) was added and the mixture incubated for 30mins (37°C), this reduces the viscosity of the solution so that it is more easily drawn through the purification filter. An equal volume of 1x dilution buffer (kit component) was added (approx 5mls) and the lysate was filtered using a 0.45µm filter (BD).

### **Stage II Virus purification**

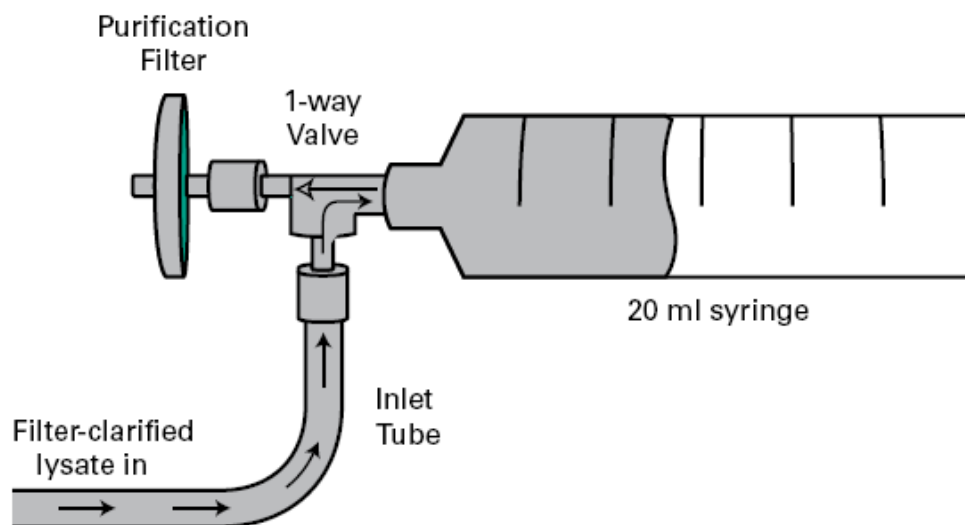
The virus was purified by selective binding and elution using the kit membrane assembly. The filter assembly was first equilibrated using 5mls of 1X equilibration

buffer which was drawn into the syringe from the inlet tube and pushed through the filter at a rate of 3mls/min (1 drop/sec, **Fig 6.2.2**).The inlet tube was used to draw up the lysate which was also pushed through the filter at a rate of 3mls/min. The inlet tube was transferred to a sterile tube containing 20mls of 1X wash buffer which was pushed through at a rate of 3mls/min. The purification filter was removed from the assembly and the virus was eluted from the filter using 3mls of 1X elution buffer into a 15ml conical flask. The virus was snap frozen in liquid nitrogen and stored at minus 80°C for further characterisation and interventional studies.



**Fig 6.2.1 HEK 293 cells in culture**

(A) 70% confluent HEK cells, (B) Cytopathic effect of virus



**Fig 6.2.2 Adenovirus purification filter assembly (Clontech®)**

#### **6.2.4 VIRAL TITRE CALCULATION**

The AdSDF1 virus was acquired at a titre of  $1 \times 10^9$ , following its subsequent growth and purification its potency had to be checked to ensure it had not declined. A precise titre can be calculated using the Adeno-X Rapid Titre kit (Clontech UK). This is a simple antibody based assay that can be used to titre any adenovirus. The hexon protein which is encoded by the adenoviral genome, is an essential component of the adenoviral capsid required for replication. Its expression is E1 gene dependent, HEK 293 cells supply this E1 encoded region, and therefore only

infected cells will produce the hexon protein. A hexon-specific antibody is then used to label infected cells. Counting the number of infected cells per high power microscope field can then be used to calculate viral titre after the cells have been incubated with varying dilutions of the virus for 48hr incubation. An overview of the protocol is shown below (**Fig 6.2.3**).



### **Stage 1) Cell infection**

1. A 12 well plate (NUNC, UK) was seeded with  $5 \times 10^5$  healthy HEK 293 cells per 1ml of growth media (DMEM+10% FBS)
2. Using PBS (pH~7.5) a 10-fold dilution of the AdSDF1 was prepared, starting at  $10^{-2}$  down to  $10^{-6}$  dilution
3. 100 $\mu$ l of each dilution was added to duplicate wells, leaving one well as a control with no virus.
4. Cells were then incubated for 48hrs in 5% CO<sub>2</sub> for 48hrs (NAPCO)

### **Stage 2) Cell staining**

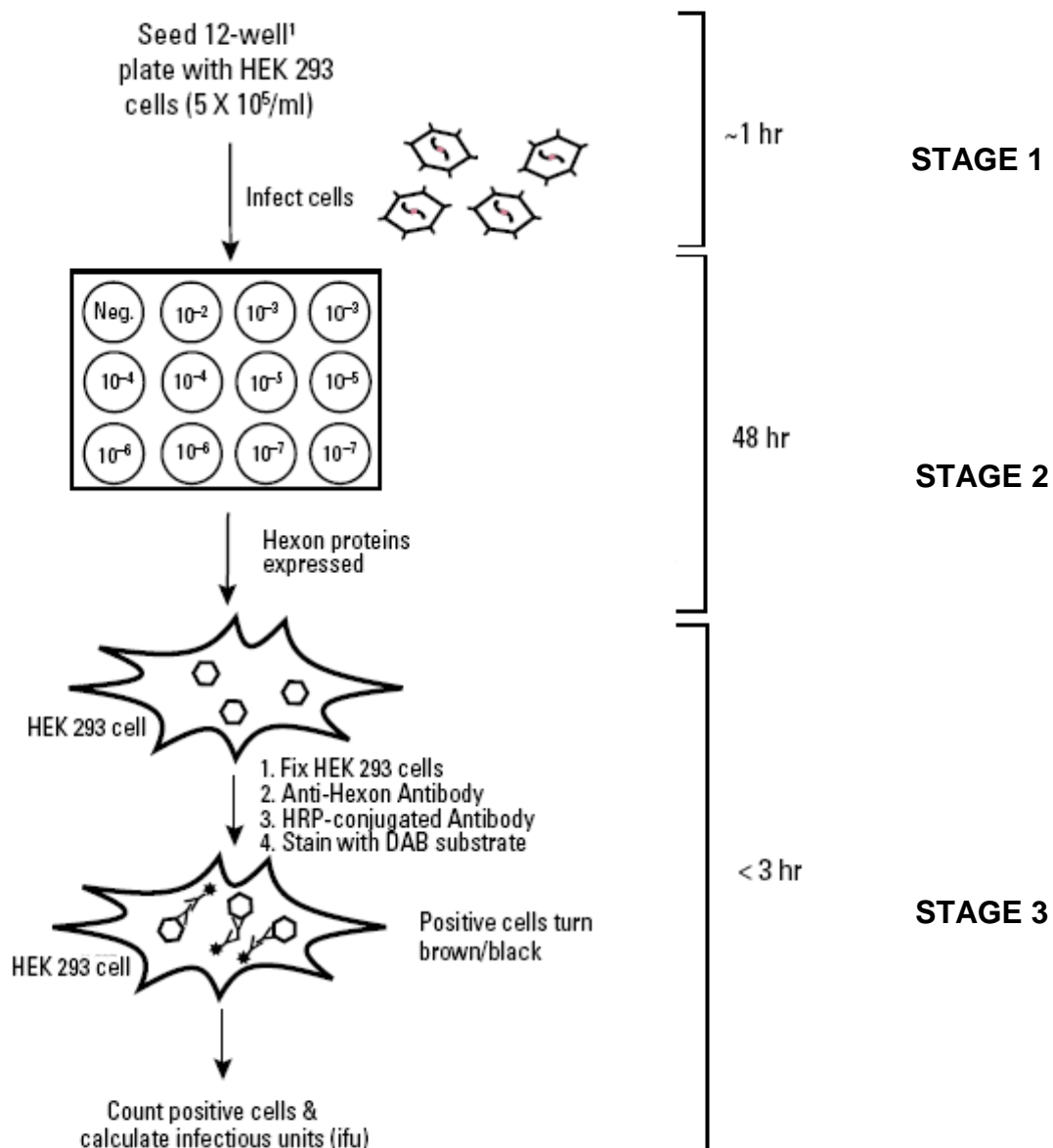
1. Cell media was aspirated and the cells dried in a convection hood for 5mins.
2. Cells were fixed using 1ml of cold (4°C) 100% methanol per well.
3. The plate was incubated for 20mins at -20°C
4. The methanol was aspirated.
5. Cells were washed X3 (PBS + 1% BSA)
6. Anti-Hexon antibody (1/1000 dilution) was added to all wells and incubated for 1hr (37°C)
7. Step 5 was repeated.
8. Rat anti-mouse antibody (HRP conjugate, kit component) was added to each well (1/500 dilution) and incubated for 1hr (37°C)
9. Step 5 was repeated.
10. 3,3'-Diaminobenzidine (DAB) substrate solution (1/10 with 1X stable peroxidase, kit component) was added to each well (500 $\mu$ l per well) for 20mins (20°C).
11. DAB solution was aspirated and 1 ml of PBS was added to each well

### **Stage 3) Titre Calculation**

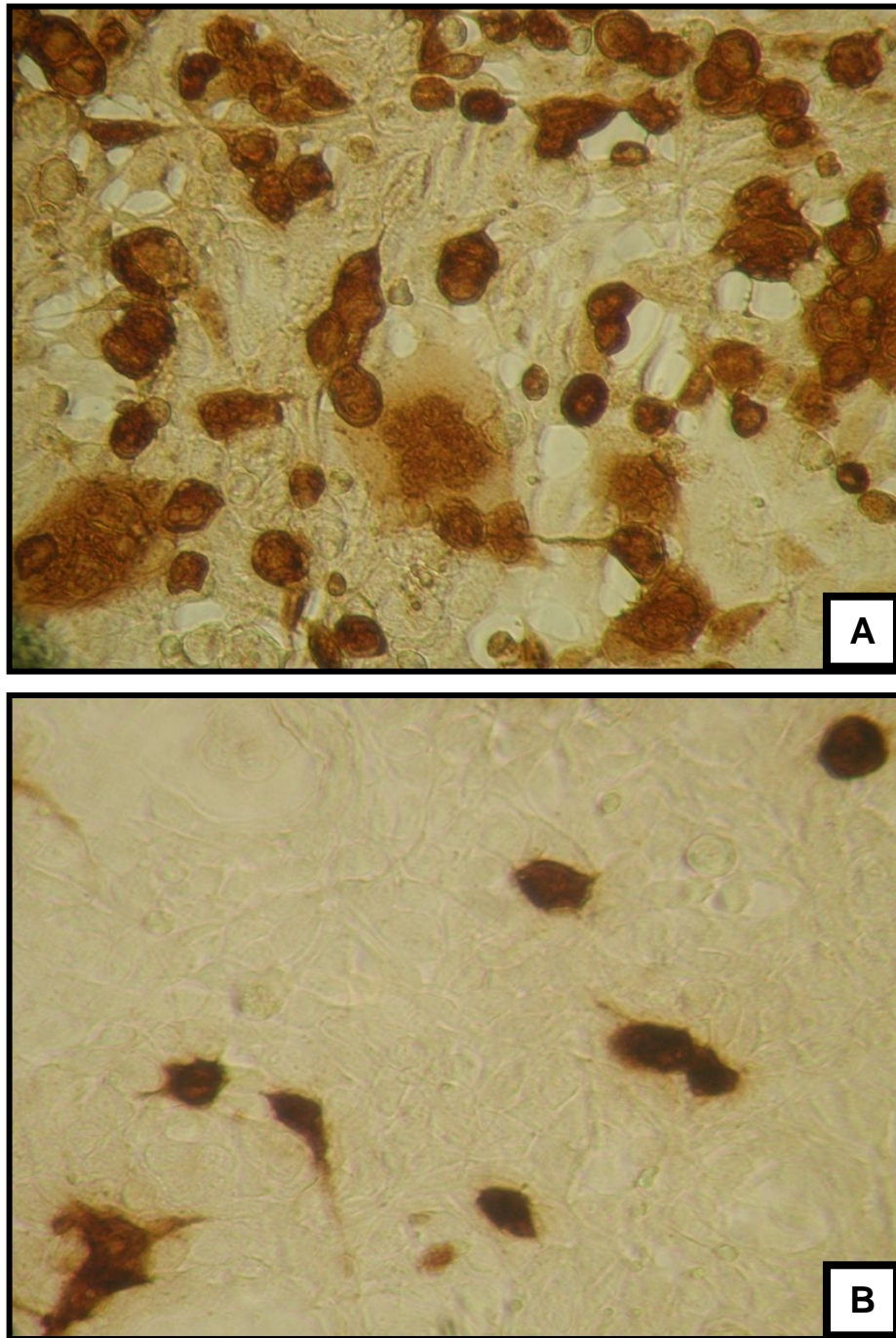
1. Using a X20 objective lens, stained cells were counted in each field (**Fig 6.2.4**), the average of several field was taken to give a more accurate titre.
2. The titre was then calculated using the following formula:

$$\text{titre} = \frac{(\text{infected cells /field}) \times (\text{fields/well})}{\text{Volume of virus (ml)} \times \text{dilution factor}}$$

Dilution factor- see **Table 6.2.1**



**Fig 6.2.3 Overview of Rapid Titre Experiment (Clontech®)**



**Fig 6.2.4 DAB substrate stained infected HEK cells**

(A) X20 objective view of infected HEK cells at  $10^{-4}$  dilution (B) X20 objective view of infected HEK cells at  $10^{-5}$  dilution

**Table 6.2.1 Fields to Well ratio as advised by Clontech®**

Table I. Derivation of Area Counted in Fields/Well						
Objective Lenses	Eyepiece Lenses (10X)			Fields/Well		
	Total Magnification	Field Diameter	Field Area (mm <sup>2</sup> )	12-Well Plate area = 3.8 cm <sup>2</sup>	24-Well Plate area=2.0 cm <sup>2</sup>	96-Well Plate area=0.32 cm <sup>2</sup>
4X	40X	5 mm	19.6	19	10	1.6
5X	50X	4 mm	12.5	30	16	2.6
10X	100X	1.8 mm	2.54	150	79	12.6
20X	200X	0.9 mm	0.64	594	313	50

### 6.2.5 REPLICATION COMPETENCY TEST

10 $\mu$ l 1X10<sup>9</sup> of purified AdSDF1 was added to 1990 $\mu$ l serum free DMEM. The media from 2 small (25cm<sup>2</sup>) flasks of human skin fibroblasts (Invitrogen, UK) was aspirated, and the diluted virus was added to one flask and serum free DMEM (as a control) to the other flask, a third flask of HEK 293 cells was also infected with the same viral dilution. Cells were incubated for 2hrs (37°C) and the media removed from both flasks and replaced with 10% DMEM. The cells were observed over 7-10 days if the virus produced any discernible cytopathic effect on the fibroblasts (compare to control cells) then it would have been destroyed (see results, **Fig 6.3.1**).

