Mediators of monocyte activity in inflammation

KEVIN JAMES WOOLLARD
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ASTON UNIVERSITY

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Summary.

C-reactive protein (CRP) is the prototypic acute phase serum protein in humans. CRP is currently one of the best markers of inflammatory disease and disease activity. One of the keys cells involved in inflammation within chronic inflammatory diseases is the monocyte. Monocytes are able to modulate inflammation through cytokine expression, cytosolic peroxide formation, adhesion molecule expression and subsequent adhesion/migration to sites of inflammation.

CRP has been previously shown to bind directly to monocytes through Fc receptors. However this observation is not conclusive and requires further investigation. The effects of incubation of CRP with human primary and monocytic cell lines were examined using monocytic cytokine expression, adhesion molecule expression and adhesion to endothelial cells and intracellular peroxide formation, as end points. Monocytic intracellular signalling events were investigated after interaction of CRP with specific CRP receptors on monocytes. These initial signalling events were examined for their role in modulating monocytic adhesion molecule and cytokine expression.

Monocyte recruitment and retention in the vasculature is also influenced by oxidative stress. Therefore the effect of 6 weeks of antioxidant intervention in vivo was examined on monocytic adhesion molecule expression, adhesion to endothelial cells ex vivo and on serum CRP concentrations, pre- and post- supplementation with the antioxidants vitamin C and vitamin E.

In summary, CRP is able to bind FcγRIIa. CRP binding FcγR initiates an intracellular signalling cascade that phosphorylates the non-receptor tyrosine kinase, Syk, associated with intracellular tyrosine activating motifs on the cytoplasmic tail of Fcγ receptors. CRP incubations increased phosphatidyl inositol turnover and Syk phosphorylation ultimately lead to Ca^{2+} mobilisation in monocytes. CRP mediated Syk phosphorylation in monocytes leads to an increase in CD11b and IL-6 expression. CRP engagement with monocytes also leads to an increase in peroxide production, which can be inhibited in vitro using the antioxidants α-tocopherol and ascorbic acid. CRP mediated CD11b expression is not redox regulated by CRP mediated changes in cytosolic peroxides. The FcγRIIa polymorphism at codon 131 effects the phenotypic driven changes described in monocytes by CRP, where R/R allotypes have a greater increase in CD11b, in response to CRP, which may be involved in promoting the monocytic inflammatory response. CRP leads to an increase in the expression of pro-inflammatory cytokines, which alters the immune phenotype of circulating monocytes. Vitamin C supplementation reduced monocytic adhesion to endothelial cells, but had no effect on serum levels of CRP. Where long-term antioxidant intervention may provide benefit from the risk of developing vascular inflammatory disease, by reducing monocytic adhesion to the vasculature.

In conclusion CRP appears to be much more than just a marker of ongoing inflammation or associated inflammatory disease and disease activity. This data suggests that at pathophysiological concentrations, CRP may be able to directly modulate inflammation through interacting with monocytes and thereby alter the inflammatory response associated with vascular inflammatory diseases.

Abbreviation.

a.u., arbitary units; AA, ascorbic acid; BCECF-AM, 2'-7'-bis-2-carboxy-5-(6)-carboxyfluorescein-acetoxymethylester; BCR; B-cell receptor; b cyt, b cytochrome; BMI, body mass index; BSA, bovine serum albumin; CARE, Cholesterol Recurrent Event Investigation; CAT, Catalase; Cu-ZnSOD, Copper/Zinc superoxide dismutase; CCR, chemokine receptors; CRP, C-reactive protein; CVD, cardiovascular disease; DCFH-DA, 2'-7'-dichlorofluorescein diacetate; DHA, dehydroascorbic acid; DHR-123, dihydrorhodamine 123; EC, endothelial cells; EGM, endothelial growth medium; ER, endoplasmic reticulum; ERK, extracellular-signal-regulated-kinase; FADD, fas-associated death domain; FcR, Fc receptor; FITC, fluorescein isothiocyanate; FS, forward scatter; GM-CFU, granulocyte/monocyte-colony forming unit; GM-CSF, granulocyte/monocyte-colony stimulating factor; GSH, glutathione; GSSH, oxidised glutathione; GSH Px, glutathione peroxidase; HO_2, hydroperoxy radical, HUVEC, human umbilical endothelial cells; HAEC, human artery endothelial cells; ICAM-1, CD54; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; sIL-6R, shed soluble interleukin-6 receptor; IL-1β, interleukin-1 beta; IL-1, interleukin-1; IL-1Ra, interleukin-1 receptor antagonist; IP_1, inositol monophosphate; IP_2, inositol diphosphate; IP_3, inositol triphosphate; IDL, intermediate density lipoprotein; JNK, jun kinase; LAD, leukocyte adhesion deficiency; LDL, low density lipoproteins; HDL, high density lipoproteins; ITAM, immunoreceptor tyrosine kinase activation motif; ITIM, immunoreceptor tyrosine kinase inhibiting motif; LPL, lipoprotein lipase; LPS, lipopolysaccharide; MnSOD, manganese superoxide dismutase; Mac-1, CD11b/CD18; MAPK, mitogen activated protein kinase; MdX, median X; MNC, mononuclear cells; MAb, monoclonal antibody; MW, molecular weight; NFκB, necrosis factor kappa B; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; O_2, oxygen molecule; O_2^-, superoxide radical; OH, hydroxyl radical; oxLDL, oxidised LDL; PECAM-1, CD31; PBS, phosphate buffered saline; PC, phosphatidylcholine; PCh, phosphocholine; PCR, polymerase chain reaction; PE, phycoerythrin; phox, phagocyte oxidase; PI, propidium iodide; P13K, phosphatidyl inositol-3-kinase; PKC, protein kinase C; PLA_2, phospholipase A_2, $_sPLA_2$, secretory phospholipase A; P/S, penicillin/streptomycin; RT-PCR, reverse transcriptase-PCR; PTK, protein tyrosine kinase; RA, rheumatoid arthritis; ROS, reactive oxygen species; RNS, reactive nitrogen species; SAP, serum amyloid P; SOD, superoxide dismutase; SS, side scatter; SH2, src homology 2

domain; SH3, src homology 3 domain; SOS, "son of sevenless" protein; SMCs, smooth muscle cells; TCR, T-cell receptor; TNF-α, tumour necrosis alpha; TNFR, TNF receptor; TRADD, tumour necrosis receptor-1 associated death domain; TrxR, thioredoxin reductase; VLDL, veryLDL; VLA, very late antigen; α-toc, α-tocopherol.

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1.0 General Introduction

1.0 General Introduction:

1.1 Inflammation:

Inflammation is strictly a normal response of the body to infection or injury. Whenever tissues are damaged then inflammation occurs, where the inflammation itself is not a disease, but an attempt to limit and eliminate the injury/infection. Inflammation can be divided into two fundamental types, acute inflammation (<14 days) and chronic inflammation (lasting months or years). Inflammation has long been recognised and the symptoms were first described in writings by Celsus in the first century AD, describing the four signs of inflammation. These four signs are: Rubor (redness), Calor (heat), Tumor (swelling) and Dolor (pain). These signs are a consequence of histological changes, which include vasodilation and increased vascular permeability. Vasodilation occurs in the arterioles, venules and capillaries. This increases blood supply to the tissue and gives the redness and the heat. Increased vascular permeability leads to oedema and swelling, as the blood vessels begin to leak protein as well as the normal fluid circulation of salts and water. But perhaps the most important interaction within acute inflammation, and is focused on throughout this thesis, is the adhesion and emigration of immune cells. White blood cells particularly neutrophils at the early stages of inflammation, normally get transported in the centre of the vessels and do not come in contact with the endothelial cells lining the vasculature. In inflammation they are able to come in contact with the vessel walls where the flow is slowed. The cells adhere in an active process involving surface charge and specific adhesion molecules. The white cells then move between the endothelial cells and migrate into the tissues towards the site of the 'injury' this is generally a chemotactic movement, regulated by the expression of chemokines both by the vessel wall and the circulating immune cells (as reviewed in; Kuby, 1997).

Chronic inflammation is not just a continuation of acute inflammation but is quite a distinct type of pathology, although it can arise from acute inflammation if the irritant persists it can also arise as a primary process without ever showing acute inflammation. Chronic inflammation differs from acute inflammation in that the white cells in the inflammatory area are mainly mononuclear cells (monocytes/macrophages and lymphocytes) rather than neutrophils. Briefly, recruitment of leukocytes in inflammation is characterised by an initial infiltration of neutrophils, which are present within an hour of the initiating stimulus. This phase is later replaced by a more sustained influx of mononuclear cells, mainly being monocytes, modulating the chronic stages of inflammation through secretions of and interactions with inflammatory mediators.

1.2 Monocytes:

Monocytes comprise 3-9% of the blood leukocytes. They are small cells, 10μm in diameter, and possess a kidney bean or horseshoe-shaped nucleus (often with a deep cleft). Monocytes are most closely related to neutrophils and, like neutrophils, monocytes and their differentiated forms, macrophages, are professional phagocytes (as reviewed in; Gordon, 1995). Activated monocytes differentiate into macrophages once they leave the blood and enter the tissues. Macrophages have a bone marrow origin (ie they were monocytes), and that only a small percentage (5%) of macrophages actually develop by local proliferation; probably representing not more than one cellular division (as reviewed in; van Furth, 1992). However the lineage of macrophages generated by local proliferation can be traced back to monocytes. In vitro both monocytes and macrophages are capable of division, but monocytes are better are more efficient, because they are less mature. In tissues, macrophages can form multinucleated giant cells capable of attacking and ingesting large particles or can form tight sheets of 'epitheliod' cells capable of walling off an offending particle (van Furth, 1992).

Tissue macrophages are larger (22μm) than monocytes in diameter. Their cytoplasm also contains granules, a well developed Golgi complex, some rough endoplasmic reticulae. The cytoplasm of monocytes and macrophages contains mitochondria and relatively few glycogen storage granules. Thus, in comparison to neutrophils, the monocyte derived cells generate energy to greater extent using oxidative metabolism (as further discussed below; Gordon, 1995).

Macrophages differentiate under the influence of local factors such as cytokines in the tissues. Two main functional populations are apparent upon histological inspection: (i) those that have differentiated in the tissues in the absence of inflammation and (ii) those that have been recruited to the site of inflammation. Those which have entered and differentiated in the tissues in the absence of inflammation, are included among the resident leukocytes, and are called 'fixed macrophages.' They include histocytes of the connective tissues (eg. Dermal dendrocytes, dendrophages), Kupffer cells of the liver, alveolar macrophages of the lung, and microglial cells of the nervous system. Those monocytic cells that enter the tissues in response to inflammation are called 'elicited' macrophages. Fixed macrophages are relatively un-reactive compared the elicited macrophages (van Furth, 1992).

Monocytes begin their lives as monoblasts. The time required for monocytic differentiation is shorter than for neutrophil differentiation (Figure 1.1). In the bone marrow, maturation from the granulocyte/monocyte precursor, GM-CFU (Granulocyte/Monocyte-Colony Forming Unit), to promonocyte to monocyte only takes about 48 hours (as reviewed in; Dexter & Spooncer, 1987). Unlike neutrophils, which exit

the bone marrow as terminally differentiated cells, monocytes leave the bone marrow relatively undifferentiated, in a functional immature condition. As a result, monocytes arrive from the blood to the tissues as multipotent cells capable of differentiating in a variety of different ways (Dexter & Spooncer, 1987).

