Platelet-monocyte Interaction: Relevance to Atherosclerosis

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Platelet-monocyte interaction: relevance to atherosclerosis

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ABSTRACT

Despite recent advances in diagnosis and therapy, atherosclerosis-related cardiovascular disease remains a leading cause of morbidity and mortality worldwide. An important challenge is to detect silent atherosclerosis in asymptomatic people at risk, so that preventative strategies can be more effectively targeted. This thesis examines the pro-inflammatory/pro-atherogenic role of platelet hyperactivity and the consequent interaction of activated platelets with circulating monocytes to form monocyte-platelet aggregates (MPA), and investigates the potential usefulness of MPA measurement and/or monocyte phenotype characterization as surrogate markers of atherosclerotic disease.

Using an experimental model of inflammation, namely that induced by influenza vaccine administration to healthy subjects, we found that MPA formation increases in the blood under pro-inflammatory conditions, and that this is accompanied by expansion of so-called $CD14^{high}CD16^{+}$ monocytes, which are distinct from "classical" $CD14^{+}CD16^{-}$ cells and exhibit higher pro-inflammatory activity. *In vitro* experiments showed that MPA formation increases CD16 expression on $CD14^{+}CD16^{-}$ monocytes.

In an animal model of atherosclerosis ($ApoE^{-/-}$ mice), expansion of the murine counterpart of human $CD16^{+}$ monocytes, namely $Ly6C^{low}$ cells, occurred with age and their levels strongly related to the extent of atherosclerotic disease seen in the brachiocephalic artery.

Finally, in a clinical study conducted in clinically healthy patients who had one or more underlying cardiovascular risk factors, levels of circulating $CD14^{high}CD16^{+}$

monocytes, but not of MPA, were found to increase in those patients who had carotid plaques compared to disease-free patients, and also showed a direct correlation with intima-media thickness (IMT) as evaluated by carotid ultrasonography. Unlike monocyte characterization, traditional cardiovascular risk stratification using the Framingham risk equation or the Joint British Societies 2 charts showed no relationship to IMT or to the presence of frank plaque disease.

In conclusion, our findings delineate a novel pro-atherogenic effect of platelet activation that is easily detectable in the peripheral blood through characterization of circulating monocytes. Measurement of $CD14^{high}CD16^{+}$ monocytes, but not MPA, offers a novel diagnostic approach to identify early atherosclerosis that gives information over and above cardiovascular risk estimation using traditional population-based assessment tools.

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Abbreviations

5-HT	5-hydroxytryptamine
AC	Adenyl cyclase
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APC	Allophycocyanin
ApoE	Apolipoprotein E
BMI	Body mass index
Bp	Base pair
BSA	Bovine serum albumin
°C	Degree centigrade
CAD	Coronary artery disease
cDNA	Complementary deoxyribonucleic acid
cAMP	Cyclic adenosine monophosphate
CCA	Common carotid artery
CD	Cluster differentiation antigen
CD40L	CD40 ligand
COX	Cycloxigenase
CRP	C reactive protein
CV	Cardiovascular
DBP	Diastolic blood pressure
DMSO	Dimethyl sulphosphide
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetra-acetic acid
eGFR	Estimated glomerular filtration rate
EP	Prostaglandin receptor
FACS	Flow activated cell sorting
FITC	Fluorescein isiothiocyanate
FSC	Forward side scatter

GMP	Granule membrane protein 140
GP	Glycoprotein
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
HbA_{1c}	Glycated haemoglobin
HDL	High-density lipoproteins
Hs-CRP	High-sensitivity C reactive protein
HUVEC	Human umbilical cord vein endothelial cells
ICAM	Intracelluar adhesion molecule
IgG	Immunoglobulin G
IL	Interleukin
IMT	Intima-media thickness
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemoattractant protein
M-CSF	Macrophage-colony stimulating factor
MFI	Mean fluorescence intensity
$MgCl_2$	Magnesium Choloride
MIP	Macrophage inflammatory protein
MPA	Monocyte-platelet aggregates
mRNA	Messenger ribonucleic acid
NIDDM	Type 2 diabetes mellitus
NK	Natural killer
PADGEM	Platelet activation dependent granule-external membrane protein
PBS	Phosphate buffer saline
PCM	Platelet conditioned medium
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	Phycoerythrin

PerCP	Peridinin chlorophyll protein complex
PF4	Platelet factor 4
PGE_2	Prostaglandin E_2
PLC	Phospholipase C
PMN	Polimorphonuclear cells
PKA	Protein kinase A
PKC	Protein kinase C
PSGL-1	P-selectin glycoprotein ligand 1
SBP	Systolic blood pressure
SNARE	N-ethylmaleimide-sensitive factor attachment protein receptor
SSC	Side scatter
TF	Tissue factor
TIA	Transient ischemic attack
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TP	Thromboxane receptor
TxA_2	Thromboxane A_2
TxB_2	Thromboxane B_2
VCAM	Vascular adhesion molecule
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor
WHO	World Health Organization

Chapter One

Introduction

1.1 Platelets

1.1.1 Platelet structure and function

"... In examining the content of such vessels (regardless of whether they are veins or capillaries) with an immersion objective, one will achieve the astonishing result that a third morphological element circulates along with red and white corpuscles. This is constituted of disc-shaped pale platelets with parallel surfaces, that rarely appear like lens-shaped, oval or round structures; their diameter is a third or an half of red cells....They are generally isolated from each other, even if they can rarely adhere one to another to form clusters of variable dimensions. However, this is already a sign of their alteration..." (citation from Bizzozero 1883, p 661) [translation is mine].

It was as far back as December 1881 when the Italian pathologist Giulio Cesare Bizzozero (1816-1901) made his scientific report to the Royal Sciences Academy of Turin (Italy), providing the first evidence that the "third morphological element" of the blood, that many other authors (Hewson in 1780; Donne in 1842; Beale in 1850; Zimmermann in 1860 and Schultze in 1865) had described since the late 18th century as a blood component of dubious biological function, had an important role in thrombosis and coagulation (Gazzaniga & Ottini, 2001). Indeed, his great achievement was the demonstration that platelets have characteristics and functions different from those of erythrocytes and white cells, and that they "*...constitute the major part of the thrombus and give rise to the accumulation of the abundant granular substance that is among leucocytes, and that was believed to derive from disrupted white cells...*"(citation from Bizzozero 1883, p 701) . On the basis of this observation, that was enabled by the development of intravital microscopy applied to

mesenteric vessels of guinea pigs and rabbits, Bizzozero called these particles "piastrine" (Italian from the Latin *emplastra*, meaning embedding substance), that the English later termed "platelets" (little plates).

More than a century of research in the field coupled with progress in laboratory techniques, have provided remarkable insight into the structural and functional complexity of platelets. These anucleate particles that circulate in the blood normally at 150-400 x 10^9 per litre, possess a composite network of membrane structures, including the plasmalemma, intracellular granules and canalicular systems, and originate from the segmentation of megakaryocytes. The different cellular compartments are regulated by an intricate cell signalling machinery that confers to platelets cellular plasticity, in terms of shape modifications in response to platelet agonists, and secretory ability in many different pathophysiological settings. For instance, specific receptors expressed on the platelet plasmalemma initiate an outside-in signalling pathway that leads to activation of protein phosphorylation cascades ultimately resulting in actin polymerization (Reed *et al*., 2000). This event, that culminates in a structural re-organization of the cytoskeleton, has been considered for many decades as the main determinant in organelle trafficking (the so-called "platelet release reaction"), due to the generation of a contractile force with consequent expulsion of intracellular granules (White *et al*.,1978; Painter *et al*.,1984; Loftus *et al*.,1989). More recent studies have revealed a process of fusion between the granule membrane and the plasmalemma, regulated by N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family proteins, chaperon molecules that direct the function of the SNARE complex (such as GTPase Rab family) and the composition of the lipid membrane (reviewed by Reed *et al*., 2000 and Flaumenhaft, 2003).

The activity of this regulatory apparatus is disconnected from the biomolecular mechanisms that direct platelet shape changes, and seems to be modulated by agonist-specific effector molecules to ensure selectivity of granule content release as appropriate. Indeed, immunocytochemical and electron microscopic studies have identified three main types of storage organelles in platelets, known as dense, alpha and lysosomal granules. Their content can be classified into three main groups on the basis of their principal function (Ren *et al.*, 2008):

- platelet agonists (adenosine diphosphate (ADP), serotonin, epinephrine, 5-hydroxytryptamine (5-HT) and calcium), contained in the dense-granules;
- adhesion molecules (P-selectin, fibrinogen, von Willebrand factor (vWF), platelet factor 4 (PF4)), stored in alpha-granules;
- lysosomal enzymes (cathepsin, hexosaminidase), within the lysosomes.

However, these molecules only represent a minority of the entire array of functional mediators stored in circulating platelets, that comprise growth factors (such as vascular-endothelial growth factor, VEGF; platelet-derived growth factor, PDGF), pro-inflammatory molecules (including inteleukin-1β and CD40L) and a variety of chemokines (reviewed by Klinger & Jelkmann, 2002).

The scenario is complicated by evidence that, despite lack of genomic DNA, platelets contain residual mRNA from megakaryocytes along with a complete translational apparatus, thus enabling the production of newly synthesised proteins (Harrison & Goodall, 2008).

To date, the study of platelet structure and the understanding of their function are far from complete. However, the evidence to date indicates multiple roles for platelets,

extending from their now well-established role in haemostasis and thrombosis to a more general pro-inflammatory action, as will be discussed later.

1.1.2 Platelet activation

The classical model of platelet activation describes a series of phenotypic and morphological changes in resting platelets such that they acquire adhesiveness to the vascular wall following exposure to pro-thrombotic molecules (e.g. collagen, vWF) (Weiss *et al.*,1978; Tschopp *et al.*,1973; Savage *et al.*,1996), forming platelet-to-platelet aggregates (homotypic aggregation) through interlinking by soluble adhesive proteins (especially fibrinogen) (Isenberg *et al.*, 1987) and releasing a number of mediators that stabilize the initial aggregate and amplify thrombus formation (Siess,1989). The widespread view that vessel injury is the sole determinant of platelet activation has been largely abandoned in light of the evidence that a variety of pathophysiological stimuli, including pro-inflammatory cytokines and infective agents (recently reviewed by Semple & Freedman, 2010) as well as shear stress (Kroll *et al.*,1996), can activate platelets with no detectable vessel damage. Evidence has also accumulated that, in these and other diverse pathophysiological settings, platelets exhibit differential biological responses due to activation of distinct biomolecular pathways. Importantly, pro-inflammatory activity mediated by interaction with circulating leucocytes (heterotypic aggregation) and release of inflammatory mediators constitutes an important aspect of platelet biology and provides strong pathophysiological links between inflammation and thrombosis (Semple & Freedman,2010; Kroll *et al.*,1996; Freedman & Loscalzo,2002). As a consequence, platelet activation is not limited to the occurrence of a local thrombotic

event triggered by vascular damage and mainly sustained by homotypic aggregation. On the contrary, it is often part of a systemic inflammatory response that can develop independently of, or in addition to, local injury-related factors. Indeed, formation of heterotypic aggregates in the peripheral circulation has been observed not only during acute thrombotic events (Furman *et al.*,1998; Michelson *et al.*,2001; Furman *et al.*,2001; Sarma *et al.*,2002; Marquardt *et al.*,2009), but also in those clinical conditions associated with high blood thrombogenicity in the absence of intravascular thrombosis, such as auto-immune disorders (Joseph *et al.*,2001), haematological disease (Jensen *et al.*,2001; Wun *et al.*,2002), and in subjects with cardiovascular risk factors (Harding *et al.*,2004a; Harding *et al.*,2004b; Gkaliagkousi *et al.*,2009). Given this, as well as the ongoing discovery of ever more biomolecular mechanisms underlying the platelet response to different agonists, it is likely that the signalling pathways that regulate thrombus formation and platelet-leucocyte interaction may operate either together or independently under different circumstances. In keeping with this, some anti-platelet drugs that effectively reduce platelet-platelet aggregation, such as aspirin, may have very little or no effect on heterotypic aggregation (Klinkhardt *et al.*,2003).

1.2 Homotypic aggregation

Homotypic aggregation occurs at the site of a vascular lesion such as a ruptured atherosclerotic plaque or a traumatic injury, where exposed pro-thrombotic molecules provide an adhesive surface for recruitment of circulating platelets, followed by their activation and thrombotic plug formation (Tschopp *et al.*,1973; Weiss *et al.*,1978; Isenberg *et al.*,1987; Siess,1989; Savage *et al.*,1998). *In vivo* and *in vitro* observations suggest that this phenomenon is a complex and dynamic multi-step process (Furie & Furie,2005; Kulkarni *et al.*,2000; Maxwell *et al.,* 2007). The initial phase of adhesion of platelets to the vascular wall as well as to each other (primary reversible aggregation) is followed by a second phase of stabilization and growth of the initial platelet plug (secondary irreversible aggregation). Platelet activation has long been assumed to have a dual role in this process, as an initiating factor in platelet arrest and as an essential mediator of the transition from reversible to irreversible aggregation (Savage *et al.*, 1996; Savage *et al.*,1998; Packam & Mustard, 1984). Technical advances in intravital microscopy and real-time perfusion studies have demonstrated that primary aggregation can also occur without the need for platelet activation under conditions of elevated shear stress (Maxwell *et al.,* 2007; Ruggeri *et al.,* 2006). However, when non-activated platelets adhere to the vessel wall they only form transient micro-aggregates that, in the absence of activation-dependent release and generation of soluble agonists (principally adenosine diphosphate, ADP; thrombin and thromboxane A_2, TxA_2), disaggregate with translocation of platelets in the direction of flow (Maxwell *et al.,* 2007). Central to homotypic aggregation is therefore the concept that platelets become activated in response to interaction with thrombogenic surfaces, and multiple ligand-receptor interactions are required to stabilize and amplify their adhesion and aggregation.

1.2.1.Biomolecular mechanisms of platelet activation leading to homotypic aggregation

Fibrinogen, vWF and collagen are able to initiate primary aggregation through the engagement of specific platelet integrins, namely glycoprotein (GP)IIb/IIIa (also designated $\alpha_{2b}ß_3$ integrin), GPIb and GPVI respectively (Savage *et al.*,1996; Nachman & Leung, 1982; Emsley *et al.*,2000). At low shear rate (<1000 s^{-1}), the interaction between GPIIb/IIIa and fibrinogen has been demonstrated to constitute the predominant biomolecular event (Savage *et al.*,1996; Ruggeri *et al.*,1997; Savage *et al.*,1998). However, since GPIIb/IIIa is expressed in a low affinity state on the plasmalemma of quiescent platelets, initial stimulation of platelets by one or more soluble agonists in the vicinity of the lesion (e.g. ADP released from endothelial cells or thrombin locally produced) is required in order to activate downstream signalling pathways (inside-out signalling) that ultimately result in platelet shape change and activation of GPIIb/IIIa (Isenberg *et al.*,1987). When the shear rate rises within the range 1000 - 10000 s^{-1}, platelet activation is not required to induce primary aggregation, since the synergistic action of GPIIb/IIIa and GPIb suffice in promoting tethering and transient aggregation of discoid-shaped quiescent platelets to the vascular wall. Nevertheless, the ensuing activation of platelets induced by integrin engagement leads to release of soluble agonists, mainly ADP, which is essential in stabilizing the initial aggregate (Maxwell *et al.*,2007). At high shear rates (>10000 s^{-1}), Ruggeri et al have shown both *in vitro* and *in vivo* that thrombus can form efficiently through a mechanism independent of platelet activation, that is solely mediated by interaction between vWF and GPIb giving rise to stable local adhesion of platelets to a thrombogenic surface and homotypic aggregation (Ruggeri *et al.*,2006).

The *in vivo* role of these ligand/receptor interactions has been evaluated in animal models selectively lacking one or more of the molecules involved in these pathways. In studies using $vWF^{-/-}$ mice, platelet accumulation and thrombus growth were markedly delayed but not absent in a model of ferric chloride-induced thrombosis (Ni *et al.*,2000), and the thrombogenic activity of platelets in laser-induced vessel wall injury was in fact comparable to that observed in wild-type mice (Dubois *et al.*,2007), suggesting that platelet thrombus formation can occur in the absence of vWF. Fibrinogen/vWF double knockout mice exhibit preserved platelet-to-platelet interaction (Ni *et al.*,2000), via a mechanism primarily triggered by thrombin and sustained by soluble agonist release (ADP) and different integrin signalling cascades (Dubois *et al.*,2007; Yang *et al.*,2006). Activation of the coagulation cascade at the site of vessel injury, with consequent generation of thrombin through the tissue factor (TF) pathway, has been proposed as a major contributor to the thrombogenic component of atherothrombotic disease (Corti *et al.*,2002; Libby, 2002). However, recent evaluation of the dynamics of thrombus formation on atherosclerotic plaques has shown that TF has a predominant role only in the amplification phase of platelet aggregation, whilst the first key event of platelet arrest and aggregation is crucially regulated by engagement of the collagen receptor GPVI (Reininger *et al.*,2010). Indeed GPVI blockade, but not plaque TF suppression, significantly inhibits thrombus development. In similar experiments performed by Penz et al (Penz *et al.*,2007), GPIb was found to be a crucial effector in plaque-induced thrombosis, a finding in agreement with the work of Ruggeri et al showing the importance of the vWF/GPIb axis in thrombus formation under conditions of high shear, and further confirmed in studies of interleukin 4-receptor/GPIb transgenic mice (Bergmeier *et al.*,2006), in which lack of activity of GPIb gives rise to a severe bleeding

phenotype. However, compared to the relatively mild effect on thrombogenic response to vascular injury observed in vWF-knockout mice, these results are strongly suggestive of an additional thrombogenic mechanism sustained by GPIb that may interact with ligands other than vWF.

Taken together, these findings imply that, independent of the initial thrombogenic stimulus and blood flow conditions, platelet-to-platelet interaction only results in irreversible aggregation once stable adhesion to the vascular wall, mediated by multiple receptor-ligand binding events, is established. Figure 1.1 summarises the main molecular interactions involved in homotypic aggregation.

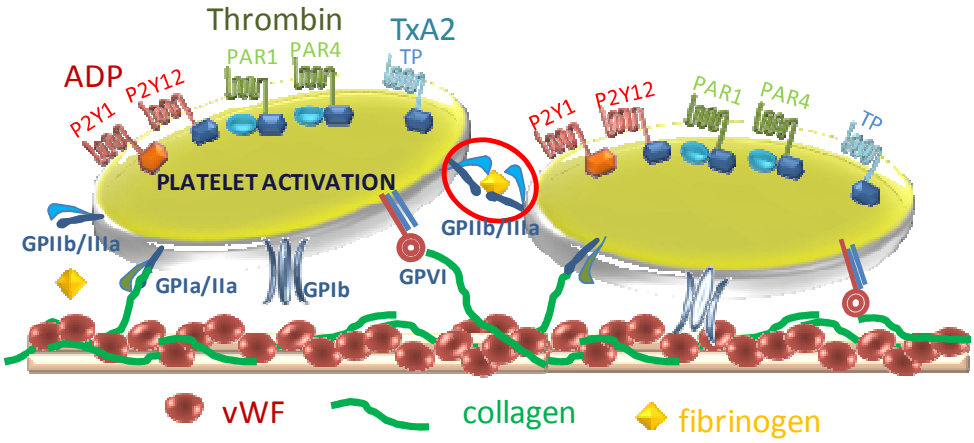

Figure 1.1 Ligand-receptor interactions sustaining platelet homotypic aggregation. Fibrinogen, vWF and collagen anchor platelets to the vascular wall by engaging specific platelet receptors (GPIIb/IIIa, GPIb and GPVI respectively). The ensuing activation of platelets induced by integrin engagement, along with the action of soluble agonists (i.e. ADP and thrombin), leads to platelet release of soluble thrombogenic molecules (ADP and TxA_2) that stabilise the initial aggregate, by inducing the recruitment of other circulating platelets and the conformational change of GPIIb/IIIa from a low- to a high-affinity state for fibrinogen that acts as a bridge between platelets (circled). $P2Y_1$ and $P2Y_{12}$: ADP- receptors; PAR-1 and PAR-4 : thrombin-receptors; TP: TxA_2 receptor.

1.3 Heterotypic aggregation

By contrast with homotypic aggregation, which is confined to the vascular wall, heterotypic complex formation occurs in circulating blood. The association of platelets with leucocytes was first reported in the late 1970s and described as "platelet satellitism". It referred to the *in vitro* phenomenon of platelets rosetting around leucocytes observed in ethylenediaminetetra-acetic acid (EDTA)-anticoagulated blood of patients with a wide variety of clinical conditions, particularly immunological disorders (Kjeldsberg & Swanson, 1974; Mant *et al.*,1975; White *et al.*,1978; Djaldetti *et al.*,1978; Peters *et al.*,1998). From these clinical observations, scientific interest rapidly moved to the study of platelet-leucocyte interaction, in order to characterize the underlying mechanisms and elucidate the potential pathophysiological implications. Due to the inflammatory nature of atherosclerosis, heterotypic aggregation in this condition has been a major area of research. In subjects with coronary atherosclerosis, leucocyte-platelet aggregation was initially identified within the coronary circulation, in close proximity to atherosclerotic lesions (Mickelson *et al.*,1996). The same study reported a higher level of heterotypic complexes in the vicinity of plaques with thrombotic complications as compared to uncomplicated ones. In further clinical studies, heterotypic complexes were observed in the systemic circulation of patients with coronary atherosclerosis (Ott *et al.*,1996; Furman *et al.*,1998; Michelson *et al.*,2001; Furman *et al.*,2001, Sarma *et al.*,2002), their levels being increased during acute thrombotic events. These findings suggest that circulating heterotypic aggregates form in parallel with thrombi, as a consequence of local platelet activation sustained by vascular damage. However, the presence of atherosclerotic lesions may not be mandatory for heterotypic aggregation, as raised levels of such aggregates have been

described also in clinically healthy subjects with cardiovascular risk factors (Harding *et al.*,2004a, Harding *et al.*,2004b, Gkaliagkousi *et al.*,2009) – although such subjects may well have subclinical atherosclerosis – as well as in patients with other inflammatory conditions (reviewed by von Hundelshausen & Weber, 2007). Adhesion between platelets and leucocytes is stable, does not require adhesion to pro-thrombotic surfaces, and is a systemic phenomenon whose extent depends on the degree of platelet activation (Table 1.1). Early *in vitro* experiments performed on whole blood showed that platelet activation by a variety of agonists results initially in homotypic aggregation mediated by fibrinogen-GPIIb/IIIa interaction, which is followed – in the absence of an adhesive vascular surface – by platelet disaggregation and subsequent platelet adhesion to leucocytes, with monocytes having a competitive advantage over other white cells in binding activated platelets (Rinder *et al.*,1991a). Inhibition of fibrinogen binding to GPIIb/IIIa is even able to enhance the interaction of activated platelets with leucocytes, and the heterotypic complexes so formed, that are mainly composed of monocyte-platelet aggregates (MPA), display a strong pro-inflammatory action mediated by newly expressed molecules such as CD40L and P-selectin on their surface, along with pro-thrombotic activity sustained by the production of TF (Zhao *et al.*,2003).

Table 1.1 Comparison between homotypic and heterotypic aggregation [From Passacquale & Ferro, 2011a].

	Homotypic aggregation	Heterotypic aggregation
Localization	Confined to the vascular wall	Detectable in peripheral blood
Platelet activation required	Not under high shear rate	Yes
Mechanism of stabilization	Multiple ligand-receptor interactions with thrombogenic surface (demonstrated)	Multiple ligand-receptor interactions with leucocyte membrane (hypothesized)
Main effector molecule(s)	Varies depending on hemodynamic status and lesion components	P-selectin, P-selectin glycoprotein ligand-1
Platelet release reaction	Required for irreversible stabilization, mainly mediated by dense granule contents	Required for initiation, mediated by alpha granule contents

1.3.1 Biomolecular mechanisms of platelet activation leading to heterotypic aggregation

The pioneering work of Jungi et al (Jungi *et al.*,1986) demonstrated that platelets acquire adhesiveness to leucocytes when stimulated with thrombin in a concentration-dependent manner, whilst non-activated platelets show little tendency to associate with white cells, any such association being explained by low-grade spontaneous activation of platelets undergoing handling *in vitro*. A number of other researchers confirmed these results (Hamburger *et al.*,1990; Rinder *et al.*,1991a; Rinder *et al.*,1991b), and it is now well established that platelet activation,

independent of the initial stimulus, is required to trigger platelet-leucocyte interaction. As part of the underlying molecular mechanism, expression of the adhesion molecule P-selectin on the plasmalemma of activated platelets appears to be crucially involved (Larsent *et al.*,1989; Hamburger *et al.*,1990). P-selectin, also designated CD62P and previously referred to as GMP-14O or PADGEM protein, is a transmembrane molecule stored in the alpha-granules of platelets. Upon stimulation, the granules fuse with the plasma membrane and P-selectin thus translocates from the cytosolic compartment to the extracellular surface, where it acts as a receptor for circulating white cells (Larsent *et al.*,1989). Alpha-granule release reaction with consequent P-selectin expression on the platelet surface is induced by a number of different stimuli, including thrombogenic molecules (collagen, vWF, fibrinogen) and strong platelet agonists (thrombin, thromboxane). However, it can also follow stimulation with weak platelet agonists or inflammatory molecules, such as epinephrine, prostaglandin E_2 (PGE_2) and chemokines, that in the absence of other agonists do not sustain platelet homotypic aggregation (Offermanns, 2006), but rather sensitise platelets to the actions of other agonists (Hjemdahl *et al.*, 1994). Hence, exposure of P-selectin on platelets can occur during effective thrombus formation, but also when sub-clinical platelet activation (i.e in the absence of a thrombotic event) is sustained by different pathogenetic stimuli. From reviewing all the existing literature, it appears likely that this phenomenon can be largely attributed to the selective and differential modulation of the platelet release reaction by different agonists (reviewed by Passacquale & Ferro, 2011a). Indeed, strong platelet agonists are able to sustain thrombus formation and concomitant platelet heterotypic aggregation by inducing full platelet degranulation, with consequent release of platelet-derived pro-thrombotic agents stored in dense-granules and

adhesive/pro-inflammatory molecules contained in the alpha-granules (Figure 1.2). For instance, thrombin and thromboxane activate G-protein coupled receptors (GPCR) that feed-back on the G_q- and $G_{12/13}$-signalling cascade whose main effector is phospholipase C (PLC) which then activates protein kinase C (PKC). On the other hand, weak platelet agonists, including ADP and epinephrine, are characterized by a common ability to bind GPCR that activate G_i-dependent transduction pathways, thus resulting in reduction of the activity of protein kinase A (PKA) via suppression of intracellular cyclic AMP (cAMP).

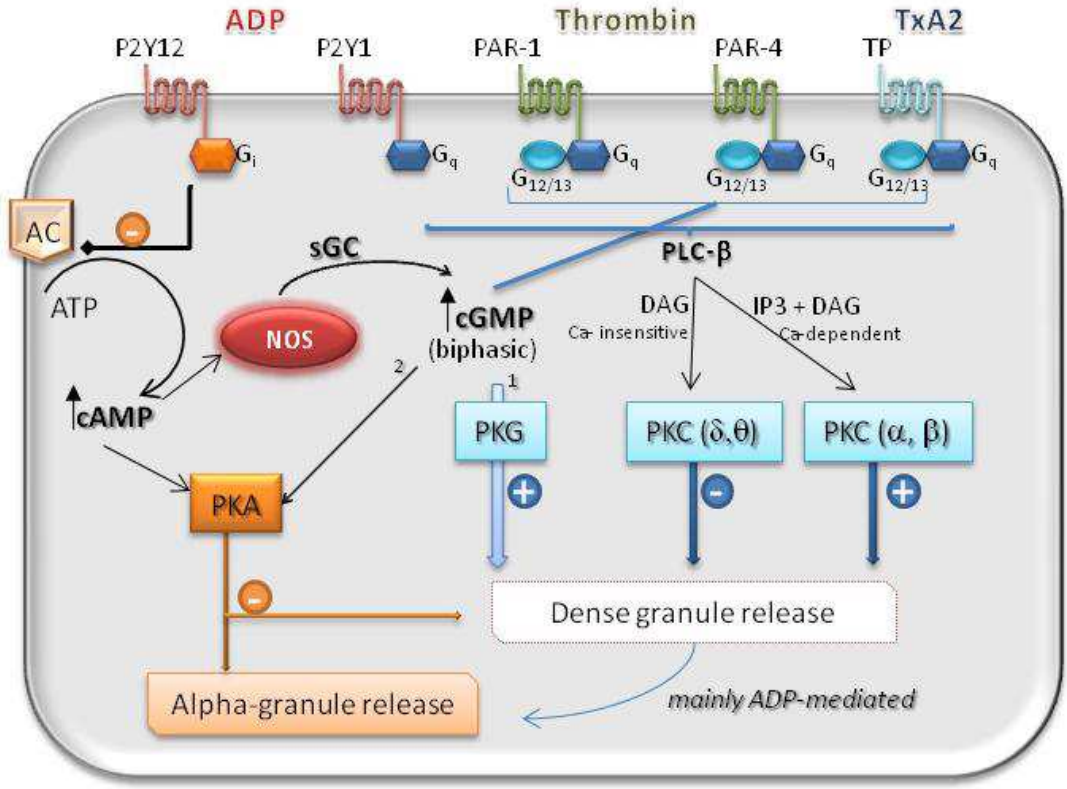

Figure 1. 2. Model of agonist-selective modulation of alpha and dense granule release in human platelets. Different platelet agonists (ADP, thrombin and TxA_2) activate distinct transduction pathways. Thrombin- and TxA_2-dependent G_q- and $G_{12/13}$-signalling cascades result in phospholipase C (PLC) activation, mainly the PLC-β isoform, that via hydrolysis of membrane phospholipids and generation of two second messengers, IP3 and DAG, leads to the activation of protein kinase C (PKC) enzymes. PKC comprises different isoforms: the classical subgroup (α, β) whose activation is calcium-dependent and mediated by the

synergistic action of IP3 ad DAG; and the calcium-insensitive isoforms (δ,θ) regulated by DAG. In human platelets activation of conventional Ca-dependent PKC isoforms (α, β) stimulates effects on dense granule release and platelet aggregation, while the Ca-insensitive isoforms of PKC (δ,θ) inhibit these (Gilio *et al.*, 2010). ADP, either produced at the site of a vascular lesion by endothelial cells or released by platelets as a consequence of dense-granule exocytosis, interacts with $P2Y_1$ receptors that, via Gq-proteins, trigger the same molecular pathways regulated by thrombin and TxA_2. In this way, ADP regulates dense granule exocytosis. Moreover, ADP interaction with $P2Y_{12}$ receptors induces a G_i-dependent transduction pathway that inhibits PKA via negative modulation of adenylyl cyclase (AC) and intracellular cAMP. The inhibition of AC, and the consequent reduction in cAMP levels, removes the self-limiting mechanism of platelet activation sustained by PKA activity, thus producing an amplification of the activator pathways through release of alpha-granule content. Reduction in cAMP levels also decreases the enzymatic activity of platelet nitric-oxide synthase (NOS). NOS inhibition down-regulates PKG and, in turn, PKA activity. [From Passacquale & Ferro, 2011a].

Hence, distinct phosphorylation cascades that act on different cellular targets appear to selectively regulate dense- and alpha-granule release reaction. The intracellular content of cAMP appears to be the main regulator of P-selectin expression and consequent heterotypic aggregation. Table 1.2 summarises the effects of several platelet agonists/inhibitors which modulate cAMP on homotypic and heterotypic aggregation.

Table 1.2. Effect of platelet agonists/inhibitors on intraplatelet cAMP and consequent effects on homotypic vs heterotypic aggregation [From Passacquale & Ferro, 2011a].

	cAMP modulation	Effect on homotypic aggregation	Effect on heterotypic aggregation
ADP	↓	Stimulatory, requires activation of both P2Y1 and P2Y12	Stimulatory, mainly sustained by P2Y12
Adenosine	↑	Inhibitory	Inhibitory
Epinephrine	↓	Very little	Stimulatory
Prostaglandin E2	*via* EP3 receptor ↓	None alone	Stimulatory
Prostaglandin E2	*via* EP4 receptor ↑	Inhibitory	Inhibitory
Prostaglandin E1	*via* EP3 receptor ↓	None alone	Stimulatory
Prostaglandin E1	*via* IP receptor ↑	Inhibitory	Inhibitory
Chemokines	↓	None alone	Likely stimulatory
β-adrenoceptors	↑	Inhibitory	Inhibitory
Cilostazol	↑	Inhibitory	Inhibitory
Selective serotonin re-uptake inhibitors	↑	Inhibitory	Inhibitory
Magnolol, Homokiol, Curcumin	↑	Inhibitory	Inhibitory

The ligand for P-selectin, namely P-selectin glycoprotein ligand (PSGL)-1, is constitutively expressed on the plasmalemma of leucocytes (Yang *et al.*,1999). Within the leucocyte population, the expression of PSGL-1 on the cellular surface of monocytes is highest (Kappelmayer *et al.*, 2001), and this could in part explain their preferential affinity to platelets over other white cells.

The preferential binding of platelets to monocytes may also be ascribed to an additional role played by other ligands specifically present on the plasmalemma of monocytes, which may play a role following on from P-selectin/PSGL-1 interaction. Indeed, PSGL-1 is not only an adhesion but also a signalling molecule, as further described in the following paragraphs.

1.3.2 Kinetics of heterotypic complex formation

The kinetics of interaction between platelets and leucocytes have been extensively explored through both *in vitro* experiments and *in vivo* investigations (Table 1.3), and demonstrate that the adhesion of platelets to circulating leucocytes is a rapid and transient event following platelet activation. In 1991 Rinder et al (Rinder *et al.*,1991b) developed a flow cytometric assay allowing the quantification of platelet-leucocyte adhesion in terms of percentage of white cells binding platelets and number of platelets per leucocyte. They co-incubated the different subpopulations of white cells purified from peripheral blood with autologous platelets previously stimulated with different agonists. A time course was performed to follow heterotypic aggregation within each white cell subtype. They showed that platelet-leucocyte complexes form within a few minutes of platelet activation (2-10 min, dependent on the white cell subtype), and that the number of platelets per leucocyte increases in a time-dependent manner.

These results appear to reproduce the *in vivo* dynamics of heterotypic aggregation. Indeed, a recent study performed in apoE$^{-/-}$ mice (Huo *et al.*,2003) showed that intravenous injection of activated platelets led to sequestration of circulating white cells within a few minutes of infusion (5 min) and persisting for up to 80 min for

neutrophils (PMN) and 180 min for monocytes. No such effect was reported for lymphocytes, confirming once more the differential binding ability of activated platelets to different leucocyte sub-populations. The same experiment repeated in baboons showed similar findings (Michelson *et al.*,2001). After injection of activated platelets, formation of circulating aggregates with monocytes and PMN occurred within 1 min. The *in vivo* half-lives of circulating monocyte-platelet and PMN-platelet complexes were 30 min and 5 min respectively. No heterotypic aggregates were detectable after 2 hours.

Table 1.3. Kinetics of heterotypic aggregation. Data are from both *in vitro* experiments and *in vivo* investigations in different animal models. Level of heterotypic aggregates obtained in vitro refers to the percentage of each type of cell binding platelets after stimulation with ADP or thrombin, while in animal models it was evaluated in the peripheral blood after intravenous injection of activated platelets.

In vitro exp (Humans)	*In vivo* exp	Cell type	Start of effect (min)	End of effect (min)	Heterotypic aggregates (%)
Isolated cell fractions (Rinder *et al.*, 1991b)		Monocytes Neutrophils Lymphocytes	1 1 -	> 60 > 60	87 ± 9 34 ± 6
Whole blood (Rinder *et al.*, 1991a)		Monocytes Neutrophils Lymphocytes	1-3 6-10 No effect	> 30 > 30	45.25 ± 3.20 38.75 ± 2.19 36.50 ± 2.20
	Mice (Huo *et al.*, 2003)	Monocytes Neutrophils Lymphocytes	5 5 No effect	120 80	
	Baboons (Michelson *et al.*, 2001)	Monocytes Neutrophils Lymphocytes	1 1 No effect	120 120	~ 70 ~ 30

However, the *in vivo* circulation time and the clearance of heterotypic complexes in humans remain to be clarified. In subjects who have suffered cerebrovascular events,

including stroke and transient ischemic attack (TIA), the circulating level of leucocyte-platelet aggregates increases dramatically during the acute phase, and returns to values comparable to those observed in healthy controls only after 3 months (Htun *et al.*,2006). High concentrations of heterotypic aggregates have also been reported during the first 4 hours following acute myocardial infarction (Furman *et al.*,2001). The number of leucocyte-platelet complexes is increased with respect to the general population also in subjects with hypertension (Gkaliagkousi *et al.*,2009), diabetes (Harding *et al.*,2004b), and in smokers (Harding *et al.*,2004a). This suggests that a certain degree of persistent and chronic heterotypic aggregation occurs in these clinical conditions, even in the absence of an acute thrombotic event.

1.4 Monocyte-platelet aggregates: linking thrombosis to inflammation

Monocyte-platelet aggregation is not a simple epiphenomenon that accompanies platelet activation but carries important pathophysiological implications. The interaction between activated platelets and monocytes, as occurs in the context of thrombus formation, contributes to thrombus stabilization by amplifying platelet activation and favouring homotypic aggregation at sites of vascular injury (Furie & Furie,2005). On the other hand, heterotypic aggregation following activation of platelets in response to infectious or inflammatory agents results in modulation of the immunological response. Of note, platelets are known to mediate phagocytic-like functions, internalizing bacteria and viruses (Zucker-Franklin *et al.*,1990; Youssefian *et al.*,2002), and to express Toll-like receptors (TLR) that are critical for regulating innate immune mechanisms (Medzitov & Janeway, 2000). In keeping with this, a

number of *in vitro* and *in vivo* pre-clinical studies, as well as clinical observations, have provided evidence of anti-inflammatory actions of anti-platelet drugs, such as aspirin and clopidogrel (reviewed by Muhlestein, 2010).

Apart from being a robust index of platelet activation, monocyte-platelet aggregation is therefore of functional importance with both pro-thrombotic and pro-inflammatory effects.

1.4.1 Monocyte-platelet aggregates: a reliable method for assessment of degree of *in vivo* platelet activation

A number of laboratory tests are available to study blood thrombogenicity. They include:

- morphological analysis of platelets (including volume and mass), as shape changes are suggestive of platelet activation;
- concentration of metabolites produced by activated platelets (urinary 11-dehydrothromboxane B2, serum TXB_2; plasma β-thromboglobulin; PF4);
- platelet aggregometry, which measures *in vitro* the clumping of platelets either spontaneously or in response to platelet agonists;
- flow cytometry analysis of molecules expressed on the platelet plasmalemma following activation, such as GPIIb/IIIa in its high-affinity form or granular products (P-selectin).

Amongst all these techniques, platelet aggregometry is regarded as the gold standard in the research setting. However, flow cytometry, through the use of monoclonal antibodies that specifically bind platelet receptors, offers the highest sensitivity and

indeed it has been applied in an aggregometry context to measure micro-aggregates in samples of platelet-rich plasma and whole blood (Abrams *et al.*,1990). Indeed, the development of whole blood flow cytometry in 1987 (Shattil *et al.*,1987) represented a major technical advance in the functional testing of platelets as it minimises sample manipulation and the unavoidable platelet activation that occurs *in vitro* during platelet-rich plasma preparation. The possibility to study platelets in their physiological environment (whole blood) and the ability to simultaneously analyse multiple activation-dependent surface markers, in only a small sample of blood (200 µl), has favoured widespread application of this technique in many clinical trials. However, its usefulness in clinical practice remains unclear, mainly due to the lack of an accepted gold standard assay available for widespread clinical use for comparison.

Flow cytometry also allows the quantification of circulating MPA, based on double immunostaining of the blood for the monocytic marker CD14 and the constitutive antigen CD42b expressed on platelets. Double-positive cells are considered to represent the population of circulating monocytes binding activated platelets. Compared to P-selectin expression on platelets, the measurement of MPA in the peripheral blood offers a number of advantages, and is now regarded as the most sensitive and robust index of platelet activation (Michelson *et al.*,2001). Indeed, following degranulation, activated platelets rapidly lose their surface P-selectin while continuing to circulate and function in the peripheral blood (Michelson *et al.*,1996). Therefore, the level of P-selectin expression on platelets may underestimate the true degree of platelet activation. Consistent with this, in two separate clinical studies in patients with acute coronary syndrome, circulating P-selectin positive platelets were not increased compared to healthy controls whereas, by contrast, the level of

circulating MPA was higher in patients with respect to normal subjects, with the degree of MPA increase being more pronounced in the presence of myocardial infarction (Furman *et al.*,1998; Michelson *et al.*,2001). These results can be in part explained by a difference in the half-life of P-selectin and MPA in the blood (Michelson *et al.*,2001). In light of this and other evidence, circulating MPA are believed to be a better indicator of platelet activation than the level of CD62P-expressing platelets.

1.4.2 Effect of platelet interaction on monocyte function

Accumulating evidence indicates that contact of monocytes with platelets regulates their function and enhances their pro-inflammatory activity (Figure 1.3). Intercellular signalling via adhesion molecule ligation stimulates monocytic production of various cytokines, including tumour necrosis factor (TNF)-α (Weyrich *et al.*,1995; Weyrich *et al.*,1996) and TF (Celi *et al.*,1994; Lindmark *et al.*,2000). The expression of chemokines, induced by thrombin-activated platelets, has been also reported (Gawaz *et al.*,1998). P-Selectin/PSGL-1 interaction sustains these events through the activation of both tyrosine-kinase-dependent signalling and transcription factor NF-κB. However, PSGL-1 engagement is not exclusively responsible for these processes. Indeed, TF expression by monocytes is reduced not only by a P-selectin-blocking antibody but also by IL-10 (Celi *et al.*,1994). Moreover, ligation of TREM-1 also induces NF-κB activity and subsequently secretion of monocyte chemotactic protein (MCP)-1, TNF-α, and IL-8 (Weyrich *et al.*,1995; Weyrich *et al.*,1996; Bouchon *et al.*,2000).

Platelet-dependent activation of NF-kB in monocytes also regulates gene transcription of the cyclooxygenase enzyme type 2 (COX-2) (Dixon *et al.*,2006). Following prolonged platelet interaction, a series of MAPK signalling events are activated in monocytes, leading to stabilization of the COX-2 transcript and increased COX-2 protein expression. COX-2 is an inducible enzyme involved in arachidonic acid metabolism and biosynthesis of prostanoids. COX-2 and its terminal products are cell- and tissue-specific. The enzymatic activity of COX-2 in monocytes principally leads to the production and release of PGE_2 (Cipollone *et al.*,2008), which not only exerts potent pro-inflammatory effects, including enhancement of monocytic migration (Panzer *et al.*, 2004), but is also able to stimulate platelet activation *per se* (Fitzgerald, 1991; Reilly & Fitzgerald, 1993).

Cross-talk between monocytes and platelets induces pro-thrombogenic activity. Expression of P-selectin, tissue factor and CD40L is higher on the surface of micro-aggregates compared to the levels expressed on activated platelets only (Zhao *et al.*,2003). At the site of thrombus formation, accumulation of TF leads to coagulation, the generation of more thrombin, and the propagation of a fibrin clot (Furie & Furie, 2005). Moreover, MPA formation is believed to have a pro-atherogenic effect due to facilitation of monocyte infiltration into the vascular wall, as further discussed in the following paragraphs.