### 6.2.6 ELECTROPHORESIS AND WESTERN BLOTTING OF ADSDF-1 PROTEIN

#### Gel electrophoresis

To demonstrate that the AdSDF-1 produced an 8kd protein and a band at the same level as the recombinant SDF-1(R&D, UK), the NuPAGE Bis-Tris MiniGel

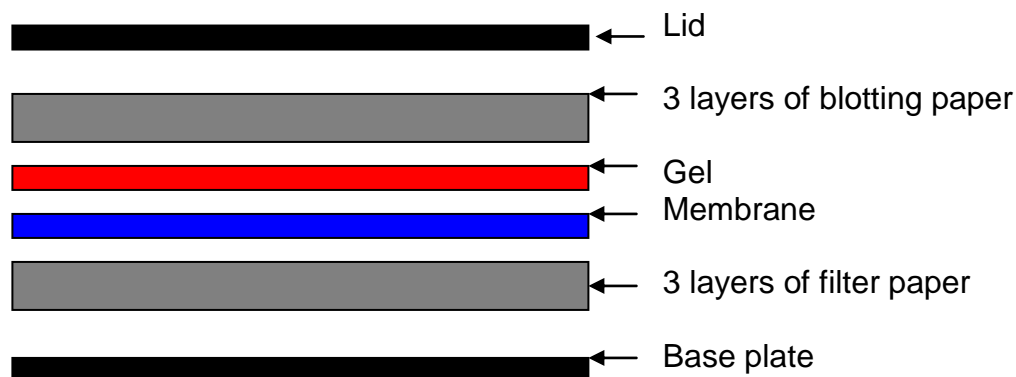
(Invitrogen, UK) was used. It comes with precise instructions but the method is briefly described below:

The gel cassette was rinsed with deionised water and the sample wells were rinsed the with 1X NuPAGE SDS running buffer (Invitrogen, UK). The X-Cell SureLock Mini-Cell electrophoresis system was used (Invitrogen, UK). Supernatant from the AdSDF and AdEGFP culture flasks, as well as normal HEK cell media were all tested in the *reduced* and *unreduced* forms, against a recombinant SDF1 protein for comparison. 25µl (max 30µg protein) of sample solutions and protein standards ((SeeBlue® Plus2 (Invitrogen, UK)) were loaded per well. The gel was run in X1 MES buffer (Invitrogen, UK) at 200 volts for 35mins at approx. 90amps with an expected current of 200V constant for 35mins (100-125mA/gel (start); 60-80 mA/gel (end)).

### **Western Blotting**

Proteins were transferred from the NuPage gel to the Hybond PVDF membrane using a semi-dry transfer system (BioRad, UK) at 10V for 45mins. *(All steps performed at 18-20°C).*

The gel was first allowed to equilibrate in transfer buffer (5.82g Tris(hydroxymethyl)methylamine (BD, UK), 2.9g glycine, 3.75ml 10% sodium dodecyl sulphate (SDS) (Gibco, UK), 200ml methanol) made up to 1litre with dH<sub>2</sub>O) (pH~9.2) for 1hr (18-20°C). The membrane was soaked in methanol and then transfer buffer before it was placed on top of the Hybond-P membrane (GE Healthcare), with filter paper on either side (**Fig 6.2.5**). The process was run at 10V for 40mins after which the membrane was placed in block buffer(0.05% Tween<sub>20</sub> (PBST<sub>20</sub>) with 2% non-fat dry milk (NFDM, Marvel™) for 1hr (18-20°C). The membrane was washed X3 with wash buffer (PBST<sub>20</sub> and 0.1% NFDM). The membrane was then probed with 0.4 µg/ml anti-SDF1 (R & D, UK) antibody diluted in wash buffer, it was washed again X3 and the membrane exposed to Streptavidin at 1/200 dilution (ELISA kit component, R&D) concentration. Reagents 1 and 2 from the Enhanced chemiluminescence (ECL™, Amersham) were allowed to come to room temperature. Reagents were mixed in equal volume, approx. 0.125 ml/cm<sup>2</sup> was required for adequate development of the blot.



**Fig 6.2.5 Placement of gel on Hybond PVDF in semi-dry transfer system (Bio Rad, UK)**

### **6.2.7 FUNCTIONAL CHARACTERISATION OF ADEONVIRUS PRODUCED SDF1**

In order to verify the biological activity of the AdSDF product, a migration assay was performed. Using a modified Boyden chamber<sup>293</sup> the effect of AdSDF1 generated protein on BMMNCs and BM derived CFUs was assessed. BMMNCs or cultured CFU cells were washed twice with PBS and incubated in a solution of DMEM, 2% charcoal treated FBS (foetal bovine serum) and 5mcg/ml of the fluorescent marker Calcein (Molecular Probes, UK) for 2 hrs. The cells were washed twice with PBS and lifted using 0.25% trypsin EDTA (Serotec, UK). The cell suspension was then washed in PBS X2 and resuspended in 1ml of 3.5mg/ml BSA in DMEM, at a density of  $30 \times 10^3$  per 300 $\mu$ l.



24hrs prior to assay, Falcon HTS Fluroblok (BD, UK) filters were immersed in a 10% solution of Fibronectin 10mcg/ml (Serotec, UK). The filters were washed with DMEM and placed into a 24well plate (BD Falcon). 1ml of either the AdsSDF1 HEK supernatant, AdEGFP supernatant test solution was placed in the lower chamber and either DMEM or recombinant SDF1 (50µg/ml) were used as controls. 300µl aliquots of cells were placed into the top chamber of each well. Fluorescence intensity was measured using a (bottom reading) fluorimeter (Perkin Elmer), at time 0, 1hr, 2hrs, 3hrs, 4hrs, 5 hrs and 6hrs, a 2-way ANOVA was used to compare migration.

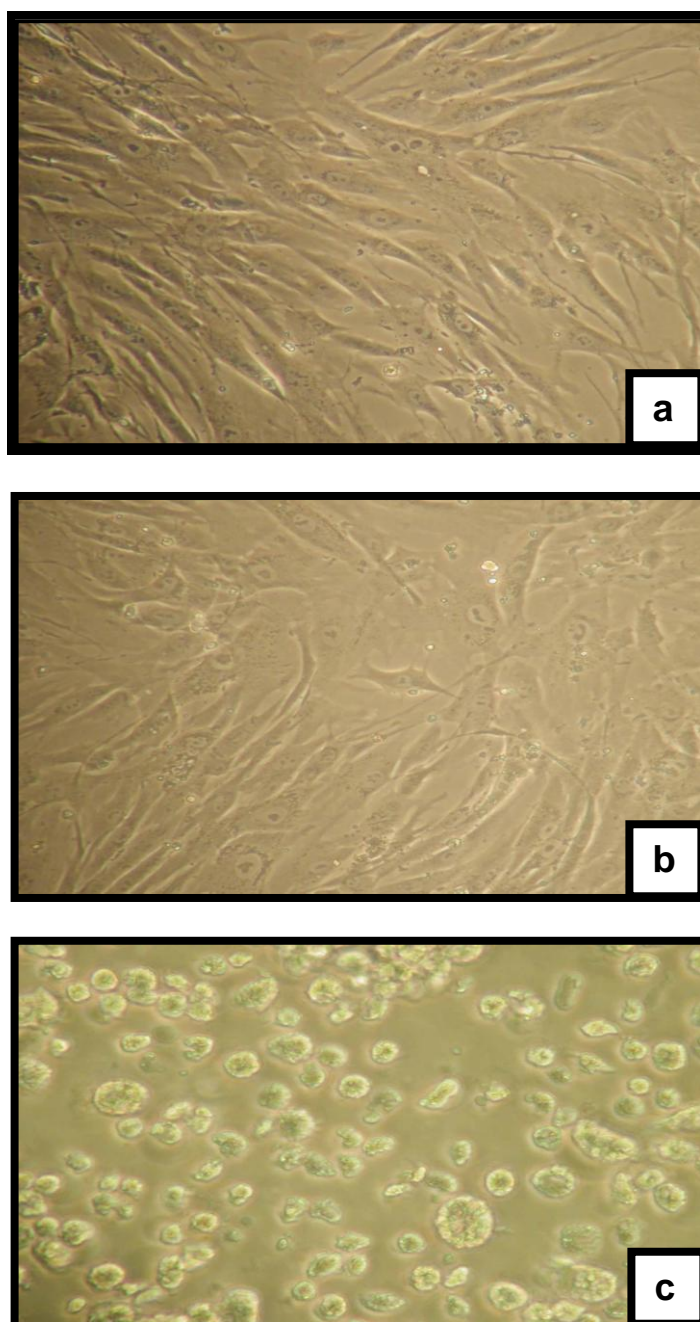
## **6.3 RESULTS**

### **6.3.1 ADSDF1 TITRE**

The AdSDF1 virus was titred at  $1 \times 10^9$  pfu/ml.

### **6.3.2 REPLICATION COMPETENCY**

After 10 days in culture the fibroblasts showed no evidence of viral cellular infection in comparison to HEK cells which had demonstrated a cytopathic effect at day 3 (Fig 6.3.1).

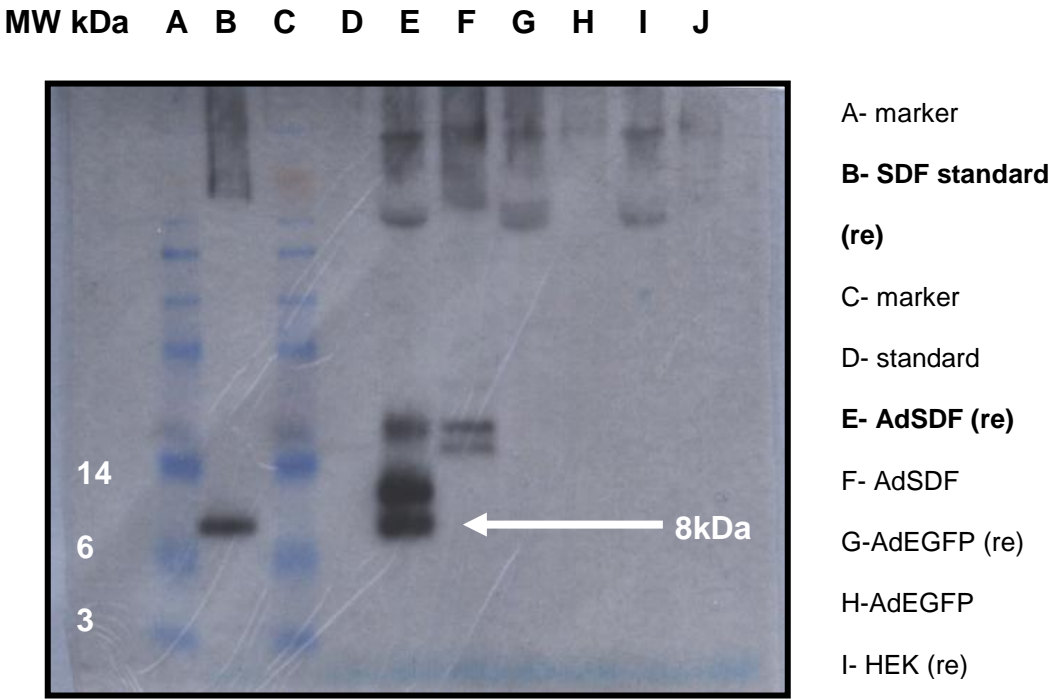


**Fig 6.3.1 AdSDF1 replication competency**

(a) Fibroblasts prior to AdSDF1 exposure (b) fibroblasts post AdSDF1 exposure (c)  
HEK 293 cells day 3 following AdSDF1 exposure

6.3.3 ADSDF1 PROTEIN WESTERN BLOTTING

The reduced supernatant produced a clear band at the same weight as the recombinant SDF1 (8kDa). This was not produced by either the control virus supernatant or HEK 293 cell culture media (**Fig 6.3.2**).