MONOCYTIC CELLS REMAIN MITOTIC THROUGHOUT LIFE, ALTHOUGH THERE IS A DECREASE WITH INCREASING DIFFERENTIATION

MARROW   BLOOD   TISSUES

† Note: This monocyte chart shows protein detected

12 h   6 h   24 h   > 17 h

Monoblast   Promonocyte   Monocyte   Macrophage

Pluripotent Stem Cell   SCF

LYMPHOCYTES   IL-7

IL-3

GM-CSF

Myeloid Stem Cell

Erythrocytes

GM-CSF

Megakaryocytes

GM-CFU

Promonocyte

M-CSF

Macrophage

Monocyte

Eosinophils
Basophils
Mast Cells
Dendritic cells

Monoblast   2 DAYS

G-CSF

LPS

Neutrophils

Figure 1.1. Maturation of the monocyte. The granulocyte/monocyte-colony forming unit (GM-CFU) is the precursor to both neutrophils and monocytes, and it develops in the presence of the growth factor, GM-CSF (granulocyte/monocyte-colony stimulating factor). Monocyte differentiation is initiated by the formation of a monoblast, in response to M-CSF (monocyte-colony stimulating factor). The differentiation from monoblast to promonocyte to monocyte only takes about 48 hours.

Like neutrophils, monocyte circulate in the blood for about a day (17hrs half-life in blood) prior to egress to the tissues (or destruction in the spleen or liver). Unlike neutrophils, monocyte derived macrophages are far longer lived in tissues (van Furth, 1992). Macrophages often survive over two weeks in the tissues (compared to the few hours of

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neutrophils). This longer life in the tissues enables macrophages to differentiate and respond to local conditions. It also affords macrophages the time necessary to communicate with lymphoid cells and other tissue cells and therefore is one of the most important cells in modulating inflammation, particularly modulating disease outcomes in many inflammatory pathologies, such as rheumatoid arthritis (RA) or atherosclerosis.

1.3 Cytokines:

Monocytic cells release a variety of hormones that are important in inflammation, (once referred to as 'monokines'), including IL-1β, IL-6. IL-8, IFN-α, TGF-β and TNF-α. These cytokines influence both tissue alterations and lymphocyte activity. As already previously discussed, the inflammatory response is a vital component of the host defence; nonetheless, it can be very harmful and potentially fatal if it is not tightly regulated in vivo, cytokines in this respect are of no exception. It is the balance of anti-inflammatory cytokines such as cytokine receptor antagonists (e.g., IL-1Ra) or direct anti-inflammatory cytokines (e.g., IL-10), which maintain the tight modulatory immune effects of cytokines in vivo. Two important pro-inflammatory cytokines IL-6 and TNF-α secreted by activated monocytes are of particular importance within the human immune system and are further characterised below and throughout this thesis, and the role of anti-inflammatory cytokines in particular IL-10, are further discussed in other sections of this thesis.

Interleukin-6 (IL-6) exerts diverse effects on a variety of different organs or cell systems. It plays a central role in the differentiation and growth of haematopoietic precursor cells, B-cells, T-cells, keratinocytes, neuronal cells, endothelial cells, osteoclasts and osteoblasts (as reviewed in; Akira *et al.,* 1993; Taga & Kishmoto, 1997). Moreover, IL-6 induces the hepatic acute phase response by modulating the transcription of several liver-specific genes during inflammation, as described previously in the introduction. Altered levels of IL-6

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have been found in a variety of different diseases including Crohn's disease (Atreya *et al.*, 2000), osteoporosis (Jilka *et al.*, 1992), cardiac ischaemia (Kukielka *et al.*, 1995), cachexia (Strassman *et al.*, 1992), rheumatoid arthritis (RA; De Benedetti *et al.*, 1994), sepsis (Waage *et al.*, 1989) and AIDS (Poli *et al.*, 1990).

The diversity of IL-6 driven biological effects is all the more surprising, since the IL-6 receptor (IL-6R) is only present on a comparatively small number of cells i.e hepatocytes, monocytes, neutrophils and some T- and B- cells, whereas gp130 is expressed by all cells of the body (Peters *et al.*, 1998). Classically, binding of IL-6 to the membrane-bound IL-6 receptor (the α-receptor) causes recruitment of two membrane-spanning gp130 molecules (β receptors) into a tetra- or hexameric, active IL-6 receptor complex (Grotzinger *et al.*, 1999). Neither IL-6 nor IL-6R alone binds or activates gp130, which effectively means that the heterodimeric complex IL-6/IL-6R constitutes the active cytokine. A soluble form of the IL-6 receptor can be formed either by alternative splicing (Oh *et al.*, 1996) or by shedding of the membrane bound IL-6R (Mullberg *et al.*, 1995). Recent evidence indicates that the pathophysiological effects of Il-6 may depend strongly on a soluble form of the receptor (as reviewed in; Rose-John & Heinrich, 1994), see figure 1.2.

Transsignalling via the soluble IL-6R
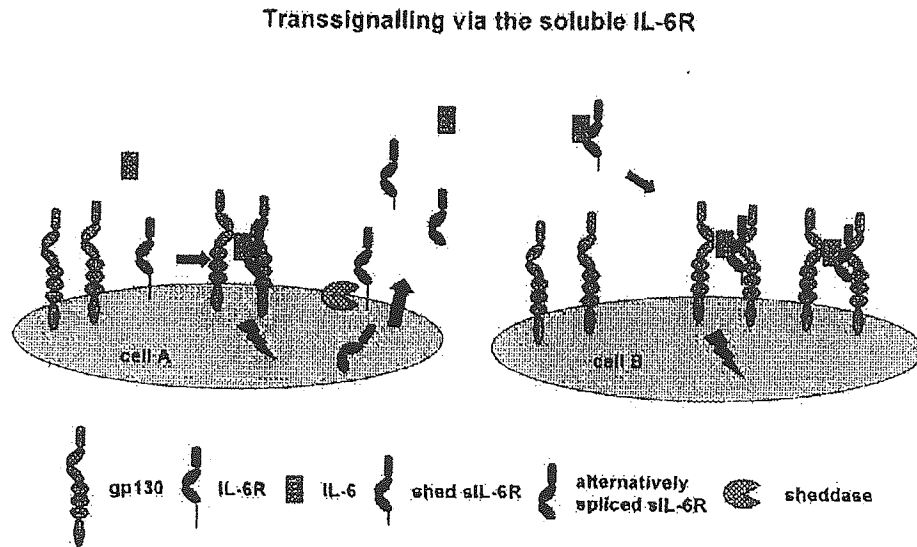
Figure 1.2 Transsignalling of the soluble IL-6R. IL-6 classically activates cells binding to the membrane-bound IL-6R followed by recruitment of two gp130 molec which activates downstream signal cascades. A soluble form of the IL-6R (sIL-6R) m be generated by proteolytic cleavage or by alternate splicing. The sIL-6R is agonistic active, so that a complex of IL-6/sIL-6R can activate cells which are norm unresponsive to IL-6, since they do not express the membrane bound IL-6R. This pro is called transsignalling.

The concept of a specific factor responsible for the necrosis of tumours has beer existence for decades, however, its true identity was first realised in 1984. Since tl TNF has been established as a major component in a number of cellular signal pathways contributing to abnormal cell growth, viral replication, immunomodulat: angiogenesis and the growth of bacteria and other parasites (as reviewed in; Aggarwa Natarajan, 1996). TNF also participates in a wide variety of clinical conditions includ cancer-induced cachexia (Beutler, 1993), delayed type hypersensitivity (Higashi et 1995), septic shock syndrome (Strieter et al., 1993), fever (Licino & Wong, 19S

hepatocyte regeneration (Cressman *et al.*, 1996) and rheumatoid arthritis (Muller-Ladner 1996).

TNF is a member of the TNF superfamily whose constituents are inducers of cellular processes such as apoptosis, proliferation, as well as transcription factor activation e.g., nuclear factor kappa B (NF-κB) and signal transduction e.g., c-Jun N-terminal kinase (JNK).

The traditional TNF signal cascade begins with its binding to members of the TNF receptor (TNFR) superfamily, whose activation has been linked directly to cellular proliferation, survival and differentiation (Locksley *et al.*, 2001). More than 20 members of TNFR family have been identified and are commonly classified into two groups based on the presence or absence of a death domain. The presence of a death domain for example TNFR1 (CD120a) or Fas (CD95), enables signal transduction via downstream proteins (eg. TNF receptor-associated death domain [TRADD] or Fas-associated death domain protein [FADD]), leading to caspase activation and apoptosis (Rathmell & Thompson, 1999; see figure 1.3). TNF receptor (TNFR)-related members without a death domain such as TNFR2 (CD30) recruit only TRAF2 and therefore regulate transcription factors NF-kB and AP-1 without the influence on TRADD-dependent apoptosis pathway (see figure 1.3).

TNF Signal Transduction
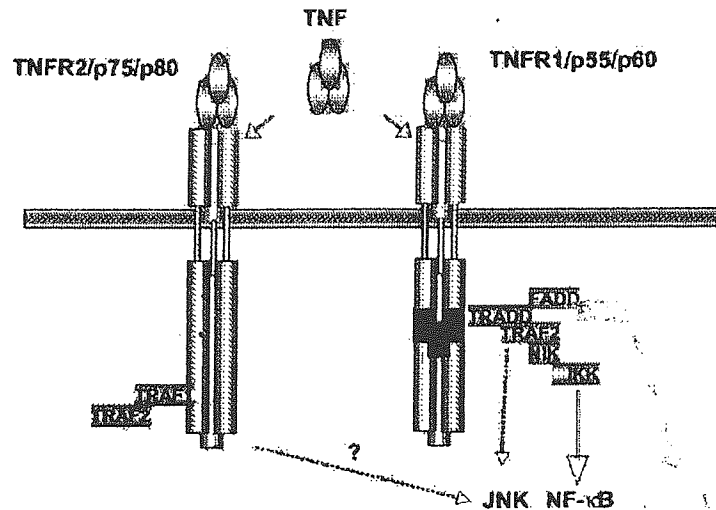
Figure 1.3 TNF signal transduction. TNF receptors containing a death domain (1 induce apoptosis via a TRADD-FADD pathway. TNF receptors without a death (TNFR2) have been shown to induce NF-κB and JNK activation via TRAF2. TNI also been shown to recruit TRAF2 and induce gene transcription.

1.4 Monocyte Adhesion:

The process of inflammation is central to protection of the host against infectious, a

potentially harmful, microorganisms, e.g., bacteria and it is the unregulated inflam

response or autoimmune response that can lead to vascular inflammatory pathologi

as aforementioned rheumatoid arthritis or atherosclerosis. In general, leu

circulating in the blood are drawn to sites of tissue injury or infection by chemoat

gradients. However, other mechanisms are required to retard and eventually 1

movement of leukocytes in order that they can exit the blood vessel and migrate i

affected areas. The major mechanism involved in arrest of leukocyte movement is

cellular adherence through contacts made between the leukocytes on the one hand

endothelial cells lining blood vessels on the other. These contacts are provided by a number of different cell surface molecules, collectively known as CAMs, which appear to act in succession to: 1) allow leukocytes to roll along the endothelial cell layer; 2) to stop the rolling; 3) to enable leukocytes to transmigrate from inside the blood vessel into the adjacent tissue (as reviewed in; Springer, 1994; Luscinakas & Gimbrone, 1996; Imhof & Dunon, 1995). Here the leukocytes and lymphocytes, release a number of mediators and enzymes to produce an inflammatory reaction. Essentially cellular adhesion molecules fall into three families of different structural architecture. They are the selectins, the integrins and certain glycoproteins included in the immunoglobulin superfamily.

The selectin molecules are characterised by a lectin-like domain attached to an 'epidermal growth factor-like' domain and a variable number of complement regulatory protein repeat sequences, which together comprise their extracellular motifs (as reviewed in; Kansas, 1996).