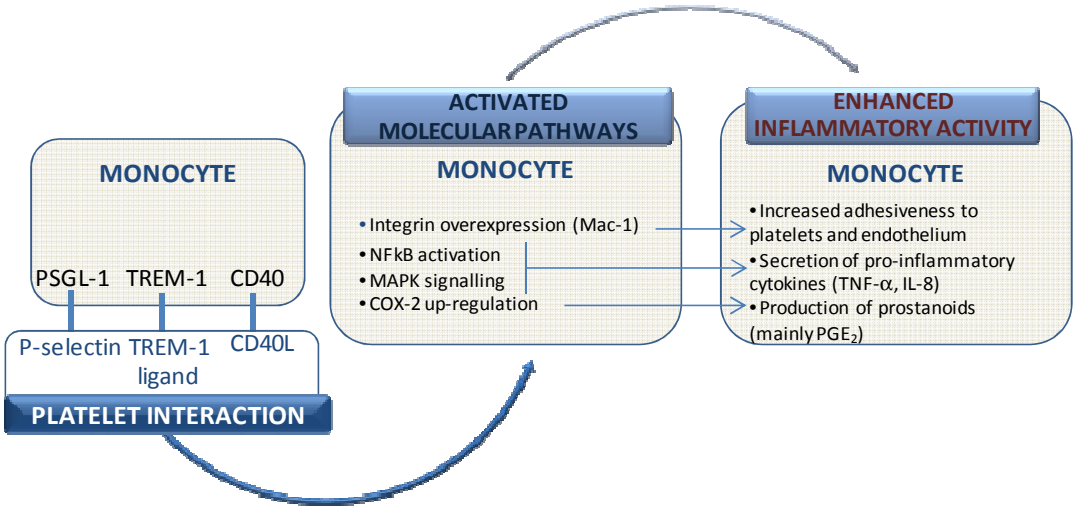

Figure 1.3 Interaction with platelets stimulates pro-inflammatory activity in monocytes. Schematic representation of the general molecular pathways activated in monocytes upon interaction with platelets and the consequent effect on their pro-inflammatory activity. Engagement of monocytic PSGL-1, TREM-1 and CD40 by platelet P-selectin, TREM-1 ligand and CD40L stabilizes cell adhesion and activates intracellular signalling leading to NFκB (nuclear factor κ B), MAPK, and COX-2 (cyclo-oxygenase 2) activation. Their activity leads to production of pro-inflammatory cytokines (TNF-α and IL-8) and synthesis of prostanoids (PGE_2 is the main prostanoid deriving from COX-2 activity in monocytes). Over-expression of integrins, particularly the β2 integrin CD11b/CD18 (Mac-1), further stabilizes the interaction between monocytes and platelets and favours monocyte adhesiveness to endothelial cells.

1.5 Monocyte-platelet aggregates in atherosclerosis

1.5.1 Evidence from *in vitro* experiments and *in vivo* animal models

Activated platelets have a dual role in atherosclerosis, since they are responsible for the thrombotic complications of "vulnerable plaques" but also contribute to lesion initiation and progression (Davi' & Patrono, 2007). The pro-inflammatory nature of platelets underlies their pro-atherogenic effect, and the ability to interact with monocytes to form MPA constitutes a central pathophysiological mechanism.

Upon activation, platelets adhere to the vascular endothelium where they act as a bridge between the vascular wall and circulating white cells, through the release of inflammatory mediators that exert a chemotactic action on circulating monocytes (i.e. soluble CD40L), and/or through direct engagement of PSGL-1 constitutively expressed on monocytes (reviewed by McEver, 2001). Therefore, adherent platelets facilitate monocyte recruitment into the vascular wall. In keeping with this, interference in platelet binding to the endothelium, by blocking the platelet adhesion molecule for vWF, namely GPIb, prevents progression of atherosclerosis (Massberg *et al.,* 2002).

On the other hand, monocytic PSGL-1 engagement by platelet P-selectin induces production of superoxide anion radicals from monocytes (Tsuji *et al.*,1994), and tyrosine phosphorylation of various cytoplasmic proteins, including pp125 focal adhesion kinase, ERK, Syk, Src kinase, and paxillin (Hidari *et al.*,1997; Haller *et al.*,1997; Urzainqui *et al.*,2002; Wang *et al.,* 2007). These biomolecular pathways lead to integrin activation on monocytes, resulting in further stabilization of cellular adhesion (monocyte-platelet and monocyte-endothelium interactions) and synthesis and release of monocytic pro-inflammatory chemokines. Further interactions are

through CD40L/CD40, TREM-1 ligand/TREM-1 (which may also promote integrin expression), and CD36/CD36 via thrombospondin (van Gils *et al.,* 2009). The multiple receptor-ligand interactions (Figure 1.4), the humoral factors released at the site of vascular injury and monocytic infiltration into the sub-endothelium amplify the pro-inflammatory milieu within the vascular wall, and this further contributes to and sustains atherosclerotic plaque development.

Direct *in vivo* evidence supporting the role of MPA in atherogenesis has been reported by Huo et al (Huo *et al.*,2003). They demonstrated that activated platelets injected into apoE$^{-/-}$ mice aggregate with circulating monocytes, thereby promoting the onset and progression of atherosclerotic lesions by favouring monocytic infiltration into the vessels.

1.5.2 Clinical observations

Increased formation of MPA in the peripheral circulation of patients with atherosclerotic disease is now well described. High levels of MPA have been reported in subjects with acute cardiovascular events as well as in asymptomatic patients with underlying vascular disease. Apart from being an index of increased blood thrombogenicity, it is believed that MPA may exert a pro-atherogenic effect in humans, but this hypothesis remains to be proved. The abovementioned experiments in apoE$^{-/-}$ mice (Huo *et al.*,2003) suggest that interaction between platelets and monocytes is not only a localized phenomenon occurring at sites of vascular damage, but occurs in the peripheral circulation with resultant MPA formation which then induces a damaging effect on blood vessels. In this context, it is noteworthy that the level of MPA observed in patients presenting with acute coronary syndrome has

been demonstrated not only to be an indicator of platelet activation, but also to have predictive value for future cardiovascular events and hospitalizations for cardiovascular disease (Ashman *et al.*,2009).

1.6 Polymorphonuclear neutrophil (PMN)-platelet interaction

As previously discussed, activated platelets also adhere to PMN to form heterotypic aggregates although with a lesser binding affinity than that displayed for monocytes, thus resulting in a lower amount and a shorter *in vivo* half-life of PMN-platelet complexes in the peripheral blood compared to MPA (Table 1.3). This has important implications as regards the sensitivity of the distinct types of heterotypic aggregate to reflect *in vivo* platelet activation (Michelson *et al.*, 2001). Nevertheless, as with MPA formation, PMN-platelet interaction modulates the biological function of PMN, and this results in a distinct but important effect of these heterotypic complexes on atherosclerosis-related inflammation.

1.6.1 Biomolecular mechanisms underlying PMN-platelet aggregation

The reasons why activated platelets preferentially adhere to monocytes remain to be elucidated. Increased expression of PSGL-1 on the surface of monocytes compared to the levels foound on granulocytes has been proposed as a potential explanation (Kappelmayer *et al.*, 2001, Bournazos *et al.*, 2008a). Moreover, adhesive cellular interactions other than PSGL-1/P-selectin binding have been demonstrated to be important in the stabilization of the initial aggregate between platelets and monocytes (as discussed above).

However, the formation of PMN-platelet complexes gives rise to unique biomolecular characteristics that are not shared by monocyte-platelet aggregation. Unlike monocytes, in which additional adhesive events seem to be secondary to, and/or sustained by, PSGL-1-dependent cellular activation, the pre-activation status of granulocytes seems to have a more decisive role in the initial interaction with platelets. Indeed, activated PMN have been observed to undergo a re-distribution of PSGL-1 on their extracellular surface, from a diffuse to a more concentrated spatial localization in uropods, and this event has been proposed to favour receptor/ligand interaction with platelets (Bournazos *et al.,* 2008a). Moreover, PMN activation as sustained by the pro-inflammatory peptide n-formyl-methyl-leucyl-phenylalanine (fMLP), induces the extracellular β2 integrin CD11b-CD18 (Mac-1) to undergo conformational changes which lead to an increase in the binding affinity of PMN to platelets, even in the absence of platelet activation (Evangelista *et al.,* 1996, Bournazos *et al.,* 2008a). Indeed, Mac-1 can either establish a bridging complex with platelet receptors through fibrinogen (Spangenberg *et al.,* 1993) or directly interact with molecules expressed on the platelet plasmalemma such as GPIb or ICAM (Cerletti *et al,* 1999). Following Mac-1 dependent PMN-platelet interaction, further stabilization of the complex requires platelet activation and the consequent engagement of leucocyte PSGL-1 by platelet P-selectin (Evangelista *et al.,* 1996). Therefore, PMN-platelet interaction requires a dual adhesive mechanism involving a co-adjuvant action of PSGL-1 and Mac-1 ligation. By contrast with monocytes, blockade of Mac-1, that does not exert any modulatory effect on MPA formation (Steiner et al., 2003), is able to prevent PMN-platelet aggregation with similar efficacy to anti-PSGL-1 or anti-P-selectin blocking antibodies (Evangelista *et al.,* 1999; Haselmayer *et al.,* 2007). Suggestions in to the differences in the biomolecular

mechanisms involved in the differential binding of platelets to monocytes and to neutrophils also come from the observed divergence in effect of the anti-coagulant sodium citrate on the levels of heterotypic complexes measured in human samples: blood collected into sodium citrate contains less MPA than that measured when blood is anti-coagulated with heparin or hirudin, due to the calcium chelating activity that distinguishes the former which reduces the calcium-dependent contact between PSGL-1/P-selectin occurring *ex vivo* (Bournazos *et al.,* 2008b). No such difference is observed on the level of PMN-platelet aggregates when these different anti-coagulants are used, thus suggesting a minor dependency of PMN-platelet interaction on the calcium-dependent PSGL-1/P-selectin binding. Figure 1.4 and 1.5 illustrate the mechanisms involved in heterotypic aggregation between platelets and both PMN and monocytes.

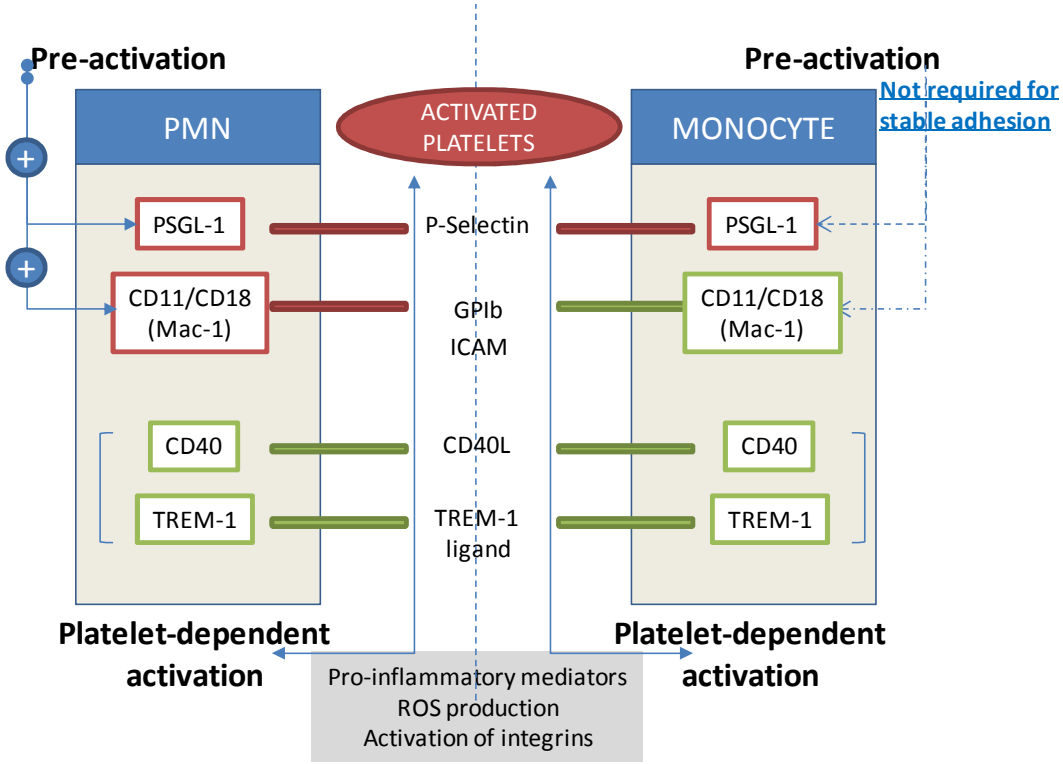

Figure 1.4 Adhesive mechanisms involved in heterotypic aggregation. Molecules in red rectangles are those necessary for heterotypic aggregate formation, while molecules indicated in green rectangles are those that participate in stabilization of the complexes.

For PMN-platelet interaction, engagement of both PSGL-1 and Mac-1 by P-selectin and GPIb/ICAM respectively is required, and blockade of each of these interactions abolishes heterotypic complex formation. Their binding affinity for platelet ligands is influenced by PMN pre-activation status, independent of platelet stimulation. For MPA formation, PSGL-1 ligation is considered the main effector, whilst Mac-1 participates in further stabilization of the initial aggregate. However, Mac-1 blockade does not exert any modulating activity on MPA formation, which is strongly inhibited by PSGL-1 or P-selectin blocking agents only. Monocyte activation status does not influence MPA formation. The molecules illustrated in this scheme activate signalling pathways as shown in Figure 1.5. The consequent release of pro-inflammatory cytokines and reactive oxygen species (ROS) positively feed-to cause both platelet and white cell activation, thus amplifying the pro-inflammatory activity of the heterotypic aggregate and its further stabilization.

1.6.2 Effect of platelet interaction on PMN function

As is the case with monocytes, contact with activated platelets modulates the pro-inflammatory activity of PMN through mutual cross-talk involving adhesion molecules and humoral factors released by activated cells. Reactive oxygen species production by PMN is induced by PSGL-1 ligation, this effect being amplified by the presence of fibrinogen (Ruf *et al.*, 1992). Since fibrinogen binds to CD11b/CD18, it can be hypothesised that the participation of Mac-1 in adhesion also has functional consequences by sustaining platelet-dependent activation of PMN. On the other hand, PSGL-1 binding enhances the adhesion of PMN to fibrinogen through the activation of β2 integrins (Xu *et al.,* 2007) that can be further sustained by the secretion of IL-8 from platelet-activated PMN (Hidari *et al.,* 1997). PSGL-1, along with Mac-1 activation, also stimulates PMN degranulation (Hidari *et al.,* 1997) and enhances their phagocytic ability (Maugeri *et al.,* 2009). Additionally, given the role of Mac-1 in mediating leukocyte interaction with endothelial ICAM-1 and VCAM-1, activation of CD11b on PMN secondary to platelet interaction carries important implications in promoting PMN adherence to the vascular endothelium and their trans-migration into inflamed tissues (Xu *et al.,* 2007). In this setting, the previously demonstrated ability of the intracellular domain of PSGL-1 to interact with, and induce a re-organization of, the cytoskeleton (Ba *et al.,* 2005) is likely to contribute to PMN adhesion, rolling and consequent migration through the vascular endothelium (Tauxe *et al.,* 2008). The interaction between CD40 and CD40L, described above as an importnat adjunctive cellular adhesive mechanism involved in the stabilization of MPA, is also a significant player in PMN-platelet interaction since lack of its expression, either on platelets or leucocytes, has been found to reduce, but not abolish, the formation of PMN-platelet heterotypic complexes

(Lievens *et al.,* 2010). Moreover, TREM-1-mediated interaction between PMN and platelets has been described as a crucial effector in the activation of PMN and consequent IL-8 release in response to platelet co-incubation (Haselmayer *et al.,* 2007). Figure 1.5 summarises the functional effects exerted by activated platelets on PMN and monocytes as a consequence of heterotypic aggregation. The enhancement of pro-inflammatory activity induced by platelets on both cell types suggests a co-ordinated and concerted action of both MPA and PMN-platelet aggregates in the pathophysiology of atherosclerosis, as further discussed in the following section.

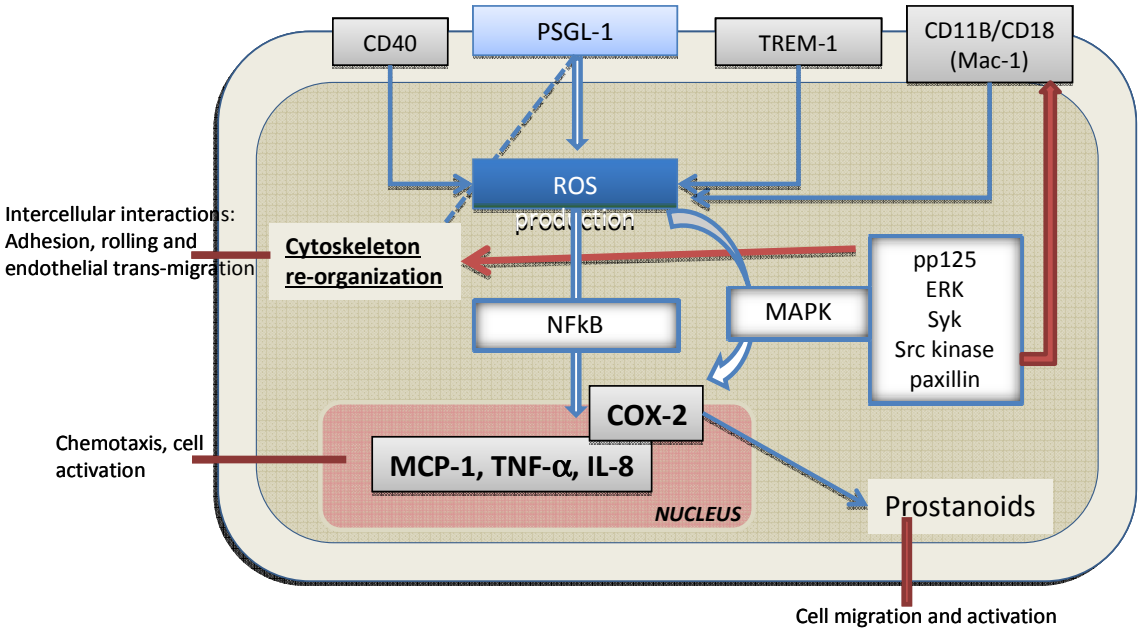

Figure 1.5. Biomolecular pathways activated in monocytes and PMN following platelet interaction and engagement of adhesion molecules. Blue arrows indicate in-cell signalling induced by leucocyte-platelet aggregation, while red arrows illustrate the effect of the main mediators that, following release/production by platelet-activated cells, exert positive feedback on the initial molecular mechanisms and consequently cell activation status. Briefly, PSGL-1 ligation induces ROS-dependent activation of NFkB that translocates into the nucleus to induce pro-inflammatory gene transcription. Cell release of pro-inflammatory cytokines amplify the reaction through MCP-1–dependent recruitment of other leukocytes and autocrine TNF-α/IL-8-induced cell activation. The concomitant activation of MAPK by PSGl-1 stabilizes the transcript of COX-2 (induced by NFkB) and promotes its protein expression, with consequent production of prostanoids deriving from its enzymatic activity. These further activate the cell and sustain cell migration. Migration is also influenced by PSGL-1 through both direct and indirect effects (this latter sustained by protein kinase activation) on the actin cytoskeleton. The resulting structural re-organization of the cytoskeleton facilitates leucocyte adhesion and rolling on the vascular endothelium and subsequent tissue transmigration. PSGL-1 dependent activation of kinases activates β2 integrins such as Mac-1. The concerted action of multiple adhesion molecules, including CD40, TREM-1 and Mac-1, further amplifies the biomolecular mechanisms underlying platelet-dependent leucocyte activation.

1.6.3 Role of PMN-platelet interaction in atherosclerosis

The role of PMN in the pathophysiology of atherosclerosis has received very little consideration compared to that given to monocytes. This is due to a relative lack of evidence concerning the recruitment of PMN into human atherosclerotic lesions, by contrast with that regarding monocyte/macrophage colonization within plaques which is observable throughout the course of the disease, in both early and advanced vascular lesions. Initial observations that PMN are detectable in atherosclerotic arteries of primates (Trillo, 1982), and the more recent descriptions of granulocytes infiltrating plaques in murine models of atherosclerosis (Zernecke *et al.*, 2008; van Leeuwen *et al.*, 2008; Rotziu *et al.*, 2010), have never been supported by similar histological findings in human specimens. Some workers (Weber & Noels, 2011; Drechsler *et al.*, 2011) have proposed that the fact that human plaques stain positive for myeloperoxidase (MPO) indicates the presence of PMN, based on the fact that PMN are particularly rich in MPO. However, this only provides indirect evidence of potential PMN infiltration in human atherosclerosis, and does not exclude the possibility that MPO could derive from monocyte-macrophage cells that also contain this enzyme, albeit in lower quantities (Daugherty *et al.*, 1994). Failure to detect PMN within plaques might be attributable to poor sensitivity of the current methodologies or to fast turnover of PMN within tissues, as recently indicated by Drechsler (Drechsler *et al.*, 2011), or even to a differentiation programme of PMN in the post-migration phase that might alter their phenotype. Regardless of this considerations, even if PMN trafficking is truly low in vascular lesions, their pathophysiological involvement in atherosclerosis may occur through mechanisms other than direct recruitment into the arterial wall. Evidence has emerged that PMN favour monocyte trans-migration through the release of intracellular granules

containing chemotactic substances such as azurocidin (Soehnlein *et al.,* 2005) and cathepsin G (Chertov *et al.,* 1997), that attract monocytes locally and increase their adhesiveness to endothelial cells by inducing integrin activation. In accordance with this, depletion of neutrophils in animal models of atherosclerosis reduces monocyte infiltration into vascular lesions in the early stage of disease (Drechsler *et al.,* 2010). In this context, it is reasonable to hypothesise that platelet-dependent activation of PMN that follows heterotypic aggregation may amplify the pro-inflammatory/pro-atherogenic activity of PMN. However, the poor stability of PMN-platelet complexes makes it problematic to detect variations in the level of these aggregates in the peripheral blood in the context of acute and sub-acute thrombotic events, making difficult the evaluation of the relationship between their dynamics of formation and the occurrence of cardiovascular events in humans. In the peripheral blood of patients with acute ischaemic stroke, MPA level increases with no change in the concentration of PMN-platelet complexes, either during the acute or convalescent phases compared to controls (McCabe *et al.,* 2004; Tsai *et al.,* 2009; Cao *et al.,* 2009). Controversial results have been reported by Marquardt (Marquardt *et al.,* 2009) who observed that PMN-platelets are more consistently elevated than MPA over the 90 days following acute stroke, although a completely different methodology was used to that in the previously mentioned studies, and this could have affected the results. In the context of coronary disease, increased formation of PMN-platelet complexes has been detected only during acute myocardial infarction (Linden *et al.,* 2007) and in unstable angina (Ott *et al.,* 1996), whilst patients with stable angina were found to have levels of PMN-platelet aggregates comparable to control subjects (Ott *et al.,* 1996; Furman *et al.,* 1998b; Linden *et al.,* 2007). On the contrary, MPA formation increases in both stable and unstable coronary disease

compared to healthy controls. Moreover, in patients with underlying cardiovascular risk factors but asymptomatic for cardiovascular disease, in whom increased MPA levels have been reported even in the absence of acute thrombotic events, the formation of PMN-platelet complexes is not affected and their levels are similar to those measured in healthy subjects (Tuttle *et al.*, 2003; Kaplar *et al.*, 2001).

Taken together, the evidence suggests a primary role of PMN-platelet aggregation in the setting of acute thrombotic events. In accordance with this, a modulatory action of PMN-platelet complexes on the pro-thrombotic/pro-coagulant activity of monocytes has been described by Halvorsen (Halvorsen *et al.*, 1993) who demonstrated enhancement in TF expression on monocytes through PMN-derived cathepsin G. However, the poor stability of PMN-platelet complexes may underestimate their level of formation during the sub-acute and chronic phases of atherosclerosis and, as a consequence, their contribution to the progression of disease. With regards to their contribution to disease progression, this can be largely ascribed to the action of PMN-platelet complexes on promoting and amplifying monocyte pro-atherogenic activity.

1.7 Monocytes

Monocytes constitute 5-10% of peripheral blood leucocytes in humans. They originate in the bone marrow from a myeloid precursor, enter the peripheral circulation and, after a short half-life in the blood (approximately 3 days), migrate into the tissues to give rise to resident macrophages as well as dendritic cells. The morphology of circulating monocytes in the peripheral blood is heterogeneous as they vary in size, granule content and nuclear morphology. Differential expression of

surface molecules has also been reported and has allowed their phenotypic classification into different subsets which display distinct inflammatory properties (Auffray *et al.*,2009). However, due to the complexity of their morphological and functional heterogeneity, monocyte characterization in humans still remains an active area of research and the precise pathophysiological relevance of each subset in different inflammatory conditions remains unclear. In the context of cardiovascular disease, one of the major challenges is to understand the pro-atherogenic role of distinct subpopulations, and to determine whether their pattern of distribution in the peripheral blood might be indicative of stage of disease.

1.7.1 Monocyte heterogeneity and characterization

Monocytes were initially identified by their expression of the differentiation antigen CD14 on the extracellular membrane. CD14 is a 55-kDa glycoprotein anchored to the membrane through glycophosphatidylinositol (Simmons *et al.*,1989). It functions as a receptor for bacterial lipopolysaccharide (LPS) and acts by transferring LPS from the circulating protein:lipopolysaccharide complex to the Toll-like receptor 4/MD-2 on monocytes. Engagement of this complex results in intracellular activation of the nuclear transcription factor NFκB and MAPK signalling, with consequent up-regulation of inflammatory genes (Wright *et al.,* 1990; Bochkov *et al.,* 2002). CD14 is then crucially involved in the adaptive immune response by promoting innate host defence mechanisms, such as release of inflammatory cytokines, and in up-regulation of co-stimulatory molecules. CD14 also recognizes apoptotic cells and favours, in this way, the phagocytosis of such cells (Devitt *et al.*, 1998).

The ability of monocytes to infiltrate various tissues and terminally differentiate into distinct cellular lines with tissue-specific functions (macrophages, dendritic and Langherans cells and also osteoclasts) raises the question of whether different subsets of monocytes in the peripheral blood commit to specific maturation programmes and/or target organs. In 1989, the identification of a novel subpopulation of monocytes was reported and characterized by double positivity for the classical marker CD14 and a different surface molecule known as CD16 (Passlick *et al.,* 1989). This latter is a low-affinity immunoglobulin G (IgG) Fc receptor initially described on the extracellular membrane of natural killer (NK) cells and neutrophils (Fleit *et al.,* 1982; Chang *et al.,* 1983). Flow cytometry analysis of the peripheral blood revealed that two subsets of monocytes are distinguishable on the basis of these two differentiation antigens, and include the "classical" monocytes highly expressing CD14 but not CD16, and those double positive for these molecules (although the level of CD14 expression is lower in this compared to the classical population). These subpopulations were then named $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$. The distinction was recently refined in 2003 (Ancuta *et al.,* 2003) when, within the group of CD16 positive monocytes, the presence of two different cellular subsets was described, characterized by differential fluorescence intensity for the CD14 signal: $CD14^{high}CD16^{+}$ and $CD14^{low}CD16^{+}$, to be distinguished from the classical $CD14^{high}CD16^{-}$ cells.

It has been demonstrated that these different subpopulations exhibit distinct functions in inflammation, in terms of phagocytic ability, cytokine production and infiltrative ability (Table 1.4). This can be in part ascribed to the distinct combinations of chemokine receptors and adhesion molecules present on their

cellular surface (Gordon & Taylor, 2005; Ziegler-Heitbrock, 2007; Ingersoll *et al.*, 2010).

Table 1.4. Different functions in inflammation displayed by human monocyte subsets in relation to distinct phenotypes.

FUNCTIONAL CHARACTERIZATION			PHENOTYPIC CHARACTERIZATION		
FUNCTION	$CD14^+CD16^-$	$CD14^+CD16^+$	IMPLICATED MOLECULES	EXPRESSION ON $CD14^+CD16^-$	EXPRESSION ON $CD14^+CD16^+$
Cytokine production	IL-10	TNF IL-12 IL-1	TLR-2 TLR-4	No data	No data
Antigen presentation	+	++	MHC class II	+	++
Adhesion to endothelium	+	++	CD11c CD11b CX3CR1 CXCR4 CCR5	+ ++ + + +	+++ ++ ++ ++ -
Migration CX3CL1 induced CCL2 induced	 + ++	 ++ -	 CX3CR1 CCR2	 + +	 ++ -

Cytokine production reported is that obtained in response to LPS stimulation of monocytes.

1.7.2 Monocytes in atherosclerosis: recruitment into the vascular wall

An initiating event in atherosclerosis is the accumulation of macrophages within the vascular wall and their modification into lipid-laden foam cells (Shashkin *et al.*, 2005) through a process regulated by the balance between uptake of modified low-density lipoprotein (LDL) cholesterol and efflux of cholesterol and other lipids. Monocytes infiltrating tissues give rise to macrophages, and therefore it is largely accepted that monocytic colonisation of the sub-endothelial space is responsible for the formation of the lipid core central to atherosclerotic plaques. However, monocyte recruitment into the human vascular wall has never been observed directly. Evidence

comes from morphological analyses that show monocytes adherent to the luminal endothelium (Gerrity, 1981), or *ex vivo* perfusion studies that demonstrate monocyte adhesiveness to atherosclerotic arteries but not healthy ones (Ramos *et al.,* 1999; Huo *et al.,* 2000). Moreover, *in vivo* animal experiments have demonstrated that silencing of different genes coding for adhesion molecules involved in monocyte-endothelium interaction results in beneficial effects on disease progression, and in particular reduces accumulation of monocyte-macrophage cells in the plaque (Wu *et al.,* 2009). However, the difficulty in phenotypic characterization of monocytes in mouse blood (as will be illustrated in Chapter 3) and in identification of homologues in the different species limit the direct translation of evidence obtained in animal models to human disease and, more importantly, confuse our understanding of what monocytic subset predominates in the pathogenesis of human atherosclerosis.

It is only recently that the homology between murine and human monocytes has been clarified (Table 1.5). Based on a microarray approach (Ingersoll *et al.*,2010) Ingersoll et al have identified the counterpart of human $CD14^{high}CD16^{+}$ cells as so-called $Ly6C^{high}CD115^{+}$ murine monocytes, to be distinguished from the $Ly6C^{low}CD115^{+}$ subset that shows a similar gene expression pattern to human $CD14^{low}CD16^{+}$ monocytes. Ingersoll's work refined the previous functional characterization of murine monocytic subsets based on their differential ability to migrate into the tissues, and particularly the vascular wall. Geissmann et al (Geissmann *et al.*,2003) demonstrated that Gri^{-} cells (corresponding to the $Ly6C^{low}CD115^{+}$ subset) have a "patrolling" role in the vascular wall as compared to Gri^{+} monocytes, these findings being in agreement with Potteaux et al who showed that atherosclerotic plaques in $ApoE^{-/-}$ mice (an established model of atherosclerosis that develops as a consequence of increased LDL-cholesterol level in these animals)

are mainly infiltrated by $Ly6C^{low}$ monocytes (Potteaux *et al.*,2011). Controversial data were obtained by Swirski et al (Swirski *et al.*, 2007) who found that $Ly6C^{high}$ dominate in $ApoE^{-/-}$ mice and infiltrate the plaques in greater number than do $Ly6C^{low}$. The different methodological strategies used in the phenotypic characterization of monocytes in these studies, coupled with different ages of the animals as well as the anatomical sites studied, might account for the variability in results. Indeed, stage of disease and differences in shear stress and/or tissue microenvironment are likely to favour preferential recruitment of one monocytic type over another through selective modulation of specific endothelial surface integrin expression, monocyte chemoattractant protein (MCP) production and chemokine ligand accumulation (i.e. RANTES). For instance, early atherosclerotic lesions are rich in endothelium-derived MCP-1 (Cybulsky *et al.*, 1991; Shyy *et al.*, 1994), while in advanced stages of disease the high level of activated platelets at plaque sites is responsible for local release of RANTES (von Hundelshausen *et al.*, 2001). Moreover, trans-endothelial migration assays performed with human monocytes have shown that $CD14^{+}CD16^{+}$ and $CD14^{+}CD16^{-}$ cells respond differently to agents such as macrophage inflammatory protein (MIP)-1α, MIP-1β, and MCP-1 (Weber *et al.*,2000). Similarly, the expression pattern of adhesion molecules including CD11b and CD11c, that bind ICAM-1, VCAM-1 and E-selectin on the endothelium, as well as of chemokine receptors, differs between the two subpopulations of human monocytes (Gordon & Taylor, 2005). The recent comparison between human and murine monocytes performed by Ingersoll et al (Ingersoll *et al.*,2010) has confirmed these observations, implying a functional distinction of monocytic subsets in both species, and a distinct responsiveness and ability to infiltrate the inflamed vascular wall depending on the micro-environment

(reviewed by Wollard & Giessmann, 2010; Ley *et al.*, 2011 and Weber & Noels, 2011).

Table 1.5 Comparison between human and murine monocyte subsets, based on phenotypic and functional characterization.

Monocytes	Human "classical"	Murine " classical"	Human "non classical"	Murine "non classical"
PHENOTYPE				
CD14	+++	++	++	+
CD16	-	+	+	++
Ly6C	ND	++	ND	+
CD115	+++	+++	+++	+++
F4-80	ND	+	ND	+
CD11b	++	+	++	+
CD11c	++	+	+++	+++
CX3CR1	+	+	++	++
CCR2	+	+	-	-
CCR5	+	+	-	+
FUNCTION				
Vascular adhesion in small blood vessel	In response to CCL2	In response to CCL2	Mainly mediated by CX3CL1	Mainly mediated by CX3CL1
Chemotaxis	Mainly mediated by MCP-1	Mainly mediated by MCP-1	Mainly mediated by RANTES	Mainly mediated by RANTES
Arterial vascular infiltration	NA	Predominate in atherosclerotic aorta	NA	Predominate in atherosclerotic brachiocephalic artery

The listed molecules are those mainly implicated in the pro-atherogenic activity of monocytes. "Classical" human monocytes are $CD14^{+}CD16^{-}$ cells and their murine counterpart is the $Ly6C^{high}$ population. Non-classical human monoocytes are $CD14^{+}CD16^{+}$ cells and their murine counterpart is the $Ly6C^{low}$ subset. CD11b and CD11c are integrins that participate in monocyte-vascular infiltration via interaction with adhesion molecules ICAM-1 and VCAM-1. CX3CR1, CCR2 and CCR5 are chemokine receptors that determine responsiveness of monocytes to different chemotactic/migratory stimuli and regulate monocyte infiltration in atherosclerotic arteries (Tacke *et al.*, 2007; Combadiere *et al.*, 2008). CXCR1 interacts with CXCL1 (otherwise known as fractalkine); CCR2 and CCR5 recognize CCL2/MCP-1 and

RANTES respectively. CD14 and CD16 expression enable distinction of human monocytes, whilst the murine subsets which are identified as $CD115^{+}F4\text{-}80^{low}$ cells, are distinguished based on Ly6C differential expression. ND=not detected; NA=no data available.

1.7.3 Monocyte heterogeneity and atherosclerosis: clinical observations

The characterization of human monocytes in the setting of cardiovascular disease is a recent area of investigation, and to date very few data on this have been published. One of the first studies was performed in a cohort of 247 patients with coronary artery disease (CAD), in whom a higher number of circulating $CD14^{+}CD16^{+}$ monocytes was found compared to controls (Schlitt *et al.*,2004). Moreover, the level of cells double positive for CD14 and CD16 showed a significant and independent association with degree of coronary atherosclerosis and serum concentration of the pro-inflammatory cytokine TNF-α. The largest population study is known as the "I LIKE HOMe study" (Rogacev *et al.*, 2010), and was designed to assess the correlation between the pattern of distribution of the different monocytic subsets in the peripheral blood and the presence of obesity (a recognized cardiovascular risk factor) and of subclinical atherosclerosis, as defined by carotid intima-media thickness (IMT). The study involved 622 healthy volunteers and showed a strong association between level of $CD16^{+}$ monocytes and body mass index (BMI) as well as IMT. These data are strongly suggestive of a potential pro-inflammatory and pro-atherogenic effect of $CD14^{+}CD16^{+}$ cells in humans. In line with this evidence, the percentage of circulating $CD14^{high}CD16^{+}$ cells appears to predict future cardiovascular events, at least in dialysis patients (Heine *et al.*, 2008) and in non-dialysis chronic kidney disease (CKD) (Rogacev *et al.*, 2011). Moreover, a reduction

in the baseline level of circulating $CD14^{+}CD16^{+}$ monocytes has been described in healthy subjects undergoing regular exercise training (Timmerman *et al.*,2008), which would be expected to protect against atherosclerosis and cardiovascular disease. Similarly, weight loss in obese subjects was found to significantly reduce the level of $CD14^{+}CD16^{+}$ cells in parallel with a reduction of IMT (Poitou *et al.*, 2011). A study in subjects with type 2 diabetes mellitus reported no difference in the different monocyte populations compared to normal people; however, increased fluorescence intensity of CD14 antigen on the whole monocytic population was observed in diabetics, and this correlated with the level of C reactive protein and the presence of arterial disease (Patino *et al.*,2000).

It is clear that the pattern of distribution of monocytic subsets in the peripheral blood is dynamic, and changes in these subsets may reflect the inflammatory status which in turn relates to atherosclerosis. However, the biological significance of the changes observed under these pathophysiological circumstances remains unclear. A recent study was conducted to investigate the phenotype of monocytes and their relationship with the clinical course and outcome of 46 patients after stroke. Soon after the acute event, patients showed a transient increase in the percentage of circulating $CD14^{high}CD16^{+}$ cells which peaked at 48h, while the subset of $CD14^{low}CD16^{+}$ decreased and the "classical" monocytes did not change. Interestingly, the level of $CD14^{high}CD16^{+}$ cells was predictive of better prognosis in terms of mortality, while increase of $CD14^{high}CD16^{-}$ cells was associated with poor outcome and increased size of cerebral infarction (Urra *et al.*,2009). These results seem to suggest a potential beneficial action of the double positive subset of monocytes, despite the fact that a number of previous investigations, performed *in vitro* and in animal models together with the clinical observations abovementioned,

support the hypothesis that they likely have a pro-atherogenic effect. Moreover, the pattern of distribution of the monocyte subtypes in the peripheral blood of the study population prior to the acute event was unknown. The authors of this study cite the high migratory and phagocytic activity as well as the pro-angiogenic activity of $CD14^{high}CD16^{+}$ cells as possible mechanisms favouring the resolution of brain damage in the area of infarction. However, the same functional features might exert detrimental effects within an atherosclerotic lesion, leading to progressive increase of plaque size and destabilization. Table 1.5 summarises the changes in circulating monocytes seen in different clinical studies, together with the association of these changes with clinical parameters.

Table 1.6 Distribution of monocyte subtypes seen in clinical studies.

STUDY POPULATION	POPULATION SIZE (n)	MONOCYTE PATTERN	ASSOCIATION
Non-dialysis CKD (Rogacev *et al.,* 2011)	119	$CD14^{+}CD16^{+}$	Future CV events
Coronary artery disease (Schlitt *et al.,*2010)	247	$CD14^{+}CD16^{+}$	Coronary atherosclerosis
Obesity (Rogacev *et al.,*2010)	622	$CD14^{+}CD16^{+}$	BMI, IMT
Dialysis (Heine *et al.,*2008)	94	$CD14^{+}$ $CD16^{+}$	Future CV events
NIDDM (Patino *et al.,*2000)	51	MFI for CD14	CRP
Stroke (Urra *et al.,*2009)	46	$CD14^{high}CD16^{+}$ $CD14^{dim}CD16^{+}$	Good prognosis

The table shows differences observed in the study population when compared to healthy controls. Nomenclature of the monocyte populations used in the table, as well as in the text, is as reported by the authors in each study. CKD: chronic kidney disease; NIDDM: type 2 diabetes mellitus; MFI: mean fluorescence intensity; CRP: C reactive protein; CV cardiovascular; BMI: body mass index; IMT: intima-media thickness.

1.8 Anti-thrombotic and anti-inflammatory properties of anti-platelet drugs

Platelet inhibition is a well established therapeutic intervention in the clinical management of atherosclerosis-related cardiovascular disorders. In patients with prior cardiovascular events, including myocardial infarction and stroke, and in those with symptomatic peripheral or stable coronary vascular disease, the use of anti-platelet drugs provides significant benefit in the prevention of further thrombotic events. More debated, and still the subject of much controversy, is whether anti-platelet therapy has any place in primary prevention in subjects presenting with cardiovascular risk factors, but who are otherwise healthy and asymptomatic for atherosclerotic disease. In such cases, any net clinical benefit of anti-platelet therapy depends on the balance between prevention of arterial thrombosis on the one hand versus bleeding complications on the other. As a general rule, anti-platelet therapy is recommended in cases where the risk of future cardiovascular events outweights the hazard of bleeding complications, as is generally the case in secondary prevention. Therefore, in primary prevention, the prescription of anti-platelet therapy requires a careful clinical evaluation on a case-by-case basis, of the risks cersus benefits of such therapy (reviewed by Passacquale & Ferro, 2011b).

Unfortunately, currently available diagnostic approaches for cardiovascular risk stratification, although useful on a population basis, carry important limitations in terms of accuracy in predicting future cardiovascular events in a given individual. This will be discussed further in Chapters 4 and 5 of this thesis. In the following sections, an overview of the current indications that guide the use of anti-platelet

drugs in clinical practice, and the effect of different anti-platelet agents on both pro-thrombotic and pro-inflammatory activity of platelets, will be given.

1.8.1. Indications for the use of anti-platelet therapy in clinical practice

Aspirin, dipyridamole and the thienopyridine derivatives clopidogrel and prasugrel, are the most widely used anti-platelet drugs in clinical practice.

Aspirin is a non-steroidal anti-inflammatory drug that acts on the enzymatic activity of cyclooxygenase (COX)-1. It acetylates a serine hydroxyl group at position 529 in COX-1, and the consequent steric modification of the enzyme inhibits the metabolism of arachidonic acid, thus inducing a defect in the synthesis of TxA_2 which is the main prostanoid deriving from platelet COX-1 activity (Patrignani *et al.,* 1982; FitzGerald *et al.*, 1983, Patrono *et al*., 2005) .

Dipyridamole reduces platelet activation through several biochemical processes, namely an increase in intracellular cyclic AMP (cAMP), alteration in the biosynthesis of prostanoids, and potentiation of the inhibitory effect of adenosine on platelets by blocking its degradation and uptake by erythrocytes and other vascular cells (Harker & Kadatz, 1983).

Thienopyridines are pro-drugs that require oxidation by hepatic cytochrome P-450 to be converted into their active metabolites. The active compounds selectively and irreversibly bind the (ADP) purinergic receptor $P2Y_{12}$ on platelets, thus preventing ADP-dependent platelet activation (Williams *et al.,* 2008). A similar mechanism of action is shared by the most recently developed $P2Y_{12}$ antagonist ticagrelor, that belongs to the cyclopentyl-triazolo-pyrimidine chemical class of drugs. Ticagrelor

induces reversible blockade of the $P2Y_{12}$ receptor, and has shown superiority to clopidogrel in reducing cardiovascular events in subjects with acute coronary syndrome (PLATO trial, 2011; Wallentin *et al.,* 2009), as well as having a potential advantage of shorter time of offset, which may be useful for example if surgery is contemplated: its action wears off in 24-72 hours compared to thienopyridines which take at least 5 days to wear off (reviewed by Cattaneo & Podda, 2010).

The differential mechanisms of action of currently available anti-platelet drugs are summarized in Table 1.10, which also illustrates current clinical indications for their use.