**Fig 6.3.2 Western blot of AdSDF1 and AdEGFP supernatant**  
(re: reduced)

### 6.3.4 BMMNC AND CFU MIGRATION

Migration of BMMNCs was unsuccessful (**Table 6.3.1, Fig 6.3.3**). Both recombinant and AdSDF1 generated SDF1 successfully migrated BM generated CFU cells at a greater rate than both controls (DMEM and AdEGFP),  $P < 0.0001$  (**Table 6.3.2, Fig 6.3.4**)

**Table 6.3.1 Fluorescence value generated by mononuclear cell migration in relation to AdSDF generated protein, AdEGFP protein and DMEM (control)**

TIME (hrs)	Fluoresence (FU)					
	DMEM	SEM	AdEGFP	SEM	AdSDF-1	SEM
0	6534	74	6547	107	7142	6860
1	6800	361	7466	902	6669	6930
2	7250	86	6885	129	7103	7121
3	7114	113	7236	251	7835	7867
4	9205	644	8220	1046	8498	9159
5	8202	529	8356	352	8569	8422

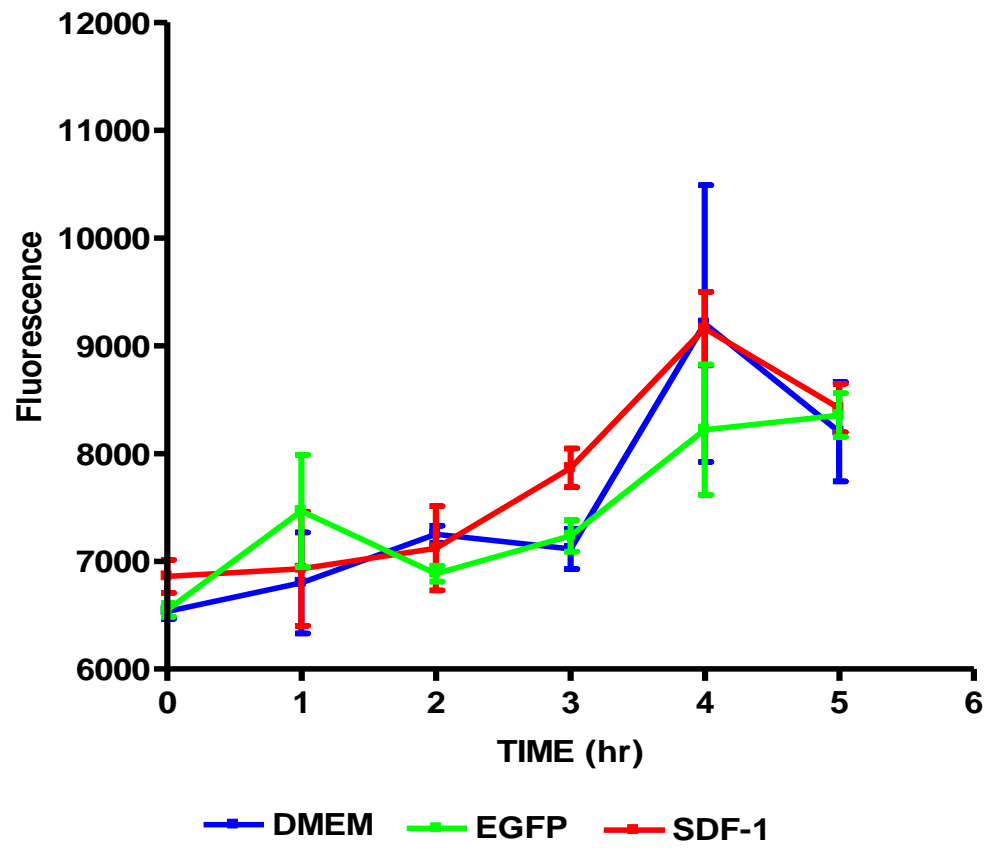


Fig 6.3.3 Migration of BM mononuclear cells to and AdSDF gradient

**Table 6.3.2 Fluorescence value generated by mononuclear derived progenitor cells migration in relation to AdSDF generated protein, AdEGFP protein and DMEM (control)**

TIME (hrs)	Fluorescence (FU)							
	DMEM	SEM	EGFP	SEM	AdSDF1	SEM	Rec SDF1	SEM
<b>0</b>	8672	397	8891	140	10790	699	9184	294
<b>1</b>	9066	589	9796	1063	14697	2451	11760	1826
<b>2</b>	11993	434	12758	115	22933	3874	17929	3145
<b>3</b>	14490	889	16239	266	26401	4557	20438	2941
<b>4</b>	17227	1571	19437	380	30947	5709	23321	3315
<b>5</b>	21818	762	23950	1433	44977	7698	37644	1585

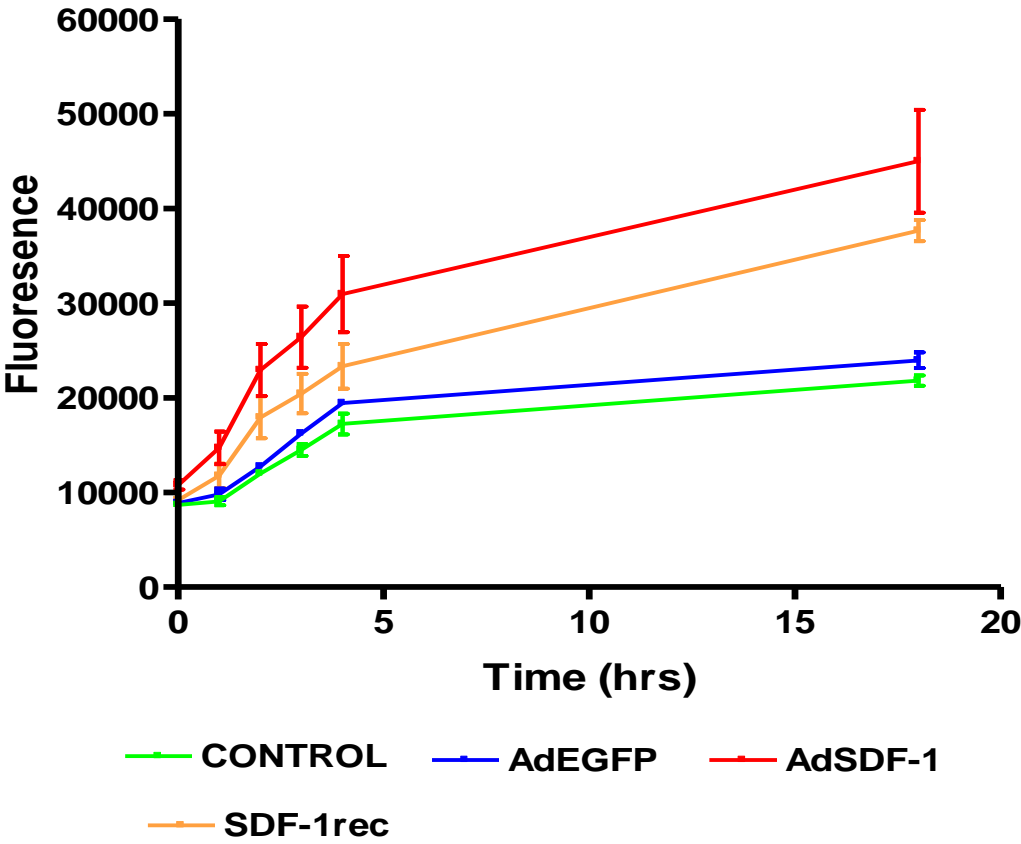


Fig 6.3.4 Migration of BM generated progenitor cells to AdSDF gradient

## **6.4 PLGF CHARACTERISATION**

### **6.4.1 ADPLGF**

This AdPIGF virus is also an Ad5 adenovirus, with the same cassette as the AdSDF1 and the EGFP virus. As this virus had been donated to the project in a fully developed and tested form, only basic testing was performed. The titre of the virus was assessed to ensure that the virus was still viable and that its potency had not fallen during transit (**Sec 6.2.4**), its replication competency (**Sec 6.2.5**) was also tested for safety reasons.

### **6.4.2 VIRAL TITRE**

The viral titre was confirmed to be  $1 \times 10^{11}$ .

### **6.4.3 REPLICATION COMPETENCY**

After 10 days in culture the fibroblasts showed no evidence of viral cellular infection in comparison to HEK cells which had demonstrated a cytopathic effect at day 3 (**Fig 6.3.1**).

### **6.4.4 ADEGFP CHARACTERISATION**

The AdEGFP control virus is an Ad5 virus with the same cassette and promoter as the AdPIGF and the AdEGFP virus.



## 6.5 IN VIVO CYTOKINE UPREGULATION

### 6.5.1 INTRODUCTION

The final aim of this project was to examine the effect of cytokine upregulation on the recanalisation of the vein following thrombosis. The cytokines chosen were SDF-1 and PlGF based on their natural profiles during thrombus resolution and previously published studies examining their effects on mobilisation of progenitor cells in response to tissue hypoxia or injury<sup>295;321;404-406</sup>. Although viral potency had been confirmed *in vitro* the ability of the virus to upregulate protein *in vivo* also had to be established. Finally the effect of adenoviral protein upregulation on caval recanalisation was assessed using image analysis

### 6.5.2 CYTOKINE UPREGULATION USING ADSDF1 AND ADPLGF

Thrombus was formed in 3 groups of 4 Balb/C mice (**Sec 2.1.2**). After 24hrs animals were re-laparotomized and thrombi were injected with 10µl of either  $1 \times 10^9$  AdPlGF,  $1 \times 10^7$  of ADSDF-1 or  $1 \times 10^9$  of AdEGFP (**Sec 2.1.3**). At day 4 following adenoviral injection, animals were culled using cardiac puncture, thrombus, vein wall and plasma were snap frozen and stored at - 80°C. Tissue samples were processed (**Sec 2.3.1**) and analysed using AdPlGF and ADSDF1 ELISAs (**Sec 2.2.3**). Statistical analysis was performed using Mann Whitney U.

## **RESULTS**

### **AdSDF-1**

Mean SDF-1 levels in thrombus were elevated day 4 post AdSDF-1 injection when compared with animals injected with AdEGFP ( $55.6 \pm 6.2$  v  $31.1 \pm 4.4$  pg/mg, **P<0.016, Table 6.5.1, Fig 6.5.1**). In the vein wall and the circulation SDF1 levels were not significantly different from AdEGFP injected animals ( $1411 \pm 205$  v  $1392 \pm 302$  pg/mg, **P>0.05, Table 6.5.2, Fig 6.5.2**) and ( $1230 \pm 75$  vs  $1644 \pm 117$  pg/ml, **P>0.05, Table 6.5.3., Fig 6.5.3**) respectively.

### **AdPIGF**

At day 4 mean PIGF levels were elevated in all tissues analysed following AdPIGF injection when compared with AdEGFP injection; in thrombus ( $21.1 \pm 3.9$  v  $8.6 \pm 0.6$  pg/mg, **P<0.05, Table 6.5.4, Fig 6.5.4**), the vein wall  $243 \pm 38$  v  $96 \pm 21$  pg/mg, **P<0.05, Table 6.5.5, Fig 6.5.5**) and the circulation ( $45.3 \pm 21$  v  $1.2 \pm 0.2$  pg/mg, **P<0.05, Table 6.5.6, Fig 6.5.6**).

Table 6.5.1 Thrombus SDF-1 levels day 4 post adenoviral injection

SDF-1 levels MEAN (SEM) pg/mg	ADENOVIRUS	
	AdEGFP (n=5)	AdSDF1 (n=4)
	31.1(4.4)	55.6 (6.2)

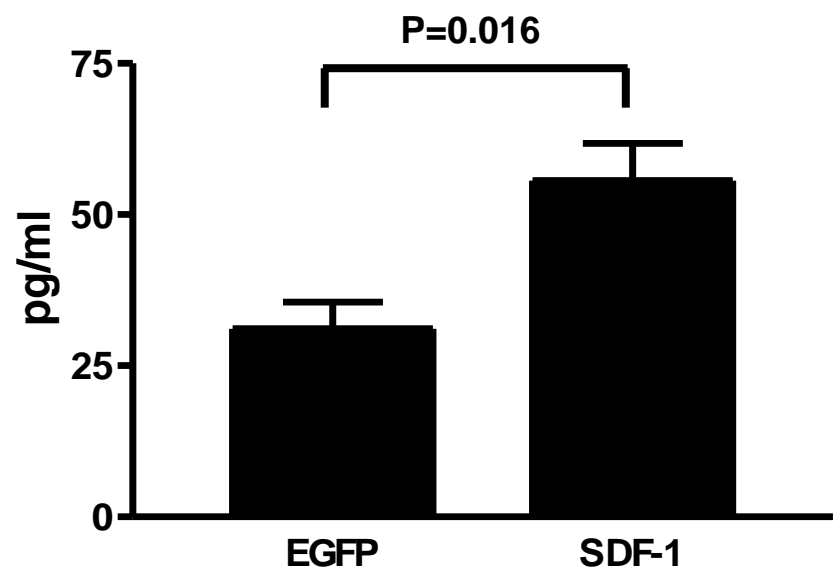


Fig 6.5.1 Thrombus SDF-1 levels day 4 after adenoviral injection

Table 6.5.2 Vein wall SDF-1 levels day 4 post adenoviral injection

SDF-1 levels MEAN (SEM) pg/mg	ADENOVIRUS	
	AdEGFP (n=5)	AdSDF1 (n=4)
	1392 (302)	1411 (205)