There are three selectins that have been shown to be particularly important in mediating cell-to-cell adhesion in the vasculature. They are L-selectin, which is constitutively expressed on leukocytes, P-selectin, which is stored pre-formed for rapid release in the α-granules of platelets or the Weibel-Pallade bodies of endothelial cells (as reviewed in; Blann & Lip, 1997), and E-selectin, which is present exclusively in endothelial cells and is only expressed following activation e.g., by cytokines (IL-1 or tumour necrosis factor-alpha; TNF-α) or gram-negative bacterial endotoxins (LPS, lipopolysaccharide; Carlos & Harlan, 1994). The lectin-like domain recognises and binds to specific sialylated carbohydrates (ligands) present on the cell surfaces of many cell types (myeloid cells and lymphocyte subsets). The ligand for E-selectin for example includes the sialyl Lewis X

group (sialic acid α 2-3 galactose β 1-4: fucose α 1-3 N-acetyl glucosamine), expressed on both neutrophils and monocytes and probably some memory T-cells (Pigott & Power, 1993). The interaction of P- and E-selectins with their carbohydrate ligands appears to be responsible for initiating leukocyte rolling on the endothelium (McEver, 1991). Although monocytes and some lymphocytes constitutively express L-selectin which may additionally bind to certain endothelial sulphated glycoproteins and glycolipids (McEver, 1991; Kansas, 1996), it is only when endothelial cells are activated to express first P-selectin and then E-selectin that adhesion forces are sufficiently strong to permit rolling and a reduction in velocity to occur (see figure 1.4).

Integrins are heterodimers consisting of non-covalently linked α and β subunits, 1100 and 750 amino acids long, respectively (as reviewed in; Gahmberg et $al.$, 1997; Chothia & Jones, 1997). There are at least 14 α subunits and eight or more β subunits, thus many combinations are possible. However, within this introduction, only the 'β_2 integrins' and the $\alpha_4\beta_2$ combinations will be considered as these are the main players in regulating cellular adhesion of monocytes to the endothelium. The surface antigen known as LFA (lymphocyte function-related antigen), which is expressed on all leukocytes, is an integrin consisting of an α subunit α_L, also defined as CD11a and the β_2 subunit, defined as CD18. Variants of this $\alpha_L\beta_2$ heterodimer are found on monocytes and granulocytes, but not lymphocytes: they are $\alpha_M\beta_2$ (also called MAC-1 or CD11b:CD18) and $\alpha_X\beta_2$ (also called CD11c:CD18). Interestingly, as CD11b is pre-synthesised and stored in intracytoplasmic storage pools situated in glutinase granules and secretory vesicles, this integrin can be rapidly expressed/upregulated on the cellular surface and hence can be an effective marker of monocyte activation (Buyon et $al.$, 1997; Carlos & Harlan, 1994). The α_4 subunit-containing integrins have been termed VLA for 'very late activation' since both VLA-1 and VLA-2 are expressed on lymphocytes some two weeks after antigen stimulation in
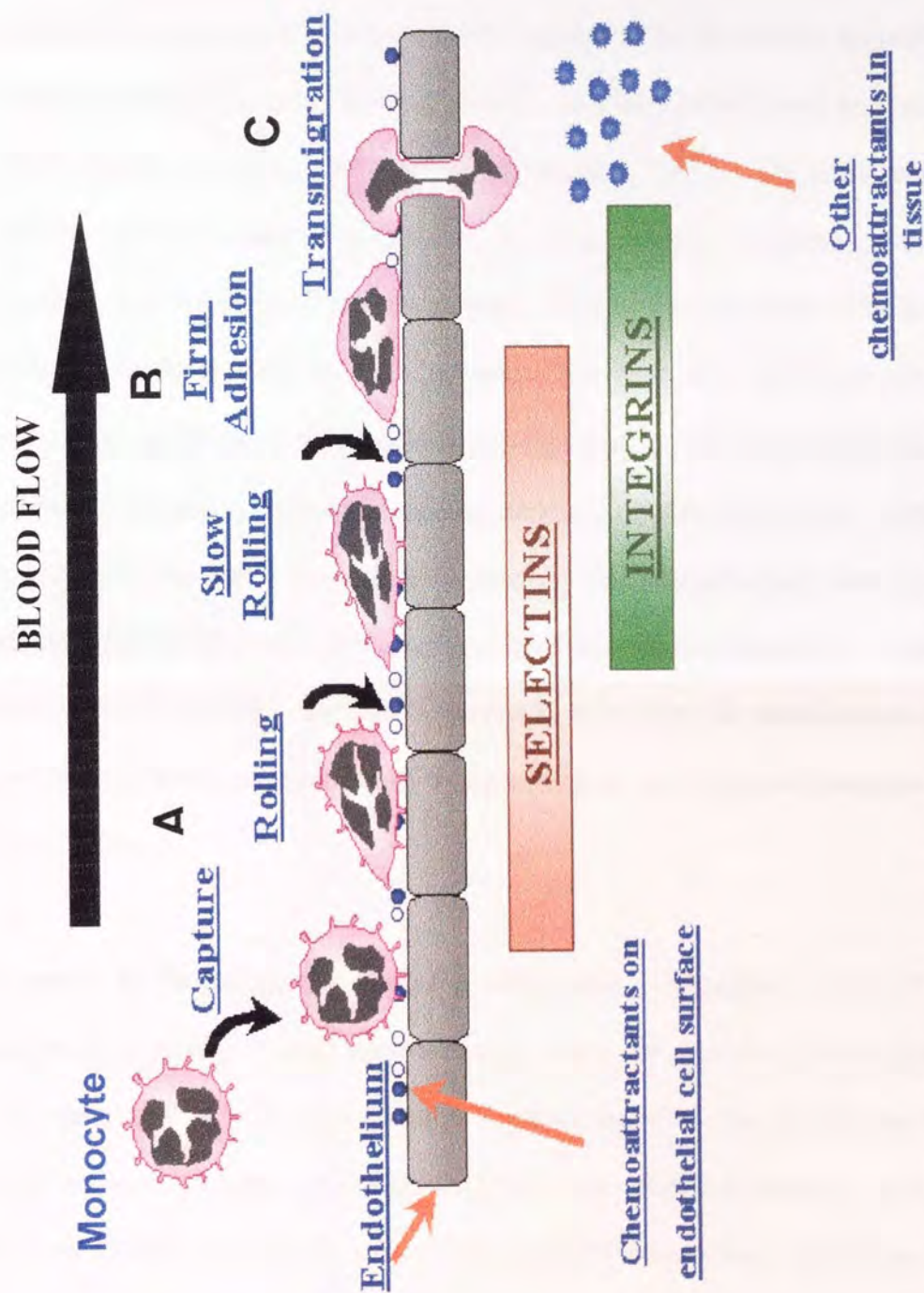
Figure 1.4 Cartoon diagram of the model of monocyte adhesion and transmigration across an endothelial cell layer at sites of inflammation. The initial steps (A) of monocyte tethering and rolling are selectin-dependent. Firm adhesion (B) follows if activating signals are encountered during monocyte rolling and this appears to be mainly mediated by monocyte integrins (CD11b/CD18; Mac-1) binding to ICAM-1 on the endothelial cells. Transmigration (C) requires CD31 expressed at endothelial cell junctions and monocytes in combination with a favourable chemotactic gradient.

vitro. Probably, the most important VLA integrin as regards cell adhesion is VLA-4 ($\alpha_4\beta_1$; CD49d:CD29) which is expressed on resting lymphocytes and monocytes but not neutrophils.

Adhesion via integrins to the endothelium appears to be due mainly to qualitative rather than quantitative changes in their cell surface expression (as reviewed in; Gahmberg *et al.*, 1997; Chothia & Jones, 1997; Bazzoni & Hember, 1998). The circulating leukocytes require activation signals, e.g., antigen, cytokine, agonist, to induce the conversion of integrins from a low- to a high-affinity state. There may be no change in surface density of integrins during this process, but the association between α and β-integrin subunits is important – mutations in the β_2 subunit prevent this association and result in the congenital deficiency known as leukocyte adhesion deficiency (LAD; Back *et al.*, 1992). Patients having LAD are highly susceptible to recurring bacterial infections that can be fatal in childhood if the deficiency is not rectified by a bone marrow transplant. Divalent cations such as Mn^{2+} and Mg^{2+} are also important in increasing the adhesiveness of integrins, probably by causing conformational changes in the α- and β-subunit structures (Chothia & Jones, 1997).

However, by far the greatest change in adhesiveness of integrins is effected by agonist activation, a rapidly induced phenomenon in which integrins are converted from a form that cannot interact with counter-ligands to a form that can. The intracellular changes that occur leading to structural alterations in the integrin subunits to cause this avidity shift are not well defined, but probably involve activation of protein kinases and phosphorylation of integrin subunits and other cellular membrane elements (Gahmberg *et al.*, 1997; Chothia & Jones, 1997; Bazzoni & Hember, 1998).

The integrins bind to receptors or counter-ligands, which are specific cell

molecules belonging to the immunoglobulin (Ig) superfamily, expressed on en

cells. The β_2 integrins primarily recognise intracellular adhesion molecule-1 (I

designated CD54; as reviewed in; Chothia & Jones, 1997). VLA-4 ($\alpha_4\beta_1$) by

recognises vascular cell adhesion molecule-1 (VCAM-1), a second distinct memb

Ig superfamily (Berlin & Berg, 1993).

In the mediation of cell adhesion, lymphocytes chiefly use LFA-1 (CD11a:CD

VLA-4 (CD49d:CD29) to tether to ICAM-1 and VCAM-1, respectively,

monocytes appear to use Mac-1 (CD11b:CD18) to attach to ICAM-1 expressi

(Gahmberg et al., 1997). These interactions are a necessary prerequisite to bring

leukocyte rolling along endothelium (see fig 1.3). Transmigration through the inte

junction of endothelial cells into injured or perceived injured or infected tissues a

require the expression of platelet/endothelial cell adhesion molecule-1 (PE

designated CD31), another member of the Ig superfamily (see fig 1.3; Newman, 19

1.5 Reactive Oxygen Species:

Reactive oxygen species (ROS) are produced at a high rate in all mammalian c

product of normal mitochondrial aerobic metabolism. The electron transport chain

of 5 protein complexes. Each complex consists of different subunits where comple

have multiple redox active prosthetic groups. Ubiquinone (Co-enzyme Q) is the o

protein component of the electron transport chain, capturing one or two electrons,

forming ubiquinol (hydroquinone). Since ubiquinone is not tightly bound to pro

may play a strategic role as a mobile carrier of electrons, transferring them to cytoc

and this is termed the Q cycle. In turn, cytochrome c shuttles electrons from compl

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complex IV of the electron transport chain (see figure 1.5). The electron transport (

also found within the endoplasmic reticulum (ER), where the reduced form of N

cytochrome c reductase leaks electrons to O_2 reducing it to O_2^- Nuclear membrar

contain an electron transport chain (as reviewed in; Cross & Jones, 1991).

The electron transport system (or respiratory chain) consumes 85-95% of O_2 util

mammalian cells, reducing it to water according to the equation:

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$$
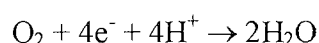
and generating energy necessary to synthesise ATP from ADP as a result of ox

phosphorylation (as reviewed in; Fernandez-Checa et al., 1998).

O_2 acts as the terminal electron acceptor during oxidative phosphorylation at comp

(as reviewed in; Buttke & Sandstrom, 1994; Halliwell & Gutteridge, 1990). Howev

process is not 100% efficient with the average person generating 200mmol O;

(Cadenas & Davies, 2000).