Table 1.7. Comparison of indications for different anti-platelet agents in cardiovascular prophylaxis. [Modified from Passacquale & Ferro, 2011b]

DRUG / MECHANISM	ACUTE CORONARY SYNDROME / PCI	STABLE ANGINA AND OTHER CARDIOVASCULAR DISEASE	PRIMARY PREVENTION FOR THOSE AT HIGH CARDIOVASCULAR RISK
Aspirin Reduces production of platelet agonist thromboxane A_2 (TxA_2) by blocking enzyme cyclooxygenase-1 (COX-1)	***Acute therapy:*** with clopidogrel or prasugrel (see below) ***Long-term therapy:*** 75mg daily	First line option for: 1. prior myocardial infarction 2. stable angina 3. transient ischaemic attack (in combination with dipyridamole: see below) 75 mg daily long-term	ONLY when calculated 10-year cardiovascular risk >20% and only after adequate control of blood pressure 75mg daily long-term
Clopidogrel Metabolites inhibit platelet activation by preventing ADP from binding to its platelet receptor $P2Y_{12}$	***Acute therapy:*** Loading dose 600mg / Maintenance Dose 75mg daily IN COMBINATION WITH aspirin 75mg daily (UNLESS aspirin is contraindicated or not tolerated) for 12 months. ***Long-term therapy:*** After 12 months discontinue (but continuing aspirin 75mg daily) or review	First line option for: 1. prior stroke 2. peripheral vascular disease 3. multivessel disease 4. prior myocardial infarction (ONLY when aspirin is contraindicated or not tolerated) 75 mg daily long-term	As for aspirin, but ONLY when aspirin is contraindicated or not tolerated 75 mg daily long-term
Prasugrel Metabolites inhibit platelet activation by preventing ADP from binding to its platelet receptor $P2Y_{12}$	***Acute therapy:*** *ONLY if required urgently, or in patients with type 2 diabetes, or if stent thrombosis occurs while taking clopidogrel, or if clopidogrel is contraindicated.* Loading Dose 60mg / Maintenance Dose 10mg daily IN COMBINATION WITH aspirin 75mg daily (UNLESS aspirin is contraindicated or not tolerated) for 12 months ***Long-term therapy:*** After 12 months discontinue (but continuing aspirin 75mg daily) or review	No data available	No data available
Ticagrelor Inhibits platelet activation by preventing ADP from binding to its platelet receptor $P2Y_{12}$	*Acute therapy:* Loading dose 180mg / Maintenance Dose 90mg twice daily IN COMBINATION WITH aspirin 75mg daily (UNLESS aspirin is contraindicated or not tolerated) for 12 months. *Long-term therapy:* After 12 months discontinue (but continuing aspirin 75mg daily) or review	No data available	No data available
Modified-release Dipyridamole 1. Potentiates inhibitory effect of adenosine on platelets by reducing its degradation and uptake 2. Increases intracellular cAMP 3. Modulates biosynthesis of prostanoids	Not indicated	First line option for: 1. transient ischemic attack IN COMBINATION WITH aspirin 75mg daily (or alone if aspirin is contraindicated or not tolerated) 2. prior stroke ONLY when clopidogrel is contraindicated, IN COMBINATION WITH aspirin 75mg daily (or alone if aspirin is contraindicated or not tolerated) 200mg twice daily long-term	Not indicated

1.8.1.1 Secondary prevention

According to the most recent guidelines for secondary cardiovascular prophylaxis, aspirin remains the first line option for long-term treatment (more than 12 months) of patients with acute coronary syndrome (encompassing STEMI, NSTEMI and unstable angina) and stable angina (CG48 NICE guidelines, 2007; CG94 NICE guidelines, 2010). On the other hand, clopidogrel is recommended in subjects with prior stroke or peripheral vascular disease (CG90 NICE guidelines, 2010). The recommended choices of one anti-platelet agent over another are based on review of the literature to identify the most effective treatment in specific clinical settings, coupled with an economic analysis of cost/benefit ratio. Direct comparison of the efficacy of aspirin and clopidogrel in long-term cardiovascular prophylaxis has shown no important differences between the two drugs (CAPRIE trial, 1996). Therefore, taking into consideration cost-effectiveness analyses, aspirin is recommended as first-line and, when contraindicated or not tolerated, clopidogrel is used as an alternative (CG48 NICE guidelines, 2007). Superiority of clopidogrel in preventing cardiovascular events in patients with peripheral vascular disease (subgroup analysis of CAPRIE trial, 1996) as well as recurrent stroke (PROFESS trial) support its preference over aspirin in these particular clinical settings (Table 1.7).

The different mechanisms of actions of anti-platelet agents might explain their differing effectiveness in distinct clinical conditions. Moreover, with their separate molecular targets, co-administration of these drugs provides for a concerted modulation of multiple signalling pathways involved in platelet activation, that results in superior anti-thrombotic efficacy compared to that obtained with

monotherapy. Dual anti-platelet intervention is restricted to the treatment of patients immediately after a thrombotic event, and is recommended for the first 12 months following ACS and PCI (Task Force for Diagnosis and Treatment of Non-ST-Segment Elevation Acute Coronary Syndrome of European Society of Cardiology *et al*., 2007; Van de Werf F *et al.*, 2008; CG182 NICE guidelines 2009; CG90 NICE guidelines 2010; CG 236 NICE guidelines, 2011), a period when more aggressive platelet inhibition consistently reduces the risk of a secondary event to a level which greatly outweighs potential heamorragic complications (CREDO trial, 2002; CURE trial, 2003; CLARITY trial 2005; COMMITT trial, 2005). Aspirin plus clopidogrel is generally recommended in cases of acute coronary syndrome, whilst prasugrel is preferred over clopidogrel in diabetic patients, in whom the greater anti-platelet effect of prasugrel has shown clinical advantages over clopidogrel (TRITON-TIMI 38 trial, 2007). A greater anti-platelet effect of ticagrelor over clopidogrel has been demonstrated in the PLATO trial (Wallentin *et al.*, 2009), and ticagrelor is now considered a valid therapeutic alternative to thienopyridines for the treatment of acute coronary syndrome (NC 236 NICE guidelines, 2011). However, the anti-platelet superiority of both prasugrel and ticagrelor over clopidogrel has been demonstrated only in one clinical trial for each drug (TRITON-TIMI38 for prasugrel and PLATO for clopidogrel) and, because of this, further trials are required to address the real benefit of these novel anti-platelet drugs in clinical practice. For the long-term management of patients with acute coronary syndrome, aspirin alone is preferred over dual anti-platelet therapy, since the use of dual therapy in the long term gives rise to a risk of bleeding that outweighs the anti-thrombotic benefit (CHARISMA trial, 2006; MATCH trial, 2004).

For the treatment of transient ischaemic attack, current guidelines indicate the use of aspirin plus dipyridamole (CG90 NICE guidelines, 2010), whilst for stroke clopidogrel alone is recommended, since it provides similar efficacy to aspirin+dipyridamole with a reduced risk of haemorrhagic complications (PROFESS trial, 2008; CG90 NICE guidelines, 2010).

Table 1.8. Efficacy and safety profile of different anti-platelet drugs and regimes. [Modified from Passacquale & Ferro, 2011b].

TRIAL	STUDY POPULATION	TREATMENT	FOLLOW-UP	END-POINT	NNT	NNH
ACUTE CORONARY SYNDROME						
UNSTABLE ANGINA, NSTEMI, STEMI:						
CREDO	2116 pts scheduled for elective PCI	Clopidogrel vs placebo Aspirin in all pts (81-325 mg)	12 months	CV death, MI, or stroke	33	48
CURE	12 562 pts with ACS with NSTEMI	Clopidogrel + aspirin vs Placebo + aspirin (Aspirin dose 75-325 mg)	12 months	CV death, MI, or stroke	53 333 26	91 167 83
CLARITY	3491 pts with STEMI	Clopidogrel + aspirin vs Placebo + aspirin (Aspirin dose 150-325 mg)	30 days	Death, recurrent MI	16	500
COMMIT	45 852 pts with MI 93% STEMI/7% NSTEMI	Clopidogrel + aspirin vs Placebo + aspirin (Aspirin dose 162 mg)	15 days	Death Re-infarction Stroke	11	333
PLATO	18,624 patients with ACS (STEMI/NSTEMI)	Ticagrelor + aspirin Vs Clopidogrel + aspirin	12 months	Death Re-infarction Stroke	59 100 -500	-333
PCI:						
PCI-CURE	2658 pts with ACS undergoing PCI	Clopidogrel vs placebo	12 months	CV death, MI, or Stroke	31 -143 53	-250 -- 83
PCI-CLARITY	1863 pts with STEMI undergoing PCI	Clopidogrel + aspirin vs Placebo + aspirin (Aspirin dose 150-325 mg)	30 days	Death, recurrent MI, or stroke	22	100
CURRENT	25086 pts with ACS undergoing early PCI	Clopidogrel double dose vs Clopidogrel standard dose	30 days	CV death, MI, or stroke	30	20.53
TRITON-TIMI 38	13,608 pts with ACS scheduled for PCI	Prasugrel vs clopidogrel Aspirin in all pts (75-162 mg)	15 months	CV death, MI, or stroke	45	45
STABLE ANGINA AND OTHER CARDIOVASCULAR DISEASE						
MATCH	7599 high-risk pts	Aspirin vs placebo Clopidogrel in all pts (75mg)	18 months	Ischaemic stroke, MI, vascular death, rehospitalization	100	77
CHARISMA	15 603 pts with multiple risk factors or evident CV disease	Clopidogrel vs placebo Aspirin in all pts (75-162 mg)	28 months	CV death, MI or stroke	200	250
CAPRIE	19 185 pts with previous CV events and/or manifest atherosclerotic disease	Clopidogrel vs aspirin	1.91 years	CV death, MI, or stroke	111	-10 000‡
STROKE						
PROFESS	20 333 pts with stroke or transient ischemic attack	Clopidogrel vs Aspirin+dipyridamole	2.5 years	Recurrent stroke	500	200

Table 1.8 [continued]

Efficacy is expressed as number needed to treat (NNT), according with to formula NNT=1/ARR, where ARR (absolute risk reduction) = CER (control event rate) – EER (experimental event rate); CER refers to aspirin and EER to the other anti-platelet drug(s) as specified for each trial. Safety is expressed as number needed to harm (NNH), calculated with the same formula as NNT but on the basis of bleeding disorders reported in each trial.

Clinical trials are divided according to study population:

- acute coronary syndrome (ACS) encompassing unstable angina, ST-elevation myocardial infarction (STEMI) and non-ST-elevation myocardial infarction (NSTEMI);

- ACS undergoing percutaneous coronary intervention (PCI);

- stable atherosclerotic disease including stable angina, peripheral vascular disease and those asymptomatic for atherosclerosis;

- stroke.

1.8.1.2 Primary prevention

As mentioned above, by contrast to the situation with secondary prevention, the place of anti-platelet therapy in patients asymptomatic for atherosclerotic disease is much debated. The JBS-2 guidelines recommend the use of aspirin in patients with a calculated cardiovascular risk of greater than 20% over 10 years (Joint British Societies' guidelines 2005); however, even here, its use for this indication should be judged on an individual basis (Barnett *et al.*, 2010), and only following correction of high blood pressure, due to the risk of major bleeding complications in patients with uncontrolled hypertension treated with any anti-platelet medication. On the other hand, the recent Antithrombotic Trialists' Collaboration meta-analysis (Antithrombotic Trialists' (ATT) Collaboration 2009), which reviewed the efficacy of aspirin in preventing serious vascular events in six primary prevention trials, showed equivocal net benefit of aspirin in subjects without prior disease, since the reduced rate of vascular events in the aspirin arm was counterbalanced by a higher risk of major gastrointestinal and extracranial bleeds compared to placebo. Therefore, the use of anti-platelet drugs in primary prevention requires a full comprehensive evaluation of the risk of experiencing future cardiovascular events versus the risk related to bleeding complications.

However, parameters other than simply the rate of bleeding events could be implicated in the lack of clinical benefit deriving from anti-platelet intervention in primary prevention trials. An importnat aspect that should be taken into account in the analysis of results is the heterogeneity of the study populations studied. Asymptomatic patients can be at a different stages of the atherosclerotic disease process, some having silent vascular lesions that might benefit greatly from anti-

platelet intervention much more than those who are at the earliest stages of atherosclerosis development. Lack of a diagnostic tool able to effectively characterize and distinguish such sub-groups of patients in relation to stage of disease makes it difficult to ascertain, in such studies, which patients do and which do not benefit from anti-platelet therapy.

Moreover, in all the clinical trials, including those designed for primary prevention, the main outcomes have been reduction of fatal and non-fatal cardiovascular events, the emphasis being on the pharmacological modulation of acute thrombus formation. It is reasonable to hypothesise that, in the early stages of atherosclerosis, when vascular lesions are developing, targeting the pro-inflammatory action of platelets, for example through therapeutic modulation of heterotypic aggregation, may produce greater benefit than a pharmacological approach mainly affecting platelet homotypic interaction, which associates with acute thrombotic complications in the later stages of disease. In the future, drugs which are designed to specifically inhibit heterotypic aggregation may prove to be more useful in primary prevention than those which inhibit homotypic aggregation.

1.8.2 Anti-inflammatory effect of anti-platelet drugs

Different anti-platelet drugs display significant divergence in the pharmacological modulation of heterotypic aggregate formation, despite similar inhibitory effects on platelet-platelet aggregation (and consequent thrombus formation) (Passacquale & Ferro 2011a). As described above, homotypic and heterotypic aggregation are sustained by distinct molecular pathways that selectively regulate the alpha and dense granule release reactions. Homotypic aggregation appears to be mainly related

to dense granule release, with alpha granule content acting as a amplifier of platelet activation and thrombus growth. Heterotypic aggregation is directed by alpha granule exocytosis and consequent P-selectin expression, an event that is not related necessarily to dense granule release and can occur also in the context of sub-clinical platelet activation. The distinct abilities of different anti-platelet agents to interfere in the biomolecular pathways that regulate alpha and dense -granule release confer on them differential anti-inflammatory and anti-thrombotic properties (Figure 1.6).

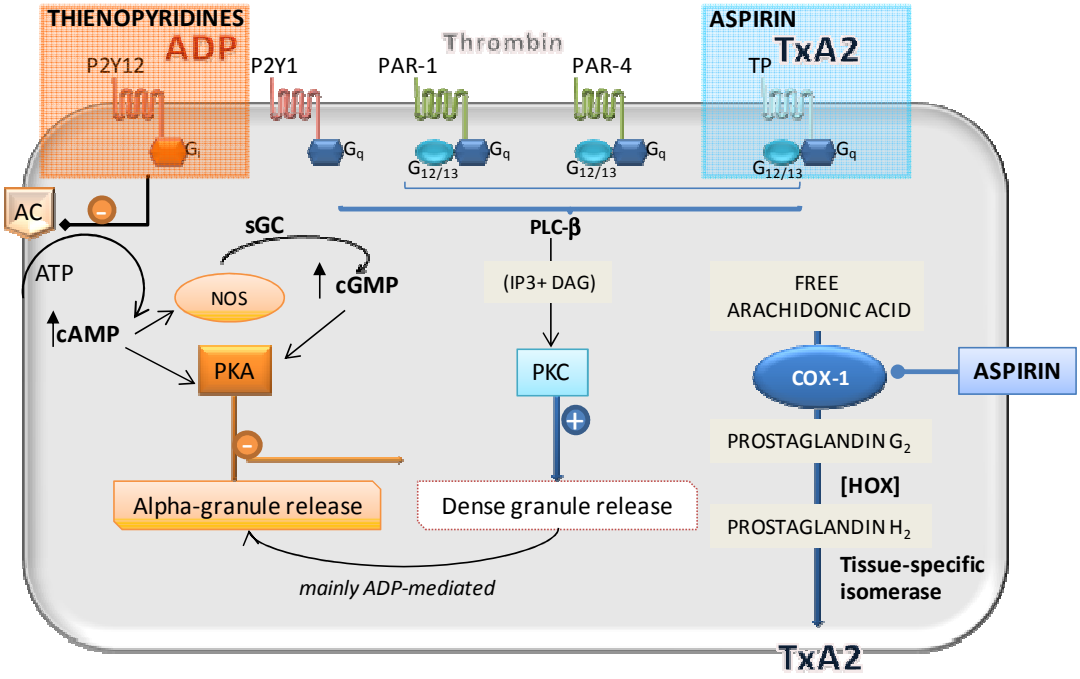

Figure 1.6. Pharmacological modulation of dense and alpha-granule release reaction. Aspirin irreversibly blocks the enzymatic activity of COX-1 which is a key enzyme in the metabolism of arachidonic acid to produce prostanoids. COX-1 converts arachidonic acid to the unstable intermediate prostaglandin G_2 (PGG_2). Further metabolism of PGG_2 by hydroperoxidases (HOX) leads to prostaglandin H_2 synthesis that is finally converted into prostanoids by tissue-specific isomerases (platelets mainly contain TxA_2 synthase resulting in production and release of TxA_2). By acting on COX-1, aspirin reduces TxA_2-dependent platelet activation, with the main effect being on dense granule release and consequent homotypic aggregation. However, aspirin does not affect the pathway regulated by ADP that acts on alpha-granule release. Indeed, although negatively modulated by aspirin, the minimal release of ADP stored in dense granules is able to stimulate platelets through $P2Y_{12}$ and

$P2Y_1$ receptors. Activation of the $P2Y_{12}$-dependent pathway leads to alpha granule exocytosis that sustains heterotypic aggregation via P-selectin translocation to the platelet plasmalemma. Thienopyridines are able to modulate this biomolecular pathway through preventing ADP from binding its specific receptor P2Y12. The resulting preservation of PKA inhibitory action on both alpha and dense granule release negatively modulates heterotypic and homotypic aggregation [Modified from Passacquale & Ferro, 2011a].

For instance, aspirin-mediated platelet inhibition is regulated by COX-1-dependent modulation of dense granule release. Indeed, an early study reported that alpha granule exocytosis, as detected by P-selectin expression, vWF and beta-thromboglobulin secretion, was not at all influenced by COX-1 blockade, in platelets stimulated with either ADP or thrombin (Rinder *et al.,* 1993). These data imply that TxA_2 signalling is primarily involved in dense granule but not alpha granule secretion. In accordance with this, Li et al. reported no effect in healthy subjects of aspirin either on platelet P-selectin expression or on platelet-leucocyte aggregation, after stimulation with ADP, thrombin or platelet activating factor (Li *et al.,* 2003). Similarly, Klinkhardt et al. found that aspirin did not affect the formation of leucocyte-platelet complexes in the circulation (Klinkhardt *et al.,* 2003), and Moshfegh et al. demonstrated a reduced effect of aspirin when compared to other anti-platelet drugs in reducing platelet P-selectin expression (Moshfegh *et al.,* 2000). Since heterotypic aggregates are believed to promote atherosclerosis progression through sustaining a pro-inflammatory state, this relative lack of effect of aspirin on heterotypic aggregation may explain at least in part the lack of any clear effect on atherosclerosis progression as well as the poorer results on clinical outcome, particularly in the setting of primary prevention.

As regards the thienopyridines, antagonism of $P2Y_{12}$ has been demonstrated to directly affect homotypic aggregation, as well as effectively reduce CD40L and P-selectin expression and consequent platelet-leucocyte aggregate formation (Storey *et al.*, 2002). In accordance with these findings, thienopyridines, but not aspirin, have been shown to reduce platelet P-selectin expression, which can be explained by their ability to inhibit ADP-induced alpha-granule release (Klinkhardt *et al.*, 2003; Storey *et al.*, 2002). Prasugrel has shown greater efficacy than clopidogrel in modulating heterotypic aggregation and P-selectin expression, in ADP-stimulated platelets obtained from subjects with atherosclerosis treated with the two different thienopyridines (Braun *et al.*, 2008).

Thus, $P2Y_{12}$ blockade may modulate vascular inflammation in addition to platelet thrombosis (Antonino *et al.*, 2009), and this peculiar action on the pro-inflammatory activity of platelets might conceivably result in more effective cardiovascular prophylaxis than aspirin in the early stages of disease. Clinical trials are needed to invesigate this hypothesis. So far, very little evidence exists to show a beneficial action of anti-platelet therapy on disease progression. In an early clinical study, Ranke (Ranke *et al.*, 1993) described reduction of carotid atherosclerosis progression in aspirin-treated patients following one year administration of 900 mg aspirin daily, which is certainly above the dose clinically used normally for cardiovascular prophylaxis; a few years later, Kodama (Kodama *et al.*, 1999) showed delayed progression of carotid intima-media thickness (IMT) in type 2 diabetics either on aspirin 81mg/day or ticlopidine 200 mg/day compared to untreated patients over a 3-year follow-up period. However, no additional evidence supporting a beneficial action of anti-platelet drugs on atherosclerosis progression has been reported in the following decade or more of research in the field. On the contrary, as

described above, doubt has been on any role for aspirin in preventing cardiovascular events. Further studies are required to establish whether and for what group of patients, aspirin and/or thienopyridines or other anti-platelet drugs can provide anti-atherosclerotic activity. However, before such studies can be done, development of innovative modalities enabling more selective and accurate cardiovascular risk stratification of individual patients is required, so that these studies can be carried out in more homogenous and selective populations.

1.9 Aims of the work

The work presented in this thesis aimed to develop novel biomarkers of silent atherosclerosis, with a view to improving cardiovascular risk stratification of asymptomatic patients at risk of future events and hence more effectively targeting cardiovascular preventative strategies. Given the relevance of platelet activation in the pathophysiology of atherosclerosis, the hypothesis was that differential levels of MPA, widely recognised as the most robust available index of blood thrombogenicity, are detectable in the peripheral blood of patients with cardiovascular risk factors according to degree of disease progression, thus enabling the distinction between those with plaques from disease-free patients. Moreover, due to the functional role of activated platelets in modulating monocyte biological activity, I felt that further investigation of MPA formation and its downstream effects could shed further important light on the underlying atherosclerosis.

Therefore, in the first part of my project, the importance of monocyte-platelet interaction in human inflammatory pathophysiology was explored, in order to test the hypothesis that formation of MPA has pro-inflammatory actions by inducing phenotypic changes in circulating monocytes (Chapter 2).

In the second part, the relevance of platelet-dependent modulation of monocyte phenotype to the pathophysiology of atherosclerosis was investigated. For this purpose, we conducted a study in an animal model of atherosclerosis (ApoE knock-out mice), in which the pattern of distribution of the different circulating monocyte subsets was analysed at different stages of disease, either in the presence or absence of anti-platelet therapy (Chapter 3).

In the third part, we investigated whether circulating MPA level and/or monocyte phenotype are altered in clinically healthy subjects with underlying cardiovascular risk factors, and whether such alterations are indicative of underlying atherosclerosis (Chapter 4).

Chapter Two

Monocyte-platelet interaction induces a pro-inflammatory phenotype in circulating monocytes

2.1 Introduction

As discussed in Chapter 1, platelet activation plays a major role in the pathogenesis of atherosclerosis and its complications through a combination of pro-thrombotic and pro-inflammatory actions that not only promotes the occurrence of acute thrombotic events but also contributes to vascular lesion initiation (Davi' & Patrono, 2007). Activated platelets can bind to the vascular endothelium through a number of receptor-ligand interactions that mediate their local accumulation at the site of a vascular lesion (Zimmerman, 2001; van Gils *et al.,* 2009). The arrest of activated platelets on the arterial wall is an event that can precede plaque formation and acts as a promoting factor for monocyte recruitment and subsequent infiltration of the sub-endothelial space (Massberg *et al.*, 2002). The participation of activated platelets in this early stage of disease is attributable to a number of different mechanisms. Adherent platelets induce endothelial cells to expose adhesion molecules on the luminal surface and synthesise chemotactic factors that recruit circulating monocytes (Massberg *et al.,* 2002). On the other hand, adherent platelets constitute an adhesive surface per se for circulating monocytes, through platelet P-selectin-mediated monocytic PSGL-1 ligation (Zimmerman, 2001), and the release of platelet-derived inflammatory mediators into the local microenvironment exerts a further chemotactic action on circulating monocytes (i.e. soluble CD40L). Additionally, their contact with platelets causes enhanced cytokine and prostanoid production by monocytes (Weyrich *et al*., 1995; Weyrich *et al*., 1996; Dixon *et al*., 2006) as well as increased adhesiveness to the vascular endothelium (da Costa *et al*., 2006) by inducing integrin over-expression and activation; this not only increases monocyte adhesiveness to the vascular endothelium but also enhances trans-endothelial migration, thus resulting in amplification of the inflammatory response

within the vascular wall. It is largely recognized therefore that local heterotypic aggregation between monocytes and platelets at the site of a vascular lesion has important pro-atherogenic effects (Zimmerman, 2001; Massberg *et al.*, 2002; Huo *et al.*, 2003; Huo & Ley, 2004). However, given that MPA are detectable in peripheral blood, it is likely that the interaction between monocytes and platelets is not a phenomenon strictly confined to the vascular wall, but also found in the systemic circulation which may potentially exert a damaging effect on the arteries.

Since circulating monocytes comprise different sub-populations with distinct infiltrative and migratory properties into the tissues, that can be identified by their differential expression for CD14 and CD16 (Weber *et al.*, 2000; Belge *et al.*, 2002), we hypothesised that contact with platelets induces a pro-inflammatory change in monocyte phenotype towards a type more predisposed to contribute to the atherosclerotic process.

2.2.Aims

The overall aim of this chaper was to investigate the effect of platelet activation and consequent formation of MPA on the phenotype of monocytes.

In the first part, we analysed levels of MPA and the normal pattern of distribution of the different monocyte subtypes in the circulation of healthy subjects *in vivo*, as well as the relationship between MPA level in the peripheral blood and circulating monocyte phenotype in response to a mild acute inflammatory stimulus, namely that

in response to influenza immunisation (Posthouwer *et al.*, 2004). Vaccine administration has been demonstrated to represent a reliable "human model" of mild inflammation and has been used by other investigators to examine cardiovascular (patho)-physiology under pro-inflammatory conditions (Clapp *et al.*, 2004; Donald *et al.*, 2006; Wallace *et al.*, 2010).

In the second part, we determined *in vitro* the underlying mechanism by which monocyte-platelet interaction modulates monocyte phenotype and adhesiveness to the vascular endothelium.

2.3 Methods

2.3.1 Effect of influenza immunization on circulating MPA and monocyte phenotype

MPA levels and monocyte phenotype were analyzed in a cohort of twelve healthy (9 male, 3 female; age 25-30 years) employees of Guy's and St Thomas' NHS Foundation Trust, who were attending the Trust's Occupational Health service to receive influenza immunization. Participants were studied immediately before and 2 days post-immunisation. At the first visit, blood (32 ml) was taken for full blood count and biochemistry screening, as well as for high sensitivity C-reactive protein (hs-CRP) assay, measurement of $CD62P^+$ platelets (reflecting degree of platelet activation), determination of MPA level and characterization of monocytes. At the second visit, blood (4 ml) was once again taken, for repeat determination of hs-CRP, $CD62P^+$ platelets and MPA, as well as monocyte characterization.

Percentage of $CD62P^+$ platelets was analyzed by flow cytometry (FACSCalibur, Becton Dickinson (BD), Oxford, UK) on whole blood immunostained with fluorescein isothiocyanate (FITC)-conjugated anti-human CD42b and allophycocyanin (APC)-conjugated anti-human CD62P (BD Bioscience, UK). 10,000 events in total were acquired within the platelet gate, as identified on forward and side light scatter plot. MPA determination and monocyte characterization were performed by whole blood flow cytometry, as described below.

The study was approved by St Mary's Hospital Research Ethics Committee, London, UK. All participants gave written informed consent.

2.3.2 Monocyte characterization and MPA measurement in peripheral blood

More detailed monocyte and MPA characterization was performed by whole blood flow cytometry analysis in a additional cohort of 15 healthy subjects (10 male, 5 female; age 25-35 years). Samples were processed immediately after venepuncture according to the following protocol. 100 μl whole blood, collected in sodium citrate (0.3% final concentration), was transferred into polystyrene tubes (BD Bioscience, UK) and incubated with saturating concentrations of the following antibodies: peridinin chlorophyll protein complex (PerCP)-conjugated anti-human CD14, FITC-conjugated anti-human CD16, APC-conjugated anti-human CD42b or CD62P, phycoerythrin (PE)-conjugated anti-human CD11b or CD11c (all from BD Bioscience), APC-conjugated anti-human Toll-like receptor (TLR)-2 or 4, and APC-conjugated anti-human CD162 (PSGL-1) (eBioscience, UK). Isotype control

antibodies were used as negative control. These were added in different combinations to whole blood immediately after collection, and the incubation continued for 20 min at 4°C. Erythrocytes were then lysed with 1ml FACS lysing solution (BD Bioscience, UK), for 10 min at room temperature, followed by two washes with phosphate buffered saline (PBS) supplemented with 0.2% bovine serum albumin and 0.1% sodium azide. Samples were fixed in 1% paraformaldehyde and kept at 4°C until analysed by flow cytometry (FACSCalibur, Becton Dickinson, Oxford, UK) within a maximum of 48 h from sample preparation.

Forward- and side-light scatter parameters were used to access the monocyte population, and a total of 20,000 events was acquired. The negative and positive delineators were determined from the isotype control fluorescence.

Monocyte subsets were identified by double immunostaining for CD14 and CD16. The percentage of $CD14^+$ cells also expressing CD42b (all platelets) or CD62P (activated platelets) was also calculated and taken as representative of total MPA (Figure 2.1 and 2.2). By gating for each monocyte subtype, we also calculated the percentage of MPA formed by the different subsets of monocytes, and we analyzed their positivity and mean fluorescence intensity (MFI) for PSGL-1, in order to identify potential differences in the ability of distinct monocyte subpopulations to bind platelets. The percentage of cells positive for CD11b/c or TLR-2/4 as well as the MFI for CD11b/c or TLR-2/4 was also analyzed within the different monocyte gates. Post-acquisition analysis was performed using FlowJo software (Tree Star, Ashland, OR). A schematic representation of the method used for flow cytometry analysis is illustrated in Figure 2.2.

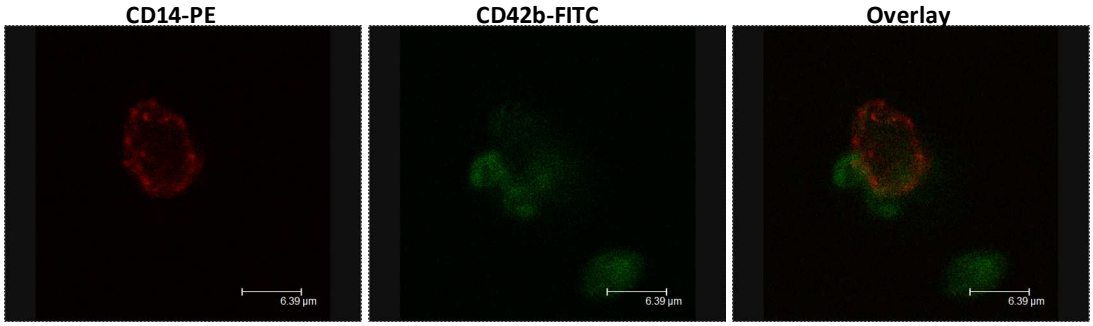

Figure 2.1 Monocyte-platelet aggregates. Detection of monocyte-platelet aggregates in whole blood by immunofluorescence confocal microscopy. Whole blood was stained with PE-conjugated anti-human CD14 (red) and FITC-conjugated anti-human CD42b (green). The overlay shows a double-stained aggregate.

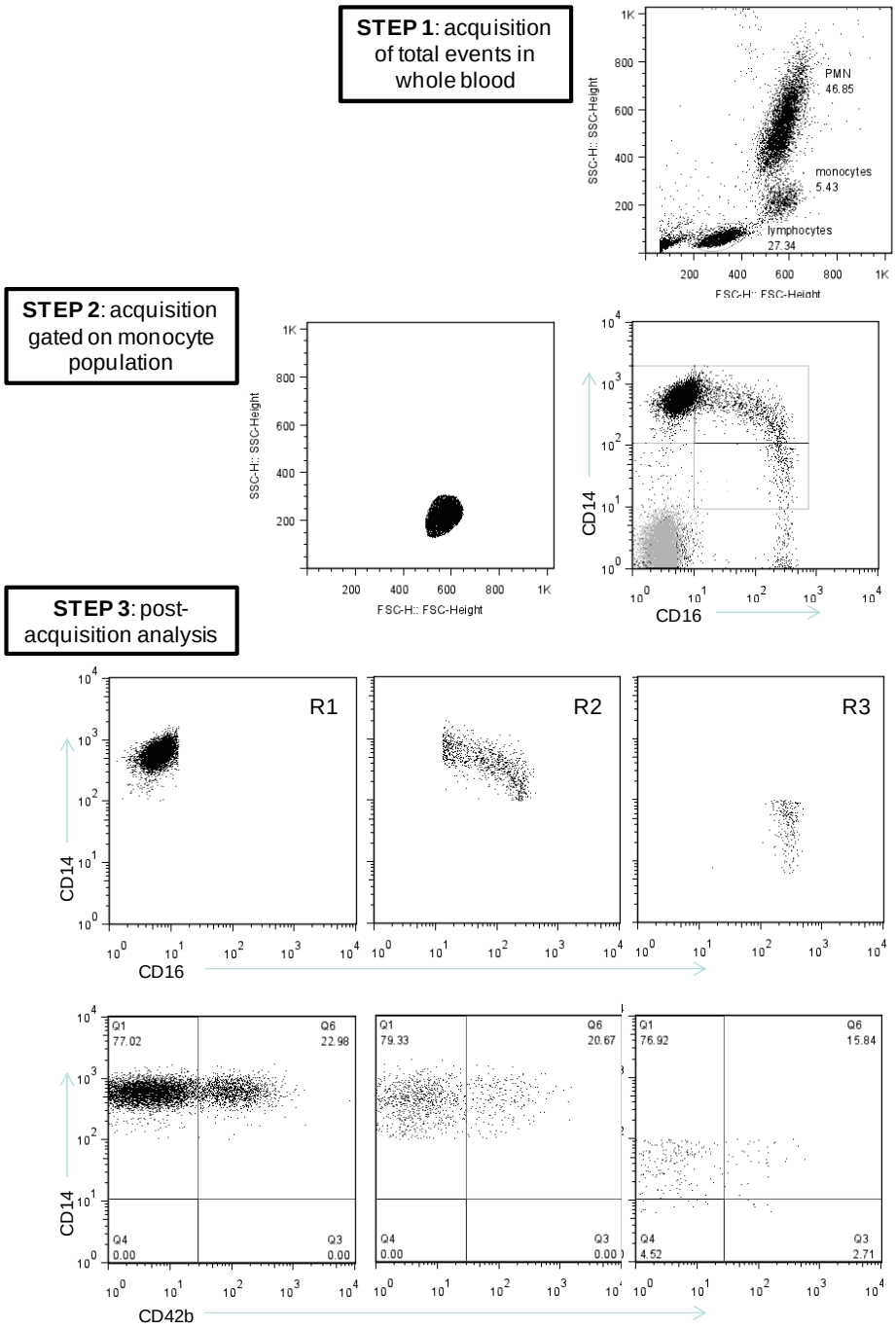

Figure 2.2 Schematic representation of flow cytometry analysis of whole blood. Samples were stained with PerCP-CD14, FITC-CD16 and APC-CD42b. The forward scatter light plot allowed the identification of the different leucocyte types. Acquisition was then gated for the monocyte population which was distinguished into three subsets on the basis of CD14 and CD16 expression: R1, $CD14^{high}CD16^{-}$; R2, $CD14^{high}CD16^{+}$; R3, $CD14^{low}CD16^{+}$. Post-acquisition analysis was performed to study the percentage of MPA formed by each subtype. Q2 (quadrant in the right corner) indicates the percentage of monocytes binding platelets (as defined by double positivity for CD14 and CD42b).

2.3. 3 Monocyte-platelet co-culture experiments

2.3.3.1 Cell isolation, MPA measurement and monocyte characterization

Whole blood (30 ml) collected in EDTA was subjected to Lymphoprep gradient centrifugation. The plasma obtained was centrifuged (1400×g, 4 °C, 6 min) to obtain platelets and platelet-conditioned medium (PCM). Platelets were resuspended, under sterile conditions, in PBS containing 5 mmol/l EDTA, centrifuged (500×g, room temperature, 5 min) and resuspended (25×10^9 platelets/ml) in serum-free RPMI-1640. PCM was obtained by stirring isolated platelets in serum-free RPMI-1640 (1200 rpm, 10 min, 4 °C), followed by removal of platelets by centrifugation (10,000×g, 3 min, room temperature) and collection of supernatant.

Mononuclear cells obtained after Lymphoprep gradient centrifugation of whole blood underwent immunomagnetic negative selection for $CD14^+CD16^-$ monocytes (kit from Invitrogen) according to the manufacturer's instructions. Purity of isolation, as determined by antigen expression ($CD14^+$, $CD16^-$, $CD3^-$, $CD4^-$, $CD8^-$, $CD20^-$) using flow cytometry, was 78-82% in all experiments. Isolated cells were resuspended in serum-free RPMI-1640 (10^6cells/ml), and transferred to polystyrene tubes.

Aliquots of purified autologous platelets or equal volumes of PCM were added to monocyte suspensions (1 ml final volume, monocyte:platelet ratio 1:100 in experiments where platelets were added) and incubated at 37°C, 4% CO_2 for 48 h. Although these experiments were performed in the absence of pharmacological platelet agonists, the platelet isolation and culture procedure prior to addition to

monocytes induced platelet activation, with P-selectin expression measured at 87±3%.

Viability of monocytes, as assessed by Trypan Blue dye exclusion, was >95% immediately after their isolation and after 48h culture, either in the presence or absence of platelets/PCM. CD16 expression and MPA formation were assessed by flow cytometry at different time points. The supernatants were also collected and soluble P-selectin measured according to the manufacturer's instructions (R&D System, UK).

2.3.3.2 Investigation of the role of platelet P-selectin binding to monocyte PSGL-1 in modulating MPA and monocyte phenotype

The above experiments were repeated by co-incubating monocytes with platelets or PCM in the presence of monoclonal anti-human PSGL-1 blocking antibody (10μg/ml; Chemicon), and the effect compared to that obtained with isotype control (mouse anti-human IgG, 10μg/ml; Chemicon).

Additionally, monocytes were treated for 48h with increasing concentrations of soluble human recombinant P-selectin (0-240 ng/ml, R&D System), and monocyte CD16 expression as well as MPA formation *in vitro* were measured at the end of the incubation time.

2.3.3.3 Monocyte adhesion to endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords obtained from uncomplicated deliveries following healthy pregnancies; these were obtained from the labour ward at St Thomas' Hospital, with written informed consent from the mothers. HUVEC isolation was performed using previously described methods (Jaffe *et al.,* 1973). When the endothelial cells were confluent at passage three, HUVEC were seeded onto coverslips in 12-well plates. The following day, HUVEC were stimulated with TNF-α (10 ng/ml, Invitrogen) for 3h at 37°C, and then washed with PBS. A cell suspension containing monocytes pre-cultured either in medium alone or with platelets for 48h was added to the coverslip cultures. After a further 2h incubation at 37°C, non-attached cells were removed by vigorous pipetting. Immunostaining using PE-conjugated anti-CD14 and FITC-conjugated anti-CD16 was then performed at 4°C for 20 min. After washing in PBS twice, HOECHST solution was added for nuclear staining and removed after 2 min incubation, following which cells were fixed in 1% paraformaldeyde. The coverslips were mounted on slides with mounting medium and analyzed by fluorescence microscopy. Cells positive for monocytic markers were counted in ten fields (20x magnification).

2.3.4 COX-2 expression in monocytes

We investigated COX-2 induction in monocytes co-incubated for 18h with either platelets or medium alone. Following such incubation, cells were permeabilized with Triton X100 0.01%, and immunostained for 20 min at 4°C in PBS/0.2% BSA

containing PE-conjugated mouse anti-human COX-2 antibody (Calbiochem,UK; 1μg/ml). After washing in PBS and fixation in 1% paraformaldeyde, cells were resuspended in 10μl PBS, seeded on a microscope slide and examined under fluorescence microscopy (40x magnification, Zeiss LSM, 510 META).

2.3.5 Thromboxane and PGE2 receptor expression on monocytes

Expression at the mRNA level of the thromboxane receptor (TP) (α isoform) and the different PGE_2 receptors (EP1, EP2, EP3 and EP4) was studied by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR), in freshly isolated monocytes and those cultured for 48h either in medium alone or with platelets. Total RNA was extracted by phenol-chloroform extraction (TRIZOL, Invitrogen) and resuspended in RNase- and DNase-free water. RNA concentration was analyzed by NanoDrop and 500 ng total RNA used for cDNA synthesis. Equal aliquots of cDNA were then PCR-amplified by Taq Polymerase (Invitrogen, UK) in a 40μl reaction containing 1x Buffer, 250 μM dNTPs, 4 mM $MgCl_2$, 1.25 μM of each primer, 2U Taq. The conditions of amplification were as follows: an initial 5min at 95°C for denaturation; 36 cycles of 95°C for 1min, 60°C for 1min and 72°C for 1min 30s; and a final extension step of 72°C for 10min. The sense and antisense primers used, and the expected product sizes, are listed in Table 2.1.

Table 2.1 Sequence of primers used for RT-PCR.

GENE	Forward primer (5'- 3')	Reverse primer (5'-3')	Product size (bp)
EP1	TTGTCGGTATCATGGTGGTG	ATGTACACCCAAGGGTCCAG	160
EP2	GTCTGCTCCTTGCCTTTCAC	CGACAACAGAGGACTGAACG	176
EP3	ATCTCAGTCCAGTGCCCAGT	TTTCTGCTTCTCCGTGTGTG	172
EP4	CTGGTGGTGCTCATCTGCT	TATCCAGGGGTCTAGGATGG	150
TP	AGGTGGAGATGATGGCTCAG	CGGCGGAACAGGATATACAC	220

2.3.6 Investigation of the role of COX-2 and PGE_2 in modulating MPA and monocyte phenotype

CD16 expression and MPA formation were analyzed following monocyte co-incubation with platelets for 48h, either in the presence or absence of different COX inhibitors: NS-398 (10μM, a COX-2-selective inhibitor) (Kato *et al.*, 2001), SC-560 (30nM, a COX-1-selective inhibitor) (Kato *et al.*, 2001) and aspirin (0.5mM, a non-selective COX inhibitor). The effect of PGE_2 receptor blockade on CD16 expression and MPA level was also examined using the EP1/EP2-selective antagonist AH-6809 (10μM; Cayman) and the EP4-selective antagonist AH-23848 (10μM; Cayman) (Meya *et al.*, 1993), either alone or in combination. In other experiments, monocytes were stimulated with PGE_2 (1μM) for 48h. NS-398, aspirin and EP antagonists were resuspended in DMSO/PBS solution (1:3), whilst SC-560 was resuspended in ethanol. PGE_2 was resuspended in PBS. In all experiments, the effects of each agonist/antagonist were compared to those obtained using the respective vehicle.

2.3.7 Statistical analysis

All data are presented as mean ± SD. Statistical analysis was performed using GraphPad Prism 4 software. Differences in level of hs-CRP, $CD62P^+$ platelets, MPA and monocyte phenotype before and after influenza vaccine were evaluated by paired Student's *t* test, and the associations between these different parameters were analyzed by least squares and multiple regression analyses. All other statistical comparisons were by ANOVA, with or without repeated measures as appropriate. In all cases, $P<0.05$ (two-tailed) was taken to indicate statistical significance.

2.4 Results

2.4.1 Influenza immunisation causes an increase in circulating MPA and a shift in circulating monocytes towards CD16 positivity

Administration of the influenza vaccine induced a mild inflammatory response in all participants, as demonstrated by an increase in hs-CRP 2 days post-immunisation (0.57±0.26 mg/L at baseline vs 2.94±1.44 mg/L two days post-immunization, p=0.002). The systemic inflammatory response was paralleled by an increase in degree of platelet activation, as reflected by $CD62P^+$ platelet positivity and level of MPA (Figure 2.3A). We also observed a change in distribution pattern of monocyte subsets, with an expansion of the pool of $CD14^+CD16^+$ monocytes (Figure 2.3B). Within this double positive pool, the subset of $CD14^{high}CD16^+$ monocytes showed the greatest increase in percentage (from 4.7±3.61% to 10.44±4.79%, p=0.003), whilst the $CD14^{low}CD16^+$ subset did not change significantly (Figure 2.3B).

In examining the relationship between the percentage of $CD14^{high}CD16^+$ monocytes and other variables examined (hs-CRP, $CD62P^+$platelets and MPA levels), a linear correlation was found between the $CD14^{high}CD16^+$ subpopulation and both hs-CRP and percentage of $CD62P^+$ platelets. However, levels of $CD14^{high}CD16^+$correlated more closely to percentage of CD62P-expressing platelets than with hs-CRP concentration (Figure 2.3C).