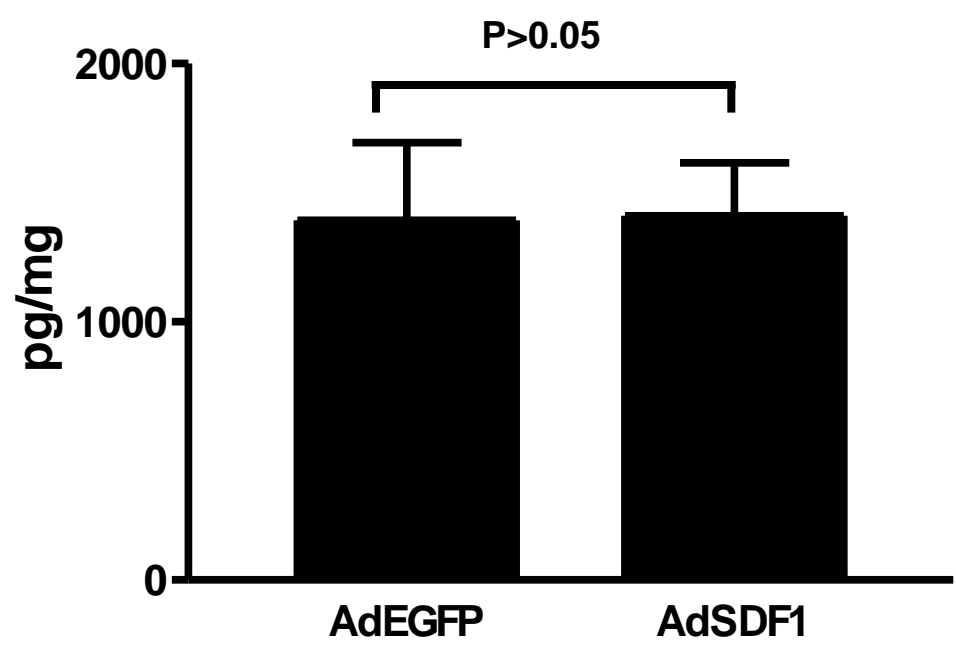


Fig 6.5.2 Vein wall SDF-1 levels day 4 post adenovirus injection

Table 6.5.3 Plasma SDF-1 levels day 4 post adenovirus injection

SDF-1 levels MEAN (SEM) pg/ml	ADENOVIRUS	
	AdEGFP (n=5)	AdSDF1 (n=4)
	1034 (42.3)	1644 (117.6)

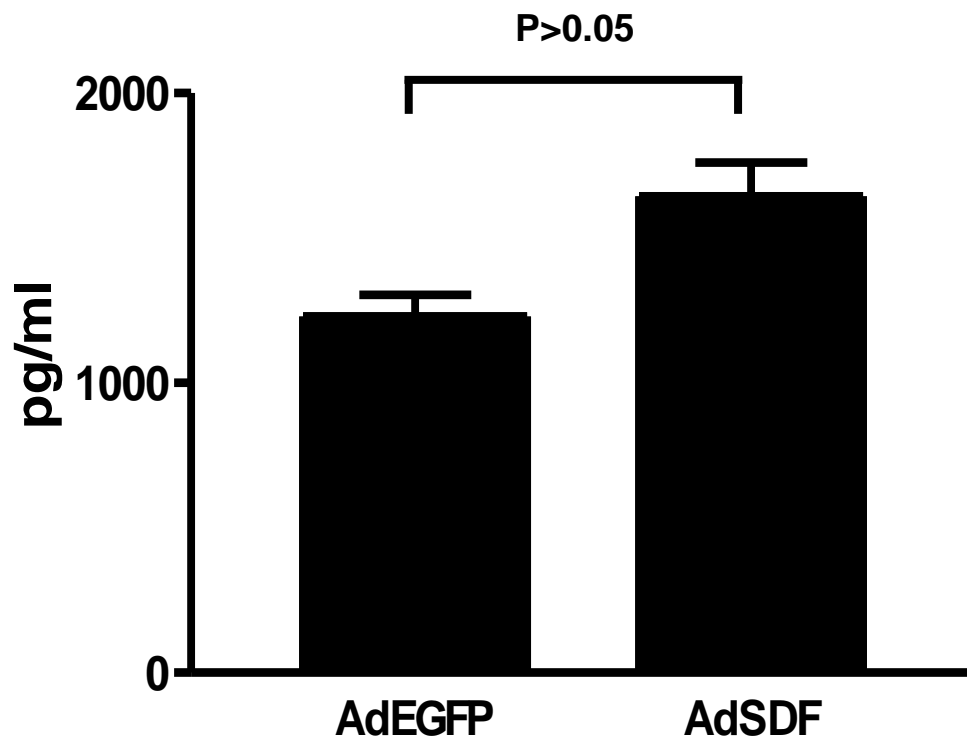


Fig 6.5.3 Plasma SDF-1 levels day 4 post adenovirus injection

Table 6.5.4 Thrombus PIGF levels day 4 post adenovirus injection

PIGF levels MEAN (SEM) pg/mg	ADENOVIRUS	
	AdEGFP (n=5)	AdPIGF (n=4)
	8.6 (0.6)	21.1 (3.85)

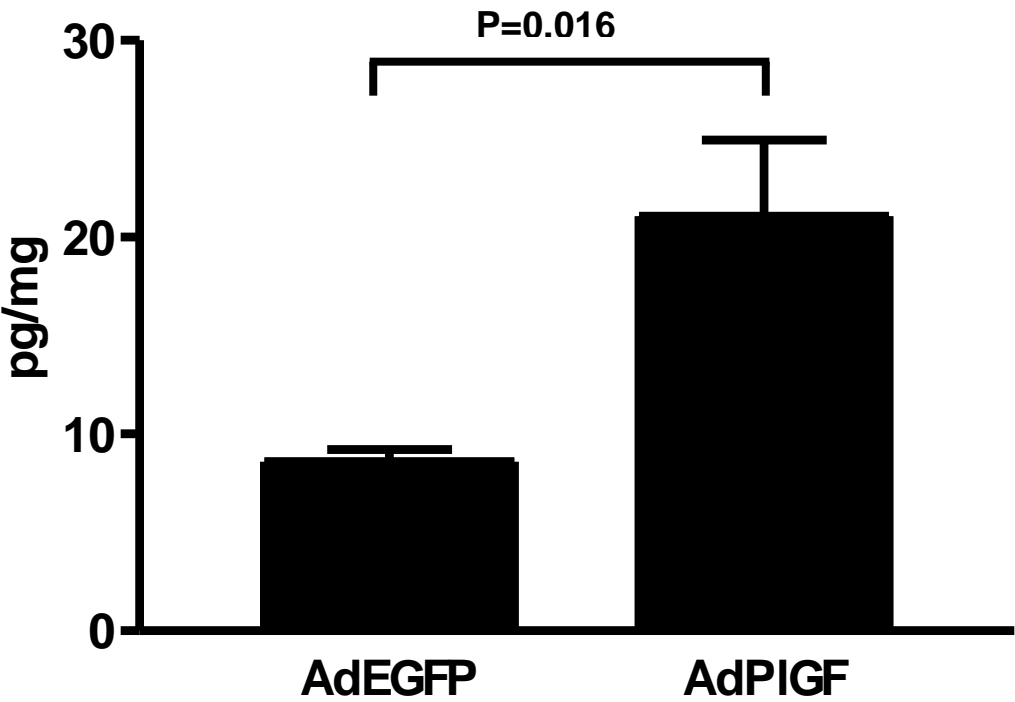


Fig 6.5.4 Thrombus PIGF levels day 4 post adenovirus injection

Table 6.5.5 Vein wall PIGF levels day 4 post adenovirus injection

PIGF levels MEAN (SEM) pg/mg	ADENOVIRUS	
	AdEGFP (n=5)	AdPIGF (n=4)
	96.4 (20.5)	242.5 (37.8)

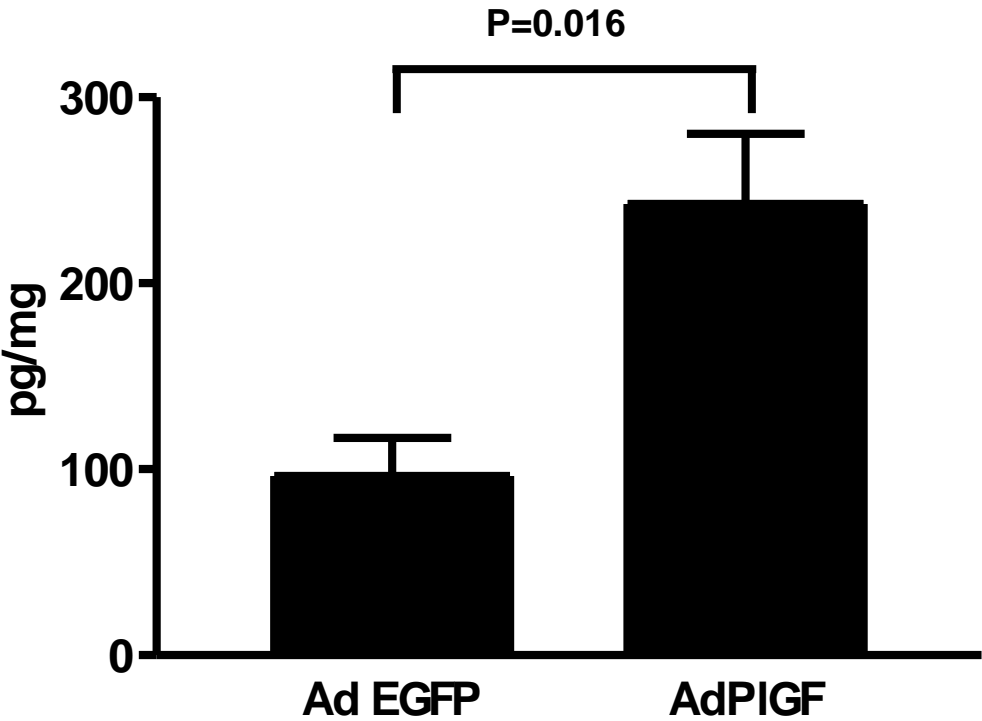


Fig 6.5.5 Vein wall PIGF levels day 4 post adenovirus injection

Table 6.5.6 Plasma PIGF levels day 4 post adenovirus injection

PIGF levels MEAN (SEM) pg/ml	ADENOVIRUS	
	AdEGFP (n=5)	AdPIGF (n=4)
	1.2 (0.2)	45 (20.4)

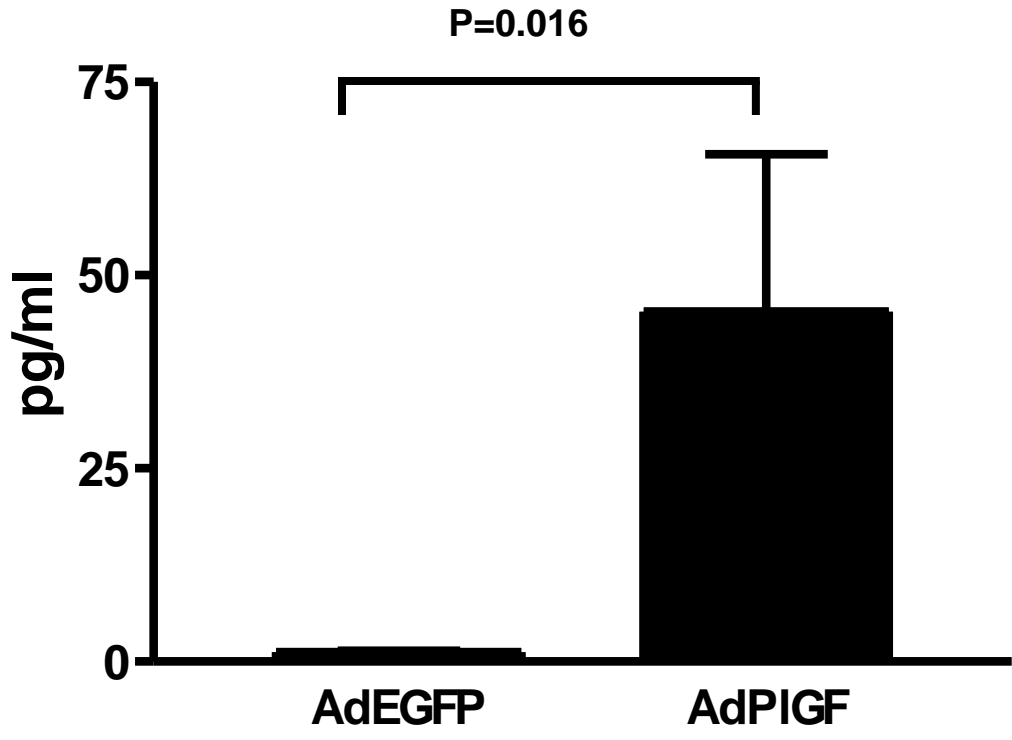


Fig 6.5.6 Plasma PIGF levels day 4 post adenovirus injection



### 6.5.3 THE EFFECT OF SDF-1 & PLGF UPREGULATION ON RECANALISATION

#### Method

Thrombus was formed in 3 groups of ten mice (**Sec 2.1.2**). After 24hrs animals were re-anaesthetized and injected with 10µl of adenovirus into the body of the thrombus (**Sec 2.1.3**). At day 7 following thrombus formation all animals were culled using cardiac puncture and tissues harvested (**Sec 2.1.4**). Thrombus and vein wall were processed for paraffin section (**Sec 2.3.2**). Sections of 5µm were taken at 300µm intervals along the entire length of the thrombus. Haematoxylin and Eosin staining were performed (**Sec 2.3.3**) and recanalisation calculated as a percentage of total vein wall area was compared between the AdSDF-1 injected and AdEGFP injected groups, as well as the ADPIGF and the AdEGFP groups using Mann Whitney U. Blood samples were analysed for HPCs (**Sec 3.2.4**).

#### Results

##### AdSDF1

AdSDF1 injection resulted in a 5-fold increase in percentage recanalisation ( $P=0.0068$ , median 2.95%,  $R=0.7-8.6$ ) v AdEGFP (median 0.62%,  $R=0.3-4.1$ , **Table 6.5.7, Fig 6.5.7**), this increased recanalisation however was not associated with a corresponding increase in circulating HPCs ( $P=0.18$ , median 0.0074%,  $R=0.0001-0.026$ ) v (median 0.0015%,  $R=0.0001-0.015$ , **Table 6.5.9, Fig 6.2.9**).