Therefore, continuous production of ROS results from approximately 1-2% of

consumed under normal conditions due to electron leakage. ROS comprise of

moieties with unpaired electrons, for example the hydroxyl radical (OH˙) a

previously mentioned superoxide radical (O_2^-), or moieties that have the ability to a

electrons from other molecules such as H_2O_2 and HOCl. ROS are derived from s

segments of the electron transport chain, mainly the ubiquinone site at complex III

catalyses the single electron transfer required for the conversion of O_2 to O_2^-,

complex I from NADPH cytochrome c oxidase. The resulting O_2^- can conse

produce other ROS.

In aqueous solutions, O_2^- is extensively hydrated and much less reactive, undergoing dismutation reaction at physiological pH catalysed by the enzyme superoxide dismuta (SOD) according to the equation:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$
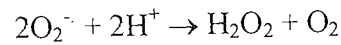
which is the sum of the two equations:

$$O_2^- + H^+ \rightarrow HO_2^-$$

$$HO_2 + O_2^- + H^+ \rightarrow H_2O_2 + O_2$$
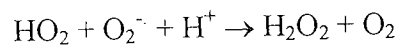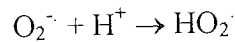
SOD catalyses this reaction 5 fold, but at the expense of producing the ROS, H_2O_2. H itself is relatively stable and has limited reactivity, but can freely diffuse across biolog membranes. O_2^- can only pass membranes slowly, unless an anion channel is present a the erythrocyte membrane or vascular endothelial cells. Similarly H_2O_2 generated wi the cell can continuously traverse across membranes to the extracellular fluid and constantly lost due to the action of catalase (as reviewed in; Evans *et al.*, 1997; Halliwe Gutteridge, 1990; Raha & Robinson, 2000).

The hydroperoxy radical (HO_2^-) is produced by the protonation of O_2^-, but at physiolog pH, only 0.025% of O_2^- exists as HO_2 and therefore its role as a cytotoxic molecu biological systems is doubtful. However, it may be an important damaging specie acidic compartments (as reviewed in; Evans *et al.*, 1997)

The hydroxyl radical (OH^-) is formed by the degradation of H_2O_2 in the presenc superoxide anion radicals, and this was first described by the Haber Weiss reaction:

$$O_2^- + H_2O_2 \rightarrow OH^- + OH^- + O_2$$
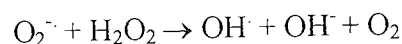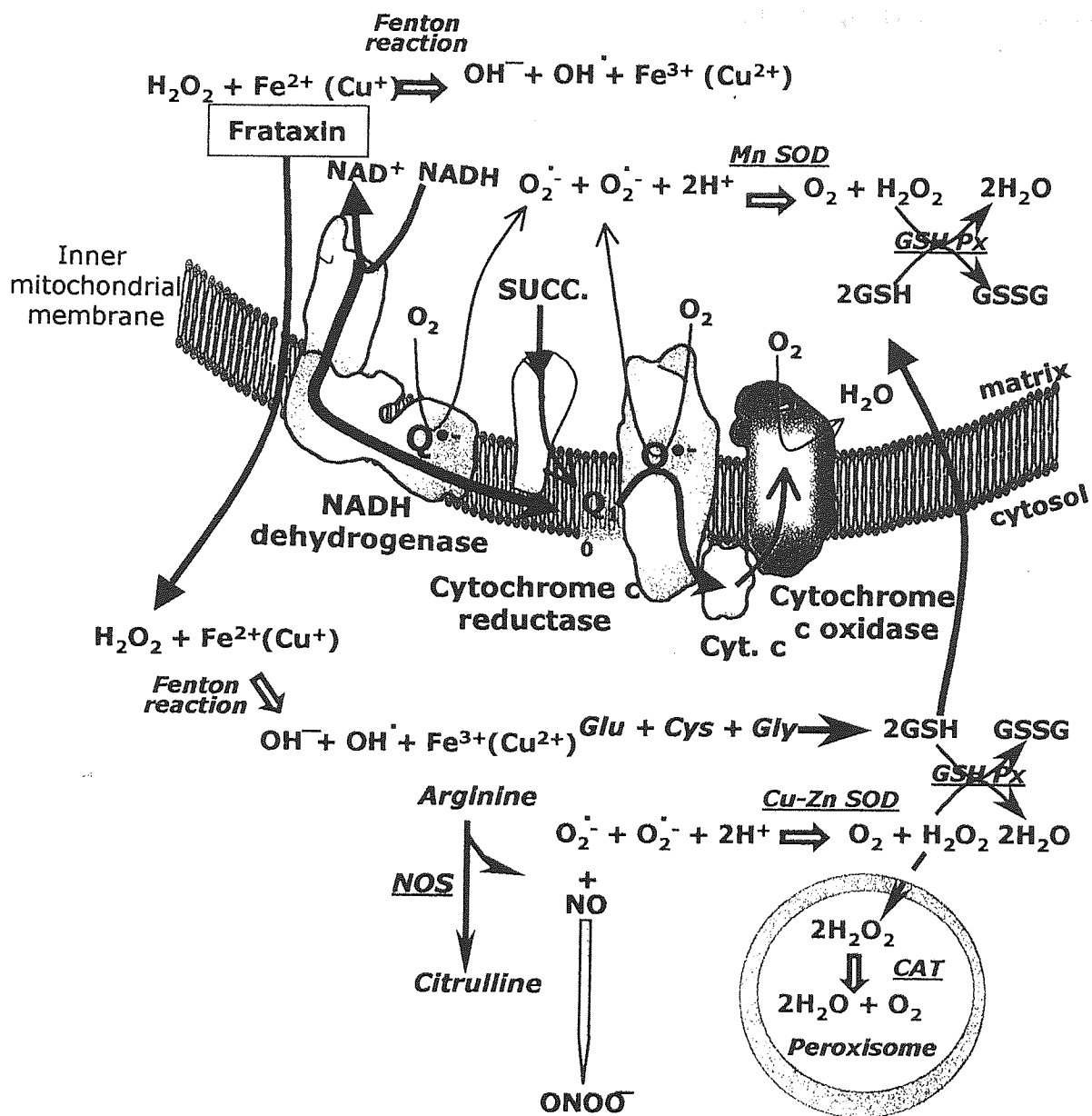
Figure 1.5. Putative sites of reactive oxygen species (ROS) formation and their associated detoxification pathways. Shown is a simplified schematic of the various components of the mitochondrial electron transport chain and sites of ROS production. Abbreviations used: Catalase, CAT; Copper/Zinc superoxide dismutase. Cu-ZnSOD; glutathione, GSH; oxidised glutathione, GSSH; glutathione peroxidase, GSH Px; manganese superoxide dismutase, MnSOD; nitric oxide synthase, NOS; superoxide dismutase, SOD; Adapted from Jackson *et al.*, (2002).

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It is highly reactive and therefore reacts close to its site of formation, eliciting sit

damage. The biological implications for the specificity of OH formation are ¡

The major determinant of the actual toxicity of H_2O_2 may be the availability or lc

metal ion catalysts for OH formation by the Fenton reaction according to th

equation:

$$H_2O_2 + Fe^{2+}(Cu^+) \rightarrow OH^- + OH + Fe^{3+}(Cu^{2+})$$
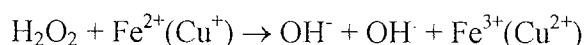
Nitric Oxide (NO) is a lipophillic molecule with a half life of between 6-30 secor

anaerobic conditions. Its reaction with O_2 forms nitrogen dioxide whicl

disproportionates to nitrate and nitrite in aqueous solutions of neutral pH. NO is ¡

by almost all nucleated cells from L-arginine by three distinct isoforms of nit

synthase (NOS), which include constitutive low NO output endothelial and

isoforms (eNOS and nNOS; as reviewed in; Nathan, 1992) and an inducible

output isoform (iNOS; Nathan, 1992; see fig. 1.5). The iNOS is induced when

stimulated by combinations of cytokines and bacterial products like lipopolysac

(Nathan, 1992). The potential for NO to react with biological targets is poor ow

short half life and propensity to react with O_2. However, the simultaneous prod

NO and O_2^- within close proximity to one another leads to their rapid reaction to

the potent oxidising and highly diffusible products peroxynitrite and peroxynitri

known as RNS (reactive nitrogen species; see fig 1.5). Peroxynitrite itself has a ¿

life, but displays far more reactivity than either of its precursors or H_2O_2 readily

with proteins and DNA (as reviewed in; Evans *et al.*, 1997; Murphy *et al.*, 1998).

While the electron transport chain of the mitochondria and those of the ER anc

have been discussed herein, other sources of ROS or peroxides quoted as the anic

or HO_2^-, or a compound that compound that contains O_2^- or HO_2^-, exist withi

These include hypoxanthine/xanthine oxidase, lipoxygenase, cyclooxygenase and gam

glutamyltranspeptidase systems (as reviewed in; Gabbita *et al.*, 2000; Sauer *et al.*, 2C

Perhaps the most important source of ROS for a monocyte is the NADPH oxidase sys

This is because the principal function of monocyte/macrophages is the destructio

invading pathogens and the removal of inflammatory debris. During phagocytosi

microbial intruders, professional phagocytes of the innate immune system increase

consumption of molecular oxygen, leading to a respiratory burst (as reviewed in; Dahl

& Karlsson, 1999). Here NADPH acts as an electron donor, converting molecular ox

to O_2^- according to the equation:

$$NADPH + O_2 \rightarrow NADP^+ + H^+ + O_2^-$$
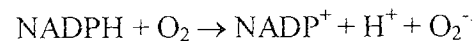
The NADPH oxidase system is dormant in resting monocytic cells (along witl

leukocytes) and is assembled and activated by a variety of mediators, such as a bacter

that interact with cell-surface receptors via intracellular pathways that are only p

known. Assembly of the oxidase requires interaction of at least four different cyto

proteins with the membrane-bound proteins of the electron transport chain (as reviewe

Babior, 1999). Briefly, the membrane-bound component consists of a unique *b*-

cytochrome referred to as cytochrome b_{-245} because of its low redox potentia

cytochrome b_{558} because of its characteristic peak in a differential spectrum. The prot

stored mainly (80-85%) in the membrane of specific granules in resting granulocytes,

is transported to the plasma membrane by fusion of granules during activation (Jesai

al., 1990). The other membrane-bound protein is a small guanine nucleotide bir

protein (G-protein), rap1A, located in the plasma membrane and in the membran

specific granules in both resting and activated cells (Mollinedo *et al.*, 1993).

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The cytosolic proteins participating in the assembly of the NADPH-oxidase consist of three proteins named by their molecular weight followed by the abbreviation *phox* for *ph*agocyte *ox*idase, and a Rac G-protein (Rac 1 and/or Rac 2). The three different cytosolic proteins, p40^{phox}, p47^{phox} and p67^{phox}, form a cytosol complex via interaction of SH3 regions and proline-rich regions. The factor p40^{phox} is tightly associated with p67^{phox} in resting cells (Nakamura *et al.*, 1998; see figure 1.6). The small GTPase Rac binds to p67^{phox}, as shown by using a fluorescent analogue of GTP, and it has been suggested that it binds also to the cell membrane and to cytochrome b_{558} (Nisimoto *et al.*, 1997; see figure 1.6).