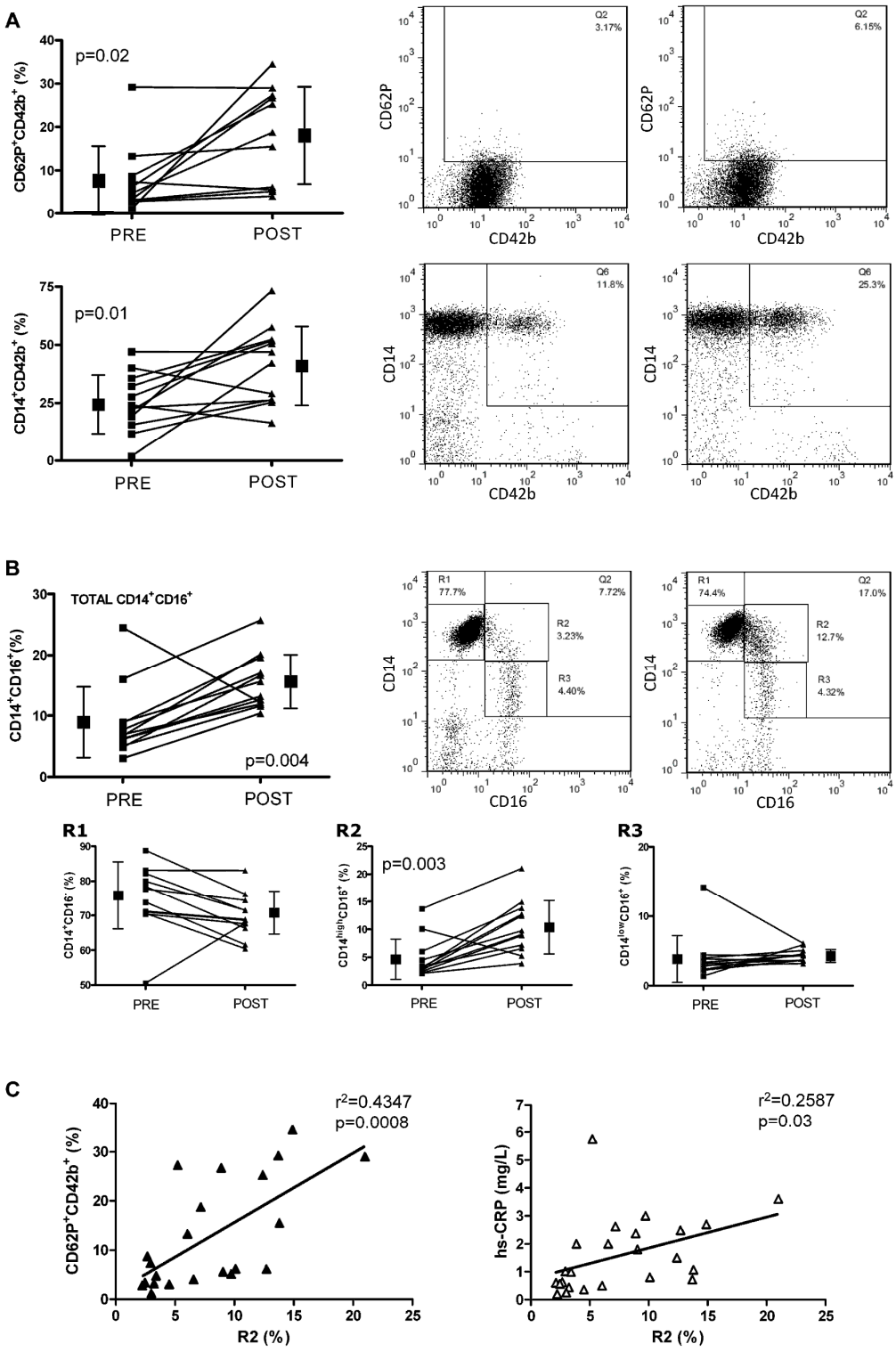

Figure 2.3 Effect of influenza immunisation on platelet activation and monocyte phenotype. Percentage of $CD62P^+$platelets and MPA (**A**), and monocyte characterization (**B**), measured at baseline (PRE) and 2 days after influenza immunization (POST). Figures show representative dot plots obtained from flow cytometry, as well as accumulated data

from n=12 experiments. Monocytic subsets are designated R1 ($CD14^{+}CD16^{-}$), R2 ($CD14^{high}CD16^{+}$) and R3 ($CD14^{low}CD16^{+}$). (**C)**, Regression analyses for $CD14^{high}CD16^{+}$ monocytes (R2) *vs* $CD62P^{+}$platelets and *vs* hs-CRP.

2.4.2 Detailed characterization of circulating monocytic subsets and MPA

As shown above, on the basis of CD14 and CD16 expression, circulating monocytes were distinguished into three subsets: $CD14^{+}CD16^{-}$, which constituted the majority (82.74±5.99% of total monocytes), with smaller contributions from $CD14^{+}CD16^{+}$ cells, comprising $CD14^{high}CD16^{+}$ (4.98±1.76%) and $CD14^{low}CD16^{+}$ subtypes (4.99±2.03%) (Figure 2.4A). All of these monocytic subsets expressed the adhesion molecules CD11b and CD11c on their extracellular surface. However, the level of expression of CD11b was higher on $CD14^{+}CD16^{-}$ cells than on $CD14^{low}CD16^{+}$ monocytes, whereas these latter cells expressed an increased level of CD11c compared to the $CD14^{+}CD16^{-}$ subpopulation; the $CD14^{high}CD16^{+}$ cells demonstrated an intermediate phenotype, expressing both CD11b and CD11c at high level (Figure 2.4B). $CD14^{high}CD16^{+}$ cells also represented the monocytic subtype with the highest expression level for both TLR-2 and TLR-4. Specifically, all the different subsets of monocytes were positive for TLR-2, but the MFI value was higher in $CD14^{high}CD16^{+}$ cells than in the other monocyte subtypes. TLR-4 was found to be expressed by 76.35±12.3% of $CD14^{+}CD16^{-}$ monocytes, 91.7±5.8% of $CD14^{high}CD16^{+}$ ($p=0.02$ vs $CD14^{+}CD16^{-}$) and 82.03±7.26% of $CD14^{low}CD16^{+}$ cells ($p>0.05$ vs $CD14^{+}CD16^{-}$). Moreover, $CD14^{high}CD16^{+}$ cells exhibited higher MFI for TLR-4 compared to the other monocytic subtypes (Figure 2.4C).

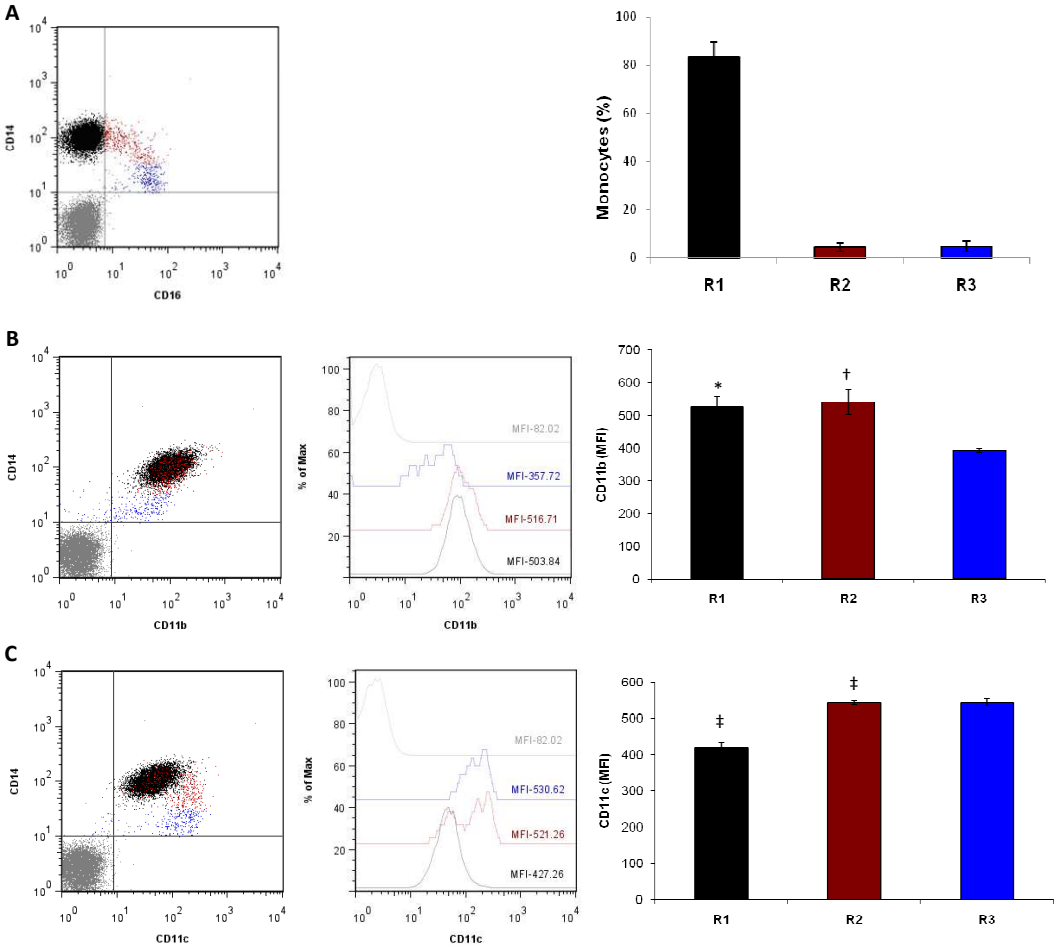

Figure 2.4 Circulating monocyte characterization. (**A**) Percentage of the different circulating monocyte subpopulations, with a representative whole blood flow cytometry dot plot showing CD14 and CD16 expression, as well as accumulated data from n=10 experiments presented graphically. (**B**) Representative flow cytometry dot plots for CD14 and CD11b (left) or CD11c (right) in the different monocyte subsets. Graph displays MFI values for CD11b (filled bars) and CD11c (open bars) in each subset (n=10). (**C**), Representative flow cytometry dot plots for CD14 and TLR-2 (left) or TLR-4 (right) in the different monocyte subsets. Graph displays MFI values for TLR-2 (filled bars) and TLR-4 (open bars) in each subset (n=10). *,**, *** $p<0.05$, <0.01 and <0.001 respectively *vs* R3. †, ‡ $p<0.05$ and <0.01 respectively *vs* R1.

Circulating MPA, as defined by double positivity for CD14 and CD42b, accounted for 15.2±6.7% of the monocytic population. No significant difference was observed in the ability of the different monocyte subpopulations to interact with platelets (Figure 2.5A). In accordance with this, a similar expression of PSGL-1 was observed in all the distinct monocyte subpopulations (Figure 2.5B). However, as expected, the number of $CD14^{+}CD62P^{+}$ events was less than the number of $CD14^{+}CD42b^{+}$ events, most likely attributable to P-selectin shedding from the plasmalemma of activated platelets, as has been previously described (Michelson *et al.*, 1996).

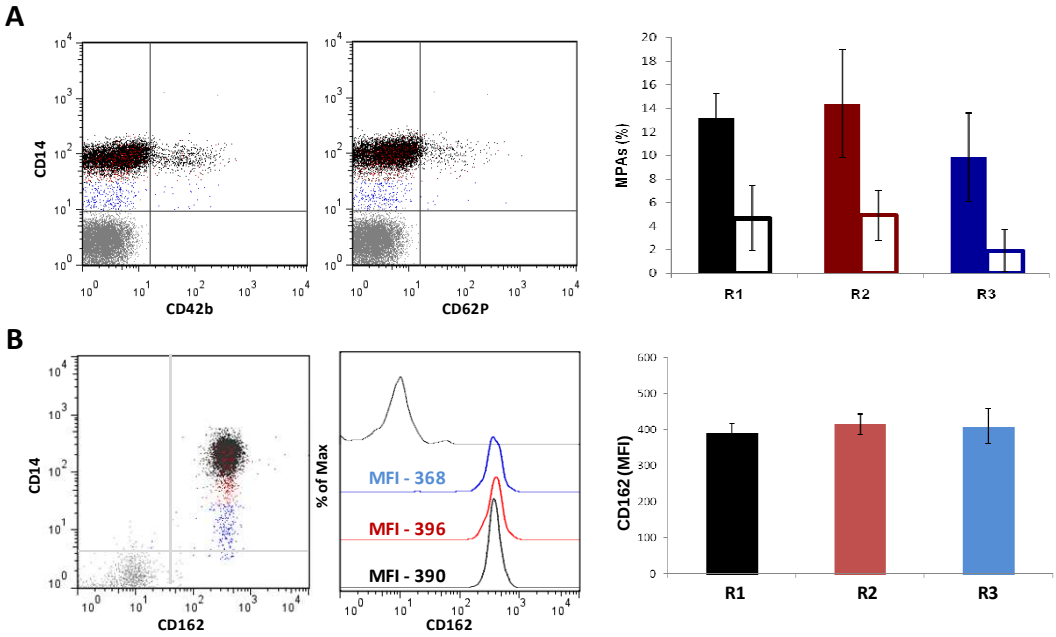

Figure 2.5 Circulating MPA characterization. (**A**) Representative flow cytometry dot plots for CD14 and CD42b (left panel) or CD62P (right panel). Graph shows data accumulated from n=10 experiments illustrating the % MPA formed by the different monocytic subsets, as determined from $CD14^{+}CD42b^{+}$ (filled bars) or $CD14^{+}CD62P^{+}$ (open bars) events (n=10). (**B**) Representative flow cytometry dot plots for CD14 and CD162 (PSGL-1) in the different monocyte subsets. Graph displays MFI values for CD162 in each subset (n=10). In black, red and blue are $CD14^{+}CD16^{-}$ (R1), $CD14^{high}CD16^{+}$ (R2) and $CD14^{low}CD16^{+}$ (R3).

2.4.3 Monocyte-platelet interaction leads to a phenotypic change of $CD14^+CD16^-$ monocytes towards $CD14^+CD16^+$

An initial experiment was performed to study the kinetics of CD16 expression and MPA formation, when monocytes in culture were co-incubated with either autologous platelets or medium alone, for up to 48h. Monocytes in medium alone showed a progressive time-dependent increase of CD16 cell surface expression. Co-incubation with platelets up-regulated CD16 expression to a greater degree from 18h onwards (Figure 2.6A). MPA formation increased in a time-dependent manner, even when monocytes were incubated with medium alone; this reflects an unavoidable degree of platelet contamination of the monocyte sample, with resultant MPA formation. Nevertheless, the addition of exogenous platelets to the culture medium substantially augmented MPA formation, at all time points examined from 18h onwards (Figure 2.6B).

On the basis of these results, in subsequent experiments the effect of platelets (or corresponding medium) on CD16 expression in isolated monocytes was investigated at 24h and 48h of co-culture, whilst the effect on MPA formation was measured at 48h. These experiments confirmed that co-incubation with platelets for 48h caused significant up-regulation of CD16 on monocytes as compared with medium alone (Figure 2.6C), and an increase in MPA formation (Figure 2.6D). Regression analysis showed a direct relationship between level of MPA formation and percentage of monocytes expressing CD16 at 48h (Figure 2.6E).

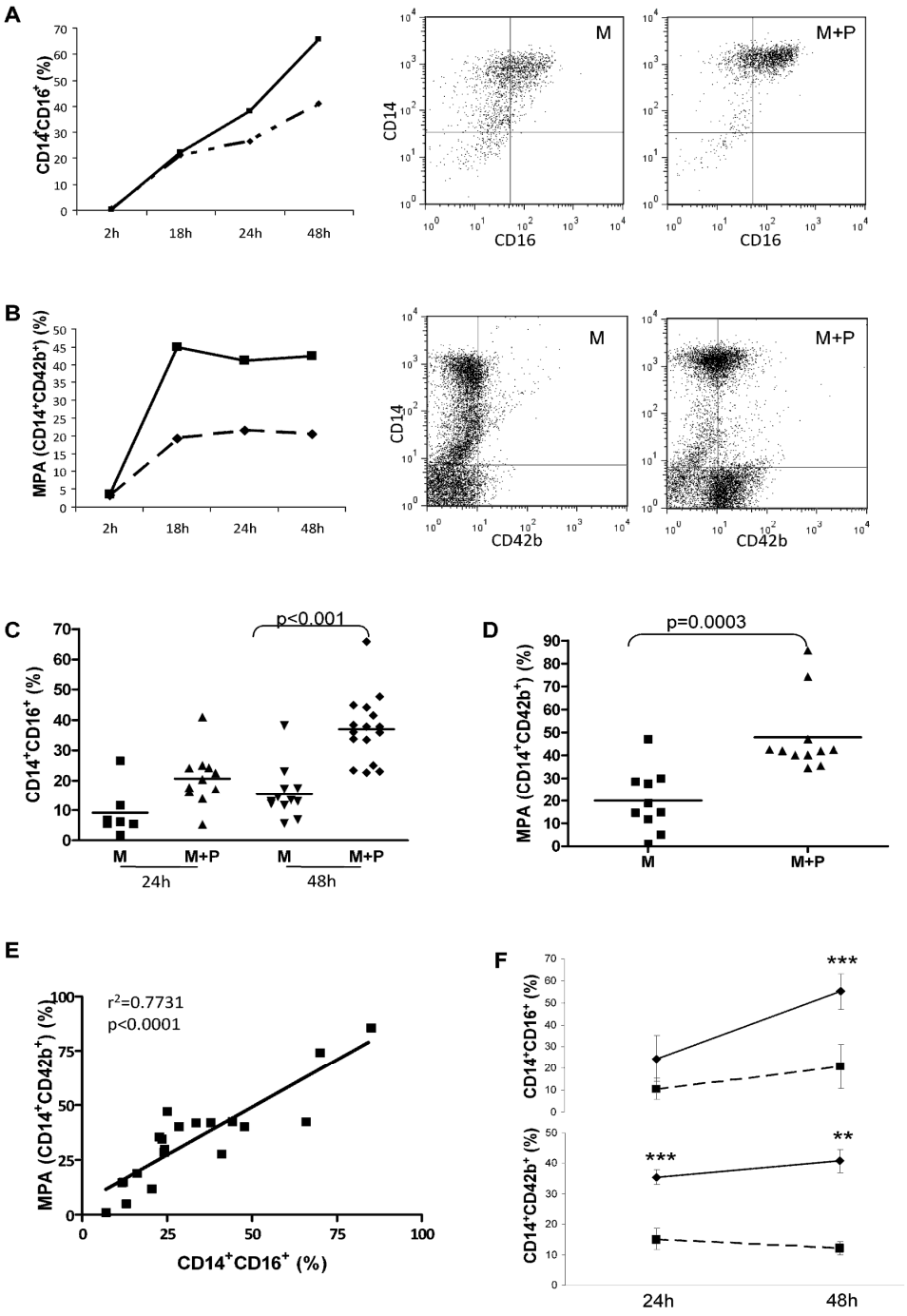

Figure 2.6 Effect of platelets on monocyte phenotype. Percentage of $CD14^+CD16^+$ monocytes (**A**) and MPA (**B**), following incubation of monocytes with platelets (M+P, solid line) or medium alone (M, dotted line) for different times (n=1). Dot plots show flow cytometry analysis after 48h culture. Accumulated results from n=10 experiments showing percentage of $CD14^+CD16^+$ monocytes (**C**) and MPA (**D**) after co-incubation of monocytes with platelets (M+P) or medium alone (M). (**E**), Regression analysis of MPA level *vs*

percentage of $CD14^+CD16^+$ cells at 48h. (**F)**, Percentage of $CD14^+CD16^+$ cells (upper panel) and MPA (lower panel) after co-incubation of monocytes with platelets for different times, either in the presence of isotype antibody (solid line) or anti-PSGL-1 blocking antibody (dotted line) (n=4). **, *** $p<0.01$ and <0.001 respectively *vs* anti-PSGL-1.

Although CD16 induction was also seen in monocytes incubated with PCM for 48h, the percentage of $CD16^+$ cells was considerably lower than that achieved by incubation with platelets (Figure 2.7A). As expected, MPA levels following monocyte culture in PCM was unchanged compared to monocytes in medium alone (Figure 2.7B), as were levels of soluble P-selectin in the medium (Figure 2.7C). However, soluble P-selectin levels were much greater following monocyte co-culture with platelets (Figure 2.7C).

In order to ascertain whether P-selectin binding to monocytic PSGL-1 is in itself sufficient to induce the phenotypic changes observed in monocytes, we examined the effect of co-incubation with anti-human PSGL-1 blocking antibody or isotype control. Blockade of PSGL-1 abrogated MPA formation from monocytes in the presence of platelets, and also markedly reduced the degree of induction of CD16 expression (Figure 2.6F). Similarly, anti-PSGL-1 blocking antibody abolished the PCM-induced up-regulation of CD16 on monocytes (Figure 2.7A). However, soluble P-selectin alone, when added to the culture medium of $CD14^+CD16^-$ monocytes, exerted no demonstrable effect on CD16 expression in monocytes, even at concentrations as high as those found in the supernatants of monocyte-platelet co-cultures (Figure 2.7D).

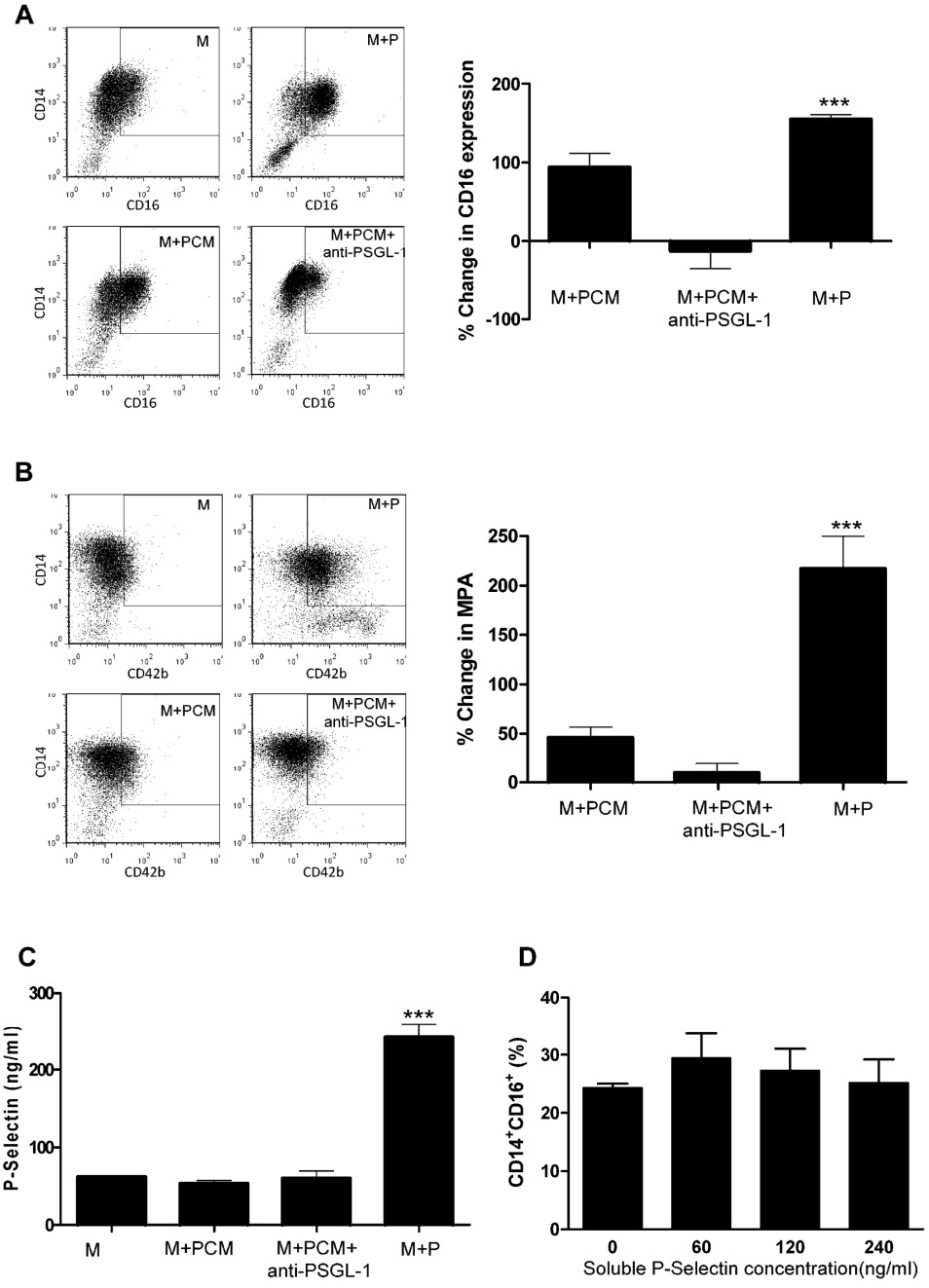

Figure 2.7 Effect of platelet-free conditioned medium on monocyte phenotype. Percentage of $CD14^{+}CD16^{+}$ monocytes (**A**) and MPA (**B**), following 48h incubation of monocytes in medium alone (M), platelet-free conditioned medium either in the absence (M+PCM) or presence (M+PCM+anti-PSGL-1) of anti-PSGL-1 blocking antibody, or platelets (M+P) (n=3 for each). (**C**), Soluble P-selectin measured in the supernatant of monocytes cultured for 48h under different experimental conditions as shown. (**D**), Percentage of $CD14^{+}CD16^{+}$ monocytes after 48h exposure to different concentrations of soluble P-selectin. *** $p<0.001$ vs M.

2.4.3 $CD14^+CD16+$ monocytes exhibit increased adhesiveness to activated endothelium

In monocytes co-incubated with platelets as well as in those maintained in medium alone, cells adhering to TNF-α-pre-activated HUVEC were almost exclusively $CD14^+CD16^+$; only occasional monocytes expressing CD14 but not CD16 were observable (Figure 2.8A). $CD14^+CD16^+$ cells showed a higher expression of both CD11b and CD11c compared with the $CD14^+CD16^-$ subtype (Figure 2.8C). Pre-treatment of monocytes with platelets increased the number of $CD14^+CD16^+$ monocytes attaching to HUVEC (Figure 2.8A and 2.8B).

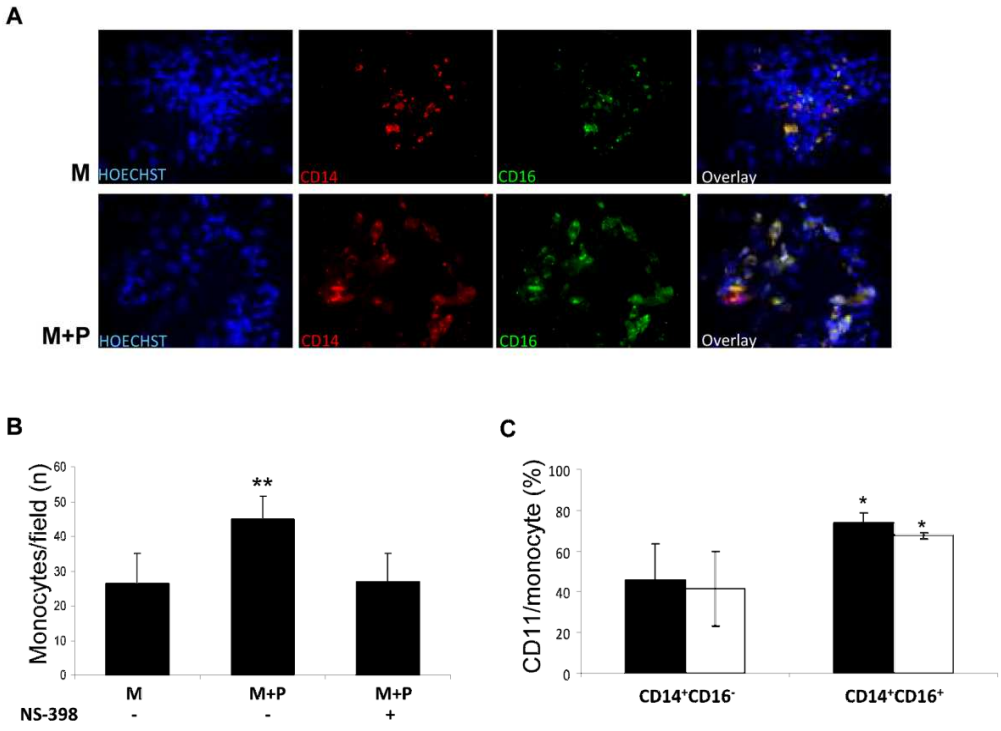

Figure 2.8 Effect of platelets on monocyte adhesiveness to endothelial cells and expression of adhesion molecules. (A), Photomicrographs showing CD14-PE (red) and CD16-FITC (green) staining of monocytes adhering to TNF-α-pre-activated HUVEC (stained with HOECHST, blue), following 48h culture in medium alone (M) or in the presence of platelets (M+P). **(B),** Number of monocytes/field following 48h pre-culture in medium alone (M), in the presence of platelets (M+P) either with or without NS-398 (n=3). ** $p<0.01$ vs M. **(C),** Expression of CD11b (filled bars) and CD11c (open bars) in the same cellular suspension tested in the HUVEC adhesion assay, in monocyte subpopulations classified according to CD14 and CD16 positivity. *$p<0.05$ *vs* $CD14^+CD16^-$.

2.4.4 COX-2 induction and consequent PGE_2 generation underlies the phenotypic changes observed in monocytes in response to interaction with platelets

As previously reported (Dixon *et al.*, 2006), co-incubation with platelets for 18h induced COX-2 expression in monocytes (Figure 2.9A). Since this effect temporally preceded the phenotypic and functional changes described above in response to monocyte-platelet co-culture (Figure 2.6), we wished to determine whether COX-2 induction was responsible for inducing these changes, by examining the effect of COX-2 inhibition on monocyte phenotype and function. Platelet-dependent up-regulation of CD16 on monocytes was markedly reduced by the COX-2 selective inhibitor NS-398, while no effect was observed in response to the COX-1 selective inhibitor SC-560; aspirin also reduced platelet-dependent up-regulation of monocytic CD16, to a degree comparable to NS-398 (Figure 2.9B). MPA formation was not affected by SC-560, NS-398 or aspirin (Figure 2.9C). Treatment with NS-398 also led to a marked reduction in the number of monocytes adhering to TNF-α-pre-activated HUVEC (Figure 2.8B) As before, we found that $CD14^+CD16^+$ cells exhibited an advantage in binding to HUVEC over the $CD14^+CD16^-$ subset.

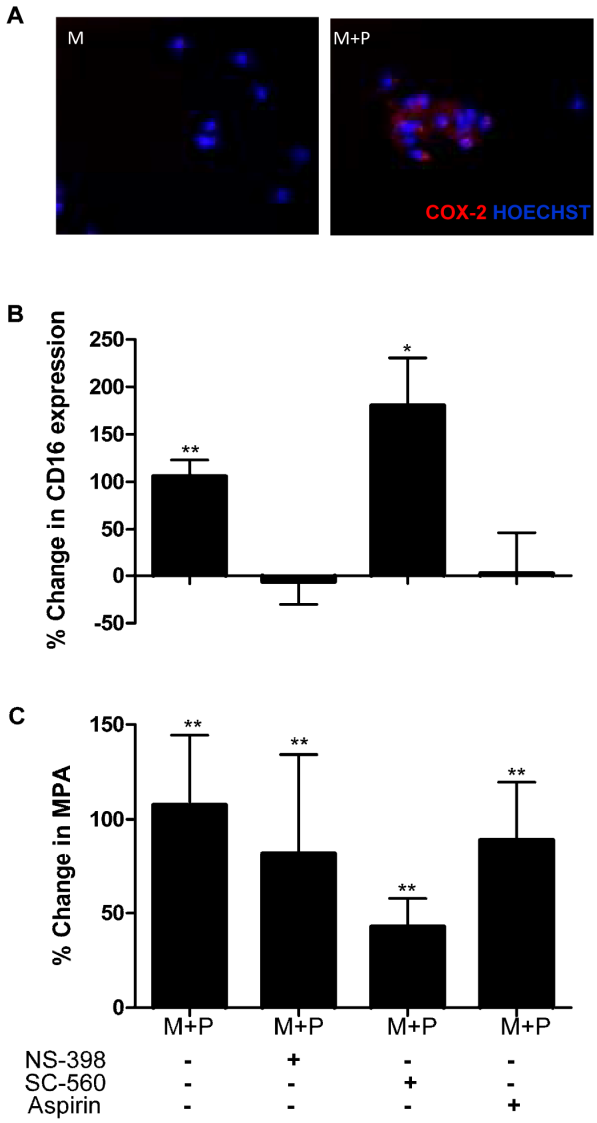

Figure 2.9 Platelet-dependent COX-2 up-regulation in monocytes and effect of COX inhibitors on monocytic CD16 expression and MPA formation. (**A**), Immunofluorescence staining for COX-2 (red) in monocytes cultured in medium alone (M) or with platelets (M+P) for 18h. Photomicrographs are representative of n=3 experiments. Change in CD16 expression (**B**) and MPA (**C**) in monocytes co-cultured with platelets, expressed as % change in $CD14^{+}CD16^{+}$ and $CD14^{+}CD42b^{+}$ cells respectively, as compared to monocytes cultured in medium alone, after 48h. Also shown are the effects of NS-398, SC-560 and aspirin addition to monocyte-platelet co-culture (n=3-4). *, ** $p<0.05$ and <0.01 respectively vs M.

In order to clarify whether the effects on CD16 expression obtained with NS-398 and aspirin, but not with SC-560, were attributable to differential responsiveness of monocytes to the COX-1- and COX-2-derived prostanoids thromboxane A_2 and PGE_2 respectively (Rothe *et al.,* 1996), the mRNA expression of TP and EP receptors was studied (Figure 2.10A). TP expression was found in $CD14^+CD16^-$ monocytes immediately after isolation, whilst after 48h incubation, either in medium alone or with platelets, it became undetectable. Freshly isolated $CD14^+CD16^-$ cells expressed PGE_2 receptors, mainly EP2 and EP4 isoforms, with only small amounts of EP1 and no EP3 mRNA detectable. After 48h incubation in medium alone, mRNA for EP2 and EP4 were still detectable, albeit at lower levels than in freshly isolated cells; moreover, EP1 mRNA also became undetectable. Notably, when monocytes were co-incubated with platelets for 48h, the decrease in mRNA for EP1, EP2 and EP4 was less than that observed in monocytes cultured in medium.

We next examined the effect of different PGE_2 receptor antagonists on platelet-dependent CD16 up-regulation in monocytes (Figure 2.10B) as well as on MPA formation (Figure 2.10C). The EP1/EP2-selective antagonist AH6809 markedly reduced monocytic CD16 up-regulation. Although the EP4-selective antagonist AH23848 appeared also to decrease CD16 up-regulation, this did not reach significance, and there was no additive effect of AH23848 and AH6809, suggesting that EP1 and/or EP2 are predominantly involved in modulating monocytic CD16 up-regulation in response to platelet interaction. Moreover, the effect of AH6809 on the CD16 increase was similar to that seen in response to NS-398, aspirin or anti-PSGL-1 blocking antibody (Figure 2.7B). The two EP antagonists also reduced formation of MPA in vitro, with this effect being significant when they were used in combination rather

than singly; and their combined effect was comparable to that in response to anti-PSGL-1 blocking antibody (Figure 2.8C).

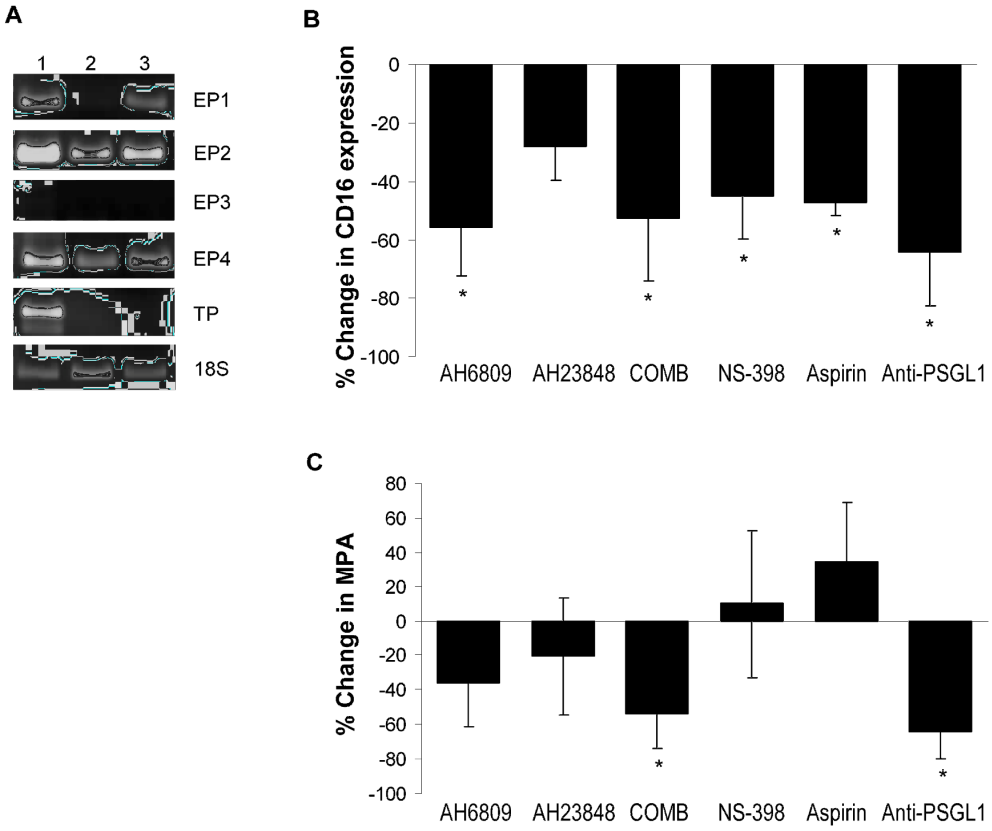

Figure 2.10 Role of COX-2 up-regulation and of PGE_2 on platelet-dependent monocytic CD16 expression and MPA formation. (**A**) mRNA level of EP and TP receptors in freshly isolated monocytes (lane 1), and after 48h culture either with medium alone (lane 2) or platelets (lane 3). 18S ribosomal RNA expression is shown as housekeeping RNA. Experiment is representative of n=3.

Effects of AH6809 and AH3848, alone or in combination (COMB), NS-398, aspirin or anti-PSGL-1 blocking antibody on CD16 expression (**B**) and MPA (**C**) in monocytes co-cultured with platelets for 48h, expressed as % change in $CD14^+CD16^+$ and $CD14^+CD42b^+$ as compared to monocytes co-cultured with platelets alone. * $p<0.05$ vs M+P.

The role of PGE_2 in determining platelet-dependent CD16 over-expression in monocytes was further explored by studying the effect of PGE_2 alone (1μM) on monocyte phenotype. No detectable modulation of level of CD16 expression was exerted by PGE_2 alone compared with vehicle-treated cells (Figure 2.11).

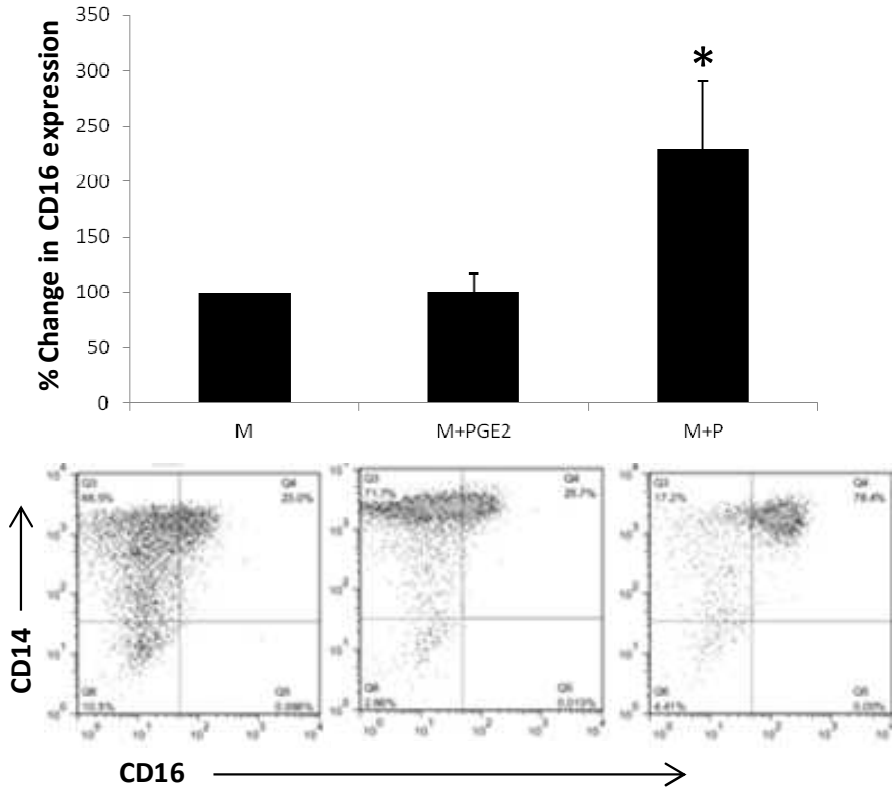

Figure 2.11 Lack of effect of PGE_2 alone on monocyte CD16 expression. Monocytes were incubated in medium alone (M), in the presence of PGE_2 (1μM) (M+PGE2) and with autologous platelets (M+P) for 48h. The graph shows the expression of CD16 measured by flow cytometry on cultured cells and expressed as % of variation in M+PGE2 and M+P as compared to untreated cells (M) (n=3). Representative flow cytometry dot plots shows the percentage of $CD16^+$ monocytes (upper right quadrant) at the end of the incubation time under the different experimental conditions. *, $p<0.001$ vs M.

2.5 Discussion

The present data demonstrate that MPA formation gives rise to a phenotypic change of circulating monocytes toward $CD16^+$ cells, which display increased endothelial adhesiveness. We have shown that this change is mediated by platelet-dependent COX-2 up-regulation and consequent PGE_2 synthesis in monocytes, and that this process can be prevented by selective blockade of either COX-2 enzymatic activity or EP receptors.

2.5.1 MPA formation drives the acquisition of a $CD16^+$ phenotype by "classical" human monocytes

Our interest in the study of CD16 expression on human monocytes stems from the finding that $CD14^+CD16^+$ cells identify a subgroup of circulating cells with higher pro-inflammatory activity than the "classical" $CD14^+CD16^-$ subpopulation (Belge *et al.*, 2002). To date, no direct evidence exists in the literature that double positive cells originate from "classical" monocytes. This study is the first to show that the presence of activated platelets, with consequent MPA formation, induces CD16 up-regulation on $CD14^+CD16^-$ monocytes. Although other groups have reported that platelets can alter monocytic CD16 expression (Ammon *et al.*,1998), this has been interpreted as an effect on terminal monocytic maturation toward macrophages; however, our *in vitro* results together with our *in vivo* data point to a change in circulating monocyte phenotype rather than a terminal differentiation event. Importantly, due to the isolation techniques used in previous studies (elutriation of peripheral blood mononuclear cells), circulating double positive monocytes were

included in the cells under investigation; and since CD16 antigen was measured in the cell supernatant, the prior presence of $CD14^{+}CD16^{+}$ monocytes is likely to have affected the results obtained in such studies. Our *in vitro* experiments were performed specifically with isolated $CD14^{+}CD16^{-}$ monocytes, using flow cytometry which allowed the direct detection of CD16 on their cellular surface, enabling us to measure the percentage of cells developing positivity for the CD16 marker over time. Moreover, we have quantified the degree of interaction between monocytes and platelets by measuring monocyte-platelet complexes formed *in vitro*. Interaction with platelets up-regulated CD16 antigen on isolated monocytes, with this effect being directly related to the level of MPA. Although simple co-incubation of $CD14^{+}CD16^{-}$ monocytes with human recombinant P-selectin did not induce CD16 expression, anti-PSGL-1 blocking antibody, which we have found to abrogate MPA formation (Gkaliagkousi *et al.*, 2009), abolished platelet-induced over-expression of CD16 on monocytes, demonstrating that physical contact of monocytes with platelets is critical in this process, most likely by P-selectin / PSGL-1 binding sustaining multiple receptor-ligand interactions between these cells which trigger the final phenotypic changes.