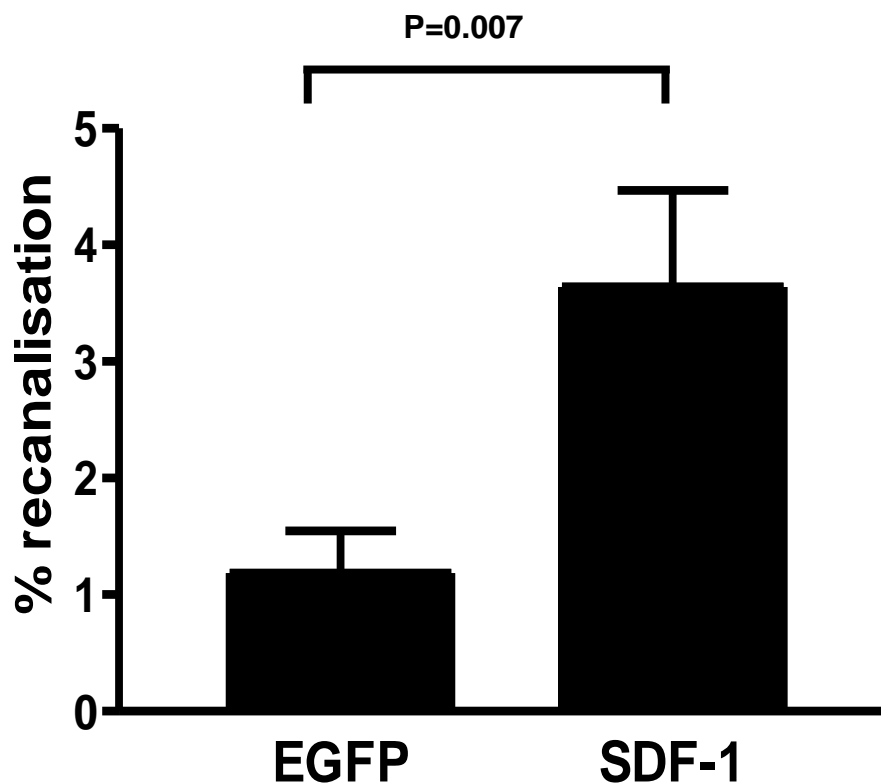
The slides shown in (**Fig 6.5.11**) are representative of the recanalisation observed under microscopy.

### **AdPIGF**

AdPIGF injection resulted in a 6-fold increase in percentage recanalisation ( $P=0.001$ , median 3.6%  $R=2.3-4.1$ ) v EGFP control (0.62 %,  $R=0.3-4.1$ , **Table 6.5.8, Fig 6.5.8**) and was associated with a 6-fold rise in circulating HPCs ( $P=0.028$ , median 0.0095%,  $R=0.0001-0.015$ ) v (median 0.0015,  $R=0.0001-0.015$ , **Table 6.5.10, Fig 6.5.10**). The slides shown in (**Fig 6.5.11**) are representative of the recanalisation observed under microscopy.

**Table 6.5.7 Percentage recanalisation at day 7 following injection with either AdEGFP or AdSDF1**

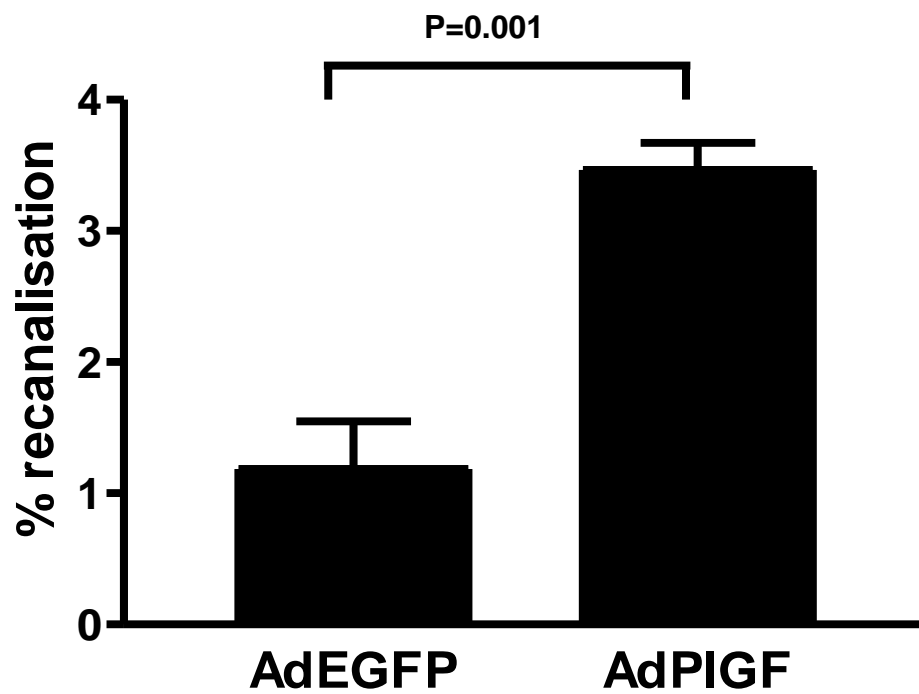
% vein recanalisation Median (SEM)	ADENOVIRUS	
	AdEGFP (n=10)	AdSDF (n=10)
	1.19 (0.36)	3.64 (0.83)



**Fig 6.5.7 Percentage recanalisation at day 7 following injection with either AdEGFP or AdSDF1**

**Table 6.5.8 Percentage recanalisation at day 7 following injection with either AdEGFP or AdPIGF**

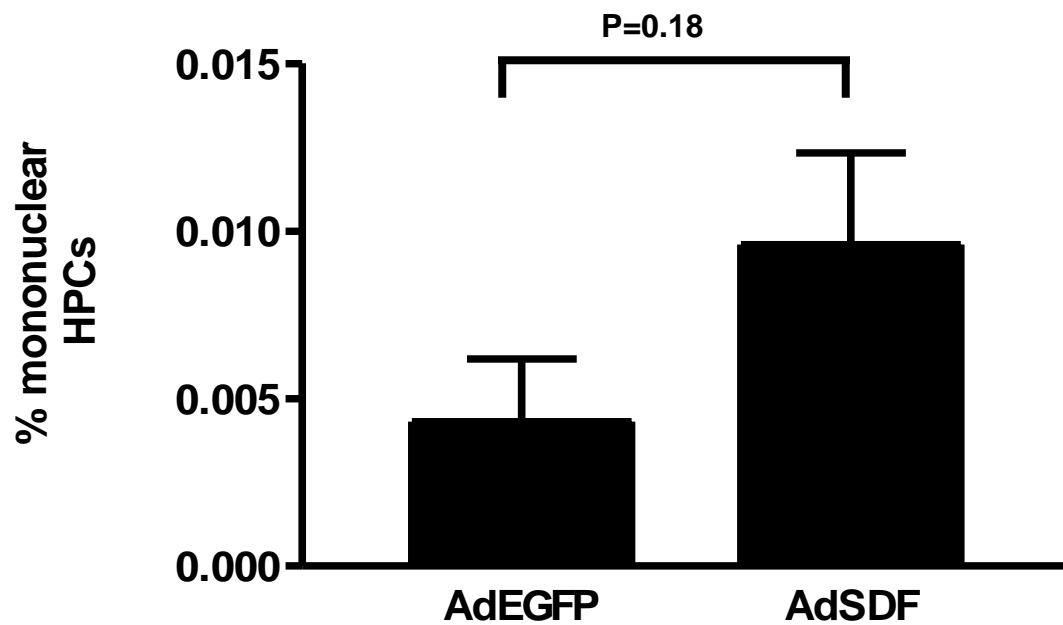
% vein recanalisation Median (SEM)	ADENOVIRUS	
	AdEGFP (n=10)	AdPIGF (n=10)
	0.21 (0.36)	3.47 (1.19)



**Fig 6.5.8 Percentage recanalisation at day 7 following injection with either AdEGFP or AdPIGF**

**Table 6.5.9 Percentage circulating mononuclear progenitor cells at day 7 post AdEGFP or AdSDF1 injection**

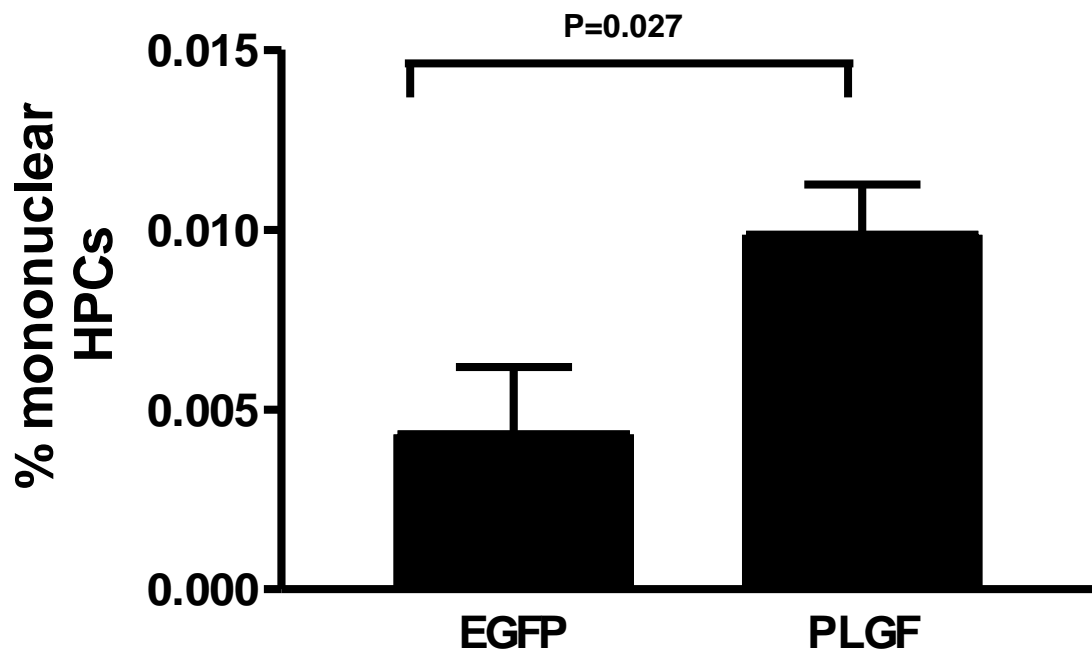
% Median Mononuclear HPCs (SEM)	ADENOVIRUS	
	AdEGFP (n=10)	AdSDF (n=10)
	0.0043 (0.0019)	0.0096 (0.0027)



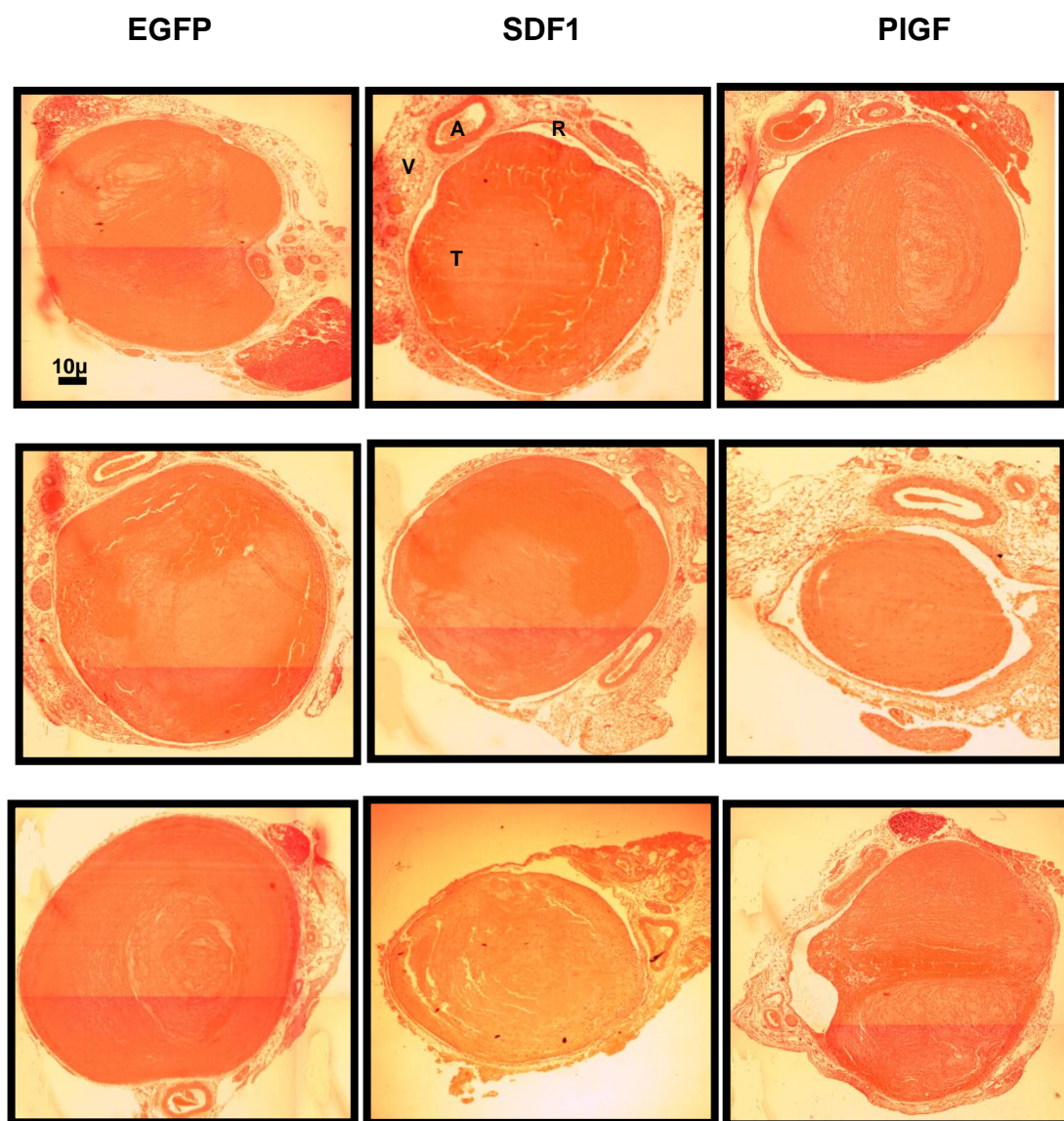
**Fig 6.5.9 Circulating HPCs at day 7 after AdEGFP or AdSDF injection**