During activation of cells, the NADPH-oxidase is assembled after phosphorylation of the b cytochrome and the p47^{phox} in the presence of high levels of Ca^{2+} in the cytoplasm. During oxidase activation, serine (ser) residues in the C-terminal portion of p47^{phox} become extensively phosphorylated, the protein acquiring up to nine phosphate residues. Of these, phosphorylation of either ser 303 or ser 304 has been previously shown to be necessary for restoration of enzyme activity in EBV-transformed, p47^{phox}-deficient, B-cells (Model *et al.*, 1998). Phosphorylation of p47^{phox} may be performed by mitogen-activated protein kinases (MAP-kinases). Three distinct classes of MAP-kinases exist, ERK, JNK and p38. Of these, the proline-directed kinases ERK and p38 phosphorylate p47^{phox} (El-Benna *et al.*, 1996), and may both be required for activation of the NADPH-oxidase (Rane *et al.*, 1997).
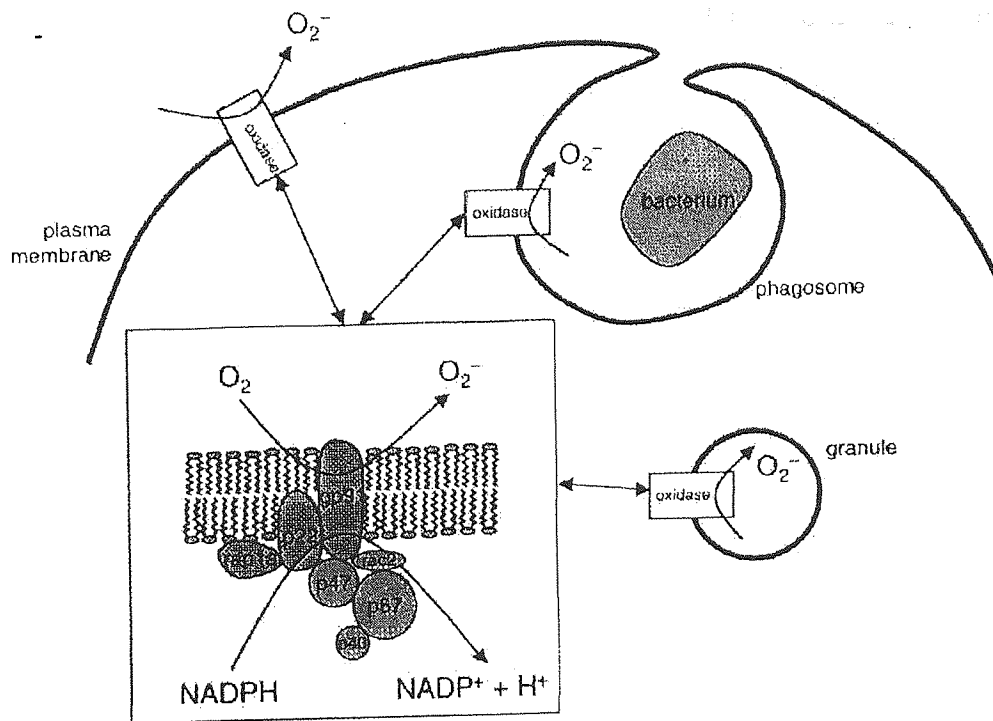
Figure 1.6. Possible sites for NADPH-oxidase assembly and activity in monocytes. Activation of cells by bacterium, leads to a translocation of cytosoli (p47, p67, p40 and rac2) to *b* cytochrome (*b* cyt) containing membranes.

ROS readily react with cellular macromolecules such as polyunsaturated fatty acic damaging them directly or setting in motion a chain reaction, where a free radical from one macromolecule to another, resulting in extensive damage to cellular s such as membranes (as reviewed in; Buttke & Sandstrom, 1994). ROS and RNS damage to proteins also leads to altered function. For example carbonyls are ul products of protein oxidation arising on amino acid side chains as well as sugars a (Berlett & Stadtman, 1997), where protein carbonyls have been strongly linked wi and associated phenomena (as reviewed in; Chevion *et al.*, 2000). Similarly, prot are readily oxidised by ROS, and oxidation of Ca^{2+}/ATPase thiols impedes enzym (Viner *et al.*, 1999). Free radical attack upon DNA generates a range of DN/

including strand breaks and modified bases. Hydroxyl radical (·OH) attack

to a large number of –pyrimidine and purine-derived base changes. ¦

modified bases have considerable potential to damage the integrity c

(Dizdaroglu, 1990; Floyd, 1990). ROS can effect intracellular signalling by

several different pathways of signal transduction, making use of signalling

as calcium, protein tyrosine kinases and protein tyrosine phosphatases, s

kinases, and phospholipases (as reviewed in; Jackson et al., 2002). Numero

factors exhibit redox sensitivity through thiol residues, thereby ROS can

expression of a variety of proteins (as reviewed in; Jackson et al., 2002).

Consequently, cells possess valuable defence systems to cope with ROS/RN

enzymes, SOD, glutathione peroxidase (GSH Px) and thioredoxin (Trx

reviewed in; Phillips, 2002). These enzymes scavenge peroxides which

NOS and prevent its production of NO. Therefore, these enzymes prevei

formation and allow NO production, which in turn facilitates endothelial

vascular health (McCarty, 1999). Tocopherols, cartenoids and ascorbic ɛ

block free radical chain reactions, while lactoferrin, transferring and/

caeruloplasmin are a group of agents which sequester transition metɛ

catalytic formation of ROS (as reviewed in; Buttke & Sandstrom, 1994).

1.6 ROS Defence:

Ascorbate is a cytosolic antioxidant that may increase the ability of the ce

ROS. Vitamin C (ascorbic acid) is fundamental to human physiology and, ৷

cannot synthesise it, it must be provided externally and transported

Vitamin C is present in human blood at concentrations of about 50 µM alr

in the reduced form, ascorbic acid. Similarly, ascorbic acid is present in ce

concentrations that can exceed by several orders of magnitude the blood levels of the vitamin (Vera et al., 1998). Transport of vitamin C is essential for its various functions as a cofactor and as an intracellular antioxidant. The relative accumulation of ascorbate over the surrounding plasma may be vital for the necessary protection of the cells against ROS generation, where previous work has shown long term vitamin C supplementation has been observed to protect against protein oxidation in human subjects (Carty et al, 2000). Studies with experimental scurvy have also shown a critical role for vitamin C in the maintenance of a normally functioning host defence system, suggesting that a tight regulation of the cellular content of vitamin C is central for normal host defence (Vera et al., 1998).

Monocytes transport the oxidised form of vitamin C, dehydroascorbic acid (DHA) through members of the GLUT-glucose transporter family, particularly the GLUT-1 transporter (Holmes et al., 2002; Daskalopoulos et al., 2002), however ascorbic acid uptake is possible through Na^+-ascorbate transporters (Malo & Wilson, 2000). Human leukocytes lack Na^+-ascorbate cotransporters but incubations of these myeloid cells with DHA in vitro increases their intracellular ascorbic acid concentration levels to those found in mature leukocytes in vivo (Vera et al., 1998). These considerations lead to the conclusion that the generation of DHA outside of the cell is essential to the cell's ability to accumulate vitamin C. Ascorbic acid undergoes reversible oxidation to DHA, a process that can be catalysed and greatly accelerated by traces of metal ions and prevented by metal chelators or reducing agents. The cells then transport the DHA down a concentration gradient through facilitative hexose transporters. Once inside the cell, the DHA is reduced to ascorbic acid, a mechanism allowing for the trapping and accumulation of high intracellular concentrations of reduced vitamin C (as reviewed in; Wilson, 2002).

Ascorbic acid contains a hydroxyl group by which it reacts with unpaired electrons and can reduce ROS. Ascorbic acid detoxifies the free radicals by releasing a hydrogen atom, which stabilises the once highly reactive molecule. The ascorbic acid is then in turn reduced back to its original state. The vitamins believed to indirectly prevent lipid hydroperoxide formation in plasma lipoproteins, e.g. in LDL, by reducing α-tocopherol radicals formed upon reaction with lipid peroxyl radicals (Figure.1.7). This is, in turn, an important function in the prevention of atherosclerotic plaque formation, through lipid oxidation protection, as discussed below. As ascorbic acid is an aqueous scavenger of ROS, previous studies have shown that intracellularly, ascorbic acid works in concert with other antioxidants such as glutathione to protect the cell from oxidative damage (Nordberg & Arner, 2001).

Vitamin E exists as at least eight naturally occurring compounds, including α, ß, γ - tocopherol and α, ß, γ -tocotrienol. α-tocopherol is the most active component of the vitamin E family and therefore has the highest biological activity in vivo. α–tocopherol contains a hydroxyl group by which it reacts with unpaired electrons and can reduce ROS (Jialal *et al.*, 2001;. See figure 1.7). It is the principal and most potent lipid-soluble antioxidant in plasma (Jialal *et al.*, 2001), therefore α-tocopherol is an essential nutrient that plays a significant role in protecting biological membranes and lipoproteins from damage caused by free radicals generated during lipid peroxidation. The antioxidant activity of α-tocopherol has persuaded many groups to study its ability to prevent chronic diseases, especially those believed to have an oxidative stress component such as cardiovascular diseases, atherosclerosis, and cancer. Several studies show that low levels of α-tocopherol are associated with increased atherosclerosis and that increased intakes of

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α-tocopherol appear to be protective against CAD (Diaz *et al.*, 1997; Jialal & Grundy, 1992). This may be since α-tocopherol is hydrophobic, it is co-transported with lipids in plasma LDL (Oram *et al.*, 2001), where oxidised LDL is involved in the formation of atherosclerotic plaques (see below); it is therefore already localised at the area in which oxidative damage could potentially occur.
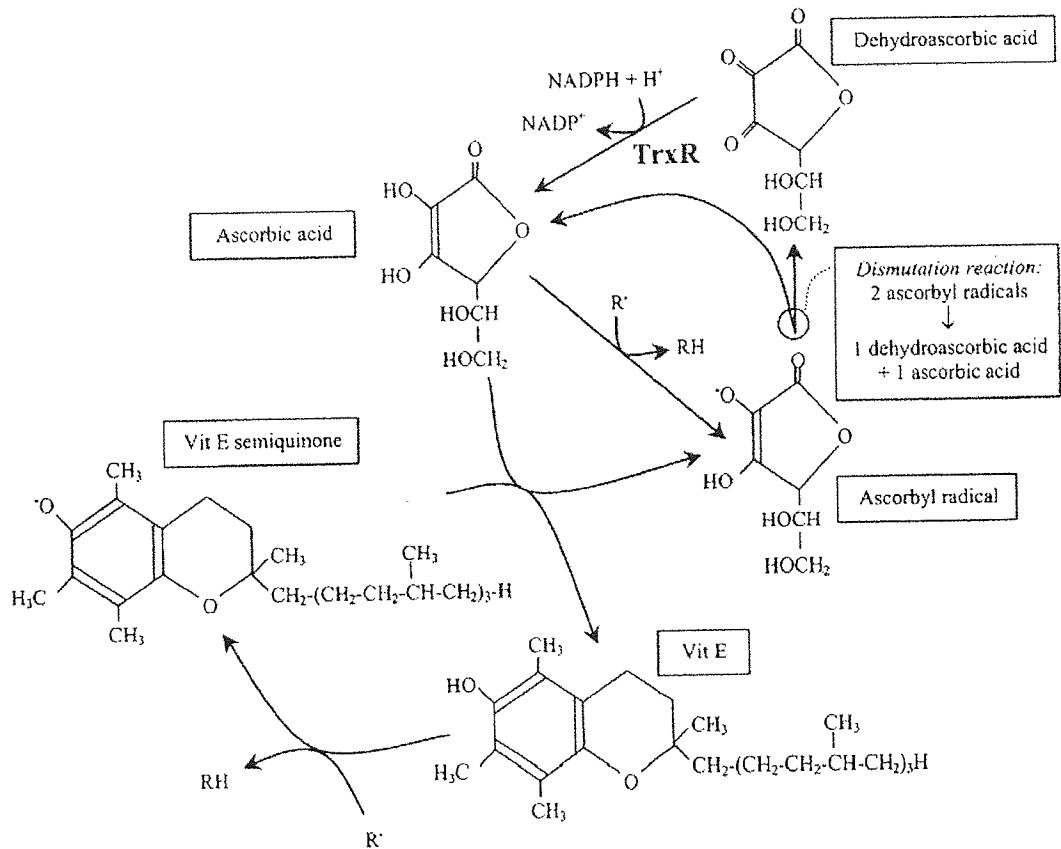
Figure. 1.7. The antioxidant properties of ascorbic acid and vitamin E. Vitamin E is the major antioxidant in biological membranes and can react with ROS (R·) to form vitamin E semiquinone, it is then reduced back by ascorbic acid. Ascorbic acid reacts with ROS to form a stable compound (RH) and it forms a ascorbyl radical which in turn is reduced directly by thioredoxin reductase (TrxR) or reduced to dehydroascorbic acid and then back to ascorbic acid.