On the other hand, our experiments suggest that soluble mediators released during the process of platelet activation also exert an important modulatory effect on monocyte phenotype, since CD16 up-regulation was induced by PCM, albeit to a much lower extent than platelets. Nevertheless, PSGL-1 blockade completely abolished both platelet-dependent and PCM-dependent CD16 up-regulation, suggesting that engagement of monocytic PSGL-1 by either soluble or platelet-associated P-selectin is a crucial factor in itself to induce platelet-dependent change

in monocyte phenotype. However, the lack of observed effect of soluble P-selectin alone on monocytic CD16 expression demonstrates that the cross-talk between monocytes and platelets is a complex process in which multiple mediators/interactions are likely to be required. In this scenario, the PSGL-1/P-selectin axis is necessary but not sufficient in itself to drive the acquisition of the CD16 phenotype by human monocytes.

2.5.2 Biomolecular mechanisms underlying platelet-dependent CD16 expression in monocytes: relevance of COX-2 activity

This study has only explored a specific aspect of the mechanism by which MPA formation modulates monocyte phenotype and function. The main focus was on the relevance of COX enzymatic activity, in particular that of COX-2 inducible isoform, based on the evidence that dysregulation in the synthesis of eicosanoids is a key factor in sustaining chronic inflammatory conditions, including atherosclerosis (Smith & Langebach, 2001; Cipollone *et al.*, 2008). Moreover, monocytic COX-2 expression has been suggested to exert pro-atherogenic effects (Cipollone *et al.*, 2008), and COX-2 up-regulation with consequent PGE_2 overproduction in circulating monocytes has been even proposed as a marker of asymptomatic atherosclerosis (Beloqui *et al.*, 2005). Nevertheless, there is not therapeutic benefit of COX-2 inhibition in cardiovascular disease, and in fact the overall effect is detrimental as will be discussed in more details in Chapter 5 of this thesis.

In accordance with the findings of Dixon (Dixon *et al.*, 2006), we have found that interaction with platelets induces COX-2 expression in monocytes, and that

monocytic COX-2 enzymatic activity, that leads mainly to PGE_2 synthesis (Cipollone *et al.*, 2008), is a crucial mediator of platelet-dependent change of monocytic phenotype. Indeed, the selective COX-2 inhibitor NS-398 prevented the increased CD16 expression on monocytes co-incubated with platelets. In parallel with these findings, selective blockade of PGE_2 receptors mirrored the effects observed with COX-2 inhibition. Of note, we found that aspirin counteracted platelet-dependent up-regulation of CD16 on monocytes co-incubated with platelets to a similar extent as NS-398. Others have demonstrated that aspirin, used at concentrations similar to that used in our experiments, modulates PGE_2 synthesis in monocytes and gives rise to similar effects as those caused by COX-2 specific inhibition (Penglis *et al.*, 2000); additionally, the COX-1 selective inhibitor SC-560 has been found to be less effective than the COX-2 selective inhibitor NS-398 in reducing PGE_2 production by bone marrow-derived dendritic cells (Harizi *et al.*, 2002). It is likely that, in our in vitro experiments, aspirin blocked monocytic PGE_2 production by COX-2 inhibition, since the same effect on CD16 up-regulation was seen with NS-398 but not with SC-560.

Our results, therefore, point at a major role exerted by COX-2-derived PGE_2 in regulating the phenotype of human monocytes in response to platelet interaction. However, PGE_2 stimulation alone did not modulate monocytic CD16 expression, most likely because of the absence of concomitant stimuli and factors, including those related to PSGL-1 ligation by platelet P-selectin which we have found to be necessary but not sufficient in itself to induce platelet-dependent change in monocyte phenotype. Based on our findings, it can be hypothesized that concomitant activation of the PSGL-1/ P-selectin axis and PGE_2 receptor stimulation play a causal role in

mediating monocytic CD16 expression following MPA formation (Figure 2.10, each necessary but not sufficient in themselves to mediate this effect). Consistent with this hypothesis, blockade either of heterotypic aggregation (using anti-PSGL-1 blocking antibody) or of COX-2 activity / PGE_2 receptors reduced platelet-dependent CD16 expression with similar efficacy. The potential relevance of these findings in the therapeutic modulation of the pro-inflammatory effects mediated by MPA will be discussed in Chapter 5.

2.5.3 Potential implications in atherosclerosis

The phenotypic changes induced by MPA formation were found to modulate adhesiveness of monocytes to endothelium. Our data suggest that $CD14^+CD16^+$ monocytes have greater potential to adhere to activated endothelium than $CD14^+CD16^-$ monocytes. In our adhesion assay, monocyte interaction with endothelial cells occurred through a mechanism independent of bridging via activated platelets. Indeed, the presence of the COX-2 inhibitor in our co-culture experiments reduced monocytic adhesion to endothelial cells, despite the fact that the same level of MPA was present in the cell suspension added. The reduction in $CD14^+CD16^+$ monocytes in response to COX-2 inhibition resulted in reduced binding of isolated monocytes to activated endothelium. The different adhesive properties of the various monocytic subsets may be attributed to differential expression of adhesion molecules on their cellular surface. CD11b and CD11c have been recognized as crucial mediators of monocyte-endothelium interaction (Kubo *et al.*, 2000; Wu *et al.*, 2009). We observed that $CD16^+$ cells, both those developed *in vitro* and those found in the peripheral blood of healthy subjects, display higher

levels of these integrins on their surface compared to monocytes positive for CD14 only.

We also observed that other molecules involved in the activation of monocytes and their pro-inflammatory activity (Zhang & Ghosh, 2001; Barton & Medzhitov, 2002), namely TLR-2 and TLR-4, are expressed on $CD14^{+}CD16^{+}$ monocytes to a higher degree than on other subsets. On the other hand, levels of PSGL-1 expression was found to be similar among the different monocyte subsets, and $CD14^{+}CD16^{+}$ cells did not differ from the other monocyte subpopulations in their ability to bind activated platelets and form MPA, at least in healthy subjects. Consistent with this, in our influenza immunization study, similar levels of MPA formed by each subset of monocytes were observed both at baseline and post-immunization. Therefore, the increased levels of MPA seen in the context of an acute inflammatory state, such as that induced by influenza immunization, are essentially attributable to increased platelet activation. In our experimental model of inflammation, we found that the increase in hs-CRP, activation of platelets and consequent MPA formation are accompanied by an increase in the percentage of $CD16^{+}$ monocytes in the circulation, mainly driven by expansion of the $CD14^{high}CD16^{+}$ subpopulation. It cannot be excluded that the vaccine *per se* drives the phenotypic changes observed on circulating monocytes. However, the level of $CD14^{high}CD16^{+}$ cells was found to be strongly and directly related to the percentage of $CD62P^{+}$ platelets, suggesting that the degree of platelet activation influences the phenotype of monocytes in the peripheral blood by shifting them towards positivity for CD16.

The fact that this phenomenon is observable in response to a non-specific pro-inflammatory stimulus is indicative of poor specificity for $CD62P^{+}$ platelet and MPA

measurement in the diagnosis of atherosclerosis-related inflammation. Nevertheless, these data provide evidence that a host of modifications occur in the phenotype of circulating monocytes in response to a pro-inflammatory stimulus, rendering them more pro-inflammatory and with increased endothelial adhesiveness, and that these are strongly related to – and are likely to be driven by – the extent of platelet activation. In the setting of atherosclerosis, the exposure to a chronic inflammatory stimulus, such as that which occurs in the presence of cardiovascular risk factors, may lead to a similar response. In keeping with this, a characteristic pattern of distribution of the different monocytic subtypes has been reported in the blood of subjects with dyslipidemia (Rothe *et al.*, 1996), obesity (Rogacev *et al.*, 2010), coronary artery disease (Schlitt *et al.*, 2004) and kidney failure (Rogacev *et al.*, 2011), with the population of $CD14^{+}CD16^{+}$ cells being expanded in those cohorts when compared to healthy controls. In similar clinical conditions, higher values of circulating MPA have been described (Furman *et al.*, 1998; Sarma *et al.*, 2002; Ashman *et al.*, 2003; Gkaliagkousi *et al.*, 2009), suggesting that the formation of heterotypic aggregates between platelets and monocytes may sustain a potential pro-atherogenic action by increasing the number of pro-inflammatory $CD14^{+}CD16^{+}$ cells in the circulating blood, and not just at the level of the atherosclerotic plaque. Figure 2.12 illustrates the potential contribution of MPA formation to atherosclerosis.

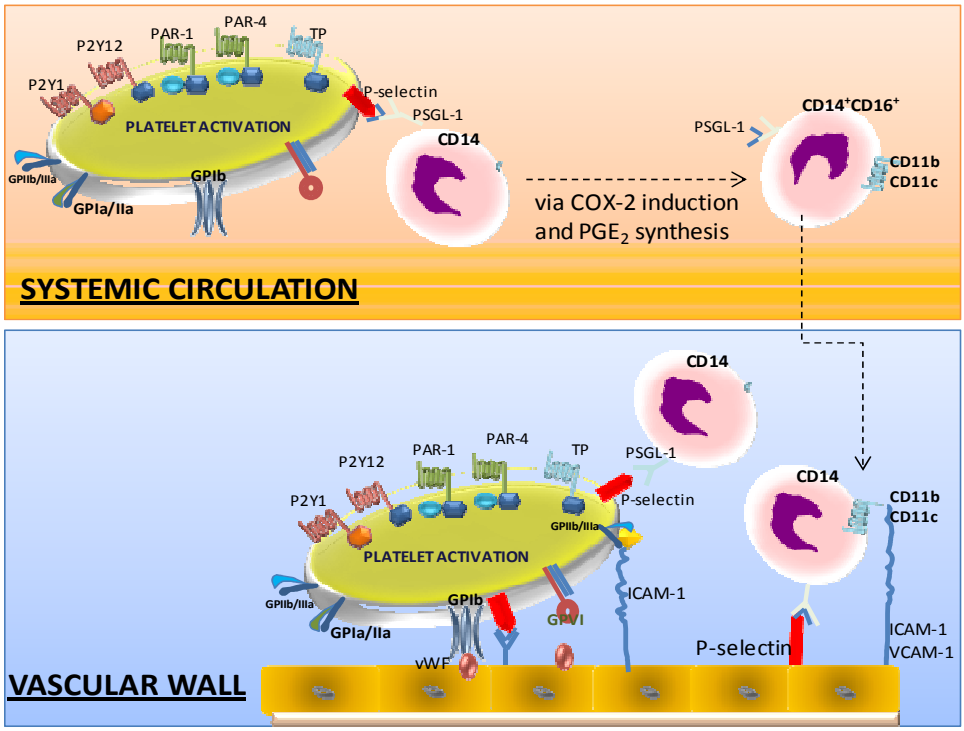

Figure 2.12 Pro-atherogenic effect of MPA formation. The diagram depicts the cellular interactions between platelets and endothelium, and between platelets and monocytes. Activated platelets interact with a dysfunctional endothelium through multiple receptor-ligand interactions. These involve adhesion molecules including vWF, PSGL-1 and ICAM-1 on endothelial cells that interact with platelet GPIb, P-selectin and GPIIb/IIIa (this latter through fibrinogen), as previously and extensively reviewed by van Gils (van Gils *et al.*, 2009). Adherent platelets act as a bridge between circulating monocytes and the arterial wall, platelet P-selectin on activated adherent platelets engaging PSGL-1 on circulating monocytes. The local formation of MPA promotes monocyte-endothelium interaction by inducing over-expression of adhesion molecules on both endothelial cells (Massberg *et al.*, 2002) and monocytes (da Costa *et al.*, 2006).

MPA formation in the peripheral circulation may also promote atherosclerosis through the induction of a $CD16^+$ phenotype on circulating monocytes. This is mediated through the P-selectin/PSGL-1 axis and consequent activation of COX-2 in monocytes, with synthesis of pro-inflammatory prostanoids such as PGE_2. $CD16^+$ cells are characterized by higher expression of adhesion molecules such as CD11b and CD11c compared with classical $CD14^+CD16^-$ monocytes, that facilitate and enhance monocyte adhesiveness to the vascular endothelium (Passacquale *et al.*, 2011c). In this way, activated platelets can stimulate

monocytic vascular infiltration through a mechanism independent of their bridging action at the site of a vascular lesion.

2.5.4 Conclusion

In conclusion, our findings shed new light on the relevance of platelet-monocyte interactions in the pathophysiology of inflammation. Of particular note, MPA formation induces a change in the phenotype/endothelial adhesiveness of monocytes that is easily detectable in the peripheral blood by measuring CD16 expression in circulating monocytes. Whether variation in degree of platelet activation and number of $CD16^+$ monocytes occurs in the context of atherosclerosis and are strictly related, and whether this phenomenon has pro-atherogenic implications remain to be clarified, and these aspects will be explored in Chapters 3 and 4.

The second noteworthy finding deriving from this study is that the series of biomolecular events that follow MPA formation, which result in a change in the biological function of monocytes, can be pharmacologically modulated. This carries potential therapeutic implications that will be discussed in details in Chapter 5.

Chapter Three

Expansion of the murine counterpart of human CD16^{+} monocytes in the peripheral blood reflects atherosclerosis progression in ApoE$^{-/-}$ mice

3.1. Introduction

3.1.1 ApoE$^{-/-}$ mice as an experimental model of atherosclerosis

Apolipoprotein E deficient (ApoE$^{-/-}$) mice were developed by two different laboratories (Piedrahita *et al.*, 1992; Plump *et al.*, 1992) as an experimental model of atherosclerosis. ApoE is a structural component of both very-low-density lipoproteins (VLDL) (Shore & Shore, 1973) and high-density lipoproteins (HDL) (Mahley, 1985), as well as a protein constituent of intestine-derived chylomicrons (Driscoll & Getz, 1986). It acts as a ligand for low-density lipoprotein (LDL) receptors expressed on cells, thus favouring the internalisation of lipoproteins and their removal from the peripheral circulation (Hui *et al.*, 1981). The scavenging action of ApoE for lipoproteins is therefore a crucial regulator of cholesterol metabolism, and lack of its expression in ApoE$^{-/-}$ mice gives rise to severe hypercholesterolemia and predisposes the animals, otherwise resistant to atherosclerotic disease (Paigen *et al.*, 1987), to fatty streak accumulation throughout the vascular tree (Piedrahita *et al.*, 1992).

An early study performed in ApoE$^{-/-}$ mice to evaluate atherosclerosis progression showed that foam cell lesions develop in the aortic sinus at 10 weeks of age in animals on rodent chow diet, to become fibrous plaques in mice aged 20 weeks. High fat diet accelerates the process (Reddick *et al.*, 1994), although this associates with extreme variability of lesion area (Meir & Leitersdorf, 2004). However, murine plaques generally fail to develop a perfectly formed fibrous cap and yet occurrence of thrombo-embolic events is unusual (Caligiuri *et al.*, 1999). Indeed, the histological appearance of a lesion, even in a more advanced stage of disease, is

typical of a xanthoma, constituted of fat-filled macrophages situated in the arterial intima (Seo *et al.*, 1997) that can reach such dimensions that it can cause vascular occlusion (although this is a rare event) (Caligiuri *et al.*, 1999). On the other hand in humans, the early atheroma generally consist of a well-defined fibrous cap overlying a necrotic core, and lesions often progress with evidence of erosion and healing of the fibrous cap, complicated by local thrombus formation with consequent distal embolisation, these events being responsible for much of the symptomatology and even death (Virmani *et al.*, 2000). However, it is worth mentioning that the majority of the histological findings described in ApoE$^{-/-}$ mice derive from studies of the aorta and aortic sinus. An extensive analysis of plaque distribution in ApoE$^{-/-}$ mice has revealed that different anatomic sites display distinct histological features (Seo *et al.*, 1997), and lesions seen within the brachiocephalic artery more closely resemble those seen in human atherosclerosis, due to the presence of an acellular necrotic core, erosion of the necrotic mass and intraplaque haemorrhage, features that are not observable in aortic lesions (Seo *et al.*, 1997; Rosenfeld *et al.*, 2000). Taken together, these considerations suggest that ApoE$^{-/-}$ mice offer a good, although not perfect, experimental model for the study of early human atherosclerosis, and provide mechanistic information mainly focused on the events that surround monocyte infiltration and lipid core formation within the vessels.

3.1.2 Monocyte characterization in ApoE$^{-/-}$ mice

Since their generation in 1992, a number of researchers have conducted studies in ApoE$^{-/-}$ mice to investigate the mechanisms involved in monocyte chemotaxis, recruitment and transmigration into the sub-endothelial space. In recent years,

following the discovery of monocyte heterogeneity, particular interest has been aroused in the identification of the distinct roles played by different monocytic subpopulations in the pathogenesis of plaque development. This area of research, however, has posed major difficulties of interpretation and comparison of data between different laboratories, mainly due to methodological issues related to the characterization of murine monocytes. Unlike humans, mice do not express a monocyte-specific marker, and the identification of this cell population is generally performed by using multiple "gating strategies" on flow cytometry based on a combination of antibodies that allow exclusion/inclusion of white cell populations according to their phenotype. For instance, the initial criteria to identify monocytes were by firstly their mononuclearity and secondly their myeloid nature. Indeed, monocytes have a low side-scatter plot profile in the flow cytometer (SSC^{low}), easily distinguishable from the high side scatter characteristic of neutrophils, and higher level of expression of CD11b ($CD11b^{high}$) (de Bruijn *et al.*, 1994; Lagasse & Weissman, 1996; Biermann *et al.*, 1999) compared to other mononuclear cells belonging to the lymphocyte class ($CD11b^{low}$ cells). Lagasse and Weissman also showed that absent or low expression of the epitope Gr-1, a myeloid differentiation antigen (Hestdal *et al.*, 1991), in $SSC^{low}CD11b^{high}$ cells is a discriminating criterion to distinguish monocytes from other white cell types (Lagasse & Weissman, 1996). Based on the finding that monocyte recruitment into lymphatic tissue is mediated by selective activation of receptors for chemokines (Palframan *et al.*, 2001), Geissmann first demonstrated the heterogeneity of circulating monocytes in mice (Geissmann *et al.*, 2003), by providing evidence that molecules involved in monocyte adhesion to endothelium (such as the fractalkine receptor CX3CR1) and chemokine receptors are differentially expressed on monocytes (as previously discussed in Chapter 1 and

summarized in Table 1.5). In accordance with Geissmann's results, Sunderkötter (Sunderkötter *et al.*, 2004) showed that murine monocytes can be differentiated into distinct populations based on differential expression of Ly-6C (ER-MP20, a monocyte/macrophage differentiation antigen). Moreover, he demonstrated that SSC^{low}CD11b^{high} are uniformly positive for the macrophage-colony stimulating growth factor (M-CSF) CD115, which has been proposed as a universal marker for mononuclear phagocytes (Hume *et al.*, 2002).

Recently, in an attempt to explore the extent of similarity between murine and human monocytes, Ingersoll (Ingersoll *et al.*, 2010) described a "gating strategy" for the characterization of murine monocytes in peripheral blood by flow cytometry, that defines the monocytic population as CD115^{+}F4/80^{low} cells (both of which are markers of monocytes-macrophages), and within this group distinguishes two cellular subsets based on differential expression of Ly6C. He reports that Ly6C^{high} are the counterpart of "classical" CD14^{+}CD16^{-} human cells, and Ly6C^{low} cells correspond to human pro-inflammatory CD14^{+}CD16^{+} monocytes.

3.2 Aims

The aims of the current work were twofold: we firstly wished to determine whether a change in the distribution pattern of circulating monocytes, similar to the expansion of pro-inflammatory CD14^{+}CD16^{+} cells observed in the clinical study of mild inflammation described in Chapter 2, is present in the context of atherosclerotic disease; and secondly, we wished to address *in vivo* the potential contribution of platelet activation in modulating the phenotype and, in turn, the pro-atherogenic

activity of circulating monocytes. ApoE$^{-/-}$ mice were used as an experimental model of atherosclerosis, and studied at different time points according to disease progression, either in the presence or absence of conventional anti-platelet treatments (aspirin and clopidogrel).

3.3 Methods

3.3.1 Mice and study design

Homozygous male ApoE$^{-/-}$ mice (B6.129P2–apoEtm1Unc/J) and wild-type C57BL/6J mice were purchased from Charles River Laboratories (Edinburgh, UK). In the first part of the study, aiming at establishing the relationship between blood monocyte phenotype and atherosclerosis progression, eight week-old ApoE$^{-/-}$ mice were switched to a Western high-fat diet (HFD) that contained 21% fat from lard and 0.15% (wt/wt) cholesterol (Special Diets Services, Witham, UK). ApoE$^{-/-}$ mice were sacrificed before (baseline) or at 4 or 8 weeks post-commencement of HFD. ApoE$^{+/+}$ mice (C57BL/6J) underwent the same procedures and were used as controls.

For the second part of the study, an additional group of ApoE$^{-/-}$ mice were kept on HFD for 8 weeks either in the absence (HFD alone) or in the presence of concomitant anti-platelet therapy: aspirin (high-dose, 300 mg/ kg/ day; or low-dose, 5 mg/ kg/day) or clopidogrel (25 mg/kg/day). The choice of drug concentrations administered in the current study is based on previously published work, in which these drug concentrations have been tested in mice, and provided evidence of consistent inhibitory effect on platelet activation when administrated at the above indicated concentrations (Cyrus *et al.,* 2002; Hoving *et al.,* 2010). Drugs were given

in drinking water. Due to the instability of aspirin in solution, water with aspirin added was replaced every other day (Cyrus *et al.*, 2002), whereas clopidogrel has demonstrated to be stable in solution up to 60 days (Skillman *et al.*, 2010), and therefore water containing clopidogrel was changed weekly.

The following parameters were examined: phenotype of circulating monocytes by flow cytometry; plaque burden and macrophage-dependent lipid content by Oil Red O staining of the brachiochephalic artery; phenotype of monocytes infiltrating the brachiochephalic artery by flow cytometry. The techniques are described in details in the following sections. All studies were performed in accordance with the guidelines of the United Kingdom Home Office (PPL 70/6646; PIL 70/20776).

3.3.2 Monocyte characterization in peripheral blood

Monocyte characterization was performed by whole blood flow cytometry as described by Ingersoll (Ingersoll *et al.*, 2010). Peripheral blood was drawn via cardiac puncture using syringes containing heparin as anticoagulant, from mice anaesthetized with isoflurane. Immediately after collection, 100 µl blood was incubated for 20 min at 4°C with the following antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD115, allophycocyanin (APC)-conjugated anti-mouse F4/80, phycoerythrin (PE)-conjugated anti-mouse Ly6C, peridinin chlorophyll protein complex (PerCP)-conjugated anti-mouse B220 (this latter was from BD Biosciences, UK, whereas all other antibodies were from eBiosciences, UK). Isotype control antibodies were used as negative control. Red blood cells were lysed at the end of the incubation time with FACS lysing solution (BD Bioscience),

and the resulting cell suspension was washed twice in PBS supplemented with 0.2% BSA and 0.1% sodium azide. Cells were finally resuspended in PBS containing 1% paraformaldehyde, and kept at 4°C until analyzed within a maximum of 48h from sample preparation.

At the flow cytometer (FACSCalibur, Becton Dickinson (BD), Oxford, UK) 50 000 events in total were acquired. Monocyte characterization was performed on a post-acquisition analysis conducted using FlowJo software (Tree Star, Ashland, OR). In accordance with Ingersoll's analysis strategy, the following steps were performed:

- gating of the SSC^{low} cells (with consequent exclusion of SSC^{high} neutrophils);
- exclusion of B220^{+} cells, representative of B lymphocytes
- identification of CD115^{+}F4/80^{low} cells as the monocyte population
- analysis of Ly6C expression within this population to distinguish the different monocytic subsets

Monocytes were therefore defined as SSC^{low} B220^{-} CD115^{+} F4/80^{low} cells. The number of cells expressing such a phenotype over the total 50 000 events acquired was taken as an estimation of monocyte count in the peripheral blood. Percentage and number of events with differential expression of Ly6C within this population identified the monocyte distribution pattern. A schematic representation of the method used for flow cytometry analysis is illustrated in Figure 3.1.

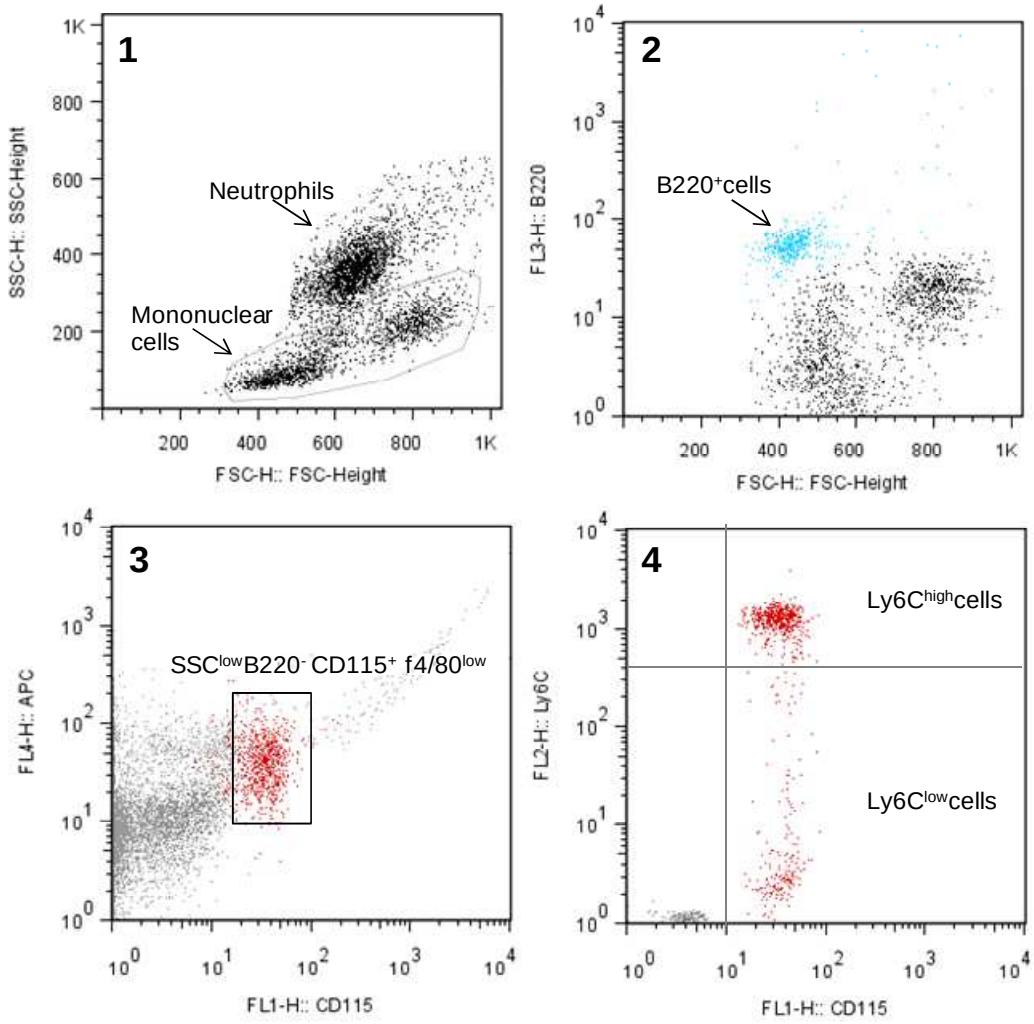

Figure 3.1 Monocyte characterization in murine blood: gating strategy A total of 50 000 events were acquired (plot 1), including mononuclear (monocytes and lymphocytes) and polymorphonuclear (neutrophils) cells. On a post-acquisition analysis, mononuclear cells were gated and the expression of B220 evaluated within this cell subgroup (plot 2). B220^{+} cells (in blue) were then excluded, and further analysis was performed on the population of B220^{-} cells (in black). Within the population of SSC^{low} B220^{-} cells the expression of CD115 and F4/80 was studied to identify CD115^{+}F4/80^{low} events (plot 3) representative of the total monocytic population (in red). Final analysis of level of expression of Ly6C within the SSC^{low} B220^{-} CD115^{+} F4/80^{low} cells (red) was carried out (plot 4) to calculate percentage and number of Ly6C^{high} and Ly6C^{low} monocytes (upper and lower right quadrant respectively). In grey is the isotype control for each combination of antibodies.

3.3.3 Measurement of *in vivo* platelet activation

Due to the requirement of four-color staining for monocyte characterization, and the availability of a four-colour flow cytometer (FACSCalibur, Becton & Dickinson, UK) for data acquisition, addition of a fifth antibody against a platelet marker (i.e CD42d), to identify MPA (CD42d-expressing monocytes) was not possible. In initial experiments, the specificity in detecting monocytes by different combinations of antibodies based on a 3-colour staining was tested in female ApoE$^{-/-}$ mice aged 8 weeks, and compared to the classical 4-colour strategy described by Ingersoll (Ingersoll *et al.*, 2010) and detailed above. We found that simultaneous detection of all 4 markers abovementioned (B220, F4/80, CD115 and Ly6C) is required for consistent interpretation. In particular, anti-mouse Ly6C was needed to distinguish the monocyte subpopulations. Exclusion of anti-mouse B220 from the antibody cocktail (which was then composed of anti-mouse F4/80, CD115 and Ly6C) gave rise to inclusion into the CD115^{+} F4/80^{+} gate of ~5% of B220^{+}cells (Figure 3.2). Therefore, we concluded that lack of detection of this cell type in the flow cytometric analysis could compromise the specificity for monocyte analysis, particularly taking into consideration that expansion of so-called plasmacytoid cells, which are B220^{+} Ly6C^{high} cells, has been described in the peripheral blood of ApoE$^{-/-}$ mice on HFD (Swirski *et al.*, 2007). In accordance with this, in our preliminary experiments to standardize the whole blood staining technique, the presence of this cell subtype was observable in samples collected from ApoE$^{-/-}$ (Figure 3.2) but not from wild-type mice (figure 3.1), although the two strains were not sex- and age-matched.

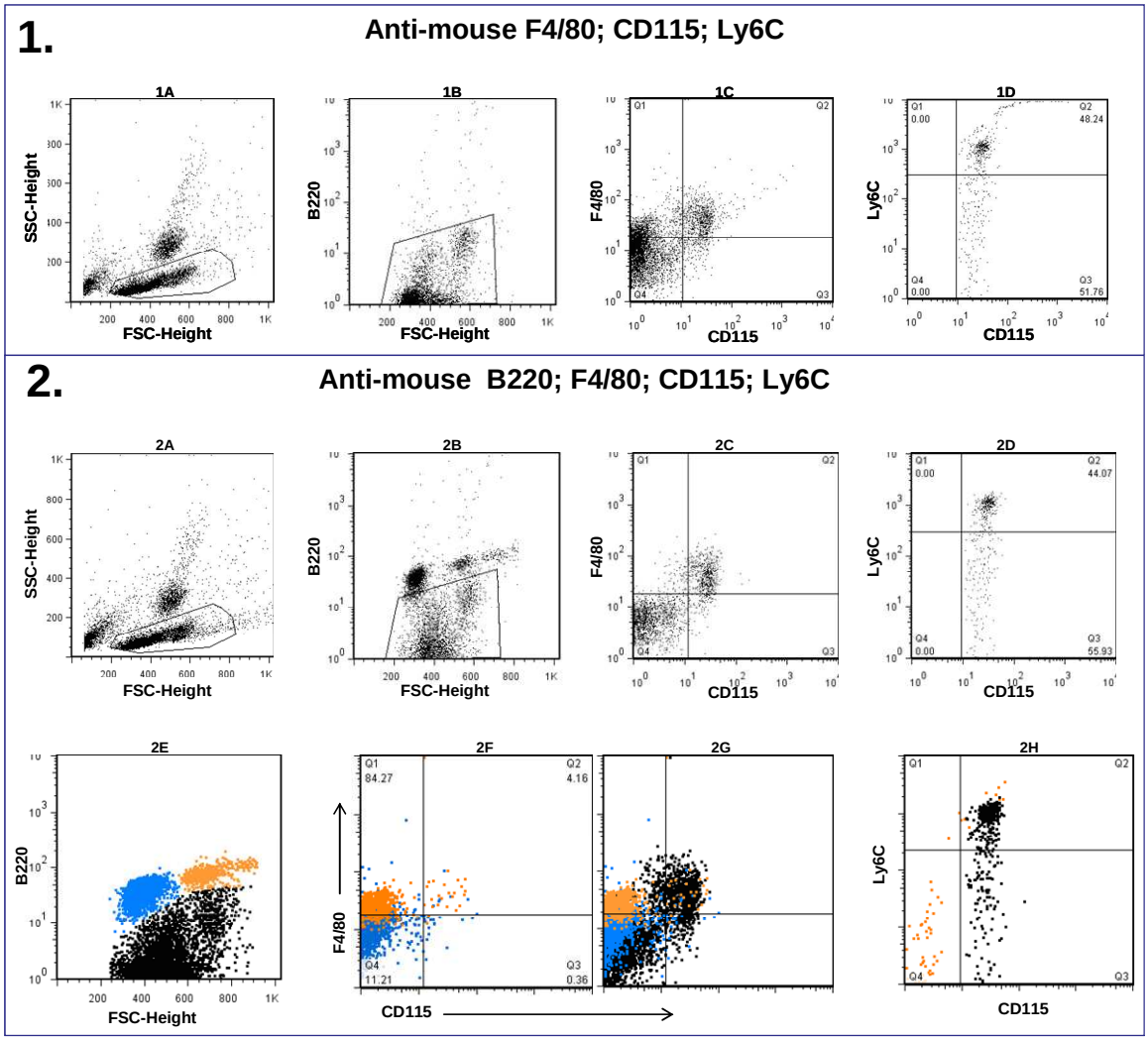

Figure 3.2 Standardization of murine whole blood flow cytometry for monocyte analysis. A preliminary experiment was conducted in female ApoE$^{-/-}$ mice to compare the specificity of two different antibody cocktails in identifying monocytes by flow cytometry. The 4-colour staining method described by Ingersoll (Ingersoll *et al.*, 2010) using anti-mouse B220, F4/80, CD115 and Ly6C (panel 2) was compared to 3-colour staining based on F4/80, CD115 and Ly6C detection only (panel 1). Acquisition was gated for mononuclear cells (gate on 1A and 2A). Presence of anti-mouse B220 in the antibody cocktail enabled the identification of a population of cells (2B) that included two different subtypes (in blue and orange in the lower plots of pnel 2). The blue cell subset was B220^{+} F4/80^{-} CD115^{-} (2F); the orange cells were B220^{+} F4/80^{low} and ~5% of them were positive for CD115 (2F). The lower

plots of panel 2 show the overlay of these two B220^{+} cells over the population of B220^{-} cells (black). This latter contain the monocytic population defined by double positivity for F4/80 and CD115 (2C and 2G). Absence of anti-mouse anti-B220 in the antibody cocktail (panel 1) led to inclusion of B220^{+}F4/80^{low}CD115^{+} cells in the monocyte count, resulting in overestimation of Ly6C^{high} number compared to the value measured in the presence of anti-mouse B220 (48.24% in 1D versus 44.07% in 2D).

In evaluating the need for the two myeloid markers F4/80 and CD115, we observed that F4/80 staining alone, in combination with anti-mouse B220 and Ly6C only, made it difficult to distinguish positive from negative cells due to the low level of expression of this marker on circulating monocytes. Addition of anti-mouse CD115 antibody was the optimal combination to distinguish monocytes from the non-monocytic population by plotting F4/80 against CD115 (Figure 3.3), and enabled the identification of a few events that were F4/80^{+} but CD115^{-}, most likely representing contaminating plasmacytoid cells (Figure 3.3).

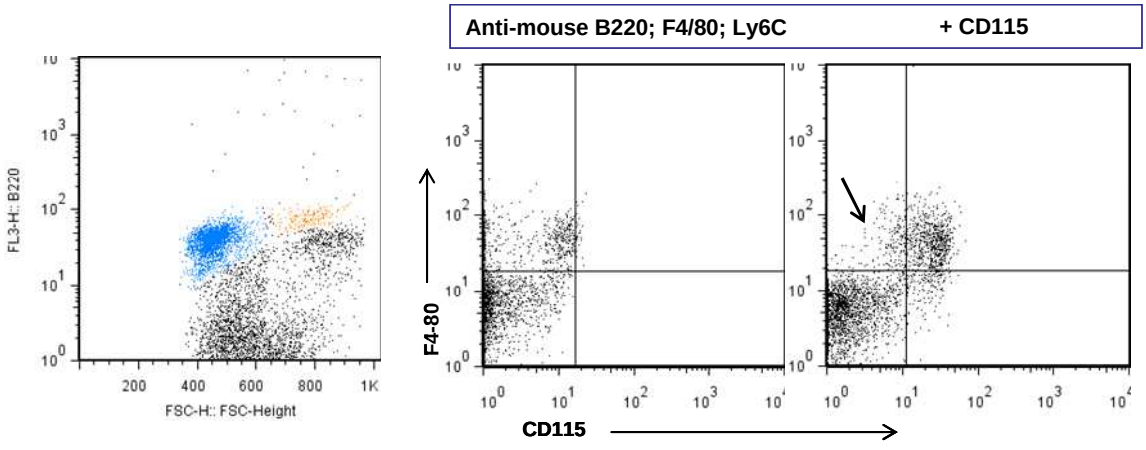

Figure 3.3. Standardization of murine whole blood flow cytometry for monocyte analysis. Addition of anti-mouse CD115 to the antibody cocktail containing anti-mouse B220, FL4/80 and Ly6C enabled the identification of a small subset of F4/80^{+} cells that were

CD115^{-} (arrow). In the blood of ApoE$^{-/-}$mice, where plasmacytoid (orange dots) and monocytic cells (within the black population) expand (Swirki *et al.,* 2007) and are in close proximity to each other (**A**), contamination from plasmacytoid cells is likely to affect the purity of the B220^{-} gate. The shift of F4/80^{+} cells along the CD115 axis (**B** and **C**) according to cell positivity for this marker facilitates the identification of monocytes (defined as B220^{-} F4/80^{low} CD115^{+}) in murine blood (upper right quadrant of **C**).

As regards the staining for CD115 in the absence of anti-F4/80 and in the presence of anti-mouse B220 and Ly6C, this gave good correspondence in the identification of monocytes compared to complete 4-colour staining. However, since it has been demonstrated that stability of CD115 staining is affected by any time delay in blood processing (Breslin *et al.,* 2011) and, more importantly, that CD115 can undergo cleavage from the extracellular membrane during inflammatory processes (Ziegler-Heitbrock *et al.,* 2010), we decided not to rely on CD115 staining alone, in the absence of an additional myeloid marker, due to the need to compare animals at different stages of atherosclerotic disease, and consequent potential differences in the level of expression of CD115. Additionally, given the phenotypic correspondence between human and murine monocytes based on Ingersoll's gating strategy, we considered that translatability of our results to the human setting would be greatest from our animal study by using his methodological approach.

Therefore, in the present experiments, the degree of *in vivo* platelet activation was defined as the percentage of P-selectin expressing platelets in the peripheral blood only. For this purpose, heparinised blood was stained with FITC-conjugated anti-mouse CD42d (a constitutive platelet marker) and PE- conjugated anti-mouse

CD62P (P-selectin), for 20 min at 4°C. After red cell lysis with FACS lysing solution (BD Bioscience) and washing in PBS supplemented with 0.2% BSA and 0.1% sodium azide, cells were resuspended in PBS containing 1% paraformaldehyde, and kept at 4°C until analyzed within a maximum of 48h from sample preparation. At the flow cytometer a total of 50 000 events was acquired on logarithmic forward- and side-scatter scales and post-acquisition analysis of P-selectin expression within the CD42d-positive gate (% of CD42d^{+}CD62P^{+} events) was performed using FlowJo software (Tree Star, Ashland, OR).

3.3.4 Tissue harvesting

For histological analyses and phenotypic characterization of monocytes infiltrating atherosclerotic tissue, immediately after blood collection for the flow cytometry studies as described above, mice were perfused through the left ventricle with physiological saline containing heparin 10U/L for 10 min, following which they were culled with a ketamine/xylazine overdose. The brachiocephalic artery together with the right subclavian artery, aortic arch and left carotid artery was removed en bloc and immediately transferred into 4% formaldehyde before undergoing Oil Red O staining. For monocyte characterization, the brachiocephalic artery only was collected and processed for flow cytometry analysis as described below.

3.3.5 Oil Red O staining

Red Oil powder (Sigma, UK) was resuspended in pure methanol at a concentration of 5%. The working solution for staining was prepared in water at a final concentration of 0.5%. Vessels collected as described above were opened to expose the intraluminal part and were incubated in the Oil Red O (ORO) staining solution for 10min. After subsequent washing firstly in pure methanol and secondly in water, they were analysed by dissection microscopy. Pictures were captured at 2.5x and 3.5x magnification and analysed using ImageJ software for total and (ORO)-positive plaque area measurement.

3.3.6 Characterization of monocytes infiltrating the brachiocephalic artery

Immediately after collection, the brachiocephalic artery was microdissected and digested at 37°C for 1 h, in an enzymatic cocktail containing 125U/ml collagenase type XI, 60 U/ml hyaluronidase type I-s, 60 U/ml DNase 1 and 450 U/ml collagenase type 1 (all enzymes were from Sigma-Aldrich), in PBS supplemented with 20 mM Hepes (Galkina *et al.*, 2006). The artery was then mashed through a 70 μm strainer to obtain a cell suspension. This latter was resuspended in PBS supplemented with 0.2% BSA, and incubated at 4°C for 20 min with a combination of the following antibodies: Per-CP anti-mouse lymphocyte markers (Lin) including B220, CD90, Ly6G, NK1.1; APC anti-mouse F4-80; FITC anti-mouse CD115; PE anti-mouse Ly6C or CD11b (all antibodies were from eBiosciences, apart from anti-mouse B220 which was purchased from Becton & Dickinson). A total of 50 000 events was acquired and, on post-acquisition analysis performed by using FlowJo

software (Tree Star, Ashland, OR), monocytes were identified as CD11b^{high} F4-80^{+} CD115^{+} Lin^{-} [B220, CD90, Ly6G, NK1.1] and distinguished into Ly6C^{high} and Ly6C^{low} subsets (Figure 3.4). Monocyte counts were normalised to sample weight. Figure 3.4 illustrates the flow cytometry analysis strategy.