**Table 6.5.10 Percentage circulating HPCs cells at day 7 post AdEGFP or AdPIGF injection**

% Median Mononuclear HPCs (SEM)	ADENOVIRUS	
	AdEGFP (n=10)	AdPIGF (n=10)
	0.0043 (0.0019)	0.0099 (0.0014)



**Fig 6.5.10 Circulating HPCs at day 7 after AdEGFP or AdPIGF injection**



**Fig 6.5.11 Cross sections of venous recanalisation following adenoviral injection.**

## 7.0 DISCUSSION

### 7.1.1 PROGENITOR CELL EXPRESSION DURING THROMBUS RESOLUTION

Historically, progenitor cells capable of vasculogenesis were thought only to exist during embryological development. It wasn't until 1997 that Asahara<sup>220</sup> first provided evidence of what he described as endothelial progenitor cells persisting into adult life. The definition of EPCs was based on the presence of two cell surface markers; CD34 and VEGFR2<sup>220</sup>. It is now known however, that using only CD34 and VEGFR2 to identify EPCs lacks the specificity required to differentiate them from mature endothelial cells<sup>221</sup>. The lack of congruity in defining EPCs has led to a large volume of literature in which the variable expression of two or more markers from CD14, CD31, CD45, CD133, VEGFR2, vWF and VE-cadherin have all been used to define EPCs<sup>325;407</sup>. This in turn has made it increasingly difficult to compare the results of the numerous studies in this area<sup>225;245;382;408-410</sup>.

At the inception of this study, it was suggested that CD133 in combination with CD34 and VEGFR2 was the gold standard triple label for EPCs<sup>227</sup>. CD133 is a cell surface glycoprotein which was initially thought to exist only in CD34+ progenitors found in blood, foetal liver and human BM<sup>411</sup>. Clinical studies have shown that circulating CD133+CD34+VEGFR2+ progenitors correlate inversely with the risk of developing cardiovascular disease<sup>229;412-414</sup>. Although this does not clarify the nature of these cells, it has imbued them with a degree of clinical significance.



Further evidence for the presence of EPCs in adult life came from Shi *et al.*<sup>415</sup> when they demonstrated that bone marrow-transplanted CD34+ cells migrated onto and covered implanted Dacron grafts and that these cells played a role in new vessel formation<sup>416</sup>. Assessment of the bone marrow transplanted CD34+ cells by conventional flow cytometry demonstrated that they were also positive for CD133 and VEGFR2 but negative for CD45, this initially substantiated their identity as EPCs and resulted in a deluge of 'EPC' research<sup>325</sup>. Since then however, polychromatic flow cytometry has shown that these cells do in fact express CD45 making them more likely to be derived from a haematopoietic or myeloid lineage<sup>417</sup>. Furthermore *in vitro* assays of angiogenesis have failed to demonstrate conclusively that CD133+CD34+VEGFR2+ cells are capable of giving rise to an endothelial phenotype, as they do not form either early or late CFUs or tubular structures in Matrigel assays<sup>250</sup>. Key *in vivo* studies have also demonstrated that following bone marrow transplantation in mice, donor bone marrow cells failed to incorporate into blood vessels when assessed by confocal microscopy (although a number of false positive cells were found because of signal overlap). Donor cells were however, found in and around the collateral vessels in much greater concentration following ischaemic insult<sup>251;418</sup>. These data suggests that these cells may play a pro-angiogenic role as HPCs and not EPCs as once thought<sup>225;238</sup>.

Having concluded that the combination of CD34 and VEGFR2 were not sufficient to adequately select an HPC population<sup>221</sup>, we investigated the effect of thrombosis

on circulating and bone marrow resident Sca1+CD34+VEGFR2+ cells. In this project Sca1 was used as a progenitor cell marker (commonly expressed on multipotent HSCs<sup>223</sup>) rather than CD133 which is not found in mice.

### **Circulating HPCs**

Circulating HPCs in the sham operated animals remained relatively constant throughout the experimental period (12hrs-14days); whereas in animals with venous thrombosis, there was a sharp early rise in circulating HPC numbers followed by a fall and then a later rise that was of a much smaller magnitude. The distinct pattern of the changes in circulating HPC numbers compared with those seen in sham controls (laparotomy wound healing) suggests an early role for these cells during venous thrombosis. Whether their role relates to thrombogenesis or early changes associated with thrombus organisation and resolution remains to be determined. Similar patterns of progenitor mobilisation are evident in other injury models; circulating CD133+VEGFR2+ cells increase by 50% in the first 12hrs followed by a rapid fall to baseline within 72hrs in patients who have suffered burns or undergone coronary artery bypass grafting<sup>247;381</sup>. Equally in a myocardial infarction study, circulating CD34+ cells peaked at day 7 following ischaemic injury<sup>381</sup>. These results point to an early progenitor response to a variety of pathological insults. The literature does not however comment on the subsequent secondary rise that occurred in the mouse model following thrombosis or its relevance to neovascularisation<sup>247;381</sup>.

### **Bone marrow resident HPCs**

There was no significant difference in bone marrow resident HPCs between the two intervention groups (sham and thrombus) and HPC levels remained low throughout the examined period. At 12hrs however, there is a clear difference between animals that have not had any intervention and both the sham and the thrombus groups. It must be remembered that the actual changes in circulating HPC levels are still a fraction of the much larger change evident within the bone marrow compartment. So small differences within this much larger pool of HPCs may be difficult to measure, secondly we must consider that cells measured within the circulation may have come from alternative progenitor sources such as the liver<sup>419</sup>. Studies examining bone marrow progenitor populations following vascular trauma are rare, one study performed on human subjects following CABG demonstrated a reduction in bone marrow CD34 progenitor cells that were CXCR4, implying an early role for SDF1<sup>420</sup>.

### **7.1.2 THE EFFECT OF HPC UPREGULATION ON THROMBUS RESOLUTION**

The discovery of circulating progenitor cells has lead to a rapid move towards interventional studies first in animal models and then in man<sup>214;382</sup>. In a hind limb ischaemia model in immunodeficient mice, *ex vivo* expanded human PBMNCs were able to recover blood flow compared with animals treated with mature endothelial cells<sup>332</sup>.

Progenitors isolated from cord blood also had a similar effect<sup>421</sup>. In murine myocardial injury models, progenitor cell upregulation reduces myocardial fibrosis and preserves cardiac function<sup>382</sup>. In order to examine the effect of progenitor cells on thrombus resolution, an appropriate source of these cells was first identified. The circulating volume in mice is too small for use as a source of PBMNCs or CFUs. Mononuclear cells derived from both bone-marrow and spleen have the ability to generate cells with an endothelial phenotype when cultured using the Hill method<sup>229</sup>. In our hands only bone marrow mononuclear cells formed early CFUs in culture; a central cluster of cells surrounded by spindle shaped cells at the periphery<sup>195</sup>. Dil-Ac-LDL was taken up by the BMMNC-CFUs which also expressed lectin binding<sup>221</sup>, but consistent with the published literature these cell did not express Sca1, CD34 or VEGFR2<sup>225</sup>.

BMMNC-CFUs similarly to early PBMNC CFUs, do not passage into expanding cell lines, suggesting that the BMMNC generated CFUs are similar in nature to early PBMNC-CFUs generated by the Hill method<sup>229;422</sup>. Lectin binds to endothelial cells, HPCs and platelets, with Ac-LDL readily taken up by monocytes and endothelial cells<sup>237;238</sup>. Proteomic analysis shows that the endothelial cell markers (CD31, VWF and lectin binding) found in early peripheral blood CFUs may be the result of platelet microparticles which also express these markers, fusing with mononuclear cells<sup>236</sup>. A large number of cells once thought to be EPCs/HPCs are therefore likely to be contaminated monocytes<sup>423;424</sup>.

Monocytes cultured under angiogenic conditions lose their monocytic lineage markers replacing CD14/CD45 with commonly accepted EPC characteristics, including LDL uptake, lectin binding and CD31 reactivity<sup>425</sup>. These cells do not incorporate directly into vascular structures but are able to produce several growth factors such as GCSF, GMCSF and VEGF, which may be the mechanism through which they promote neovascularisation<sup>422</sup>.

In the present study, systemic injection of isolated BMMNCs rich in Sca1+CD34+VEGFR2+ cells following thrombus induction caused a 2-fold increase in recanalisation. This is in keeping with data showing that left ventricular function and collateral flow are improved in response to transendocardial administration of BM or PBMNC cells in a swine model of myocardial infarction<sup>333;426</sup>. In hind limb ischaemia models both intramuscular and systemic injection of BMMNCs and PBMNCs result in increased capillary density, muscle perfusion and exercise tolerance<sup>332;427</sup>. Clinical studies have since examined the impact of BMMNC or CD133+ cell treatment after myocardial infarction or in critical limb ischaemia but the outcomes of these studies have been equivocal<sup>328;353;428-430</sup>. The results achieved in animal models in which young healthy animals are used doesn't always correlate well with the clinical setting in which patients are often older with several concomitant comorbidities<sup>280;431</sup>.

Several animal models have reported homing of progenitor cells to sites of injury or hypoxia<sup>194;220;282;432;433</sup>, my aim was to examine the post-transplantation location of BMMNCs, at 1 and 7 days. Systemic injection of PKH26-labelled cells revealed no evidence of cells in the thrombus 1 day after injection but a small number of cells were seen in the thrombus vein wall interface by day 7. Less than 0.1% of the labelled BMMNCs were detected in thrombus, suggesting that only a small number of unselected BMMNCs have the capacity to home to the site of thrombosis. Although several studies have examined the impact of BMMNC injection on cardiac function<sup>434;435</sup>, only a limited number have examined bone marrow cell engraftment following intra-cardiac or coronary vessel injection<sup>436-438</sup>. Injection of radiolabelled autologous bone marrow mononuclear cells via the coronary artery into 10 patients acute or chronic myocardial ischaemia showed that at 2hrs all but one patient had evidence of radiolabelled cells could be found in the heart but at 20hrs BMMNCs were present in the myocardium of only 3 patients (all 3 had acute ischaemia)<sup>439</sup>. The majority of injected labelled bone marrow cells migrated to the liver spleen or lung tissues<sup>439</sup>. The findings in this study are consistency with published data in that only a small fraction of bone marrow or progenitor cells engrafted to the site of myocardial ischaemia irrespective of the mode of delivery<sup>438-440</sup>.

### 7.1.3 CYTOKINE EXPRESSION DURING THROMBUS RESOLUTION

#### Circulating levels

Having demonstrated a significant rise in circulating HPCs following thrombosis, the possibility that cytokine expression within the thrombus or vein wall preceded HPC expression was examined. Cytokine levels (GMCSF, GCSF, VEGF, PIGF and SDF1) were measured both in the thrombus, thrombosed and control vein walls and the laparotomy wound. The latter was done to elucidate whether wound healing and thrombus resolution are orchestrated by similar cytokine expression patterns. In this study HPC mobilisation into the circulation was associated with early rises in the circulating concentrations of GMCSF, VEGF and PIGF following thrombus induction. In animals with thrombus circulating GMCSF was approximately 9-fold greater than sham levels at 12hrs, VEGF levels were 3-fold greater and PIGF levels were also 3-fold greater but remained at this level until 24hrs by which time both GMCSF and VEGF and already fallen to sham levels. At 48hrs following thrombus induction the fall in circulating HPCs coincided with a fall in GMCSF, VEGF and PIGF to sham levels. These cytokines are all known to affect the mobilisation and recruitment of HPCs<sup>301;317;432</sup>. There was however, no direct correlation between the numbers of HPCs measured and the circulating concentrations of the measured cytokines. Others have found a positive correlation between VEGF<sup>433</sup> and SDF1<sup>441</sup> levels and progenitor cell numbers in the circulation, but these findings have not been consistent<sup>442;443</sup>.

The lack of correlation with HPCs in this study may have been the result of low sample numbers and hence a type II statistical error.

### **Vein wall levels**

The levels of GCSF, GMCSF, VEGF and PIGF in the vein walls of animals with thrombus were greater than those from sham operated animals. GCSF levels were consistently 2-fold greater from 12hrs to 48hrs after which they fell to those of sham animals, similar patterns were demonstrated by GMCSF and VEGF. Interestingly, although SDF1 is regarded as a cytokine that mobilises bone marrow progenitor cells, the concentration of this factor only exceeded sham levels at 24hrs after thrombus induction but this was not statistically significant on Bonferroni post-testing. In the cytokines measured here, only levels of PIGF levels remained persistently higher in the vein surrounding thrombus compared with same area of vein wall in sham operated animals. Whether this imparts a greater importance to the role of PIGF during thrombus resolution is, however, unclear. PIGF does appear to be more important in pathological angiogenesis, with loss of PIGF resulting in impaired retinal, limb and myocardial revascularisation<sup>301;311;444</sup>.

### **Thrombus and wound tissues**

Patterns of cytokine expression in wound tissue and thrombus were similar for GCSF, GMCSF, VEGF and SDF1. PIGF expression however, peaked at day 7 within the thrombus, with no discernible expression pattern in the wound tissue.