A second mechanism has been suggested to account for the beneficial effects of α-tocopherol, where α-tocopherol may also inhibit the activity of protein kinase C (PKC; Azzi *et al.*, 1998). PKC plays a major role in signal transduction; it is implicated in a variety of events ranging from respiratory burst, to platelet aggregation and cellular differentiation. Many oxidant-initiated signalling processes are known to involve PKC. Li

& Cathcart, (1994) showed that superoxide generation and lipid oxidation in activated human monocytes appears to be regulated by PKC. PKC inhibition causes p47*phox* translocation and phosphorylation to be inhibited, and these assemble to form NADPH oxidase, as previously described (Babior, 1999). Therefore α-tocopherol inhibition of ROS is at least in part, related to a functional impairment of the NADPH-oxidase assembly.

1.7 Atherosclerosis & Lipid Oxidation:

Atherosclerosis, the principal cause of heart attack, stroke and gangrene of the extremities, accounts for 50% of mortality in the USA and Europe. The American Heart Association estimates that cardiovascular diseases affects 57 million Americans, and each year causes 954,000 deaths costing 259 billion dollars (as reviewed in; Zimmet, 2001).

Atherosclerosis is a pathological process that begins in the first decade of life. It gives rise to fatty streak lesions within arteries that are foci of macrophage–derived foam cells trapped beneath the endothelial cell lining of the artery (as reviewed in; Libby, 2001). Continued recruitment of mononuclear cells and the attendant proliferation and migration of smooth muscle cells (SMCs) results in the growth of fatty streak lesions into larger fibro-fatty plaques. Growth of atherosclerotic plaques could cause significant narrowing of the arterial lumen, which leads to chronic syndromes angina or limb claudication. However, atherosclerotic plaques may develop necrotic lipid cores with increased numbers of macrophages and fewer SMCs that predispose to increased expression of tissue factor and matrix metalloproteinases. These may trigger plaque instability due to degradation of the collagen matrix within the plaque (Herman *et al.*, 2001). This can lead to platelet activation, thrombosis and embolism, resulting in acute clinical syndromes, such as unstable angina, myocardial infarction or stroke (Libby, 2001). The natural history of

atherosclerotic plaque development can be very different depending on the site of the lesion within the arterial tree, the genetic predisposition of an individual to atherosclerosis and the spectrum of environmental risk factors for CVD, such as smoking, hypertension, diabetes and diet.

Atherosclerosis and inflammation share similar mechanisms in their early phases, when the interactions between the vascular endothelium and circulating leukocytes are increased. Two key initial events within the arterial wall during early atherogenesis are the recruitment and differentiation of circulating monocytes, and the uptake of oxidised low-density lipoproteins (oxLDL) or LDL by tissue macrophages to form lipid-foam cells, involved in atheroma plaque generation (as reviewed in; Greaves & Channon, 2002).

During its transport in the blood, cholesterol, an important component of blood lipids and essential part of cellular plasma membranes, is primarily in the form of LDL, a compound with a cholesteryl ester core and a coat of phospholipids, unesterified cholesterol and a molecule of apolipoprotein B-100 (apoB; as reviewed in; Kadar & Glasz, 2001). The precursor of LDL, very low-density lipoprotein (VLDL) secreted by the liver, is a larger particle with a number of triglycerides and three apolipoprotein molecules (apoB, apoC, apoE). In the capillaries of adipose and muscle tissues VLDL matures by the hydrolytic activity of the endothelial lipoprotein lipase (LPL) first into intermediate-density lipoprotein (IDL), then further into LDL (Thyberg et al., 1998). High-density lipoprotein (HDL), another lipoprotein produced by the liver, consisting of phospholipids, cholesterol, triglycerides and three apolipoproteins (apoAI, apoAII, apoE), is thought to aid the backward transport of cholesterol, i.e., from extrahepatic tissues to the liver, for further metabolism (Oram & Yokoyama, 1996). In the vascular wall LDL is transported through

the endothelium by small vesicular carriers (transcytosis; Vasile *et al.,* 1983). Lipoproteins are either trapped in the intimal matrix (Wright, 1996) or pass through fine fenestrations of the internal elastic lamina and move further into the media (Penn, *et al.,* 1997), where they again may become fixed to the matrix or metabolised by smooth muscle cells (Hajjar & Haberland, 1997). In the normal media lipoproteins are degraded by the contractile type smooth muscle cells are thought to be responsible for metabolism (Thyberg, 1998). Even in the early stages of atherogenesis, extracellular (matrix-bound) lipids begin to accumulate (Kruth, 1997) in the form of small liposome-like structures possibly formed from LDL by apoB degradation and cholesterol ester hydrolysis (Chao *et al.,* 1992). Proteolytic fragmentation of apoB on the other hand makes LDL unstable and it fuses into larger pools (Piha *et al.,* 1995). In an attempt to digest this disproportionate amount of lipid, macrophages and smooth muscle cells become foam cells and often die of lipid overload, contributing to the generation of the lipid core of advanced lesions (Stary *et al.,* 1994). Macrophages are normally protected from the toxic effects of excess cholesterol by multiple mechanisms, including the down regulation of surface LDL receptor molecules in response to replete intracellular cholesterol stores (as reviewed in; Brown & Goldstein, 1986).

The atherogenic effect of oxLDL has been reduced by antioxidants such as vitamin E and C in animal models (Carew *et al.,* 1987), emphasising the central role of oxLDL in atherogenesis. However an alternative hypothesis is that there is a potential for LDL oxidation within the circulation, and this oxLDL is taken up by monocytes/macrophages within the vascular tissue leading to foam cell formation (see fig 1.8). In contrast to LDL uptake, negative regulation of oxLDL uptake through scavenger receptors is absent, whose surface expression (e.g., CD36) is not diminished upon exposure to excess cholesterol (as

reviewed in; Steinberg, 1997; Chisolm *et al.*, 1999). Accumulation of oxLDL in the vascular wall recruits more blood monocytes through its chemotactic effect on these cells, leading to a greater progression of atherosclerosis. Isoprostanes are also important mediators within the atherogenic effects of lipid peroxidation. Isoprostanes are free radical oxidation products of arachidonic acid (a polyunsaturated fatty acid). Isoprostanes have potent biological activity in the vasculature, where they are believed to interact with a novel class of prostaglandin receptors exerting discrete effects on platelets and the endothelium (Minuz *et al.*, 1998).

As previously discussed, adhesion molecules participate in leukocyte-endothelial interactions and the extravasation of monocytes. Indeed adhesion molecules such as ICAM-1, VCAM-1 and E-selectin are strongly expressed within atherosclerotic plaques (Davies *et al.*, 1993) and the disruption of their expression may protect against atherosclerosis (Nageh *et al.*, 1997). Thus, the initiation and early phases of atherosclerosis, like fatty streak and fibrous cap formation, depend mainly on the specific conditions of cellular contact, particularly with the monocyte, and the transient or repeated synthesis of soluble monocytic mediators, such as cytokines, expressed by monocytes.

Figure 1.8. Atherosclerosis – Foam cell formation. Two key initial events within the arterial wall during early atherogenesis are the recruitment and differentiation of circulating monocytes, and the uptake of cholesterol via oxidised low-density lipoproteins (LDL) by tissue macrophages to form lipid-foam cells, involved in atheroma plaque generation 1. LDL migrate through vasculature and oxidised, which can be inhibited by antioxidants. 2. Macrophages, play an important role in LDL oxidation. 3. Uptake of oxLDL by scavenger receptors expressed by macrophages.

1.8 C-reactive protein: History, Structure & Function:

C-reactive protein (CRP) is an acute-phase protein that was discovered in 1930 by William S. Tillet and Thomas Francis at the Rockefeller Institute for Medical Research (Tillet & Francis, 1930). Studying the immune response of patients with pneumococcal pneumonia, they found that a component from sera of these patients precipitated with a soluble extract of the *Pneumoccus pneumoniae*. This soluble extract was called fraction C, later identified as a polysaccharide of the cell wall. After resolution of the pneumonia, no precipitation reaction occurred when the serum of recovered patients was mixed with fragment C, while in sera of patients with a fatal outcome, the precipitation reaction remained positive. Later, they found the same positive tests in patients with *Staphylococcus* osteomyelitis, rheumatic fever, subacute bacterial endocarditis and lung abscesses. However, sera of patients with viral infections, malaria and tuberculosis all gave negative results. In 1933, Rachel Welsh

found strongly positive precipitation tests with gram-negative microorganisms, her youngest patient being 6 months of age. Two arguments pointed in the direction of a non-specific physiochemical reaction to bacterial infection instead of a specific antigen-antibody reaction: first, the disappearance of a positive test after recovery of the infection and, second, a positive test result in a 6-month-old baby (as reviewed in; Thompson *et al.*, 1999). In 1941, OT Avery and TJ Abernethy found that the reactive substance responsible for the precipitation with fraction C was a protein: CRP. In addition, they discovered that Ca^{2+} is an essential participant in the reaction (as reviewed in; Gotschlich, 1982).

The CRP gene has been mapped to human chromosome 1, between 1q21 and 1q23. It contains 2263 nucleotides and has a single intron (Woo *et al.*, 1985). The CRP transcript is characterised by the presence of a long, 1.2kb, 3'-untranslated region, which perhaps mediates its rapid degradation following restoration of tissue structure and function. Synthesis in the liver is responsible for blood CRP (Gabay & Kushner, 1999), but extrahepatic expression has also been documented (Dong & Wright, 1996).

The swift rise of the serum concentration of CRP during the acute phase, the magnitude of the response approaching 1000-fold increase within 24-48 hours, with a biological half life of 19 hours (independent of CRP level), and the equally quick return to the very low normal concentration of a few μg/ml are the most impressive biological characteristics of CRP. Transcriptional regulation of CRP has been studied extensively both in vitro and in vivo. The combined results have established that IL-6 is the principal inducer of the CRP gene, while IL-1, glucocorticoids and certain other factors, including complement activation products, act synergistically with IL-6 to enhance its effect (Toniatti *et al.*, 1990; Ganapathi *et al.*, 1991; Szialai *et al.*, 2000). cis-Acting DNA elements necessary for

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constitutive and inducible liver specific expression of the gene have been defined. Regions of 540bp upstream and 1.2kb downstream of the gene are sufficient for liver specific inducible expression. Extended 5'- and 3'-flanking regions are required to suppress expression in the absence of induction (Murphy et al., 1995). Two sites for IL-6 inducible CCAAT/enhancer binding proteins (C/EBP) have been mapped in the proximal region of the promoter (Li et al., 1990; Ramji et al., 1993). In a close proximity to those sites are located two HNF-1 sites and a STAT3 response element (Toniatti et al., 1990b; Zhang et al., 1996). A site in the immediate vicinity of the poly(A) signal is also necessary for expression of the gene in transgenic mice (Murphy et al., 1995). Studies using IL-6 deficient mice have demonstrated that IL-6, although necessary, is not sufficient for induced expression of the CRP transgene (Weinhold et al., 1997). An additional factor, which in mice can be substituted or be induced by testosterone was reported to be required for expression of the human CRP transgene (Szalai et al., 1998). IL-6 stimulation results in binding of C/EBPβ and δ to multiple sites upstream of the promotor. A critical site for C/EBPβ binding is located at position −53 (Majello et al., 1990; Li & Goldman, 1996). Recent studies have shown that binding of C/EBPβ to its cognate site at that position requires the presence of the Rel protein p50, which binds to an overlapping non-consensus sequence (Cha-Molstad et al., 2000). The two proteins and their corresponding DNA elements form a ternary complex, which perhaps directly interacts with the promoter (Agrawal et al., 2001).