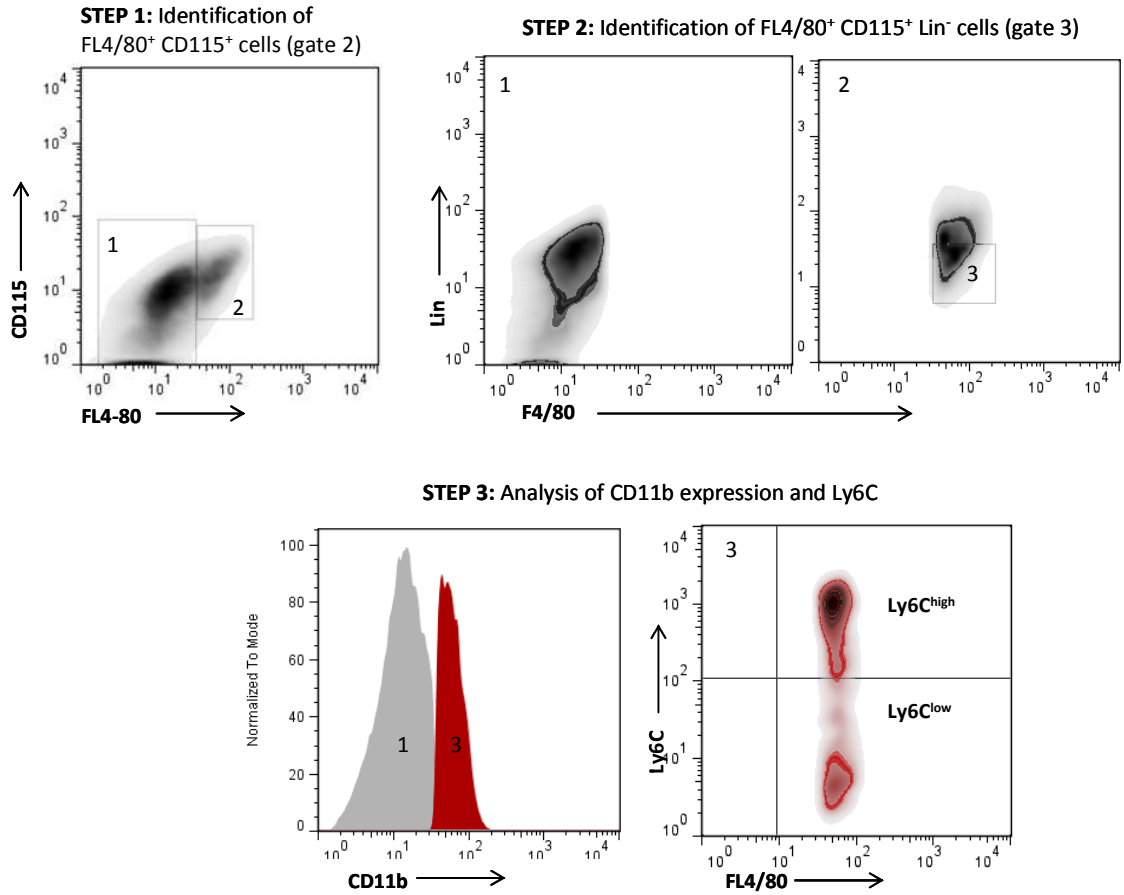

Figure 3.4 Monocyte characterization in the brachiocephalic artery. Monocytes were defined as CD115^{+} F4-80^{+} CD11b^{high} Lin^{-} cells. The figure schematises the "gating strategy" used to identify cells. Step 1: a first distinction of double positive cells (gate 2) from those negative (gate 1) for the myeloid markers F4-80 and CD115. Step 2: by plotting F4-80 against Lin markers, exclusion of Lin^{+} cells from gate 2 was performed and monocytes were identified as F4-80^{+} CD115^{+} Lin^{-} cells (gate 3 on right hand plot). Analysis of cells in gate 1 confirmed positivity for Lin markers and negativity for myeloid F4-80 (left hand plot). Step 3: analysis of CD11b expression in both gate 1 (grey histogram, non monocytic cells) and gate 3 (red histogram, monocytes) confirmed the myeloid nature of cells identified as monocytes (red). Monocytes were further characterized by differential expression of Ly6C into Ly6C^{high} and Ly6C^{low}.

3.3.7 Statistical analysis

All data are presented as mean ± SD. Statistical analysis was performed using GraphPad Prism 4 software. Differences in percentage and total number of different monocytic subsets between ApoE$^{-/-}$ and C57BL/6J mice were evaluated by paired Student's *t* test. Comparison of monocyte characterization at the different time points was conducted by ANOVA, with or without repeated measures as appropriate. In all cases, $p<0.05$ (two-tailed) was taken to indicate statistical significance.

3.4 Results

3.4.1 Ly6C^{high} monocytes predominate in the peripheral blood of ApoE$^{-/-}$ mice at baseline

A preliminary analysis was performed to compare monocyte count and phenotype in ApoE$^{-/-}$ mice with control C57BL/6J animals at baseline. For this purpose, four mice per group (aged 8 weeks, prior to initiation of HFD) were sacrificed and monocyte characterization was performed as described above.

The number of monocytes circulating in the peripheral blood was similar between the two strains (1926.5 ± 178.8 and 1862.7 ± 612.8 per 50 000 total events in ApoE$^{-/-}$ and C57BL/6J respectively; p=ns). However, ApoE$^{-/-}$ mice showed a predominance of Ly6C^{high} over Ly6C^{low} cells (93.0 ± 4.7% and 7.0 ± 4.7% respectively of total monocytes; p<0.001), whereas C57BL/6J mice demonstrated similar proportions of Ly6C^{high} and Ly6C^{low} monocytes (45.9 ± 10.6% and 54.0 ± 10.6% respectively; p=ns). Consequently, the absolute number of circulating Ly6C^{high} cells was higher in ApoE$^{-/-}$ than control mice, and conversely absolute Ly6C^{low} monocyte numbers were lower in ApoE$^{-/-}$ than C57BL/6J mice (Figure 3.5).

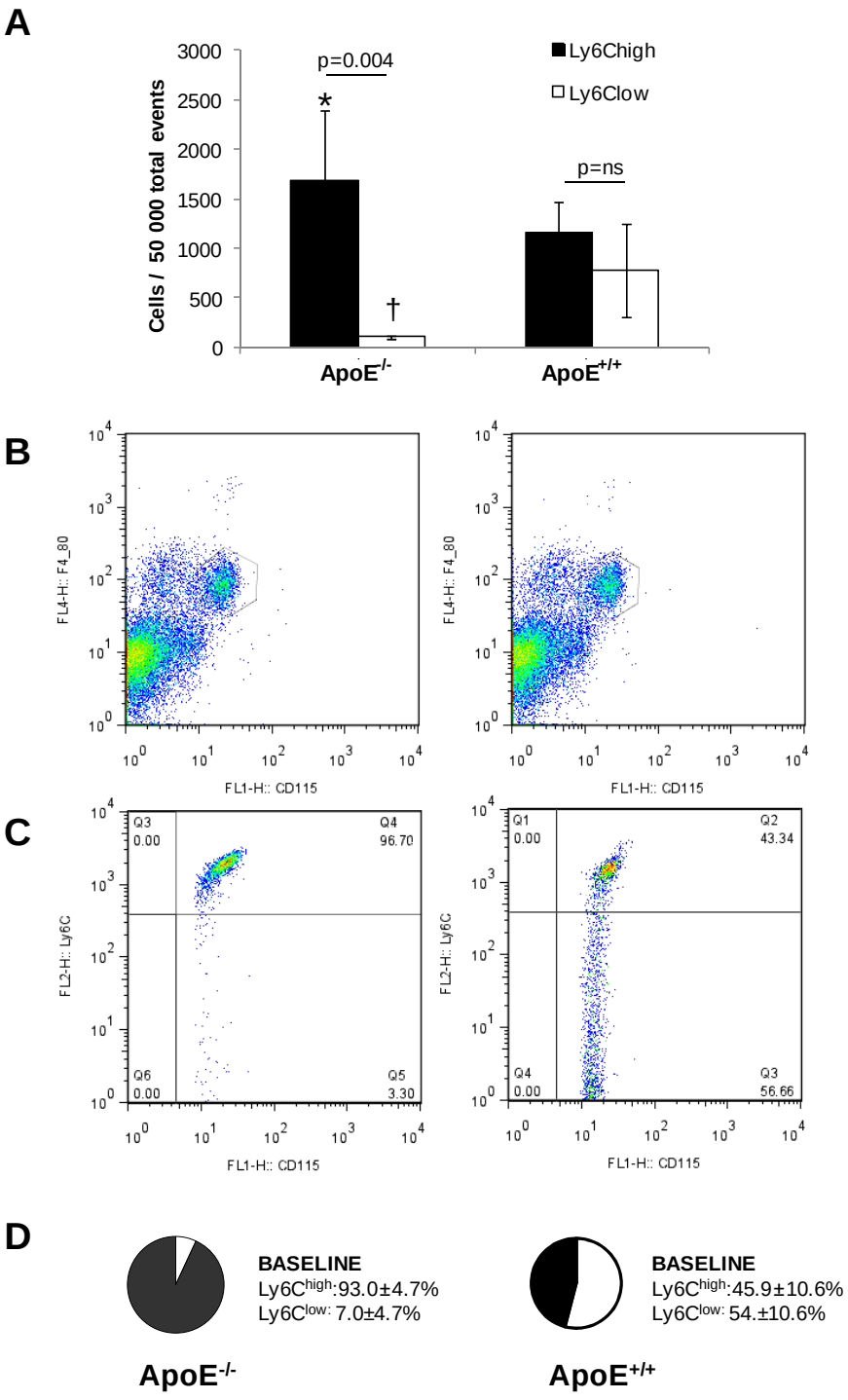

Figure 3.5 Monocyte characterization in ApoE$^{-/-}$ and C57BL/6J mice at baseline (before commencing HFD) (**A**) Absolute number of Ly6C^{high} (black bars) and Ly6C^{low} (white bars) in ApoE$^{-/-}$ and C57BL/6J (ApoE$^{+/+}$) mice; *p=0.02 vs ApoE$^{+/+}$ mice; † p=0.03 vs ApoE$^{+/+}$ mice; (**B**) Representative flow cytometry dot plots showing SSC^{low}B220^{-}CD115^{+}F4/80^{low} cells in ApoE$^{-/-}$ (left) and ApoE$^{+/+}$ (right) mice; (**C**) Representative flow cytometry dot plots showing distribution pattern of Ly6C^{high} and Ly6C^{low} cells in ApoE$^{-/-}$ (left) and ApoE$^{+/+}$

(right) mice; (**D**) Monocyte characterization in ApoE$^{-/-}$ (left) and ApoE$^{+/+}$ (right) mice aged 8 weeks; the pie charts show accumulated data from 8 animals. Black= Ly6C^{high}; white= Ly6C^{low}.

3.4.2 Monocyte count increases with age in ApoE$^{-/-}$ mice

ApoE$^{-/-}$ mice studied at 4 and 8 weeks post-commencement of HFD exhibited a time-dependent increase in the total number of circulating monocytes. As a consequence, the absolute number of both Ly6C^{high} and Ly6C^{low} cells circulating in the peripheral blood increased over the 8-week period of HFD, with the increase being especially marked in the Ly6C^{low} population (Figure 3.6).

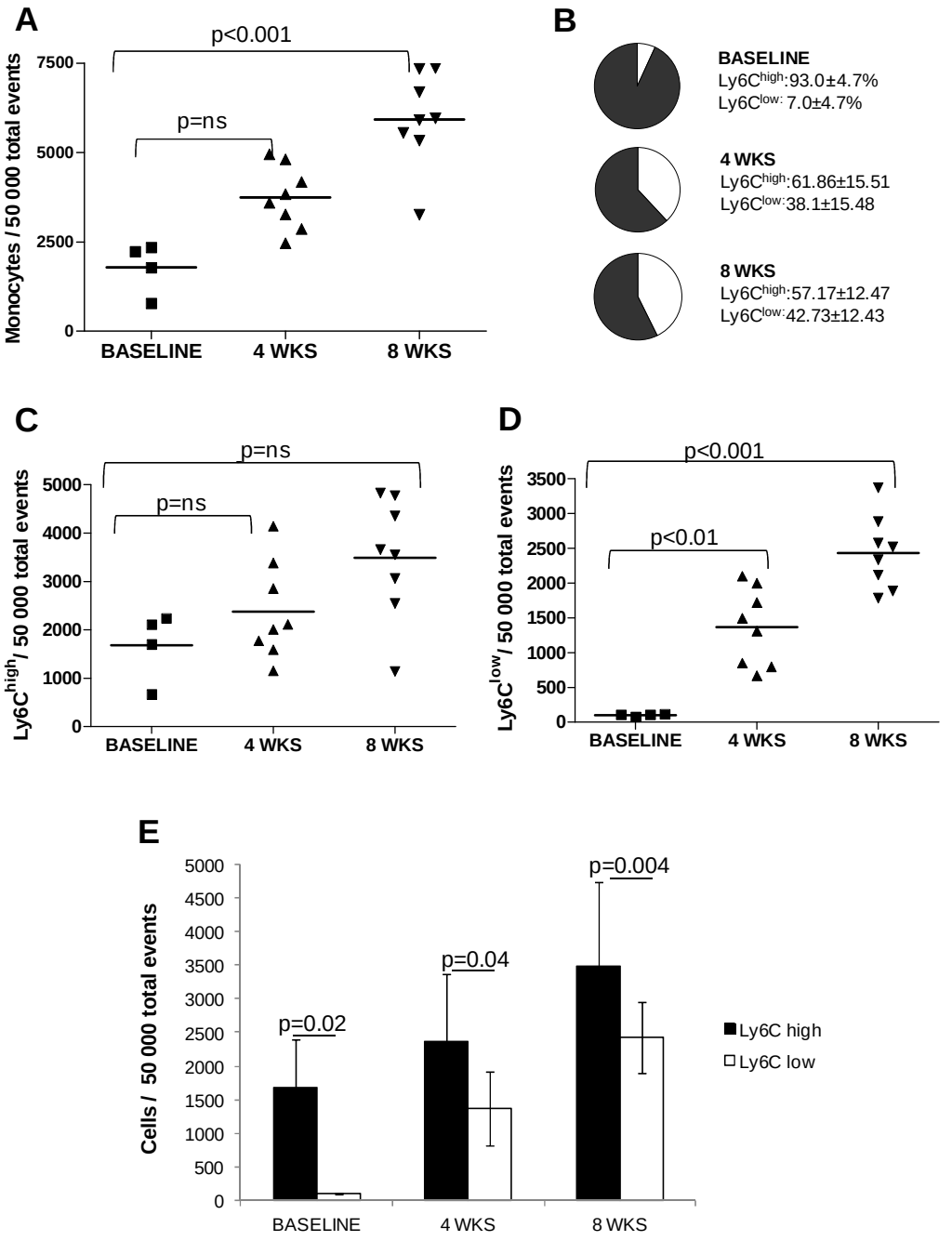

Figure 3.6 Monocyte characterization in ApoE$^{-/-}$ mice after different times on HFD
Monocyte count (**A**) and distribution pattern of Ly6C^{high} (black) and Ly6C^{low} (white) (**B**) in the peripheral blood of ApoE$^{-/-}$ mice at baseline and after 4 and 8 weeks of HFD. Absolute number of Ly6C^{high} (**C**) and Ly6C^{low} monocytes (**D**) at the different time points. Panel **E** compares Ly6C^{high} and Ly6C^{low} numbers directly at the different time points.

3.4.3 Measurement of Ly6C^{low} is a surrogate marker of extent of atherosclerosis

In order to ascertain the association between circulating monocyte count/phenotype and disease progression in ApoE$^{-/-}$ mice, we harvested the brachiochepalic artery to analyze plaque burden by Oil Red O staining at the same time points as circulating monocytes were characterized: baseline (n=4, Group 1) and after 4 (n=4, Group 2) and 8 weeks (n=4, Group 3) of HFD.

As expected, atherosclerotic lesions were observable in mice fed with HFD for 4 and 8 weeks, but not at baseline. The extent of disease measured as lesion area was highly variable within each group; medians and inter-quartile ranges were 0.05 (0.02-0.43) mm^2 and 0.20 (0.08-0.42) mm^2 in Groups 2 and Group 3 respectively (p= ns comparing Groups 1 and 2) (Figure 3.7).

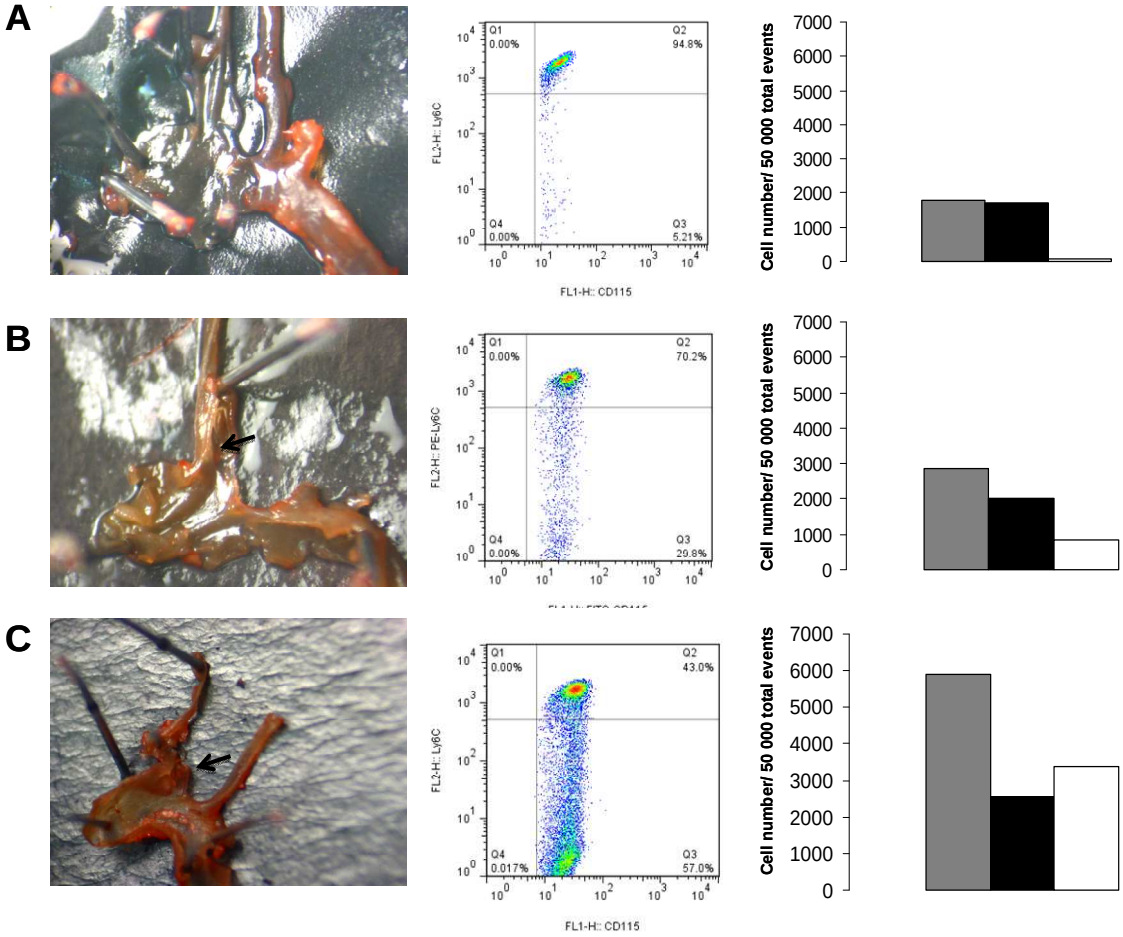

Figure 3.7 Atherosclerosis development in ApoE$^{-/-}$ mice. Pictures are representative of Oil Red O staining of the brachiocephalic artery of ApoE$^{-/-}$ mice at baseline (**A**), and after 4 (**B**) and 8 weeks (**C**) from commencing HFD (3.5x magnification). The arrows indicate plaques. Corresponding flow cytometry dot plots illustrating monocyte characterization in these 3 animals are also shown, along with data showing total monocyte count (grey bars), Ly6C^{high} (black bars) and Ly6C^{low} (white bars) in these same animals.

In accordance with the results described above, the monocyte analyses performed in the three animals shown in Figure 3.7 demonstrated progressive monocytosis and increase in the absolute number of both Ly6C^{high} and Ly6C^{low} cells after 4 and 8 weeks of HFD compared to baseline (Figure 3.8). A shift was also observed in the distribution pattern of monocyte subsets in the blood, with an increase in the

percentage of Ly6C^{low} and simultaneous decrease in the percentage of Ly6C^{high} cells compared to the predominance of Ly6C^{high} monocytes at baseline. However, as described above for extent of plaque development, great variability was seen within each group of animals on HFD, and comparison between Group 2 and Group 3 therefore showed no significant difference in terms of percentage of Ly6C^{high} (65.07 ± 12.8% vs 59.95 ± 11.3% respectively; p=ns) or of Ly6C^{low} (34.87 ±12.81% vs 40.05 ± 11.3% respectively; p=ns) cells. Absolute numbers were also not different between Groups 2 and 3 (Ly6C^{high}: 2317 ± 723.2 vs 3844 ± 990.3 per 50 000 total events respectively, p=ns; Ly6C^{low}: 1216 ± 461.9 vs 2528 ± 620.6 per 50 000 total events respectively, p=ns).

On the other hand, in examining the relationship between lesion area and circulating monocyte characteristics, a direct correlation was found between the lesion area and both percentage and absolute number of Ly6C^{low} cells. By contrast, no relationship was observed between lesion area and either total monocyte count or Ly6C^{high} monocyte count (Figure 3.8).

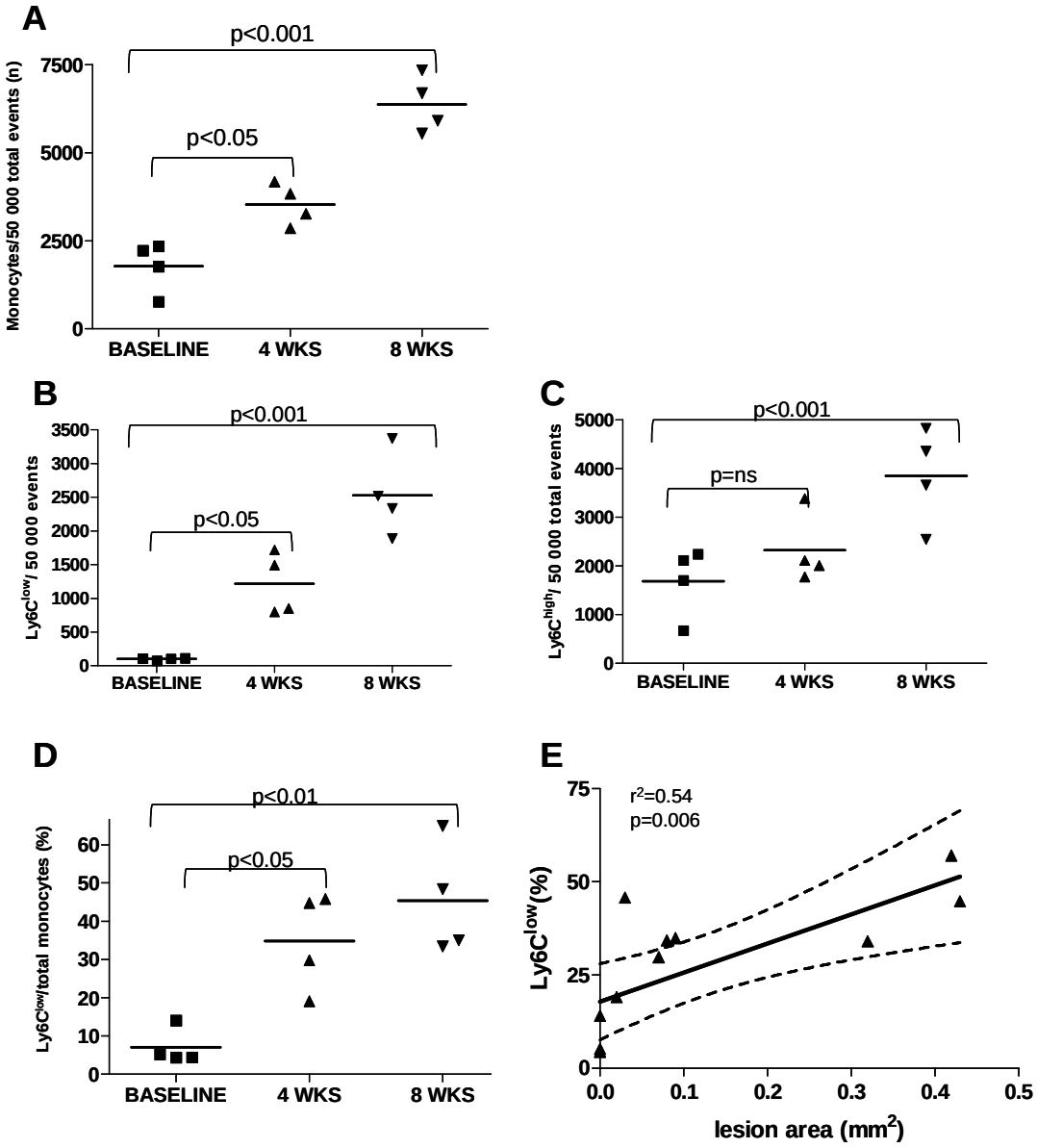

Figure 3.8 Monocyte characterization and relationship to plaque burden in ApoE$^{-/-}$ mice. Monocyte count (**A**) in the peripheral blood of ApoE$^{-/-}$ mice on HFD (n=12 in total) in which atherosclerosis was evaluated by means of Oil Red O staining of the brachiocephalic artery at different time points as shown. Absolute number of circulating Ly6C^{low} (**B**) and Ly6C^{high} (**C**) at the same time points. Panel **D** shows % of monocytes which are Ly6C^{low} at these same time points. Panel **E** illustrates the correlation between lesion area and percentage of Ly6C^{low} cells measured in the peripheral circulation.

We performed similar experiments in parallel in C57BL/6J mice, as controls for the ApoE$^{-/-}$ mice that do not develop atherosclerosis on HFD.

As described above, C57BL/6J displayed similar numbers of circulating monocytes to ApoE$^{-/-}$ mice at baseline (i.e before commencing HFD). The two subsets of monocytes were equally represented in the blood, with Ly6C^{high} and Ly6C^{low} accounting for 45.90 ± 10.56% and 54.00 ± 10.57 % respectively of total monocytes, whereas Ly6C^{high} cells were predominant in ApoE$^{-/-}$ mice at baseline (Figure 3.9).

In order to ascertain the effect of Western diet on monocyte count/phenotype independently of the presence of atherosclerosis, we studied wild-type mice after 4 and 8 weeks of the HFD regime. As expected, C57BL/6J did not develop atherosclerosis over the 8 week period of the study. However, the phenotype of circulating monocytes progressively changed to acquire, after 8 weeks of HFD, a pattern similar to that observed in ApoE$^{-/-}$ mice at baseline (Figure 3.6). Indeed, HFD induced a progressive increase in both the percentage and absolute number of Ly6C^{high} cells, with concomitant decreases in both the percentage and count of Ly6C^{low} monocytes. A simultaneous expansion of the total monocytic population was observed, with the degree of monocytosis being similar to that seen in ApoE$^{-/-}$ mice by the end of the study, whilst – unlike in ApoE$^{-/-}$ mice – the vast majority of these comprised Ly6C^{high} cells by 8 weeks of HFD (Figure 3.9).

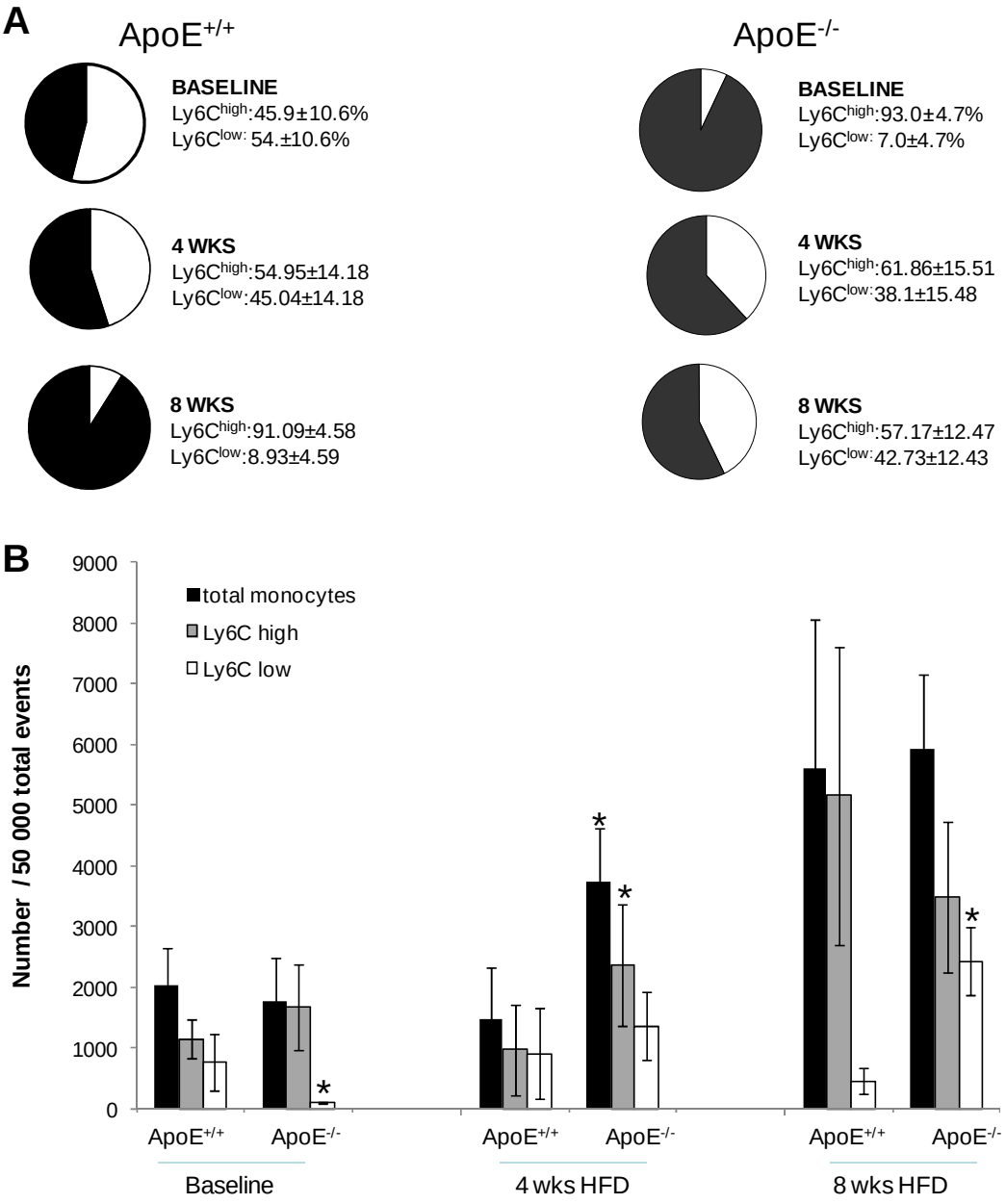

Figure 3.9 Effect of HFD on total monocyte count and Ly6C^{high} vs Ly6C^{low} distribution pattern in wild-type (ApoE$^{+/+}$) compared with ApoE$^{-/-}$ mice. (A), The pie charts show the distribution pattern of Ly6C^{high} (black) and Ly6C^{low} (white) over the 8 week-period of HFD in both strains. (**B**), Total monocyte count (black bars), Ly6C^{high} (grey bars) and Ly6C^{low} (white bars) count in wild-type and ApoE$^{-/-}$ mice at the same time points. *p < 0.05 vs ApoE$^{+/+}$ mice.

3.4.4 Anti-platelet therapy abolishes atherosclerosis-related blood monocytosis

Atherosclerosis-related monocytosis observed in ApoE$^{-/-}$ mice at the end of the 8-week HFD regime was significantly reduced by the concomitant treatment with either clopidogrel or aspirin; in the case of aspirin, efficacy was similar when administered at high- (300 mg/kg/day) or low-dose (5 mg/kg/day) (Figure 3.10). All these anti-platelet regimes reduced the absolute number of both Ly6C^{high} and Ly6C^{low} cells in the peripheral blood to a level that was comparable to that seen in ApoE$^{-/-}$ mice before commencing HFD. Moreover, the distribution pattern of the different monocytic subsets also was modulated by the anti-platelet drugs, inasmuch as the progressive increase in percentage of Ly6C^{low} over Ly6C^{high} cells was attenuated, although not to baseline levels (Figure 3.10). Measurement of P-selectin-expressing platelets, as a marker of *in vivo* platelet activation, demonstrated that both aspirin and clopidogrel inhibit platelet P-selectin expression in ApoE$^{-/-}$ mice on HFD, and the efficacy of clopidogrel in reducing P-selectin positive platelets was superior to that of aspirin at either dose (Figure 3.10).

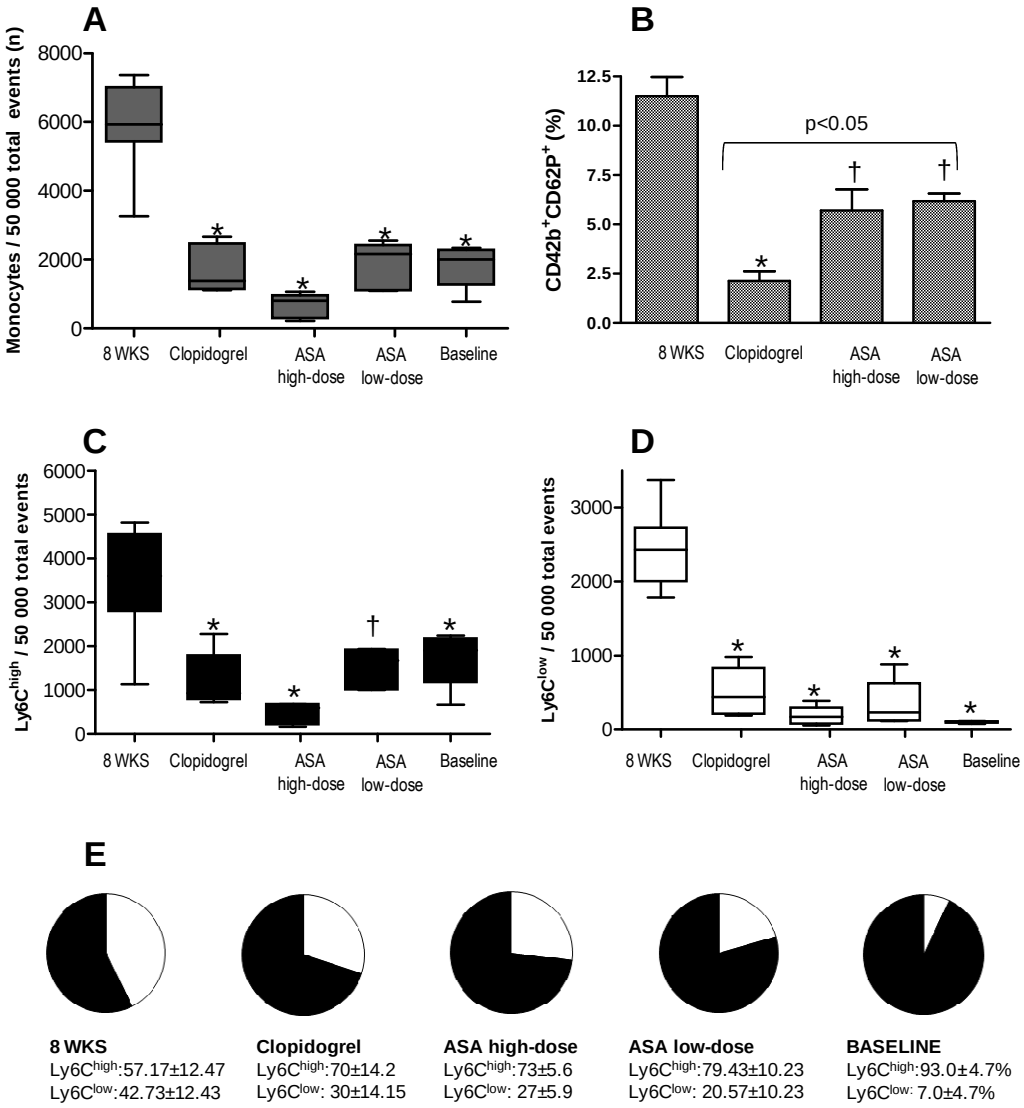

Figure 3.10 Effect of aspirin and clopidogrel on circulating monocytes. Total monocyte count (**A**); percentage of P-selectin expressing platelets (**B**); absolute number of Ly6C^{high} (**C**) and Ly6C^{low} cells (**D**) in the peripheral blood of ApoE$^{-/-}$ mice at the end of the 8 week HFD-period, either in the absence (8 WKS) or in the presence of concomitant anti-platelet treatment with aspirin or clopidogrel as specified in the graphs. Aspirin was administrated at high (300 mg / kg /d) and low (5 mg / kg /d) doses. Clopidogrel was given at a dose of 25 mg /kg /d. Level of monocytes in the peripheral blood of ApoE$^{-/-}$ mice before commencing HFD (baseline) is also shown. The pie charts (**E**) show the distribution pattern of Ly6C^{high} (black) and Ly6C^{low} (white) in the different experimental conditions/time points. *, $p<0.001$ vs 8 WKS; †, $p<0.01$ vs 8 WKS.

3.4.5 Aspirin and clopidogrel do not inhibit plaque development but differentially regulate monocyte and lipid composition of plaques

Neither clopidogrel nor aspirin inhibited atherosclerotic plaque development in the brachiocephalic artery of ApoE$^{-/-}$ mice on HFD. Lesions were observable in both treated and untreated animals at the end of the 8 week HFD-period, and the total plaque area was similar between all groups (Figure 3.11). However, the atherosclerotic brachiocephalic artery of mice on aspirin- but not clopidogrel - therapy displayed a reduction in the macrophage-dependent lipid content of plaque, as evaluated by ORO-positive plaque area (Figure 3.11). On further characterization of plaque composition performed by tissue flow cytometry analysis to specifically assess monocyte content, the brachiocephalic arteries obtained from ApoE$^{-/-}$ mice treated with aspirin- but not clopidogrel - exibited less total monocyte infiltration compared to untreated animals. Moreover, the phenotype of monocytes within plaques was different between the groups. ApoE$^{-/-}$ mice on HFD only had the highest degree of monocyte plaque infiltration, to which the two subpopulations almost equally contributed, with a slight predominance of Ly6C^{low} over Ly6C^{high} cells. Aspirin-treated animals developed plaques with a reduced content of total monocytes compared to untreated animals, this being mainly attributable to a suppressed recruitment of Ly6C^{high} cells into the lesions. By contrast, clopidogrel did not alter total monocytic infiltration into the plaques, but did significantly reduce the percentage of Ly6Clow monocytes (46.94±6.72% in clopidogrel-treated animals vs 55.21±4.4% in untreated animals; $p<0.05$) (Figure 3.11).

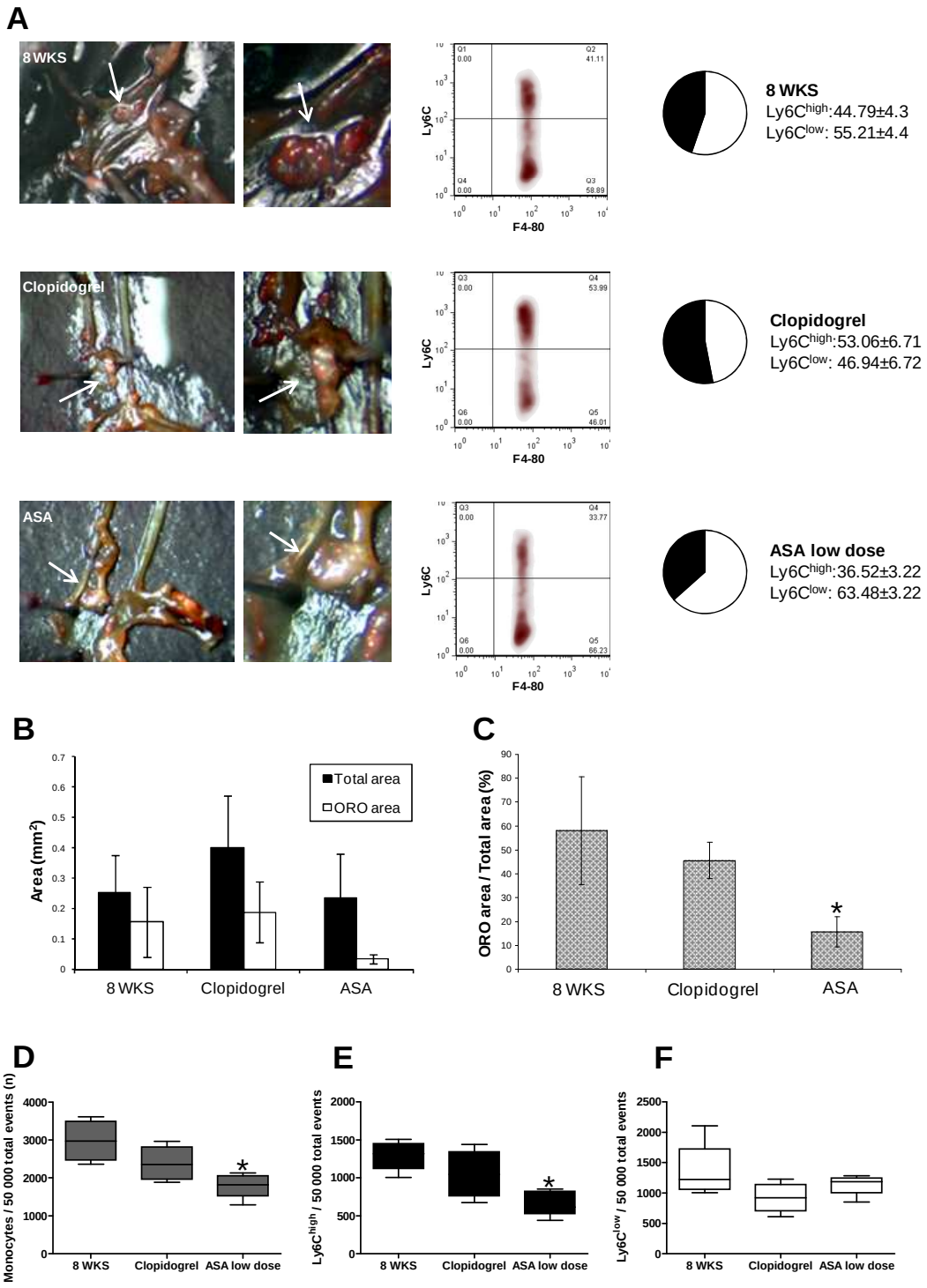

Figure 3.11 Effect of aspirin and clopidogrel on plaque development and composition. (**A**), Pictures are representative of Oil Red O staining of the brachiocephalic artery of ApoE$^{-/-}$ mice at the end of 8-week HFD-period in untreated animals (8 WKS) and those treated with either clopidogrel (25 mg/kg/d) or aspirin (low dose, 5 mg/kg/d) as indicated (3.5x magnification, pictures on the left hand side). For better visualization, images were zoomed

and cropped at the level of plaques (pictures on right hand side). The flow cytometry dot plots are representative of monocyte characterization in the brachiocephalic artery of each group of animals (upper right quadrant and lower right quadrant show Ly6C^{high} and Ly6C^{low} respectively within plaques). Accumulated data (8 WKS n=4; clopidogrel n=4; and aspirin n=5) are shown in the pie charts, which show percentage of Ly6C^{high} (black) and Ly6C^{low} cells measured in the brachiocephalic artery of each group of animals. (**B**), Total (black bars) and Oil Red O (ORO) positive area (white bars) of plaques in the different experimental conditions. (**C**), ORO positive area expressed as percentage of total plaque area in the different groups of animals. (**D**), (**E**), and (**F**) report the absolute numbers of total, Ly6C^{high} and Ly6C^{low} monocytes respectively in untreated animals (8 WKS) and those treated with either clopidogrel (25 mg/kg/d) or aspirin (low dose, 5 mg/kg/d). *, $p<0.05$ vs 8 WKS.

3.5 Discussion

3.5.1 Monocyte characterization as a biomarker of atherosclerotic disease

Our results show that expansion of circulating monocyte numbers occurs in the peripheral blood of both wild-type and ApoE$^{-/-}$ mice on HFD with age. In accordance with previous reports (Swirski *et al.*, 2007) predominance of Ly6C^{high} over Ly6C^{low} cells was observed at baseline in ApoE$^{-/-}$ but not in wild-type mice. However, the major expansion with time was in Ly6C^{low} cells in ApoE$^{-/-}$ mice; and Ly6C^{low} numbers, but neither Ly6C^{high} numbers nor total monocyte count, were predictive of extent of atherosclerotic disease in the brachiocephalic artery.

Unlike wild-type mice, ApoE$^{-/-}$ animals are predisposed to develop atherosclerosis. In our study, we observed divergence in the phenotype of circulating monocytes between the two strains when both were fed with rodent chow diet. The compromised cholesterol metabolism in ApoE$^{-/-}$ mice is likely to account for the distinct monocytic pattern seen at baseline compared to C57BL/6J mice. Indeed, it has been demonstrated that elevated levels of cholesterol induce an increase in Ly6C^{high} cells, by inhibiting their differentiation into Ly6C^{low} and enhancing their survival (Swirski *et al.*, 2007). Our results demonstrate that HFD in wild-type mice leads to a shift in the distribution of the two monocytic subpopulations in the blood, from an equal amount of Ly6C^{high} and Ly6C^{low} cells towards a dominance of Ly6C^{high} over Ly6C^{low} cells. In ApoE$^{-/-}$ mice this pattern was observable at baseline, before commencing HFD, while in C57BL/6J this only developed after an 8 week-period of HFD. In both cases, the change in monocytic phenotype preceded plaque development, since neither ApoE$^{-/-}$ mice at baseline nor C57BL/6J at the end of the

8-week period of HFD had atherosclerotic lesions detectable in the brachiocephalic artery. Therefore, predominance of Ly6C^{high} over Ly6C^{low} cells is likely to be a predisposing factor for plaque development.