The lack of PIGF upregulation in the wound tissue was not expected and does not corroborate the findings of Odoriso<sup>445</sup>, who demonstrated that PIGF mRNA levels peaked at day 2 in a murine wound healing model. One explanation for this may be that skin contains hair follicles which are physiologically regulated by PIGF during anagen, this may have contributed to overall PIGF expression during cutaneous wound healing<sup>445</sup>. The wound tissue samples used in the present study were devoid of dermis and therefore would not be affected by follicular PIGF levels, which may account for the discrepancy between these studies. Furthermore it is well known that mRNA expression does not always correlate with actual protein expression<sup>446</sup>.

Although there was no direct correlation between thrombus cytokine levels and circulating HPCs, cytokines such as VEGF, SDF1 and PIGF have all been implicated in the mobilisation and recruitment of progenitor cells into tissues<sup>301;447-449</sup>. The complex picture of cytokine expression involved in thrombus resolution might suggest that there is a common trigger such hypoxia inducible factor (HIF)<sup>450</sup>. Following venous thrombosis in the mouse, the hypoxic environment within the thrombus inversely correlates with HIF1 $\alpha$  levels<sup>451</sup>. Treatment with L-mimosine (HIF1 stabiliser) increases HIF1 levels and also results in concomitant increase in VEGF and PIGF and the promotion of venous recanalisation and thrombus resolution<sup>451;452</sup>. We speculated that these cytokines in particular PIGF may represent the impetus for the recruitment of progenitor cells from the circulation

into the thrombus, possibly via the vein wall as previously shown for the recruitment of inflammatory cells<sup>119</sup>.

#### **7.1.4 PLGF UPREGULATION**

There is evidence supporting the role of PIGF in angiogenesis following a pathological insult<sup>453</sup>. Natural upregulation of PIGF and VEGFR1 occurs in hind limb and myocardial ischaemia models<sup>317;318</sup>. In animal models PIGF administration not only reverses diminished revascularisation in the ischaemic limbs and myocardium of PIGF knockout animals, but also increases neovascularisation in wild type animals<sup>301</sup>.

The unique PIGF profiles found in thrombus and vein wall both suggested that PIGF plays an important role in thrombus resolution. Previous studies have shown that adenovirus gene constructs (AdVEGF) injected directly into the thrombus, transfects cells in the thrombus and local vein wall, with little loss to the liver or other organs<sup>313;454</sup>. To further assess its importance in the venous recanalisation we used AdPIGF. Treatment with AdPIGF injected directly into the thrombus after induction was associated with an over 40-fold increase in circulating PIGF levels, and a doubling in the expression of PIGF in both the thrombus and surrounding vein wall. Although there was over a 3-fold increase in vein recanalisation following PIGF upregulation, the percentage increase in recanalisation of the vein was small (<5%). This small but significant increase in vein recanalisation may, however, be enough to provide sufficient flow through the vein, reducing the likelihood of post-

thrombotic complications following a DVT<sup>455</sup>. Greater luminal thrombosis and delayed thrombus resolution both result in an increased incidence of post thrombotic syndrome<sup>456;457</sup>. It is therefore reasonable to speculate that smaller thrombi and earlier resolution should reduce post thrombotic sequelae. The ATTRACT trial is currently randomising patients to either standard treatment with anticoagulation versus catheter directed thrombolysis<sup>458</sup>. This will help to determine the long-term benefits of early thrombus removal<sup>458</sup>.

PIGF is a member of the VEGF family that binds to the tyrosine kinase receptor, VEGFR1 (Flt1)<sup>301;315</sup>. The attributes of VEGF have been heavily explored in both animal models and the clinical setting, our understanding of PIGF however, remains limited. PIGF and VEGF play a synergistic role in ischaemic angiogenesis when compared to the actions of either molecule in isolation<sup>301;447</sup>. VEGF has three receptor subtypes, only one of which (VEGFR1) it shares with PIGF. Although both VEGF and PIGF bind to VEGFR1 resulting in its phosphorylation, they induce phosphorylation of different tyrosine molecules, suggesting differential actions through the same receptor<sup>459</sup>. Aside from VEGFR1 phosphorylation, several mechanisms of action have been suggested for PIGF including displacement of VEGF from VEGFR1 allowing increased VEGF-VEGFR2 interaction and mobilisation of bone marrow progenitor cells. The data from this study showed that plasma PIGF and VEGF peaked early, vein wall levels of PIGF remained continuously elevated throughout, even though VEGF levels had dropped back to

baseline by day 7. Thrombus VEGF also peaked early at day 2 compared with a later peak observed with PlGF at day 7. The prolonged expression of PlGF in the vein wall and its late expression in the thrombus suggest that it works independently of VEGF in thrombus resolution. PlGF upregulation did however upregulate the number of circulating HPCs compared with the AdEGFP (control), suggesting a progenitor based mechanism for resolution. In a rat model of venous thrombosis the expression of VEGF was similar to that shown in this study<sup>167</sup>. Adenovirus mediated upregulation of VEGF in the rat model resulted in a 4-fold increase in venous recanalisation<sup>121;313;460</sup>. Although by comparison, the effect achieved with PlGF was somewhat disappointing, it is difficult to equate the efficacy of PlGF and VEGF treatments in promoting recanalisation in different animal models. PlGF treatment has however, resulted in significantly greater neovascularisation than VEGF in both coronary and hind limb ischaemia models in mice<sup>317</sup>.

#### **7.1.5 SDF1 UPREGULATION**

SDF1 has been implicated in the migration, proliferation and differentiation of several cell types including HPCs and EPCs through its CXCR4 G-protein coupled receptor<sup>450;461</sup>. During natural thrombus resolution SDF1 expression in the circulation was not elevated above sham levels, vein wall levels only exceeded sham levels after 48hrs and thrombus levels peaked at day 7. Although in the *in vitro* setting CFU cells migrated towards an SDF1 gradient, the latent SDF1 expression profile in the thrombus and circulation suggest that this factor is not

responsible for the primary HPC response following thrombus formation. Interestingly, Upregulation of SDF1 in the thrombus by direct injection of an AdSDF1 construct resulted in a 50% increase in thrombus SDF1 without a discernible change in circulating or vein wall SDF1. Treatment with AdSDF1 increased recanalisation by approximately 2-fold and whilst the number of circulating Sca1+CD34+VEGFR2+ cells increased above sham levels, this did not reach statistical significance. The variability in HPC numbers combined with the relatively small number of animals assessed, may have given rise to a type II error.

SDF1 is produced by multiple bone marrow stromal cell types and by epithelial cells in many organs<sup>462</sup>. Thrombus was stained for SDF1 and although we demonstrated SDF1 staining in thrombus tissues, this was not associated with any specific cell type. In acute coronary syndrome increased platelet bound SDF1 correlates with circulating CD34+ progenitor cells, platelets may also be the source of SDF1 in venous thrombi<sup>463</sup>. The expression of SDF1 is upregulated during ischaemia and this cytokine causes both HPC engraftment and recruitment to ischaemic tissue<sup>448;462</sup>. Neutralising anti-CXCR4 (SDF1 receptor) antibodies reduces *in vivo* homing of myeloid progenitor cells to limb tissue in a hind limb ischaemia model<sup>464</sup>. Upregulation of SDF1 is thought therefore to occur secondary to a hypoxic environment<sup>449</sup>. Oxygen tension in the day 1 murine thrombi is known to be significantly lower than that of the normal venous circulation<sup>451</sup>. In the mouse model SDF1 levels rise late within the thrombus (day 14) and therefore it seems

that the trigger in this instance is unlikely to be hypoxia which is no longer present by day 14<sup>451</sup>.

GCSF was the only cytokine to peak in the first 12hrs after thrombus formation and to coincide with the low oxygen tension as measured by Evans *et al.*<sup>451</sup>. GCSF is known to mobilise bone marrow resident HPCs and is used clinically for this purpose<sup>465</sup>. GCSF mobilises HPCs through the disruption of the SDF1/CXCR4 axis within the bone marrow resulting in an egress of HPCs into the circulation<sup>278</sup>. There is some evidence that GCSF levels correlate more significantly with circulating progenitors following tissue ischaemia than other cytokines such as SCF, VEGF and SDF1<sup>466</sup>. In the current study the GCSF peak corresponded to the lowest oxygen tension measured by Evans *et al.*<sup>451</sup> and preceded the early rise in HPCs. The cytokine milieu in upregulating progenitor release is almost certainly complex in nature and this is the likely reason that clinical studies have not been met with overwhelming success<sup>366</sup>.

### 7.1.6 CURRENT STUDY LIMITATIONS AND FUTURE STUDIES

#### i. Analysis of progenitor cells and cytokines during natural thrombus Resolution

Phenotyping of circulating cells was hampered by the lack of availability of a multi-laser flow cytometer. The FACS Calibur® machine used in this study had only a single laser, allowing measurement of only three markers for any cell solution. Current research into progenitor cell activity in disease is continually faced with evolving definitions of what characterises progenitor cells (e.g. EPCs, HPCs or MSCs)<sup>245;382;409;467</sup>. The use of a multiple laser flow cytometer that is able to identify 12-16 cell markers simultaneously would allow a more refined, simultaneous phenotyping of multiple epitopes. This is crucial given that many of the different haematopoietic lineage cells can express subtly different surface markers<sup>221;225;245</sup>.

Although we have demonstrated that venous thrombosis mobilises HPCs, this does not show whether there is local HPC engraftment. It is now possible to analyse the cell content of tissues such as thrombus using a flowcytometric approach. The tissues have to first however, be enzymatically digested<sup>468</sup> and care must therefore be taken to validate this technique for the isolation of specific cell types, as there is some evidence to show that enzymatic dissolution of tissues can modify cell surface markers<sup>469</sup>. Use of multiple laser flow cytometers would result in a more refined and quantitative characterisation of the cells in the tissue isolates and regardless of their functional roles could provide a more efficient way

of cataloguing changes within the various cell populations during thrombus resolution and recanalisation<sup>120;179;217</sup>. Once specific populations have been highlighted the onus would then be to determine their functional relevance during thrombus resolution. Using both knockout and transgenic animal models, the impact of different progenitor phenotypes could be assessed in much the same way as monocytes/macrophage heterogeneity has been elucidated<sup>470-473</sup>.

Lack of access to fluorescence activated cell sorting (FACS) at the time of this study also prevented discrete isolation of specific cell populations. Cell sorting may have facilitated the *in vitro* analysis of cell specific cytokine expression, amongst other functional characteristics in a variety of environments including those associated with the thrombosis (e.g. low oxygen tension<sup>474</sup> or VEGF rich media<sup>475</sup>). The more refined the cell separation technology the more it would facilitate *in vivo* characterisation of the effects of HPCs and other cell types on thrombus resolution. One cell population that might prove important in thrombus resolution are mesenchymal stem cells, characterised as Sca1+/c-Kit-/Lin-/CD34- cells, they are able to form both endothelium and smooth muscle cells and can also generate capillary like structures *in vitro*<sup>474</sup>. Similarly having produced CFUs from mouse bone marrow CFU cells could also be better classified following isolation from non-CFU forming spindle cells. These cells could then be better analysed with *in vitro* migration and Matrigel™ assays before being used *in vivo*.



## ii. Cytokine analysis and thrombus resolution

In these studies the mouse model was preferred to the rat model as there was a greater availability of antibodies for flow cytometry, ELISA and immunohistochemical analysis. The lower volume of plasma and tissue available from mice reduced the number of analytes that could be examined and even the adoption of a multiplex ELISA system did not fully compensate for this problem. For example, the multiplex ELISA system used in this study did not have the antibody pairs required for the analysis of GCSF and there was insufficient plasma volume to allow the measurement of GCSF using conventional ELISA. Analysis by gene array, although limited to measuring mRNA expression, may allow us to develop a more comprehensive 'picture' of the temporal changes in the expression of genes encoding the whole cytokine family (and their receptors). Current technology in this area is now common place and affordable<sup>476</sup>.

A number of cytokines are known to control the recruitment of progenitor cells<sup>406</sup>. Some such as VEGF and SDF1 have been studied in a variety of different animal models<sup>406</sup>. The literature suggests that VEGF and PIGF work synergistically in pathological states<sup>321;447;453</sup>. VEGF has previously been studied in the rat thrombosis model and had a greater effect on thrombus resolution than PIGF in the current mouse model<sup>121;209;313</sup>. Studies combining both cytokines would be informative as to the synergistic nature of these proteins.

### iii. Interventional studies

Having successfully cultured bone marrow-derived CFUs, they could not be used in any interventional studies as the double positive Dil-Ac-LDL lectin labelled cells from these cultures could not be adequately passaged. The cell based interventional study was therefore carried using the BMMNCs, which contain a large variety of undefined progenitor/stem cells, although a significant proportion of these cells were Sca1+CD34+VEGFR2+. Only a small number of BMMNCs were observed within the body of the thrombus suggesting that the cells important for recanalisation represented a very small proportion of the total BMMNC population. A flow assisted cell sorter would have allowed isolation and therefore concentration of specific cell types more likely to have an effect in the interventional studies.

The AdSDF1 virus had a lesser effect on the circulating HPCs when compared with PlGF. It is difficult to compare the efficacies of these constructs as I was unable to match the pfu/ MOI used in the treatments as the viral titre for AdSDF1 construct ( $1 \times 10^9$ ) was lower than that achieved for the AdPlGF ( $1 \times 10^{11}$ ). This was despite several attempts to grow the construct and the use of different separation methods<sup>313</sup>. It is also difficult to compare the efficacy of the virus constructs as some gene products may have a greater potency at lower concentrations<sup>477</sup>.