Post-transcriptional mechanisms also contribute to the regulation of CRP gene expression. Specifically, it has been shown that the rate of secretion of the protein is greatly accelerated during the acute phase (Macintyre et al., 1985). Under low physiological conditions, CRP is synthesised at decreased rates, the pentamer is assembled within the

endoplasmic reticulum (ER) and is largely retained there by two resident carboxylesterases (Macintyre *et al.*, 1994). During the acute phase, the half life for exit of pulse-labelled CRP from the ER is reduced from 18 hours to 75-minutes. The pronounced acceleration of secretion is apparently due to decreased affinity for CRP of one of the esterases, which has been attributed to a conformational change (Yue *et al.*, 1996).

The structure of CRP has been determined by X-ray crystallography at 3 Å resolution (Shrive *et al.*, 1996; Thompson *et al.*, 1999). Like serum amyloid P (SAP) component, the other pentraxin of known structure (Emsley *et al.*, 1994), CRP consists of five noncovalently associated protomers arranged symmetrically around a central pore. The overall dimensions of the CRP pentamer are about 102 Å outside diameter with a central pore diameter of 30 Å and a protomer diameter of 36 Å. The protomer consists of 206 amino acids folded into two antiparallel β sheets with a flattened jellyroll topology (see fig. 1.9), with a molecular weight of approximately 23,000 Da. The structure is very similar to that of SAP, although certain unique features characterise each protein.
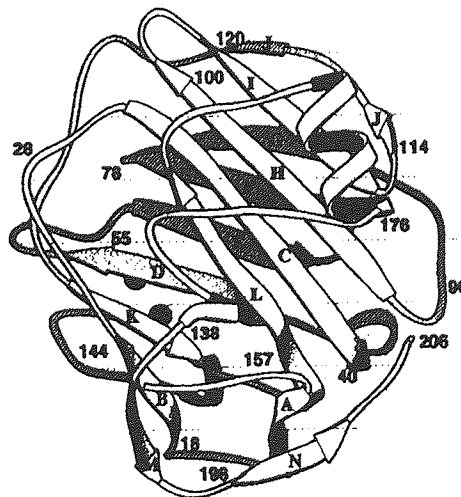
Figure 1.9. Structure of the CRP protomer. β-Strands are labelled A-N and the positions of key amino acid residues are indicated. The two ligated calcium ions are shown as spheres (adapted from Shrive *et al.*, 1996).

A long α-helix (residues 168-176) lies folded against one of the two β-sheets. The carboxyl terminal end of the helix along with loop 177-182 form one of the two sides of an unusual cleft that extends from about the centre of the protomer to its edge at the central pore of the pentamer (see Fig 1.10). The other side of the cleft is formed by parts of the amino and carboxyl termini of the protomer. The cleft is deep and narrow at its origin, but it becomes shallower toward the pentamer's pore. The side chains of the two CRP His residues (His 38 and 95) and that of Trp205 protrude from the cleft floor at the shallow end, while the side chains of Asn160 and 158 are located more centrally in the floor of the cleft. The C1q-binding site (Agrawal *et al.*, 2001b) and perhaps also the FcR-binding site (Marnell *et al.*, 1995) are associated with the cleft. On the opposite side of the protomer, two Ca^{2+} ions are ligated to side chains and main chain carbonyls of the polypeptide chain at a distance of 4 Å from each other. Both Ca^{2+} ions participate in ligand binding. Electron microscopic (Roux *et al.*, 1983) and crystallographic data indicate that in the assembled CRP pentamer all protomers have the same orientation. Thus, the molecule has two faces, a 'recognition' face exhibiting the five PCh binding sites and an 'effector' face containing the C1q and presumably the FcR binding sites (see fig 1.10).
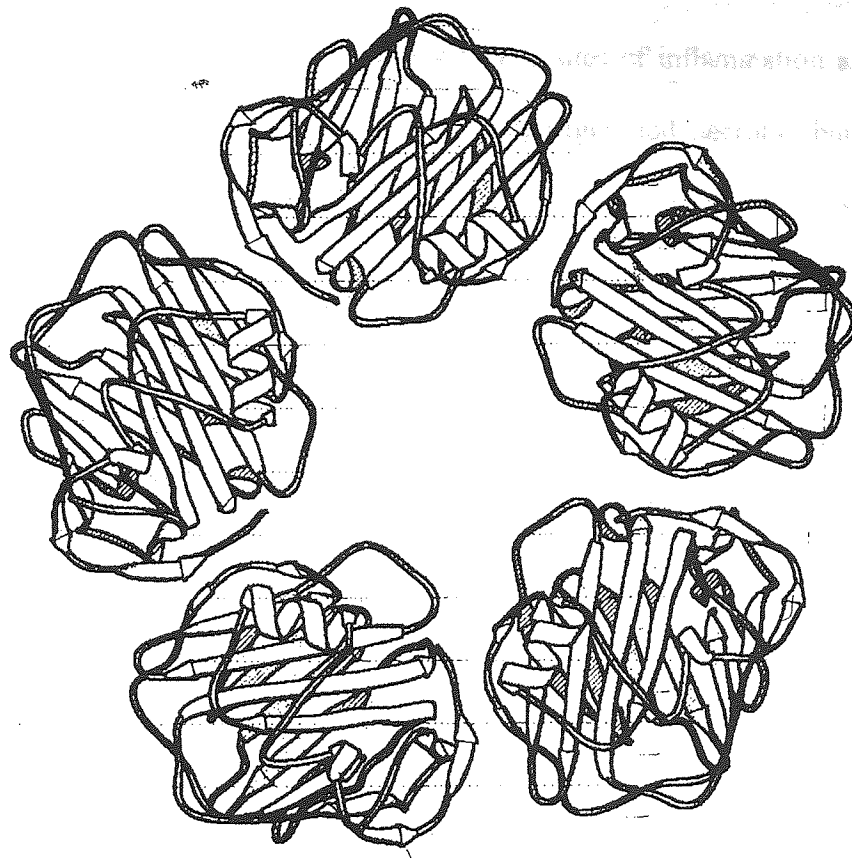
Figure 1.10. Pentameric structure of CRP viewed down the 5-fold symmetry axis. The effector face of the molecule is on top, while the calcium and PCh binding sites are on the opposite 'recognition' face (adapted from Shrive *et al.*, 1996).

The main biological function of CRP is its ability to recognise pathogens and damaged cells of the host and to mediate their elimination by recruiting the complement system and phagocytic cells. Phosphocholine (PCh), the principal CRP ligand, is widely distributed in teichoic acids, capsular carbohydrates and lipopolysaccharides of bacteria and other micro-organisms. Its presence has been reported in *Streptococcus pnemoniae* (Brundish & Baddiley, 1968), *Haemophilus influenzae* (Weiser *et al.*, 1997), *Pseudomonas aeruginosa*, *Neisseria meningiti* *s* and *Neisseria gonorrhoeae* (Serino and Virji, 2000), *Proteus morganii* (Potter, 1971) and *Aspergillus fumigatus* (Longbottom & Pepys, 1971). PCh is also present in the outer leaflet of most biological membranes as the polar head group of lecithin and sphingomyelin. Initial evidence for binding of CRP to cell membranes was

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provided by experiments demonstrating that at sites of inflammation and tissue necrosis CRP is associated with cell membranes of damaged and necrotic, but not normal cells (Kushner & Kaplan, 1961; Kushner et al., 1963). It was subsequently shown that CRP could react with emulsions of the PCh-containing phospholipids lecithin and sphingomyelin (Kaplan & Volanakis, 1974). Using lecithin liposomes and unimellar vesicles, Volanakis & Wirtz (1979) demonstrated that binding of CRP to the PCh polar head group of lecithin in lipid bilayers required the addition of submicellar concentrations of lysolecithin, a finding consistent with the previously established requirement for cell damage for binding of the protein to cell membranes at inflammatory sites. These observations were confirmed using red cells: binding of CRP was observed only after addition of sublytic concentrations of lysolecithin or treatment of the cells with phospholipase A_2 (PLA$_2$) from Naja naja (Narkates & Volanakis, 1982). PLA$_2$ hydrolyses the fatty acid ester bond in position 2 of lecithin and other sn-3-phosphoglycerides. It thus produces lysophospholipids without lysing the red cells. More relevant to the function of CRP than the snake enzyme is human secretory phospholipase A_2 (sPLA$_2$), an enzyme secreted by the liver as an acute phase protein (Hack et al., 1997). Like its snake homolog, sPLA$_2$ acts on phospholipids generating lysophospholipids, but unlike the snake enzyme, sPLA$_2$ cannot hydrolyse phospholipids of the outer membrane leaflet of normal cells, where most of lecithin is present (Fourcade et al., 1995). Cell damage causes an exchange of phospholipids (flip-flop) between the outer and inner membrane leaflets, resulting in enrichment of the outer leaflet in phosphatidyserine and phosphatidylethanolamine, which are normally present in the inner leaflet. As a consequence of their redistribution, phospholipids become susceptible to hydrolysis by sPLA$_2$, which generates lysophospholipids, including lysolecithin. In turn, the presence of lysolecithin within the outer leaflet allows binding of CRP to the PCh polar head on the cell surface (Hack et al.,

1997). Membrane flip-flop also occurs in cells undergoing apoptosis (Martin *et al.*, 1995), which therefore also becomes targets for sPLA$_2$ and CRP. Experimental evidence for binding of CRP to apoptotic cells has previously been reported (Gershov *et al.*, 2000). Kinetic analysis indicated that binding of CRP to cells undergoing apoptosis occurred later than that of annexin V, which binds to phosphatidylserine. Thus flipping of inner leaflet phospholipids and probably also generation of lysolecithin by the action of sPLA$_2$ precedes CRP binding. Another example of intact injured cells capable of binding CRP is provided by cells that have been subjected to sublytic attack by complement (Li *et al.*, 1994). The distribution of CRP on the surface of such cells is similar to that of the complement membrane attack complex. It is not known if CRP binding in this case is due to a disturbance of the phospholipids packing caused by the inserted complement protein complex (McCloskey *et al.*, 1989) or to generation of lysolecithin by sPLA$_2$. In addition to the membrane of intact injured cells, CRP also binds to membranes and nuclear constituents, including histones (Du Clos *et al.*, 1988), small nuclear ribonucleoproteins (Du Clos *et al.*, 1989) and ribonucleoprotein particles (Pepys *et al.*, 1994) in a calcium dependent, PCh inhibitable fashion. Furthermore, deposition of CRP to nuclei of necrotic cells at sites of inflammation has been observed (Gitlin *et al.*, 1977).