Previous studies have evaluated the pro-atherogenic effect of the distinct monocytic subpopulations in mice, by analysing the phenotype of monocytes infiltrating the vascular wall. Swirski found that Ly6C^{high} cells enter plaques preferentially over Ly6C^{low} cells (Swirski *et al.,* 2007), whereas Potteaux observed a preferential influx of Ly6C^{low} cells into the lesions (Potteaux *et al.,* 2011). The distinct anatomic site evaluated by the two groups might have determined the differences seen in their results (aortic root vs brachiocephalic artery), since the two cell subsets appear to have different abilities to colonize atherosclerotic lesions in different regions of the arterial wall (Teupser *et al.,* 2004), with Ly6C^{low} cells mainly implicated in monocytic infiltration in the brachiocephalic artery whilst Ly6C^{high} cells being predominant in the colonization of the vascular wall at the aortic root. These findings are in accordance with our results, that show a strong direct relationship between expansion of Ly6C^{low} cells in the blood and progression of atherosclerotic disease as evaluated at the brachiocephalic artery. Indeed, over the course of 8-week HFD period, ApoE$^{-/-}$ mice but not C57BL/6J developed plaques, and this was paralleled by a concomitant increase in the Ly6C^{low} subtype, such that from representing less than 10% of total monocytes in ApoE$^{-/-}$ mice at baseline they progressively expanded to constitute almost half of the total circulating monocytic population at 8 weeks. In particular, the increase in absolute number of Ly6C^{low} cells over time, rather than the simple percentage of this subpopulation over the total monocytes, was found to be the main determinant of disease progression.

The increase in Ly6C^{low} cell number in ApoE$^{-/-}$ mice seemed to be an effect unconnected to the HFD regime, even though this accelerates atherogenesis in this animal model (Reddick *et al.*, 1994). Wild-type mice on HFD displayed an increase of Ly6C^{high} cells and a concomitant reduction of Ly6C^{low} cells, an effect opposite to that observed in ApoE$^{-/-}$ mice fed on HFD. HFD induced marked monocytosis in both strains to similar levels, although the effects on Ly6C^{high} vs Ly6C^{low} were divergent.

3.5.2 Anti-inflammatory effect of anti-platelet therapies

In accordance with the *in vitro* data described in Chapter 2, we have found that *in vivo* platelet activation has an important effect on circulating monocytes. Indeed, treatment with either aspirin or clopidogrel resulted in suppression of atherosclerosis-related expansion of all monocytes in the circulation, both Ly6C^{low} and Ly6C^{high}, in our model. The current study did not address the mechanistic basis underlying the suppressive effect of anti-platelet drugs on blood monocytosis. This mechanistic basis may be attributable to inhibition of monocyte precursor proliferation within the bone marrow, or of cell mobilisation from the bone marrow to the peripheral circulation, or to a modulation of the survival of these cells in the peripheral blood. Indeed, all of these may occur together. Figure 3.12 shows in schematic form the potential mechanisms of action of anti-platelet treatment on monocyte count and phenotype. Whatever the mechanism, the effects demonstrated here of pharmacological platelet inhibition on circulating monocytes are novel and the first time *in vivo* demonstration that modulating platelet activity has important effects on circulating monocyte in the context of atherosclerosis. Clopidogrel is

likely to act through inhibition of P-selectin expression on the platelet plasmalemma and consequent formation of MPA, although our study only examined the former because of the methodological limitations abovementioned that precluded the direct estimation of MPA level in the peripheral blood. The well described ability of clopidogrel to decrease MPA formation *in vivo* (Storey *et al.,* 2002; Klinkhardt *et al.,* 2003; Braun *et al.,* 2008), along with our previous demonstration that interference in the physical interaction between monocytes and platelets inhibits platelet-dependent change in the phenotype and function of human monocytes (Passacquale *et al.,* 2011c; Chapter 2), support this hypothesis. A similar action may be ascribed to aspirin, which also decreased P-selectin expression on circulating platelets, although to a lesser degree than clopidogrel. However, our *in vitro* experiments previously conducted in human samples (Passacquale *et al.,* 2011c; Chapter 2), suggest an additional mechanism by which aspirin may affect monocyte phenotype, that relates to ability to inhibit COX activity. Even the lower dose used in our animals (5 mg Kg^{-1} day^{-1}, which would correspond to a daily dose of 180-240 mg in humans) may act on both COX-1 and COX-2 isoforms (Patrono *et al.,* 2005), thus producing a simultaneous inhibition of platelet COX-1 activity with consequent defective TxA_2-mediated platelet-activation, and of monocytic COX-2 activity which we have previously found to be strongly involved in platelet-mediated alteration in monocyte phenotype (Passacquale *et al.,* 2011c; Chapter 2). Therefore, it is reasonable to hypothesise that aspirin may be acting through complementary effects on both platelet and monocyte COX activity.

3.5.3 Pro-atherogenicity of Ly6C^{high} and Ly6C^{low} monocytes

As previously mentioned, several authors have investigated the pro-atherogenic activity of phenotypically distinct monocyte subsets by monitoring the trafficking of these cells into atherosclerotic lesions, in order to identify whether a particular cell population displays a preferential influx into plaques. The conflicting data obtained in different laboratories has made it difficult to establish whether and how Ly6C^{high} and Ly6C^{low} cells differentially contribute to lesion development (Teupser *et al.*, 2004; Swirski *et al.*, 2007; Potteaux *et al.*, 2011). The evidence provided by Combadiere and colleagues (Combadiere *et al.*, 2008), who showed that only concomitant inhibition of chemokine receptors involved in both Ly6C^{low} and Ly6C^{high} endothelial trans-migration can completely abolish vascular lesion onset and progression, suggests an equal contribution of these monocyte subsets in atherosclerosis development. In this context, the strong correlation found in the first part of our study between expansion of Ly6C^{low} cells and plaque size is suggestive of a greater pro-atherogenic activity exerted by this monocyte subpopulation over the Ly6C^{high} subtype. However, the tissue phenotype characterization we carried out appears to contradict this hypothesis. Indeed, although showing perhaps a slight predominance of Ly6C^{low} over Ly6C^{high} cells, no significant differences were detectable by vessel flow cytometry analysis between the content of the two cell type within plaques. Therefore, the increase of Ly6C^{low} cells in the peripheral blood seems to provide a good biomarker of disease in this atherosclerotic animal model, whereas both Ly6C^{high} and Ly6C^{low} cells appear to be involved in atherosclerosis at the level of the plaque itself.

3.5.4. Effect of anti-platelet drugs on plaque composition

In our anti-platelet-treated ApoE$^{-/-}$ mice the marked reduction in blood monocytosis, with simultaneous decrease of both monocytic subpopulations to a level similar to that observed in disease-free animals, did not result in a reduction in plaque size. The multi-factorial and multi-cellular nature of atherosclerosis (Ross 1999; Libby 2002) suggests that monocytes, although being fundamental players in vascular lesion development, are not the sole mediators of disease. Similarly, the activity of platelets, that is strongly related to atherosclerosis onset and progression by virtue of their pro-thrombotic and pro-inflammatory actions, is contributory but again only part of the larger picture. As a consequence, the therapeutic modulation of these two cell types, as occurs with an anti-platelet treatment, is not enough to abolish development of disease in this animal model. Endothelial damage sustained by chronic lipid dysmetabolism (Gaudreault *et al.,* 2012) is likely to exert a fundamental role in plaque formation, independent of the phenotype of circulating monocytes and platelets, through the release of chemotactic stimuli and expression of adhesion molecules that promote inflammatory cell recruitment and colonization of the vascular wall.

However, we found that a reduction in monocyte count in the peripheral blood as a consequence of anti-platelet therapy, especially with aspirin, dramatically decreased the monocyte-macrophage content of plaques, most likely through decreasing the number of monocytes in the blood available for vascular infiltration. Therefore, even if not sufficient in reducing plaque size, as also previously reported by other investigators in different animal models (Tous *et al.*, 2004; Schulz *et al.*, 2008), our data demonstrate that anti-platelet therapy can have an important effect on plaque

composition. These findings are in agreement with those of previous researchers (Cyrus *et al.*, 2002; Afek *et al.*, 2009), and our data add important information as regards the mechanism underlying this pharmacological effect, namely that it is mediated by a systemic action of these agents on circulating monocytes, either through an indirect mechanism sustained by platelet inhibition, or a direct action on monocytes themselves (Figure 3.12).

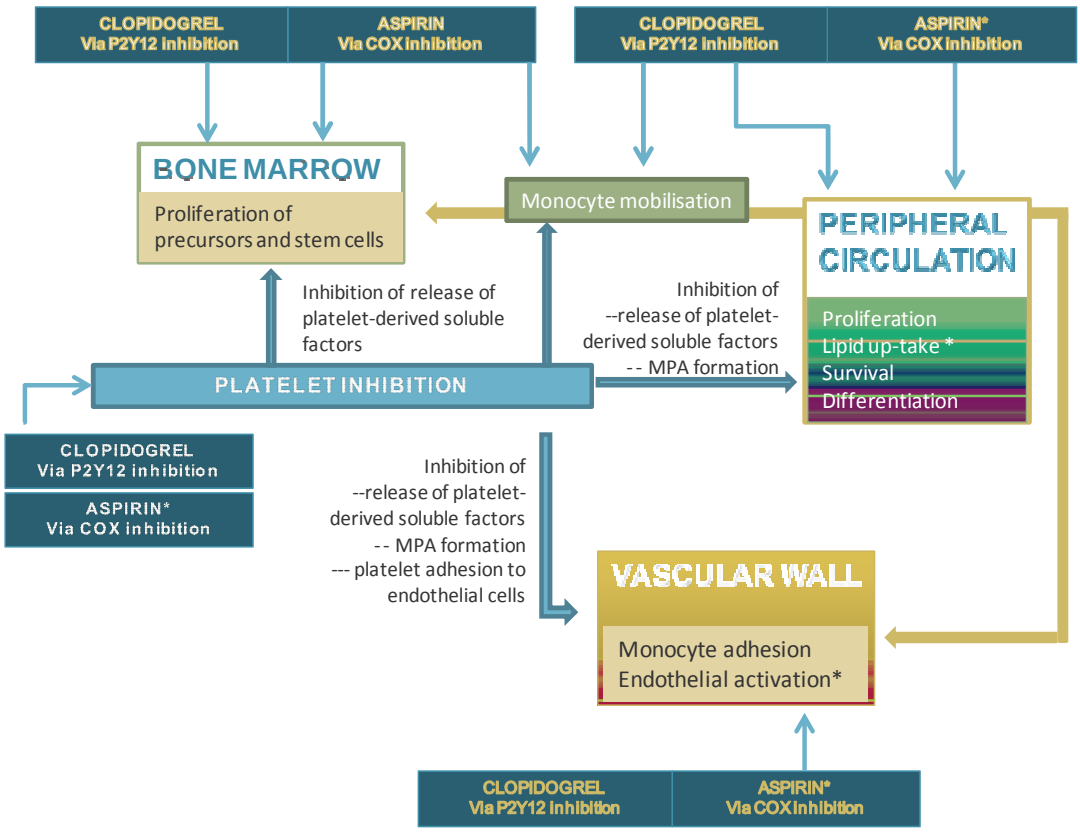

Figure 3.12 Anti-inflammatory activity of aspirin and clopidogrel. The diagram shows the multiple levels at which action of aspirin and clopidogrel may exert their effects on monocytes, which after originating from the bone marrow are released into the peripheral blood and reach atherosclerotic lesions through the systemic circulation. Release of pro-inflammatory mediators from the vascular lesions may feed back on bone marrow monocyte proliferation, stimulate mobilisation of monocytes from the bone marrow to the peripheral blood, and locally recruit these and other inflammatory cells (Shi & Pamer, 2011). Aspirin

and clopidogrel may modulate any or all of these steps, through either direct or indirect pharmacological actions, the latter being mediated by platelet inhibition.

1) A direct effect of both drugs could be exerted at the site of origin of monocytes, in the bone marrow, where aspirin-dependent COX-inhibition or clopidogrel-dependent $P2Y_{12}$-receptor blockade could potentially inhibit stem cell proliferation (mRNA for $P2Y_{12}$ receptor has been detected in stem cells and leukocytes by Wang *et al.*, 2004).

2) Aspirin and clopidogrel could regulate monocyte mobilization from the bone marrow by acting on their phenotype, particularly on the expression of chemokines involved in their trans-endothelial migration at the level of the bone marrow and their consequent release into the systemic circulation.

3) Aspirin and clopidogrel could directly act on circulating monocytes by modifying proliferation, survival, and differentiation via COX-inhibition or $P2Y_{12}$-receptor blockade respectively. Moreover, aspirin, but not clopidogrel, could exert an additional effect on the capability of circulating monocytes to take up lipids in the peripheral blood (asterisked).

4) At the site of a vascular lesion, aspirin, but not clopidogrel, could reduce endothelial inflammation and the consequent recruitment of Ly6C^{high} monocytes (asterisked).

5) Both drugs could exert indirect effects on bone marrow, peripheral circulation and vascular wall mediated by platelet inhibition, with consequent suppression of release of platelet-derived factors, MPA formation and platelet adherence to the endothelial monolayer that in turn affect monocyte phenotype and endothelial trans-migration.

The reduced plaque content of monocyte-macrophage cells, which are the main determinants of plaque vulnerability (Shashkin *et al.*, 2005), strongly suggests that aspirin and clopidogrel may exert beneficial effects by reducing the susceptibility of atherosclerotic lesions to rupture, and hence to develop superadded thrombosis. However, the detailed mechanisms by which the two drugs may do this is likely to be different. Although both exert a systemic effect on circulating monocytes, aspirin and clopidogrel seem to exert an additional action at the site of plaques, where the two drugs differentially regulate the influx of the two distinct monocyte

subpopulations. Based on the fact that the drugs have a similar effect on the count and phenotype of circulating monocytes, it is reasonable to hypothesise that they have a distinct effect on the interaction of monocytes with the vascular wall, favouring the influx of one cell type over another depending on differential modulation of endothelial adhesive phenotype. However, how the different monocytic subsets may relate to plaque vulnerability is unclear. The ORO staining data show that aspirin, but not clopidogrel, can suppress foam cell content compared to untreated animals. Given the reduced level of Ly6C^{high} cells in plaques from aspirin-treated animals, it is conceivable that lipid-laden macrophages mainly derive from this subset of monocytes rather than Ly6C^{low} cells. In accordance with this, Swirski has shown that Ly6C^{high} monocytes give rise to macrophages within plaques (Swirki *et al.*, 2007; Weber & Noels, 2011). On the other hand, Ly6C^{low} monocytes, that were suppressed by clopidogrel, have been demonstrated to have a greater tendency than Ly6C^{high} cells to terminally differentiate into resident dendritic cells (Peng *et al.*, 2009; Giessman *et al.*, 2010; Weber & Noels, 2011). The precise role of these cell types in vascular wall remodelling is uncertain. Given the patrolling behaviour of Ly6C^{low} cells in non-inflamed tissues compared to the predominant infiltration of Ly6C^{high} cells within inflamed areas reported by Geissmann in the microcirculation (Geissmann *et al.*, 2003), one could hypothesise that aspirin may exert a greater vascular anti-inflammatory activity than clopidogrel in the large arteries, leading to reduced Ly6C^{high} numbers which in turn may decrease the macrophage-dependent lipid content of plaques . On the other hand, lipid uptake by monocytes also occurs in the peripheral circulation, before such cells enter into plaques (Gaudreault *et al.*, 2011). Therefore, the possibility cannot be excluded that

aspirin may act at a systemic level to modify the capability of circulating monocytes to phagocytose lipids in the blood (Figure 3.12).

3.5.5 Conclusion

This study demonstrates that expansion of the murine counterpart of human CD16^{+} monocytes in the peripheral circulation occurs during the pro-inflammatory process that sustains and accompanies atherosclerosis, and the level of this monocyte subpopulation in the blood reflects the extent of vascular disease in the ApoE$^{-/-}$ model of atherosclerosis. The anti-platelet drugs aspirin and clopidogrel reduce atherosclerosis related-monocytosis with similar efficacy, and this associates with reduced monocyte-macrophage content in atherosclerotic plaques. However, the two anti-platelet agents, despite having a similar systemic effect on circulating monocyte phenotype and count, differentially regulate the influx of Ly6C^{high} and Ly6C^{low} cells into lesions, thus altering plaque composition in a divergent manner. In particular, the reduced trafficking of Ly6C^{high} cells into the atheroma observable with aspirin but not clopidogrel is associated with a decrease in lipid / foam cell content. This may have important consequences on plaque vulnerability and hence predisposing to superadded thrombosis.

Chapter Four

Characterization of circulating monocytes: a novel diagnostic tool for improved stratification of cardiovascular risk

4.1 Introduction

Atherosclerosis is a chronic and diffuse inflammatory process within the arterial wall that promotes the formation of atheromatous plaques composed of a lipid core with an overlying fibrous cap (Ross, 1999). The atherosclerotic process is accompanied by increased thrombogenicity of the blood, which (as reviewed in Chapter 1) not only gives rise to the thrombotic complications (which occur when a vulnerable plaque fissures or ruptures) but also contributes to lesion initiation by favouring monocyte recruitment into the sub-endothelial space and, in turn, the development of a pro-inflammatory milieu within the vascular wall (Davi' & Patrono, 2007). Atherosclerosis progression is by and large a slow and long-term process, which generally takes many years before it becomes clinically manifest, when occlusive vascular symptoms occur; although thrombotic complications can occur at any stage of the disease, largely dependent on plaque composition, degree of inflammation and haemodynamic factors.

The need to develop more effective strategies for both prevention and early diagnosis of atherosclerosis has gained increasing attention in recent years, due to the recognition that atherosclerotic sequelae, including ischaemic heart disease and cerebrovascular events, remain major causes of mortality and morbidity, accounting for 30% of all deaths worldwide (World Health Organization, 2007). Moreover, health projection studies on the global burden of disease for the next ten years, predict a dramatic increase in the prevalence of atherosclerosis-related cardiovascular disease (Murray & Lopez, 1997; World Health Organization, 2007).

In patients with established atherosclerotic disease that is clinically evident, addressing cardiovascular risk factors vigorously (smoking cessation, blood pressure and glycaemic control to target, cholesterol lowering with statins) and reducing blood thrombogenicity with anti-platelet therapy give rise to important reductions in myocardial infarction and stroke, and retard the progression of atherosclerotic disease (Joint British Societies – JBS-2 Guidelines 2005). However, a major challenge is the difficulty in early diagnosis in asymptomatic patients, in whom recognition of early disease and consequent vigorous risk factor reduction will help to retard progression of atherosclerosis as well as consequent occlusive/thrombotic arterial events.

At present, management of asymptomatic patients is based on clinical assessment, aiming to evaluate classical cardiovascular risk factors (age, sex, smoking, presence of diabetes, lipid profile) and the presence/absence of target-organ damage such as left ventricular hypertrophy and proteinuria (JBS-2 Guidelines 2005). A number of cardiovascular risk calculators, such as the risk charts provided by the JBS (JBS-2 Guidelines, 2005) or by the WHO/ISH (World Health Organization, 2007), or the Framingham equation (Anderson *et al.*, 1991), combine these clinical parameters to estimate the probability for a given individual to experience a future cardiovascular event (including ischaemic heart disease/myocardial infarction and stroke) over a period of time. Asymptomatic patients can therefore be distinguished into different categories of cardiovascular risk profile ranging from low (<10%) to high (>20%) risk over the next 10 years, and such stratification guides decision-making on the

intensity as well as the nature of preventative strategies to be adopted in individual patients.

However, since these tools are based on statistical risk in populations, they are of limited usefulness in individual patients. In order to determine extent/severity of atherosclerosis in a given individual, it is necessary to image the vasculature using either invasive (including angiography) or non-invasive methods (such as CT coronary calcium score or CT angiography). These are expensive and laborious investigations, and therefore can only realistically be used in specific groups of patients with strong clinical indications, who often turn out to be at an advanced stage of disease. There is therefore an urgent need to develop simple, non-invasive, relatively inexpensive tests that are widely applicable in the population for early diagnosis, so that prevention strategies can be more effectively targeted.

4.2 Aims

The current study aimed to investigate whether analysis of platelet activation by MPA measurement and/or characterization of the phenotype of circulating monocytes can provide a useful, non-invasive, inexpensive and generally applicable measure of silent atherosclerotic disease, and whether such a measure can give information regarding presence of atherosclerotic disease over and above standard cardiovascular risk calculators.

4.3 Methods

4.3.1 Subject recruitment and characteristics

The study was approved by the St Thomas' Research Ethics Committee, London, UK. All participants gave written informed consent. Forty-five patients were recruited sequentially from the Hypertension (n=23) and Diabetic (n=22) Clinics at Guy's & St Thomas' Hospitals, London, UK.

Inclusion criteria:

- 18 years of age or older
- able and willing to comply with study procedures
- no previous history of cardiovascular events and/or symptomatic atherosclerotic disease.

Exclusion criteria:

- patients with diagnosed secondary hypertension
- clinical evidence of atherosclerotic disease or other significant co-morbidity
- previous cardiovascular events
- cardiac dysrythmia
- significant renal impairment (eGFR < 30 mL/min/1.73 m^2)
- pregnancy or breastfeeding
- mental disorders or inability to give informed consent.

Patients were studied at the Clinical Research Facility of St Thomas' Hospital. Each participant underwent a full history and clinical examination including measurement of blood pressure by Omron 705CP, body weight and height for calculation of body

mass index (BMI). 12-lead electrocardiogram was performed, and blood (32 ml) collected by venepuncture for measurement of full blood count, full blood biochemistry, including renal and liver profiles, lipids, glycated hemoglobin (HbA_{1c}) and high-sensitivity C-reactive protein (hs-CRP). 100 µl of this blood was also taken for analysis of circulating monocyte phenotype and MPA according to the methods described in the following section. The Framingham equation and JBS-2 risk charts were used for cardiovascular risk stratification. Patient characteristics are summarized in Table 4.1.

4.3.2 Measurement of circulating MPA and monocyte characterization

Monocyte characterization and MPA measurement were performed by flow cytometry analysis on whole blood collected in sodium citrate (0.3% final concentration). Immediately after venepuncture, 100 µl blood was incubated in the dark and at 4°C with a combination of the following antibodies: phycoerythrin (PE)-conjugated anti-human CD14, fluorescein isothiocyanate (FITC)-conjugated anti-human CD16 and allophycocyanin (APC)-conjugated anti-human CD42b (all antibodies were purchased from BD Bioscience). Isotype control antibodies were used as negative control. After red cell lysis using FACS lysing solution (BD Bioscience), samples were washed twice in PBS containing 0.2% BSA and 0.1 sodium azide, and then fixed in 1% paraformaldehyde and kept at 4°C until analyzed within a maximum of 48h from sample preparation.

Using flow cytometry (FACSCalibur, Becton Dickinson (BD), Oxford, UK), forward and side light scatter parameters were used to access the monocyte

population, and a total of 20,000 events were acquired within the monocyte gate. The negative and positive delineators were determined from the isotype control fluorescence.

Monocyte subsets were identified by double immunostaining for CD14 and CD16 which allowed the identification of three cell subpopulations: $CD14^{+}CD16^{-}$, $CD14^{high}CD16^{+}$ and $CD14^{low}CD16^{+}$ monocytes. The percentage of $CD14^{+}$ cells expressing CD42b (a constitutive platelet marker) was taken as representative of circulating MPA. Post-acquisition analysis was performed using FlowJo software (Tree Star, Ashland, OR).

4.3.3 Carotid ultrasonography.

Carotid ultrasonography was performed by Dr Ben Yu Jiang at the Clinical Research Facility of St Thomas' Hospital. Carotid intima-media-tickness (IMT) was measured by ultrasonography (Accuson Sequoia 512 machine with an 8-MHz transducer). IMT was taken as the distance between the blood/intima borderline and the media/adventitia borderline, and evaluated on the far wall of the left and right common carotid artery (CCA), specifically in the distal 1 cm of the artery just proximal to the bulb. Mean carotid IMT was measured using a semi-automated computer analysis system over this 1 cm segment, and confirmed by the mean of five manual readings at 20mm intervals along the CCA. Mean values of IMT in the near and far walls of both arteries were used for analysis.

4.3.4 Statistical analysis

All data were expressed as median ± inter-quartile range. Statistical analyses were performed in GraphPad Prism. Between-group comparisons were made using one-way ANOVA with Dunn's correction. Multivariate linear regression analysis was conducted to examine the correlation between IMT and all the variables measured in the study: age, systolic and diastolic blood pressure (SBP and DBP respectively), total cholesterol and low-density and high-density lipoprotein fractions (LDL and HDL), triglycerides, HbA_{1c}, hs-CRP, BMI, estimated glomerular filtration rate (eGFR), MPA levels and percentage of different monocyte subsets. Associations were analysed by least squares and multiple regression analyses. In all cases, $p < 0.05$ (two-tailed) was considered significant.

4.4 Results

4.4.1 MPA level and monocyte CD16 positivity do not relate to cardiovascular risk stratification as assessed by standard risk calculators

Using the Framingham equation, patients were divided into three categories of global cardiovascular risk: Group 1, low (n=22); Group 2, moderate (n=15); and Group 3 high (n=8) representing 10-year calculated cardiovascular risk <10%, 10% - 20%, and >20% respectively. These three sub-populations differed in age, prevalence of diabetes, SBP and renal function (Table 4.1). The low risk group had the lowest median age and SBP; 87.5% of patients in the high-risk group were diabetic compared to 31.25% and 59% in the moderate- and low-risk groups respectively; and median eGFR in the high-risk group was lower than that in the other two groups.

No differences were seen between groups in total monocyte numbers, MPA levels and the distribution pattern of the distinct monocyte subpopulations (Table 4.1, Figure 4.1).

Table 4.1 Characteristics of study patients. Participants were distinguished into different categories of cardiovascular risk profile based on the Framingham equation (LR, low-risk; MR, moderate-risk; HR, high-risk, corresponding to <10%, 10-20% and >20% 10-year calculated cardiovascular risk respectively).

SBP: systolic blood pressure; DBP: diastolic blood pressure; HDL: high-density lipoproteins; BMI: body mass index; hs-CRP: high-sensitivity C reactive protein; eGFR: estimated glomerular filtration rate; ns: non significant.

Variables are presented as percentages or medians with inter-quartile ranges, as appropriate.

	LR (n=22)	MR (n=15)	HR (n=8)	p
Age (years)	38 (25-57)	52 (47-68)	62 (51-73)	<0.001 (HR vs LR MR vs LR)
Women (%)	7 (31.81%)	7 (43.75%)	3(37.5%)	
Smokers (n)	0	1	0	
Diabetes mellitus (%)	13 (59%)	5 (31.25%)	7 (87.5%)	
HbA_{1c} (%)	7.6 (5.2-11.10)	5.9 (4.9-9.5)	8.05 (5.6-10.5)	ns
SBP (mmHg)	135(110-165)	147 (126-190)	145.5(115-175)	0.03 (MR vs LR)
DBP (mmHg)	76(56-108)	90 (65-130)	78.5 (64-103)	ns
Total cholesterol (mmol/L)	4.6 (3.2-6.88)	4.23 (2.75-6.27)	4.2 (3.41-5.9)	ns
HDL-cholesterol (mmol/L)	1.29 (1.05-1.7)	1.4 (1.2-1.63)	1.14 (1.14-1.3)	ns
BMI (kg/m^2)	28.40 (20.2-40)	31.85 (21.7-37.5)	32.95 (26.1-37.2)	ns
hs-CRP (mg/dl)	2.72 (0.23-12.5)	1.66 (0.4-6.55)	3.13 (2.5-4.08)	ns
eGFR ($ml/min/1.73\ m^2$)	102.5 (85-133)	91.5 (73-123)	80 (37-100)	<0.05 (HR vs MR) <0.001 (HR vs LR)
Triglycerides (mmol/L)	1.4(0.53-2.07)	1.15 (0.57-2.67)	1.58 (1.2-2)	ns
Monocytes ($10^9/L$)	0.63 (0.45-0.65)	0.45 (0.27-0.78)	0.69 (0.34-0.78)	ns
Anti-platelet therapy (%)	0	4 (25%)	2 (28.57%)	

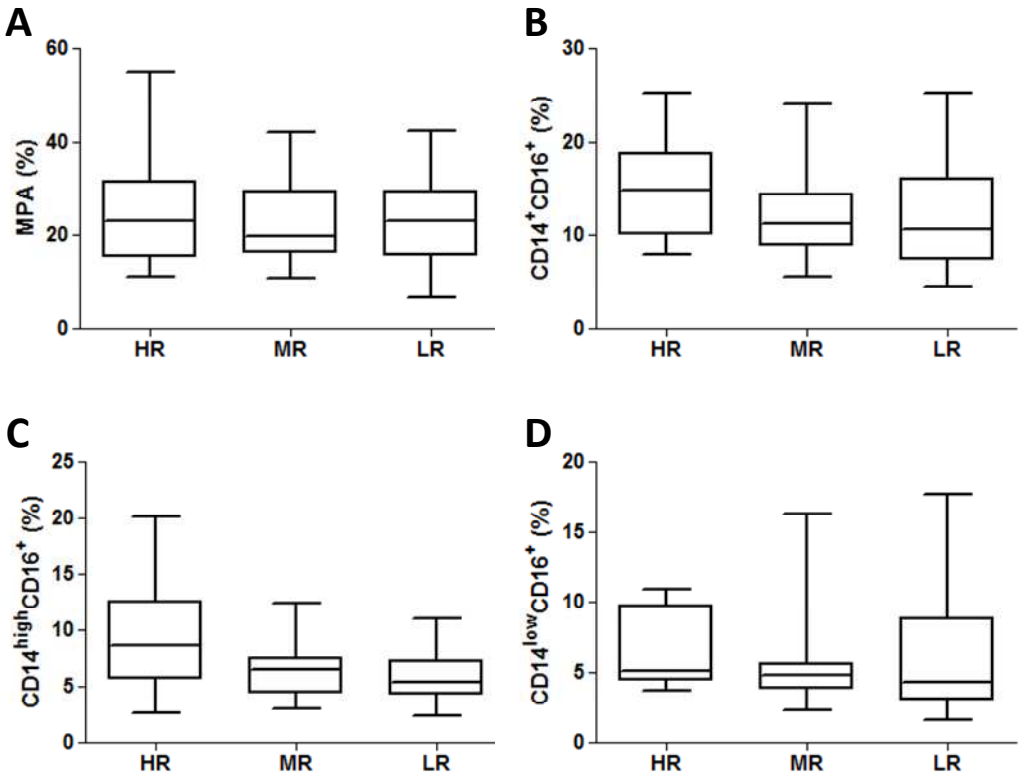

Figure 4.1 Level of MPA (measured as $CD14^{+}CD42b^{+}$ events) (**A**) and the distinct monocyte subpopulations comprising "classical" $CD14^{+}CD16^{-}$ (**B**), $CD14^{high}CD16^{+}$ (**C**) and $CD14^{low}CD16^{+}$ (**D**) in the study population, stratified according to risk as calculated using the Framingham equation. HR: high-risk; MD: moderate risk; LR: low risk corresponding to <10%, 10-20% and >20% 10-year calculated cardiovascular risk. Graphs show medians and inter-quartile ranges of each parameter.

When the population study was stratified according to the JBS-2 risk charts, all diabetic patients were classified as being at high cardiovascular risk. In this case, no differences were observed in age and eGFR between groups. However, the low-risk group had the highest median DBP and total cholesterol (Table 4.2), most likely because of a less aggressive preventative strategy adopted in these patients by their treating clinicians compared to the other groups.

A similar level of MPA was measured in the high-, moderate- and low-risk categories. On the other hand, monocyte characterization showed an increase in the level of $CD14^{high}CD16^{+}$ cells in the peripheral blood of patients at high- compared to those at low-cardiovascular risk (Figure 4.2).

Table 4.2 Characteristics of study patients. Participants were distinguished into different categories of cardiovascular risk profile based on the JBS-2 risk charts (LR, low-risk; MR, moderate-risk; HR, high-risk, corresponding to <10%, 10-20% and >20% 10-year calculated cardiovascular risk respectively).

SBP: systolic blood pressure; DBP: diastolic blood pressure; HDL: high-density lipoproteins; BMI: body mass index; hs-CRP: high-sensitivity C reactive protein; eGFR: estimated glomerular filtration rate; ns: non significant.

Variables are presented as percentages or medians with inter-quartile ranges, as appropriate.

	LR (n=7)	MR (n=8)	HR (n=30)	p
Age (years)	38 (27-57)	54.5 (45-67)	50 (25-73)	ns
Women (%)	4 (57.14%)	4 (50%)	12 (40%)	
Smokers (n)	0	1	0	
Diabetes mellitus (%)	0	0	25 (83.3%)	
HbA_{1c} (%)	5.4 (5.2-5.9)	5.6 (4.9-6.1)	8.2 (5.6-11.10)	<0.0001 (HR vs MR; HR vs LR)
SBP (mmHg)	137(110-190)	145.5 (134-167)	137.5(110-190)	ns
DBP (mmHg)	98 (85-130)	87 (76-98)	75.5 (56-115)	0.001 (HR vs LR)
Total cholesterol (mmol/L)	3.48 (3.12-5.46)	3.12 (1.97-4.58)	2.11 (1.7-3.47)	0.05 (HR vs LR)
HDL-cholesterol (mmol/L)	1.25 (1.05-1.7)	1.39 (1.2-1.63)	1.31 (1.14-1.68)	ns
BMI (kg/m^2)	30.48 (23.7-34.4)	30 (21.7-37.24)	29.85 (20.2-50)	ns
hs-CRP (mg/dl)	2.72 (0.23-12.5)	1.66 (0.4-6.55)	3.13 (2.5-4.08)	ns
eGFR (ml/min/1.73 m^2)	94(85-105)	91.5 (75-107)	100 (37-170)	ns
Triglycerides (mmol/L)	1.37(0.53-2.07)	1.27 (0.57-1.53)	1.3 (0.73-2.67)	ns
Monocytes (10^9/L)	0.55 (0.45-0.65)	0.52 (0.27-0.69)	0.59 (0.46-0.78)	ns
Anti-platelet therapy (%)	0	0	6 (28.57%)	

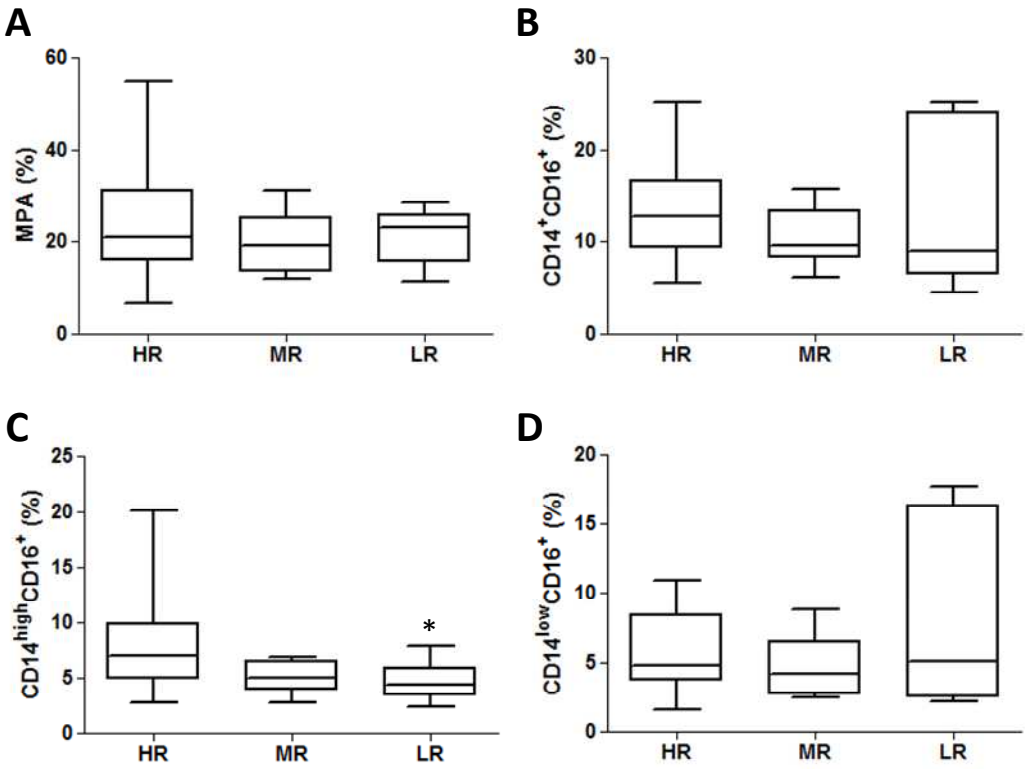

Figure 4.2 Level of MPA (measured as $CD14^{+}CD42b^{+}$ events) (**A**) and the distinct monocyte subpopulations comprising "classical" $CD14^{+}CD16^{-}$ (**B**), $CD14^{high}CD16^{+}$ (**C**) and $CD14^{low}CD16^{+}$ (**D**) in the study population, stratified according to risk as calculated using the JBS-2 risk charts. HR: high-risk; MD: moderate risk; LR: low risk corresponding to <10%, 10-20% and >20% 10-year calculated cardiovascular risk. Graphs show medians and inter-quartile ranges of each parameter. *, p=0.02 vs HR.

Due to the high prevalence of diabetes in our study population, a further sub-group analysis was conducted by dividing the high-risk group into three categories of patients: type 2 diabetic patients (T2; n=15), type 1 diabetic patients (T1; n=10) and non-diabetic high-risk patients (n=5). Their clinical characteristics, as well as MPA levels and monocyte phenotype were compared using one-way ANOVA multiple comparison test with Dunn's correction also including data from moderate (n=8) and low-risk non-diabetic patients (n=7).

Groups differed in age, renal function and blood pressure. T1 diabetic and low-risk patients had similar age (medians were 30 (25-52) and 35 (27-57) years respectively) and were younger than the other categories. No differences were detected in eGFR between diabetic and non-diabetic patients, although renal function was better in T1 than in T2 (eGFR 108 (37-123) ml/min/1.73m^2 vs 92 (37-123) ml/min/1.73m^2 respectively; p=0.01). Levels of SBP and DBP were higher in non-diabetic compared with diabetic groups (Figure 4.3). All other clinical parameters, including lipid profile, hsCRP, BMI and total monocyte count, were similar between groups.

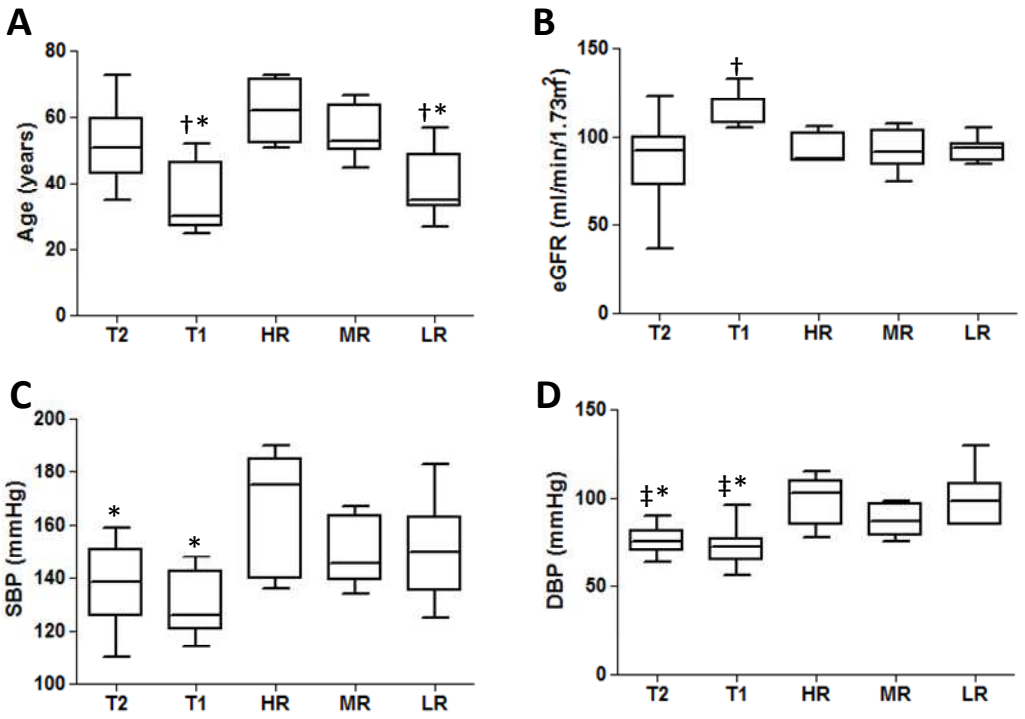

Figure 4.3 Characteristics of patient sub-groups. Graphs display differences in clinical parameters when the study population was divided into diabetic (type 1 (T1) and type 2 (T2)) and non-diabetic subjects, this latter stratified further into high- (HR), moderate- (MR) and low- risk (LR) in accordance with the JBS-2 risk charts. HR, MR and LR correspond to <10%, 10-20% and >20% 10-year calculated cardiovascular risk.

Graphs show medians and inter-quartile ranges of each parameter. *, $p<0.05$ vs HR; †, $p<0.05$ vs T2; ‡, $p<0.05$ vs LR.

Although MPA levels were similar between groups, a significantly higher level of $CD14^{high}CD16^{+}$ monocytes was found in the peripheral blood of type 2 diabetic compared with that measured in low-risk subjects. Comparison between type 2 and type 1 diabetic patients, as well as between the other groups, revelead no other detectable inter-group differences in the level of pro-inflammatory monocytes (Figure 4.4).

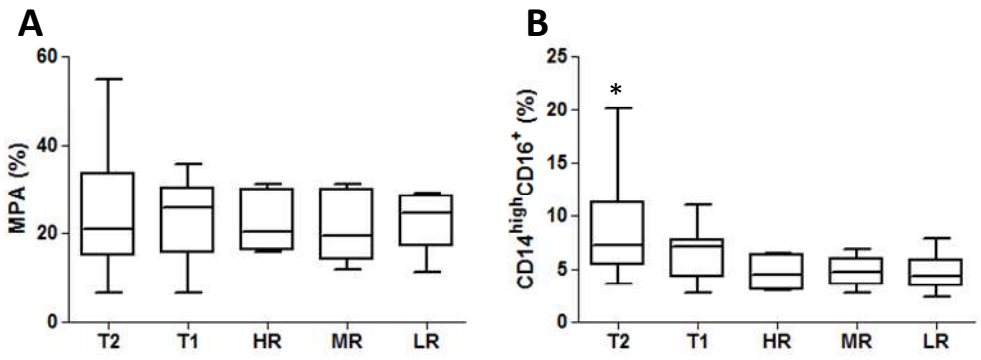

Figure 4.4. MPA and monocyte characterization in patient sub-groups. Level of MPA (measured as $CD14^{+}CD42b^{+}$ events) (**A**) and $CD14^{high}CD16^{+}$ monocytes (**B**) in patients with type 1 (T1) and type 2 (T2) diabetes, and in non-diabetic subjects stratified into high- (HR), moderate- (MR) and low-risk (LR) according to the JBS-2 risk charts. HR, MR and LR correspond to <10%, 10-20% and >20% 10-year calculated cardiovascular risk.*, p=0.01 vs LR.

4.4.2 Carotid atherosclerotic disease correlates with levels of $CD14^{high}CD16^{+}$ monocytes but not with cardiovascular risk as assessed by standard risk calculators

Twenty-two of these forty-five patients underwent carotid ultrasonography for evaluation of IMT. These included three patients of the eight classified into the high risk group, nine of the fifteen in the moderate risk group, and ten of the twenty-two in the low-risk group (as assessed using the Framingham equation). Atherosclerotic plaque, as defined by a focal lesion with IMT > 1.5 mm, was found in ten patients: two low-risk subject, six moderate risk subjects, and two high-risk subjects. These findings suggest that the presence of carotid plaque disease has no – or very little – relation to calculated cardiovascular risk.