End-point measures in these studies essentially focus on the following:

(a) Measurement of thrombus weight (that does not provide information regarding the area of recanalisation of the vein or the cellular composition /organisation of the thrombus). (b) Measurement of thrombus composition and the degree of recanalisation by histological means can introduce variability from differences in tissue processing and is also costly in terms of animal usage. A method that facilitates longitudinal imaging of both physical structure and cellular/molecular composition of the thrombus would reduce this variability and dramatically increase the power of the analysis of future studies. Over the last decade new imaging modalities have become available for use with small animal models have started to limit some of these problems. Ultrasound in the form of 'Photoacoustics' allows non-invasive real time imaging and is ideal for measuring thrombus volume, it would also allow the measurement of blood flow (vein recanalisation) through the thrombosed vessel during the course of resolution. This would enable the longitudinal collection of recanalisation data *in vivo* and reduce the number of mice required for analysis<sup>478;479</sup>. The drawback of this technology is the need for expensive micro-bubbles reagents to measure the discrete areas in and around the thrombus. Recent advances in micro-computerised tomography (MicroCT) using contrast reagents specifically designed for rodent use have shown that it is possible to measure structures down to 20µm in size, this would be invaluable in measuring venous recanalisation and blood flow through developing channels<sup>480;481</sup>.

In this work fluorescent labelling of BMMNCs was used in order to monitor their recruitment into the vena cava, this again required the use of *post mortem* histological analysis. The migration of progenitor cells once introduced into the animal can now be measured by nanotechnology<sup>482</sup>. Magnetic Resonance Imaging (MRI) can be used to track cells with magnetic nanoparticles such as superparamagnetic iron oxidase (SPIO) which are internalised by cells to produce a magnetic contrast<sup>483</sup>. Alternatively, positron emission tomography (PET) and single-photon emission tomography (SPECT) use radioisotopes with long half lives to track cells over hours and days<sup>484</sup>.

#### **iv) Post-viva amendment**

Following my viva the examiners felt that a further limitation of this study was lack of control for inter-observer error. This they felt was particularly relevant to analysis of histological sections following intervention. Therefore an acknowledgement of this has been made in the final appendix of this work.

### 7.1.7 SUMMARY

Thrombus resolution is a complex process that involves tissue remodelling and angiogenesis. The cytokine and bone marrow response to DVT may be critical in orchestrating these processes<sup>485</sup>. Venous thrombosis leads to the rapid mobilisation of cells expressing haematopoietic markers (Sca1, CD34 and VEGFR2), this was associated with an early elevation in circulating cytokine levels. The pattern of PIGF expression in the thrombus is different to that in organising wound tissue. Upregulating PIGF or the putative progenitor mobilisor SDF1 within the thrombus, leads to enhanced venous recanalisation.

A better understanding of the mechanisms that lead to the mobilisation of progenitor cells and their recruitment in response to thrombosis, as well as a more refined characterisation of these cells should lead to the development of novel strategies to promote thrombus resolution. This will be particularly important in patients in whom conventional treatments such as anticoagulation or thrombolysis are contraindicated.

## APPENDICES

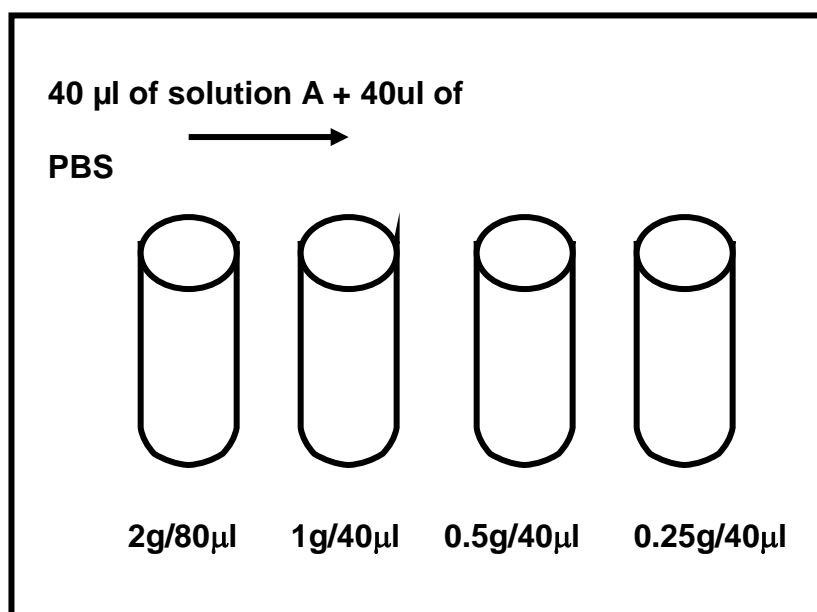
### (1) Bradford assay

The Bradford assay is a colorimetric protein assay, which is based on the absorbance shift in the dye Coomassie. The red form of the Coomassie reagent changes and stabilises into Coomassie blue by the binding of protein. The absorbance of the Coomassie blue complex is then read on an absorbance plate reader. The (bound) form of the dye has an optimal absorption at 595 nm. The increase in absorbance at 595 nm is proportional to the amount of blue dye, and thus to the concentration of protein present in the sample.

#### ***All steps were carried out at (18-20°C)***

- (1) A standard curve using known concentrations of albumin was created using a serial dilution method. 80µl of the top standard i.e. 2g of albumin was first aliquoted and then 40µl of the top standard was diluted with 40µl of PBS, this process was repeated down to a low standard of 0.31 g (**Fig A1**).
- (2) 10µl of solution of interest was placed in duplicate in a 96 well plate (NUNC, UK)
- (3) 300µl of Coomassie (Thermo Scientific) reagent was then added to each well and the solution agitated for 30secs.

- (4) The plate was read in a spectrophotometer (Spectra Max, UK) which calculates the protein contents of sample based on the signal generated and compared to directly the protein standards.

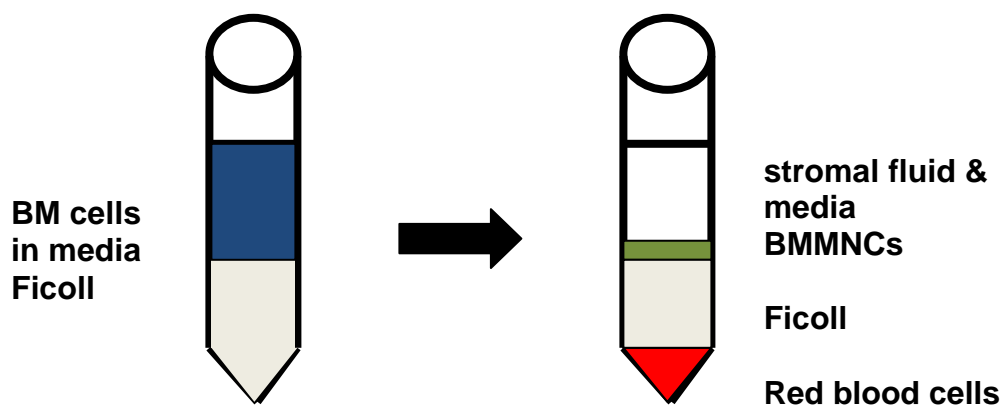


**Fig A1 Serial dilution method used in Bradford Assay**

## **(2) Ficoll density gradient**

The Ficoll-Paque<sup>TM</sup> PLUS (Ficoll, Miltenyi Biotec) was mixed thoroughly before use by inverting the bottle several times. The Ficoll was then placed in a 15ml conical tube (BD, UK). The bone marrow to Ficoll ratio was kept equal i.e. 6mls of Ficoll and 6mls of BM cells in (PBS + 10% FCS).

The BM cells were layered carefully onto the Ficoll to avoid mixing. The tubes were balanced and centrifuged (swing bucket rotator) at 400 x g for 30mins (no break, 18-22°C). This separated the BMMNCs into a single white layer which was aspirated (**Fig A2**). The BMMNCs were then washed to remove any residual Ficoll.



**Fig A2 Ficoll separation of BM cell into mononuclear fraction**

### **(3) Optimal Cytopathic Effect (CPE)**

The optimal CPE is reached when the largest number of cells is infected but not yet lysed, this occurs when 50% of cells have lifted off the bottom of the well, whilst 50% are rounded but still attached to the plate.



In order to achieve the optimal CPE the number of virus particles per cell should be between one or two times the number of cells in the flask. This is also known as multiplicity of infection or MOI.

If the viral titre is known, then the amount of virus added to a flask of HEK cells should equate to equal the number of cells in flask in order to achieve an MOI of equal to or double the number of cells for an MOI of 2. MOI can be calculated using the following formula:

$$\text{Vol. of virus required (mls)} = \frac{\text{number of cells} \times \text{MOI}}{\text{Viral titre}}$$

Viral titre

Therefore if the viral titre is  $10^9$  and a 175cm flask contains approximately  $1.46 \times 10^7$  cells. In order to achieve an MOI of 1 the amount of virus required can be calculated from the following formula:

$$\begin{aligned} \text{Vol. of virus} &= \frac{1.46 \times 10^7}{1 \times 10^9} \\ &= 0.01 \text{ ml} / 10\mu\text{l} \end{aligned}$$

If the viral titre is not known then the amount of virus added should be calculated by performing a test titre experiment:

1.  $3.5 \times 10^5$  HEK cells aliquoted into each of the wells of a twelve well plate
2. various concentrations of virus were then added to each well and the cells plate was incubated
3. the optimum CPE should occur in 2-4 days

Based on this the amount of virus required for an effective CPE in a 175cm flask is roughly equivalent to forty times what is required for a single well of a twelve well plate.

#### **4) Inter-observer error**

*Following my PhD viva, the examiners concluded that an amendment should be put into the Appendix regarding the omission of data pertaining to interobserver error. This was in specific reference to the calculation of venous recanalisation in the two interventional studies within the thesis; the role of peripheral mononuclear cell upregulation in thrombus resolution (Chapter 5) and direct adenoviral upregulation in thrombus resolution (Chapter 6).*

In this thesis the impact of interventional studies on thrombus resolution was measured using Image Analysis software®. This software allows the user to

highlight areas of thrombus recanalisation which are then subtracted from areas containing thrombus. By repeating this at 8-10 levels along each thrombus a mean percentage recanalisation and standard error of mean was calculated. The observer in this case (myself) was blinded to which slides were from mice that underwent treatment or control intervention. Blinding in this fashion aimed to remove observer bias in favour of the intervention group, this however does not compensate for operators subjective margin of error in measuring the regions of recanalisation using the image analysis drawing tool.

### **Inter observer error**

Interobserver error is of significant importance when dealing with subjective measurement data. An obvious example of this would be the measurement of abdominal organs using ultrasound<sup>486</sup>. The ability of individual observers to independently come to the same conclusion after interpretation of a result is described as *precision* (interobserver agreement) which is not to be confused with accuracy. Accuracy in contrast refers to the ability of an investigation or an observer to judge something to be a certain way and that that judgment is correct<sup>487</sup>.

There are several approaches to measuring interobserver error; one of the simplest indexes of error is the 'Agreement coefficient' (percentage). This is essentially the number of times the two observers agree on the unit data. This method has two flaws; firstly it does not take into account that two observers could

find agreement purely by chance and secondly the binary nature of this method doesn't explore varying degrees of agreement. For instance when using ultrasound to measure the length of a kidney, one observer may find it to be 11cm, whilst another observer may measure it to be 10.9cm, strictly this would be counted as different but the clinical significance of this difference would likely be minimal.

A better method is one that accounts for the fact that two observers would agree by chance and then measure the relative agreement. This measure of agreement is known as **Kappa**<sup>487</sup>. It is given as value between 0 and 1, 0 signifying no agreement and 1 indicating complete agreement. Although there is no exact cut off for what is deemed an adequate value for Kappa, it is suggested that above 0.61 is good and above 0.81 is very good<sup>488</sup>. This value has to be interpreted in light of the research area being analysed.

**Kappa= (observed agreement-chance agreement)/ (1-chance agreement)**

A variation on the Kappa statistic is the 'weighted kappa statistic' which also engages with the degree of agreement and disagreement. This would be ideal for this study where the exact agreement is not sought but degrees of agreement are more valuable.

### **Graphical representation of interobserver error**

When graphically displaying interobserver error the Pearson correlation coefficient is often used in an incorrect manner. This analysis is useful when showing

dependent linear relationships, when assessing interobserver error however, it can be misleading. An example of this would be two observers measuring the size of the abdominal aorta using US, It would be likely that both observers would report greater measurements as the size of the aorta increased (i.e. correlation) but the actual agreement between the two observers may still be significantly different.

Another more recently accepted approach to continuous variables is the Bland-Altman plot<sup>489</sup>. This was initially used to compare two methods that measured the same parameter e.g. computerised tomography and magnetic resonance imaging to measure aortic diameter. It can however also be used to assess interobserver error. In order to construct the plot, the mean of the two items measured is plotted on the x-axis and the difference between the two values on the y-axis. The main aim of this graphical representation is to detect bias. This bias might be derived from a new technique when compared to the gold standard or bias generated from flawed observer technique. In this method if the mean difference moves away from zero then the two techniques or observers are not equivalent. The best use of the Bland Altman plot appears to be as an adjunct to other more standard graphical representations of linear modelling<sup>490</sup>. It provides additional data allowing the reader to judge if either one method can be substituted with another or if data collected by one observer agrees with that collected by another. It also allows the user to set confidence limits appropriate to the data being analysed.

In this thesis the interventional studies which produced a small but significant difference would have been better validated by accounting for Interobserver error. There are several techniques as described that might have been utilised here, however as the data was only examined by a single observer, this has been accepted as a weakness in the validity of the interventional studies.

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