We then compared level of MPA and monocyte distribution pattern between patients with carotid plaque disease (n=10) and those without (n=12). MPA levels were similar between the two groups: 22.09% (10.89% - 31.32%) vs 18.22% (12% - 29.23%) in patients with and without plaques respectively, p=ns. By contrast, the percentage of circulating $CD14^{high}CD16^{+}$ monocytes was higher in patients with carotid plaque disease compared to those without plaques: 7.53% (3.55%-20.12%) vs 3.95% (2.29%-7.5%) respectively; p=0.008 (Figure 4.5).

In a multivariate linear regression analysis incorporating age, SBP, DBP, lipid profile, HbA_{1c}, hs-CRP, eGFR, BMI, MPA level, cardiovascular risk as calculated with the Framingham equation, and IMT, percentage of circulating $CD14^{high}CD16^{+}$ monocytes was found to strongly correlate with age and IMT and, to a minor extent, with eGFR (Figure 4.5).

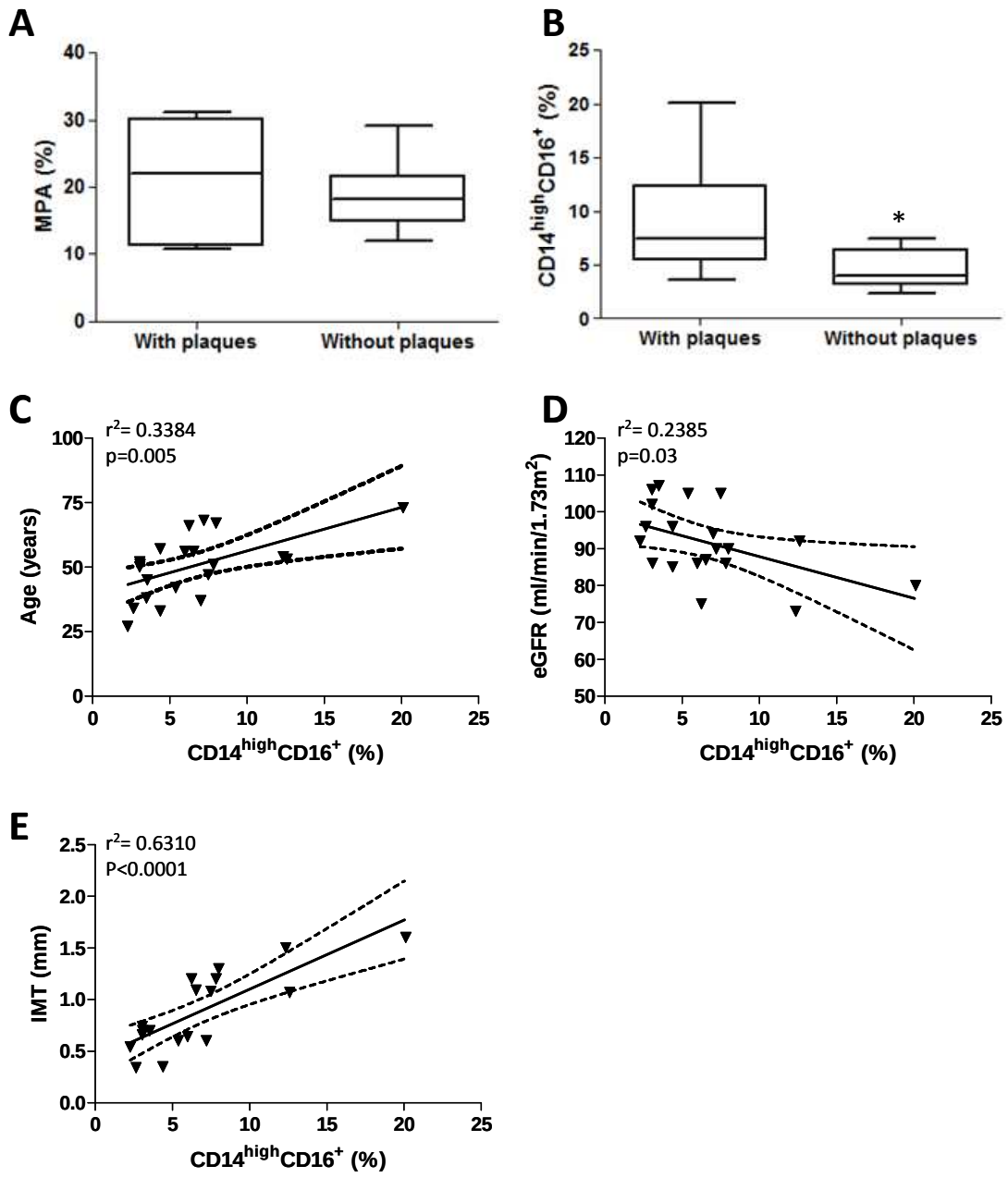

Figure 4.5 Level of MPA (measured as $CD14^{+}CD42b^{+}$ events) (**A**) and $CD14^{high}CD16^{+}$ monocytes (**B**) in patients with (n=10) and without (n=12) carotid atherosclerotic plaques. Also shown are the relationships of $CD14^{high}CD16^{+}$ monocytes with age (**C**), eGFR (**D**) and IMT (**E**).

Patients with and without plaque disease differed in age, DBP and eGFR as shown in table Table 4.3.

Table 4.3 Characteristics of patients with and without carotid atherosclerotic plaques. Variables are presented as percentage or medians with inter-quartile ranges, as appropriate. SBP: systolic blood pressure; DBP: diastolic blood pressure; HDL: high-density lipoproteins; BMI: body mass index; hs-CRP: high-sensitivity C reactive protein; eGFR: estimated glomerular filtration rate; ns: non significant.

	With plaques (n=10)	Without plaques (n=12)	p
Age (years)	61.5 (51-73)	44.5(33-56)	0.002
Women (n)	5	6	
Smokers (n)	0	1	
Diabetes mellitus (n)	2	1	
HbA_{1c} (%)	6.15 (4.9-8.2)	5.7 (5.2-7)	ns
SBP (mmHg)	143.5 (125-167)	154.5 (135-190)	ns
DBP (mmHg)	85.2 (77-98)	100.5 (76-130)	0.02
Total cholesterol (mmol/L)	4.31 (2.5-5.57)	5.23 (3.57-6.88)	ns
HDL-cholesterol (mmol/L)	1.49 (1.05-1.63)	1.34 (1.14-1.7)	ns
BMI (kg/m^2)	34 (21.7-37.5)	29.38 (23.7-50)	ns
hs-CRP (mg/dl)	3.45 (0.4-12.5)	2.32 (0.237-3.34)	ns
eGFR ($ml/min/1.73\ m^2$)	85.5 (37-92)	96 (86-107)	0.002
Triglycerides (mmol/L)	0.84 (0.53-2.67)	1.3 (0.63-2.07)	ns
Monocytes (10^9/L cells)	0.45 (0.27-0.6)	0.63 (0.41-0.78)	ns
Anti-platelet therapy (n)	4	0	

We next performed multivariate regression analysis using a model incorporating age, SBP, DBP, lipid profile, HbA_{1c}, hs-CRP, eGFR, BMI, MPA level, monocyte subsets and cardiovascular risk as calculated with the Framingham equation. IMT was found to correlate strongly with age (r^2=0.4426; p=0.001), and, to a minor extent, with eGFR (r^2=0.2973; p=0.01). However, no correlation was found between IMT and other classical cardiovascular risk factors or cardiovascular risk assigned as calculated using the Framingham equation. On the contrary, IMT was found to be linearly related to percentage of total $CD14^+CD16^+$ monocytes (r^2=0.5247; p=0.0002), particularly the level of the $CD14^{high}CD16^+$ subset (r^2=0.5505; p=0.0001), whilst no relationship was found with the subpopulation of $CD14^{low}CD16^+$ cells. Similarly, no relationship was observed between IMT and classical $CD14^+CD16^-$ monocytes or MPA levels (Figure 4.6).

Using the JBS-2 charts to calculate cardiovascular risk in those patients who were found to have carotid plaques, two were stratified at low cardiovascular risk, five at moderate cardiovascular risk and three at high cardiovascular risk. Again, therefore, there appeared to be no– or very little - relationship between the presence of carotid plaque disease and calculated cardiovascular risk.

Concerning the level of hs-CRP, only five of the ten patients with plaques and two of the twelve disease-free subjects had levels above the normal range (0.2-3 mg/dL).

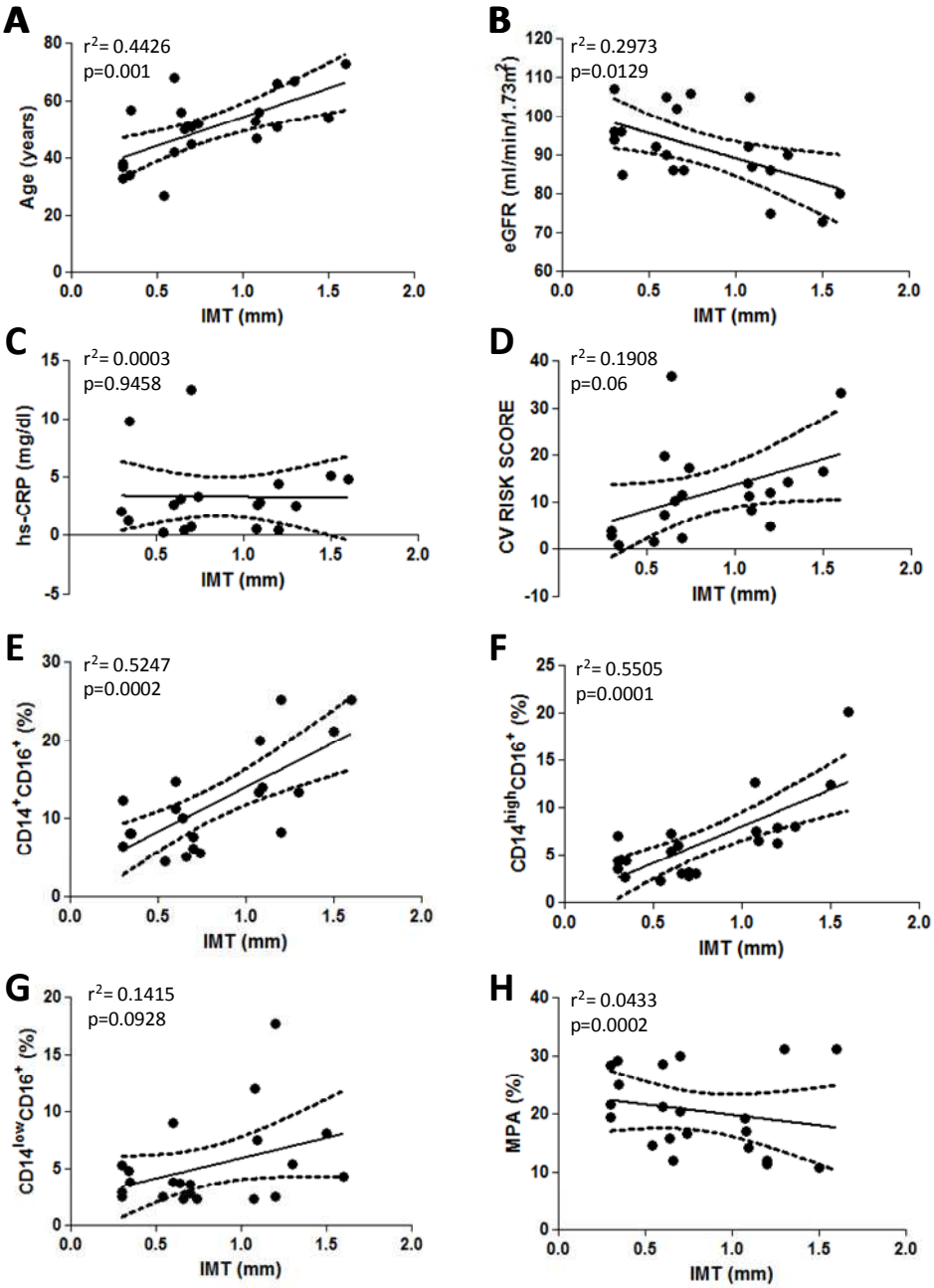

Figure 4.6 The relationship of IMT to age (**A**), eGFR (**B**), hs-CRP (**C**), cardiovascular risk score according to the Framigham equation (**D**), total pro-inflammatory $CD14^{+}CD16^{+}$ monocytes (**E**), $CD14^{high}CD16^{+}$ monocytes (**F**), $CD14^{low}CD16^{+}$ cells (**G**) and MPA (measured as percentage of $CD14^{+}CD42b^{+}$ cells) (**H**) IMT: intima-media thickness, eGFR: estimated glomerular filtration rate; hsCRP: high-sensitivity C reactive protein; CV: cardiovascular.

4.4.3 $CD14^{high}CD16^{+}$monocytes and MPA levels: relationship to classical cardiovascular risk factors

In all forty-five patients enrolled in this study, the relationship was studied between levels of $CD14^{high}CD16^{+}$ monocytes and other parameters: age, blood pressure, lipid profile, hs-CRP, HbA_{1c}, BMI and cardiovascular risk as calculated using the Framingham risk equation. We found that $CD14^{high}CD16^{+}$ levels correlated positively with age (r^2=0.1132; p=0.03) and negatively with DBP (r^2=0.1627; p=0.01), but not with the other parameters examined (Figure 4.5). No correlation was found between MPA levels and any of the parameters examined, in our study population.

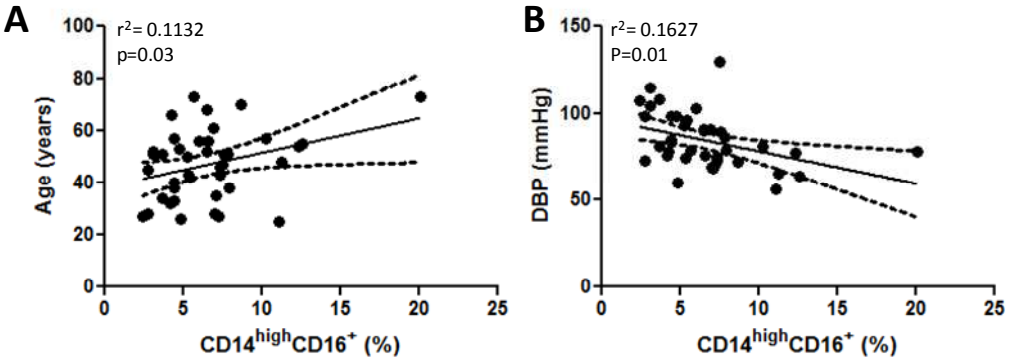

Figure 4.7 Correlation between $CD14^{high}CD16^{+}$ monocytes and age (**A**) and DBP (**B**) in the whole study population.

4.5. Discussion

This study demonstrates that the standard cardiovascular risk calculators examined here (the Framingham equation and JBS-2 risk charts) have limited ability to predict the presence of atherosclerotic disease at an individual level, when applied to a cohort of patients with one or more cardiovascular risk factors who are asymptomatic for atherosclerotic disease. Classical biomarkers currently used in clinical practice to predict future cardiovascular events, in particular hs-CRP, also did not reliably identify those subjects with silent disease. This highlights the need to develop novel biomarkers able to refine the stratification of cardiovascular risk in a population of clinically healthy patients with cardiovascular risk factors, in order that the clinical management of such subjects can be improved and preventative strategies be targeted in a more effective manner.

Our study suggests that evaluation of the phenotype of circulating monocytes, and particularly the measurement of levels of pro-inflammatory $CD14^{high}CD16^{+}$cells, provides a useful adjunct for cardiovascular risk stratification, over and above the standard approach based on the clinical assessment of traditional cardiovascular risk factors.

4.5.1 Monocyte characterization as a surrogate marker of silent atherosclerosis

In agreement with Rogacev (Rogacev *et al.*, 2010) who has previously demonstrated a strong correlation between levels of $CD14^{high}CD16^{+}$ monocytes and IMT in a population of healthy subjects, a similar relationship between percentage of pro-inflammatory monocytes and asymptomatic atherosclerosis was found in our study

population, comprising patients with different cardiovascular risk profiles. Of note, a major expansion of $CD14^{high}CD16^{+}$ cells was found in the peripheral blood of those patients with carotid plaque disease, who were by and large older and had a lower eGFR than disease-free subjects. Accordingly, levels of $CD14^{high}CD16^{+}$ cells were strongly related to age and renal function, and this is likely to reflect the well-established effect of chronic exposure to cardiovascular risk factors, as well as impaired kidney function, on development of a pro-inflammatory phenotype that, in turn, favours development and progression of atherosclerosis.

In keeping with this, among all the parameters evaluated in our study, age and renal function were also related to IMT. The detrimental effect of ageing on function and anatomical integrity of the vasculature, and the consequent age-related increase in cardiovascular disease, is well recognized (Remsberg & Siervogel, 2003). Elderly people have increased probability of developing cardiovascular disease, independently of the presence of other cardiovascular risk factors. However, stratifying patients based only on their age carries no cardiovascular predictive value without concomitant clinical assessment of other cardiovascular risk factors, including hypertension, dyslipidaemia, smoking and presence of diabetes. The correlation between IMT and age found in our study is likely purely to reflect the chronic nature of atherosclerotic disease and its progressive development over the years.

Similarly, the relationship between IMT and eGFR observed in our study cohort is likely to reflect the well established increased susceptibility of patients with impaired kidney function to accelerated atherosclerosis (Hemmelgarn *et al.*, 2010).

However, eGFR measurement does not provide a good marker of atherosclerosis due to the lack of a recognized threshold above which further clinical investigations, including cardiovascular imaging, are required in an individual. Levels of eGFR, especially where they show normal or mild impairment of kidney function as observed in our patients, are not reliable indicators of the presence of atherosclerotic disease. In keeping with this, two of the patients with evidence of carotid plaques on ultrasonography had eGFR>90 mL/min/1.73 m^2, and the remainder had levels of eGFR ranging between 73 and 86 mL/min/1.73m^2.

In this context, monocyte characterization has the potential to offer a reliable diagnostic tool widely applicable to a population at risk of future cardiovascular events, enabling the early detection of silent atherosclerosis with a superior predictive value compared to the classical inflammation biomarker hs-CRP and to traditional cardiovascular risk scoring based on either the Framigham equation or JBS-2 risk charts. Coupled to the fact that silent carotid disease was prevalent in subjects classified at low and moderate cardiovascular risk, this is a strong case that introduction of monocyte characterization in clinical practice might be of particular benefit for these two categories of patients. Indeed, although preliminary, our clinical data have shown under-estimation of probability of disease by the Framingham and JBS-2 risk charts in half of the patients with silent disease.

Conversely, the high risk group was mainly composed of diabetic patients, and only a few of them (three out of twenty-five patients in total) underwent carotid ultrasonography. Therefore, it is difficult to establish if any clinically relevant advantage may derive from the introduction of monocyte phenotypic

characterization into cardiovascular screening of such patients. Our data showed that type 2, but not type 1 diabetes, associates with expansion of pro-inflammatory $CD14^{high}CD16^{+}$ monocytes Furthermore, a negative linear relationship was found between pro-inflammatory monocytes and DBP, so that increased numbers of $CD14^{high}CD16^{+}$ cells were seen in those patients with the lowest levels of DBP, and these patients were in fact diabetic. However, the increase in $CD14^{high}CD16^{+}$ cells observed in type 2 diabetic patients reached statistical significance only when compared to levels measured in the low-risk group. This finding could be ascribed to the well established higher predisposition of type 2 diabetics to develop atherosclerosis compared with non-diabetic patients (Joint British Society Guidelines, 2005), and might therefore reflect the higher prevalence of asymptomatic disease in type 2 diabetes. Lack of any difference between type 2 diabetics and high- or moderate-risk subjects in terms of pro-inflammatory phenotype of circulating monocytes might be explained by the heterogeneity of patients in these latter categories, which comprised patients both with and without silent carotid disease.

4.5.2 Study limitations

The number of subjects studied here was relatively small, and in future studies large numbers will be requested to confirm the findings postulated here, as well as to establish the usefulness of this approach over and above current clinical practice.

Additionally, although carotid ultrasonography gives useful information about atherosclerotic burden, new non-invasive imaging modalities such as cardiovascular

magnetic resonance imaging (CMRI) will give further useful information and need to be incorporated in future studies, to overcome the multiple technical limitations associated with carotid ultrasonography that negatively impact on data reproducibility. Indeed, the strong dependency of IMT data on operator experience, the nature of scanner and the technique for IMT measurement can give rise to much variability of data between different researchers, and thereby affect the predictive value of IMT for coronary atherosclerosis and future cardiovascular events (reviewed by Komorovsky & Desideri, 2005; Passacquale *et al.*, 2008; Coll & Feinstein, 2008). In this context, extension of the present imaging study to other vascular territories would shed light on the potential prognostic ability of monocyte phenotypic characterization to detect silent atherosclerosis in other vascular beds.

4.5.3 Conclusion

In conclusion, our data demonstrate that expansion of $CD14^{high}CD16^{+}$ monocytes occurs in the peripheral blood of patients with cardiovascular risk factors and asymptomatic carotid atherosclerosis, and the measurement of this subset of monocytes in the peripheral blood has strong predictive value for silent atherosclerotic disease over and above the standard cardiovascular risk stratification methods currently used in clinical practice. The potential clinical applicability of these findings, as well potential limitations, will be further discussed in Chapter 5.

Chapter Five

Discussion

5.1 Introduction

This thesis has explored the clinical usefulness of measuring circulating MPA, and the resultant changes in monocyte phenotype, for the early diagnosis of asymptomatic atherosclerosis. The methodological approach used was translational, in which initial *in vitro* experiments (Chapter 2) were followed by a pre-clinical investigation in a murine model of atherosclerosis (Chapter 3) and finally by a preliminary clinical study in a population of patients at risk of cardiovascular disease (Chapter 4). The project was designed to address the dual questions of whether abnormal levels of MPA can provide a useful biological indicator of disease and whether if they play a pathophysiological role in atherosclerosis development, thus representing a potential therapeutic target for cardiovascular prevention. Taken together, the evidence provided in this thesis supports two major conclusions. Firtsly, MPA measurement *per se* is of limited usefulness in the early diagnosis of atherosclerosis. Hence, this research has refuted our initial hypothesis level of MPA in the peripheral blood reflects the stage of vascular disease. However, secondly, increased MPA formation, although of limited diagnostic value, leads to a systemic pro-inflammatory state mediated by a change in the phenotype and function of bound monocytes toward a type more prone to infiltrate the vasculature. This carries important pro-atherogenic implications and, importantly, can be easily detected *in vivo* by flow-cytometry-based monocyte characterization. Unlike MPA assessment, this latter has demonstrated to have high potential as a good biomarker for early atherosclerosis. Moreover, the possibility to intervene pharmacologically on the sequence of events leading from MPA formation leads to the acquisition of a pro-

inflammatory monocyte phenotype has been described in this thesis. All these aspects will be discussed in detail in the sections below.

5.2 Need for novel biomarkers of atherosclerosis

The development of predictive models of cardiovascular risk incorporating classical risk factors (for example the Framingham equation and JBS2 risk charts) provided a significant improvement in the clinical management of atherosclerosis-related cardiovascular disease, as they offered a standardised method to predict long-term cardiovascular risk for a given patient, based on which the nature and intensity of preventive measures needed could be assessed (JBS-2 Guidelines, 2005). Nevertheless, multiple large population-based studies have shown the limitations of this approach in predicting future cardiovascular events in individuals (Brindle *et al.*, 2003; Coleman *et al.*, 2007; Ramsay *et al.*, 2011), and attempts to discover novel biomarkers of atherosclerosis with higher predictive ability for cardiovascular events have been the subject of much research over the past twenty years.

In this context, a better understanding of the pathophysiology of atherosclerosis, and in particular its pro-inflammatory nature, led to much attention being focussed on markers indicative of inflammation. Measurement of C reactive protein (CRP), produced by the liver in response to the pro-inflammatory cytokines IL-6, IL-1 and TNFα (Hurlimann *et al.*, 1966; Mackiewicz *et al.*, 1991), has been the test most largely applied in many clinical trials as indicative of atherosclerosis-related

inflammation, due to the availability of a standardized methodology and the fact that it is relatively inexpensive to measure (Pearson *et al*., 2003). Levels of CRP have been shown to predict future cardiovascular events (Ridker *et al*., 2007; Sabatine *et al.*, 2007; Vidula *et al*., 2008). However, addition of CRP measurement to the standard Framingham risk calculation was not found to improve the estimation of future risk consistently (Shah *et al.*, 2009). Moreover, despite a strong correlation with future cardiovascular events in patients with known atherosclerosis, level of CRP has been reported to be a poor predictor of atherosclerotic burden (Khera *et al.*, 2006) thus suggesting that CRP assessment is of limited clinical utility in clinically healthy patients with underlying cardiovascular risk factors, for whom detection of silent atherosclerosis has important implications as regards their clinical management.

With this backdrop in mind, the work described here set out to firstly identify a novel biological indicator of atherosclerosis that would be easily measurable non-invasively and inexpensively, and hence potentially applicable to at-risk asymptomatic patients as a diagnostic tool; and secondly to verify the ability of this biomarker to specifically detect silent atherosclerosis with applicability over and above traditional cardiovascular risk stratification based on clinical assessment and standard cardiovascular risk prediction tools.

Our interest in the study of MPA as a potential novel biomarker of silent atherosclerosis stemmed from two important considerations: the first relates to the role of platelet activation in promoting inflammation and hence participating in the

pathophysiology of atherosclerosis; the second concerns the fact that MPA measurement involves a simple blood test where analysis by flow cytometry is relatively cheap and straightforward.

5.3 MPA and CD16-positive monocytes: from identification of their role in atherogenesis to identification of a novel biomarker of silent atherosclerosis

We found *in vitro* that MPA formation is pro-inflammatory, such that it leads to changes in the phenotype of circulating monocytes toward $CD16^+$ positivity, which exhibit increased capability to interact with the endothelium as well as increased expression of Toll-like receptors. Our results also provide mechanistic insight into how this occurs, inasmuch as we have found that the observed changes in monocyte phenotype and function in response to MPA formation are mediated by platelet-dependent COX-2 up-regulation and consequent PGE_2 synthesis in monocytes. These findings provide novel insight into both the molecular and cellular mechanisms underlying atherosclerosis, whilst at the same time offering the possibility to measure a biomarker which reflects the atherogenic process.

The change observed in the phenotype of circulating monocytes mediated by heterotypic aggregation suggested that assessment of the different monocytic subpopulations, along with MPA measurement, both of which are easily measurable in the peripheral blood by flow cytometry, could provide a novel diagnostic approach for detection of silent atherosclerosis even at an early stage of disease. We

found that increased platelet activation and elevated levels of circulating pro-inflammatory $CD14^{high}CD16^{+}$ monocytes reflect active inflammation and, whilst not necessarily specifically related to atherosclerosis (since we observed the same changes in healthy subjects following influenza immunization), are seen in subjects with underlying cardiovascular risk factors in the presence of atherosclerotic disease as documented by carotid ultrasonography. Indeed, the data postulated here from our clinical study conducted in patients at different levels of cardiovascular risk (Chapter 4) strongly support the validity of monocyte phenotype analysis in the identification of early atherosclerosis. Unlike MPA measurement, assessment of monocyte phenotype offered predictive information in terms of presence of silent disease, over and above both classical cardiovascular risk stratification tools and the traditional inflammation marker hs-CRP. This is not to imply that traditional cardiovascular risk calculators do not have a place, but rather that their value can be augmented considerably by also taking into account circulating $CD16^{+}$ monocyte numbers.

Our preliminary clinical data as presented here has demonstrated good correspondence between monocyte phenotype analysis and carotid ultrasonography in detecting subclinical atherosclerosis. Considerable evidence supports the add-on value of carotid ultrasononography to population-based cardiovascular risk estimation in asymptomatic subjects, particularly if non-invasive imaging is applied to individuals classified at moderate risk according to classical algorithms (Peters *et al.,* 2011). However, the clinical usefulness of cardiovascular screening based on a combination of classical clinical assessment and non-invasive imaging is

counterbalanced by the high cost as well as the logistic possibility of such an approach. In this context, monocyte characterization offers potential advantages inasmuch as it provides a relatively cheap biomarker, easily applied clinically and with high predictive ability. Such an approach could be useful to select patients in whom further imaging studies (invasive or non-invasive) are indicated.

As mentioned in Chapter 4, asymptomatic non-diabetic patients at low or moderate cardiovascular risk were those in whom carotid ultrasonography along with monocyte characterization led to re-classification of many individuals, based on detection of asymptomatic carotid plaques, such that adjustment of their therapy (addition of statin and/or aspirin) was then indicated. Our data suggest that such patients would benefit considerably by the introduction of a novel biomarker of atherosclerosis. Figure 5.1 illustrates the potential applicability of monocyte characterization into cardiovascular screening for primary prevention.

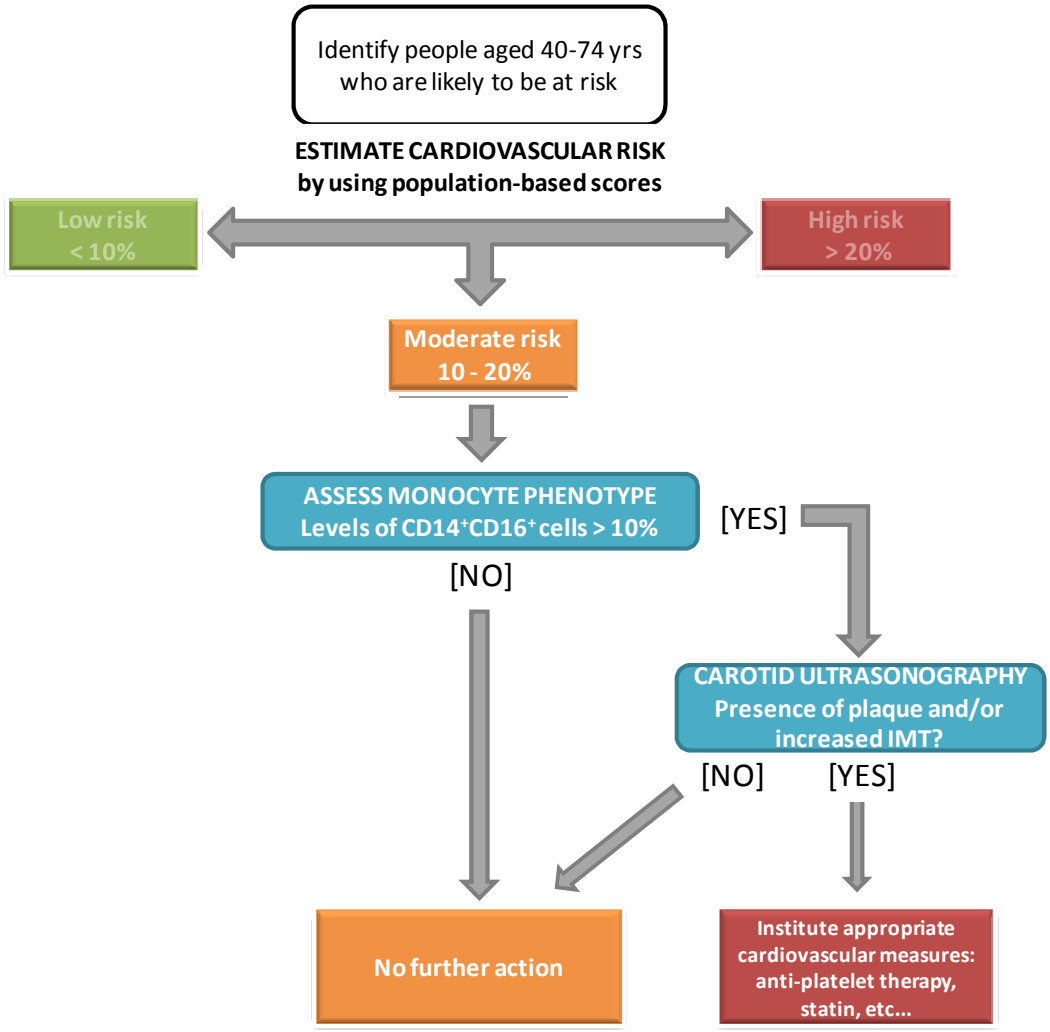

Figure 5.1 Potential applicability of monocyte characterization into cardiovascular risk assessment of asymptomatic patients. Full cardiovascular risk assessment should be performed in all people aged between 40 and 74 years who present with cardiovascular risk factors (hypertension, dyslipidemia, smoking habit). People aged 75 years and older, and those with diabetes, left ventricular hypertrophy, peripheral vascular disease or previous cardiovascular events are already considered to be in the high risk category and are not included in such risk assessment (NICE guidelines 67, 2010). Population-based tools estimate the risk of future cardiovascular events based on clinical assessment, and provide a classification of patients into three categories: low, moderate and high cardiovascular risk, defined by a probability of <10%, 10-20% and >20% of developing a future event in 10 years. People classified as being at low and moderate risk would benefit from additional diagnostic aids to indicate presence or absence of disease. An initial monocyte

characterization would identify those with increased level of $CD16^+$ monocytes, and such patients could then be selected to undergo non-invasive imaging by carotid ultrasonography, which would then guide the need for appropriate cardiovascular preventative measures.

5.4 Pro-atherogenicity of different monocyte subsets

Our findings *in vitro* that human $CD16^+$ monocytes exhibit augmented adhesiveness to the vascular endothelium and that their level increases in patients with atherosclerosis, together with our data in $ApoE^{-/-}$ mice that the murine homologue of $CD16^+$ monocytes, namely $Ly6C^{low}$, are elevated in relation to the extent of atherosclerotic disease present, are highly suggestive of a pro-atherogenic effect resulting from increased levels of pro-inflammatory monocytes. However, a definitive answer to the question of whether $CD16^+$ cells are more pro-atherogenic than the classical $CD14^+CD16^-$ subset has not yet been provided. We have partially addressed this topic in the pre-clinical study conducted in $ApoE^{-/-}$ mice, by comparing the phenotypic pattern of monocytes circulating in the peripheral blood to those infiltrating the plaque. Our data showed that, although it is the increase in $Ly6C^{low}$ cells in the blood that mainly reflects plaque size, both monocytic subpopulations are able to infiltrate arterial lesions. This would imply that expansion of human $CD16^+$ cells is a biological indicator of atherosclerosis only, without this subset of monocytes being preferentially implicated in plaque development despite its increased endothelial adhesiveness compared to the classical $CD14^+CD16^-$ subtype. Moreover, the evidence that aspirin decreases the trafficking of classical $Ly6C^{high}$ monocytes into lesions, thus reducing the macrophage-dependent lipid component within plaques, suggest that $Ly6C^{low}$ and their human counterpart $CD16^+$

cells have a minor effect on plaque vulnerability compared to classical monocytes. However, a direct translation of such data from murine to human disease would be inappropriate, since major differences exist between the two species in their inflammatory pathways. For instance, lymphocytes prevail over neutrophils in murine blood even under physiological conditions, whilst humans exibit predominance of PMN over mononuclear cells (Doeing *et al.,* 2003). Moreover, $ApoE^{-/-}$ mice develop monocytosis during atherosclerosis progression, which is absent in the human disease. Additionally, although not confirmed by functional assays, discrepancies has been identified between murine and human monocytes (Ingersoll *et al.,* 2010) in the expression profile of genes involved in phagocytosis of apoptotic cells and lipid-dependent activation. Such differences in white cell count and monocyte function can alter the inflammatory milieu and give rise to distinct inflammatory responses. As discussed in Chapter 3, $ApoE^{-/-}$ mice provide a good animal model of atherosclerosis in which to investigate monocyte vascular infiltration, and this has provided a reliable model for preliminary investigation of the *in vivo* effect of platelet activation on monocyte phenotype and trafficking into lesions. However, all the results collected in $ApoE^{-/-}$ mice would require confirmation in future clinical studies, as further discussed in the following sections. Table 5.1 summarizes and compares important findings in mice and humans, as collected in the current thesis, and the parameters that still need to be investigated in the human setting.

Table 5.1 Comparison between murine and human findings in normal physiology and atherosclerosis

	Mice	Humans
White cell count in blood	Predominance of mononuclear cells	Predominance of neutrophils
Atherosclerosis-related monocytosis	YES	NO
Monocyte subset predicting extent of disease	Non-classical Ly6C^{low} only	Non -classical CD16^{+} only
Monocyte subset infiltrating plaque	Both classical Ly6C^{high} and non-classical Ly6C^{low}	Unknown
Effect of anti-platelet drugs on circulating monocytes	*In vivo*: reduction of both total monocytosis and Ly6C^{low} expansion (aspirin and clopidogrel)	- *In vivo*: effect unknown -*In vitro:* inhibition of CD16 over-expression (aspirin)
Effect of anti-platelet drugs on plaque composition	- Reduction of total number of monocytes (aspirin and clopidogrel) - Reduction of Ly6C^{high} infiltration and macrophage-dependent lipid component (plaque stabilisation) (aspirin)	Unknown

5.5. Pharmacological modulation of monocyte phenotype in cardiovascular prophylaxis

The *in vitro* study described in Chapter 2 has provided evidence that platelet activation and consequent formation of MPA influences the acquisition of a $CD16^+$ monocyte phenotype in humans, this effect being sustained by the combined action of multiple biomolecular mechanisms involving adhesion molecules and humoral factors released by both platelets and monocytes in the microenvironment. Among those, we have identified monocytic PSGL-1 ligation by platelet P-selectin and enhanced synthesis of the COX-2-derived prostanoid PGE_2 as key events. In accordance with this, either interference in MPA formation, such as that induced by anti-PSGL-1 blocking antibody, or pharmacological inhibition of COX-2 activity, as obtained with NS-398 or aspirin, counteracted platelet-dependent CD16 over-expression on isolated monocytes with similar efficacy (Passacquale *et al.*, 2011c). These findings were confirmed by our *in vivo* study in $ApoE^{-/-}$ mice, in which administration of aspirin and clopidogrel suppressed atherosclerosis-associated monocytosis and reduced the expansion of $Ly6C^{low}$ cells through COX inhibition and interference in MPA formation respectively. Aspirin and clopidogrel are widely used in cardiovascular prevention (discussed in Chapter 1). Our demonstration that they can modulate phenotype/count of murine monocytes during atherosclerosis progression suggests that they exert additional anti-inflammatory activity, aside from their anti-thrombotic action, which may play a role in counteracting the progression of atherosclerosis. Moreover, given the effect of both drugs, particularly of aspirin, on plaque composition observed in $ApoE^{-/-}$ mice, it is conceivable to hypothesise

that a major effect of anti-platelet therapy is on plaque stabilization with consequent reduction of acute pro-thrombotic events triggered by plaque rupture. In future work, we plan to investigate whether administration of aspirin and clopidogrel to healthy subjects undergoing influenza immunization negatively modulate the expansion of $CD16^+$ monocytes induced by such immunisation. If this will prove to be the case, interventional studies might then be considered to explore the anti-atherosclerotic potential of aspirin and clopidogrel in the treatment of those patients with asymptomatic disease or those at high risk of such disease (especially if their $CD16^+$ monocyte count is high). As discussed in Chapter 1, the dubious role of, and conflicting results reported with, anti-platelet interventions in primary cardiovascular prevention might be attributable to the large heterogeneity in the study populations that have characterized previous trials, and monocyte characterization might overcome this problem in a future study.

Unlike the analysis of circulating monocyte phenotype, it is more difficult to evaluate the effect of anti-platelet drugs on plaque composition. Indeed, lack of a diagnostic modality enabling the molecular and cellular characterization of atherosclerotic lesions limits our ability to understand the impact that change in the phenotype of circulating monocytes toward a more pro-inflammatory subset might have in terms of disease progression in human disease. We have nw started already working in mice on the development of a novel magnetic resonance (MR)-based technique that would be applicable eventually to humans and enable the *in vivo* study of the monocyte/macrophage component of atherosclerotic plaques. However, this will require many years of research before it can be realistically be applied to

humans. Meanwhile, it will be useful to conduct prospective large population-based studies to evaluate the prognostic value of monocyte characterization for future cardiovascular events, as well as interventional clinical trials to explore the beneficial effects of pharmacological modulation of monocyte phenotype on atherosclerosis progression.

5.6 Anti-platelet drugs versus selective COX-2 inhibitors

Our preliminary data suggest that inhibition of platelet heterotypic aggregation or COX-blockade, as obtained with clopidogrel or aspirin respectively, might have a place in the prevention of atherosclerosis progression over and above their anti-thrombotic action, by virtue of their ability to counteract the acquisition of a pro-inflammatory phenotype by circulating monocytes. The anti-inflammatory effect of aspirin observed in both the *in vitro* and pre-clinical studies was most likely attributable to inhibition of the COX-2 isoform, which would have occurred with the high doses used in our investigations (the smallest dose of aspirin administrered to mice would correspond to the equivalent of ~200mg daily in humans). The administration of 75mg aspirin daily as recommended for long-term primary and secondary prevention would not be enough to modulate the enzymatic activity of COX-2 in the systemic circulation (Patrono *et al.*, 2005). Therefore, comparison of standard doses of clopidogrel and aspirin (75mg daily of both drugs) would merit consideration, since it could highlight differential anti-inflammatory (and hence anti-atherosclerotic) effectiveness of the two drugs. On the other hand, it would not be

ethical to test the efficacy of selective COX-2 inhibitors due to the well recognized detrimental role of these drugs in patients with (or at risk of) cardiovascular disease (Konstam *et al.,* 2001; Ray *et al.,* 2002; Warner *et al.,* 2002; Kearnery *et al.,* 2006). Indeed, systemic COX-2 blockade removes protective cardiovascular mechanisms, in particular release of endothelium-derived PGI_2 (McAdam *et al.,* 1999; Belton *et al.,* 2000) that are not affected by low-dose aspirin (Fitzgerald *et al.,* 1983). Our *in vitro* experiments demonstrate solely the pro-inflammatory effect sustained by monocytic COX-2, thus suggesting that monocyte-targeted rather than systemic COX-2 inhibition could be of potential benefit in cardiovascular prevention, . Similarly, systemic PGE_2 receptor antagonism could be harmful due to the variety of PGE_2 actions in the cardiovascular system depending on the EP receptor isoform expressed on cells/tissues and the concentration of the prostanoid in the microenvironment (Warner *et al.,* 2011). For instance, low levels of PGE_2 inhibit whilst high levels stimulate platelet activity, through a differential selectivity for distinct EP receptors and activation of distinct signaling pathways (Table 1.2, Chapter 1). Similarly, activation of endothelial EP2 and EP4 receptor isoforms, that in our co-culture experiments we found to mediate platelet-dependent CD16 over-expression in monocytes (Passacquale *et al.,* 2011c), induces protective vasodilatation (Warner *et al.,* 2011). More detailed characterization of the mechanisms underlying platelet-dependent changes in the phenotype of monocytes was beyond the scope of this thesis, but should be a focus of future research work in this area, since this will help in the identification of novel therapeutic strategies of potential clinical usefulness.

5.7 Future Directions

Further clinical investigations are required to extend our preliminary findings in a larger population-based study. Moreover, it would be useful to ascertain if measurement of $CD14^{high}CD16^{+}$ monocytes leads to improvement of the estimation of future cardiovascular events to a clinically important degree, in a longitudinal long-term follow-up study. Indeed, although we found that monocyte characterization was superior in identifying silent atherosclerosis compared to classical tools for cardiovascular risk stratification, this does not necessarily imply that early identification of plaque and consequent intensification of preventive measures will result in significant benefit in terms of reduction of future cardiovascular events in a given individual, compared with the classical approach. Finally, we only evaluated carotid atherosclerosis, so further clinical investigations are required to define the usefulness of monocyte characterization in detecting early asymptomatic disease in other vascular territories, in particular the coronary vasculature. We are now planning to examine this question, using cardiac MR to image coronary plaque burden as well as composition, and in turn to relate these to circulating monocyte phenotype.

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