CRP bound to a multivalent ligand is recognised by C1q and can efficiently initiate the formation of a C3 convertase through the classical complement pathway (Kaplan & Volanakis, 1974; Siegal *et al.*, 1975). Structural data (Shrive *et al.*, 1996) indicate the presence of one C1q binding site per protomer (see below). However, consideration of the relative sizes of CRP and the C1q globular head led to the proposal that only one site per pentamer is available for binding C1q (Agrawal *et al.*, 2001b). Therefore, it appears that more than one CRP pentamer in close proximity to each other is necessary for activation of

the classical pathway, which is also a condition for complement activation by IgG. Assembly of the C3 convertase by complexed CRP proceeds similarly to activation initiated by immune complexes. Like immune aggregates, insoluble CRP- PCh precipitates are solubilised by complement and fragments of C3 bind covalently to CRP and PCh during the solubilisation reaction (Volanakis, 1982). Also, formation of covalent complexes between CRP and the α' chain of C4 has been demonstrated (Volanakis & Narkates, 1983). Thus, CRP-initiated activation of the classical pathway leads to decoration of the activating surface with opsonic fragments of C3 and C4, which is consistent with the proposal that CRP mediates phagocytosis of its ligands.

Functional activities of CRP attributable to direct interactions with phagocytic cells have been known for many years (Hokama *et al.*, 1962; Kindmark, 1971; Mortensen *et al.*, 1976), but it was only relatively recently that CRP receptors were identified. Work in Du Clos' laboratory (1988-2003) has provided strong evidence that Fc receptors for IgG are also the receptors for CRP. It was initially demonstrated that CRP binds to the high affinity IgG receptor FcγRI, on mononuclear cells and on COS cells transfected with a cDNA encoding this receptor (Crowell *et al.*, 1991; Marnell *et al.*, 1995). More recently, transfection experiments using FcγRIIa cDNA and parallel studies on monocytic cell lines expressing this receptor, demonstrated that it is the main high affinity receptor for CRP (Bharadwaj *et al.*, 1999). Experiments using FcR γ-chain deficient and FcγRII-deficient mice indicated that similarly to man, murine FcγRI and FcγRII are the receptors for CRP (Stein *et al.*, 2000; Mold *et al.*, 2001).

FcRs exist for every antibody class: FcγRs bind IgG, FcαRs bind IgA, FcεRs bind IgE, FcμRs bind IgM and FcδRs bind IgD (as reviewed in; Hulett & Hogarth, 1994). The main FcR discussed throughout this thesis is the FcγR. FcγRs (FcγRI, FcγRII and FcγRIII) traditionally recognise overlapping non-identical interaction sites in the lower hinge region of the C_H2 domain of the IgG molecule (as reviewed in; Jefferis *et al.*, 1995). FcR capable of triggering cell activation possess one or several intracytoplasmic activation motifs, which resemble those of BCR and TCR signal transduction subunits (Reth, 1989). These motifs, composed of twice repeated YxxL sequence flanking seven variable residues (or 12 in the case of FcγRIIa), are designated immunoreceptor tyrosine based activation motifs (ITAM; Cambier *et al.*, 1994; figure 11). FcR that do not trigger cell activation have no ITAM. One FcR category constitutes a family of single-chain IgG receptors, collectively referred to as FcγRIIB, whose intracytoplasmic domain possesses a motif that inhibits cell activation by receptors capable of triggering cell activation (figure 11). This motif contains a single YxxL sequence that was designated immunoreceptor tyrosine based inhibition motif (ITIM; as reviewed in; Daeron, 1997; figure 1.11).
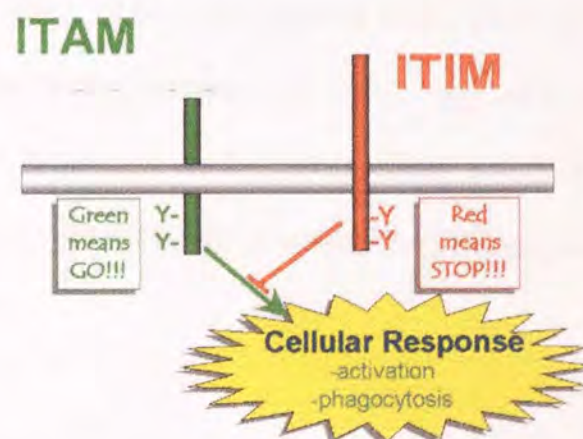
Figure 1.11 ITAM / ITIM cellular signalling. ITIM inhibits ITAM activation signalling.

The human FcγRIIa is the only single chain FcR with an ITAM. FcγRIIa and FcγRI are encoded by two related genes, located on the long arm of chromosome 1 (1q23-24 and 1q21.1 respectively). Cells of the myeloid lineage, such as monocytes, macrophages, primarily express FcγRIIa, whereas FcγRI is expressed by macrophages, monocytes and neutrophils.

An early intracellular event following aggregation of FcR with ITAM is the phosphorylation of the receptors themselves. Within the first 15 seconds following FcRγ engagement, ITAMs become tyrosine phosphorylated (as reviewed in; Daeron). Phosphorylation of ITAM is correlated with the activation of several sets of cytoplasmic protein tyrosine kinases. FcγRI activates Lck, Lyn and Hck in U937 monocytic cell line treated with IFNγ (Durden et al., 1995). FcγRIIa activates Lyn and Hck in monocytic THP-1 cells (Ghazizadeh et al., 1994) and Fgr in neutrophils (Hamada et al., 1993). Lyn and Hck also coprecipitate with FcγRIIa following its aggregation in THP-1 cells (Ghazizadeh et al., 1994). A second set of kinases that become activated upon aggregation of FcR are the Syk family kinases. Syk was found to be activated in human pulmonary macrophages and cultured monocytes (Darby et al., 1994) triggered via FcγRI; in THP-1 cells (Kiener et al., 1993), HL60 cells (Agarwal et al., 1993), platelets (Chacko et al., 1994) and human neutrophils (Unkeless et al., 1995) triggered by FcγRIIa; and in mouse macrophage cell line transfected with FcγRIIa (Shen et al., 1994). Syk was also coprecipitated with FcRγ in THP-cells following FcγRI aggregation and with FcγRIIa (Durden & Liu, 1994).

Syk binds to phosphorylated FcRγ ITAM by its SH2 domains. An efficient binding requires the two SH2 domains of syk and the two phosphorylated tyrosines of ITAMs.

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When bound to phosphorylated ITAMs, Syk becomes a substrate for lyn, and once activated, it undergoes autophosphorylation. Activated Syk may then leave the receptor and phosphorylate downstream substrates. Once activated by Syk, PLCγ generates metabolites that activate PKC and the inositide phosphate cycle. IP_3 (inositol triphosphate) triggers the mobilisation of intracellular Ca^{2+} stores by binding to IP_3 receptors on the endoplasmic reticulum. The resulting transient increase in the intracellular Ca^{2+} concentration is believed to open Ca^{2+} channels in the plasma membrane, and this results in a sustained peak in the intracellular concentration of Ca^{2+}. An increased intracellular concentration of Ca^{2+} is a constant feature of cell activation triggered by all FcR with ITAM (as reviewed in; Daeron, 1997). Some signals triggered by FcR reach the nucleus via the Ras pathway. FcγRIIa activates the Ras pathway via the exchange factor Sos, bound to the adaptor Grb2 that is recruited upon phosphorylation of Shc. Ras phosphorylates Raf, which phosphorylates the MEK kinases, which eventually phosphorylates MAP kinase (Galandrini et al., 1996). MAP kinase has also previously shown to be activated following aggregation of FcγRI (Durden et al., 1995). Once in the nucleus, MAP kinase activates transcription factors, and activation then leads to gene expression, for example FcγRI and FcγRIIa aggregation has been shown to induce NFκB in human neutrophils (Tsitsikov et al., 1995), resulting in the transcription of cytokines (Oh & Metcalfe, 1994).

1.10 CRP & Monocytes:

In view of current understanding of the biology of FcRs and particularly of their ability to deliver either activating or inhibitory signals to effector cells, it seems possible that the apparently contradictory results on the effects of CRP on leukocytes that have been

reported over the years can be attributed, at least in part, to differential expression and/or differential aggregation patterns of various FcRs.

Interaction of cell- or particle-attached CRP with phagocytic cells leads to phagocytosis of the cell or particle. The opsonic properties of CRP have been demonstrated for both macrophages (Mortensen et al., 1976; Mortensen & Duszkiewicz, 1977) and neutrophils (Kindmark, 1971; Kilpatrick & Volanakis, 1985). Phagocytosis of CRP-opsonised particles by mouse macrophages and neutrophils was shown to proceed through FcγRI (Mold et al., 2001). Enhancement of phagocytosis by CRP is also mediated indirectly by opsonic complement fragments attached to CRP ligands as a result of CRP-initiated complement activation (Edwards et al., 1982). In addition to phagocytosis, interaction of CRP with monocytes/macrophages has been reported to induce tumoricidal activity (Barna et al., 1987; Tebo & Mortensen, 1991), a respiratory burst (Zeller et al., 1986), production of hydrogen peroxide (Tebo & Mortensen, 1991), and secretion of IL-1 and TNF (Galve-de Rochemonteix et al., 1993). In addition, CRP has been reported to induce clevage and shedding of L-selectin from the surface of neutrophils resulting in reduced adhesion to endothelial cells and thus decreased migration to tissues (Zouki et al., 1997). There is also evidence that activated complement and CRP are present in atherosclerotic lesions. CRP is found to bind PCh group of degraded, nonoxidised LDL (LDL) within early atherosclerotic lesions. The LDL-Ca^{2+}-CRP complex is internalised by macrophages and activates complement via the classical route enhancing the inflammatory process (Bhakdi et al., 1999). Finally, CRP mediates shedding of membrane-bound IL-6 receptor from neutrophils, an effect that could result in modulation of IL-6 activity (Jones et al., 1999).

In recent years a plethora of studies have demonstrated a direct association between slightly elevated serum levels of CRP and the risk of developing cardiovascular disease (as reviewed in; Haverkate *et al.*, 1997; Koenig *et al.*, 1999; Rohde *et al.*, 1999; Ridker *et al.*, 2000). Furthermore, strong associations have been demonstrated between CRP concentration on admission for myocardial infarction and long-term clinical outcome (as reviewed in; Tommasi *et al.*, 1999) including risk of dying (as reviewed in; Lindahl *et al.*, 2000; Nikfardjam *et al.*, 2000). CRP has also been proven to be an objective measure of disease in RA and is useful to the physician for monitoring effects of drug therapy in this disease. Moreover, persistent high levels of CRP are a risk factor for continuing joint deterioration (as reviewed in; Otterness, 1994).

The simplest interpretation of this data is that CRP is just a sensitive marker of inflammation, a process known to underlie many vascular inflammatory pathologies. However, CRP may in fact be modulating inflammation directly through interaction with circulating monocytic cells. Previous work does indicate this, however, there are many questions still raised in these studies as to how exactly CRP is signalling and affecting monocyte phenotype. Therefore there is a strong case for further examination of the binding and effect of CRP mediated changes on monocyte in the context of monocyte-endothelial cell interactions and their relevance to vascular inflammatory pathologies, such as atherosclerosis.

1.11 Aims:

The purpose of this thesis is to examine the functional intracellular and extracellular responses of monocytes to CRP challenge in the context of monocyte involvement in

inflammation. This thesis will also examine the consequences of antioxidant intervention on monocytic phenotype and adhesion to endothelium in healthy normal subjects.

Specific Aims:

- Examine the effect of CRP challenge on monocyte derived cytokine expression and secretion.

- Investigate the changes in monocytic adhesion molecule expression and adhesion to endothelial cells after CRP treatment.

- Identify the binding of CRP to monocyte receptors and characterise the CRP receptor.

- Examine the monocytic intracellular signalling events and peroxide changes after CRP receptor engagement.

- Describe the consequences for CRP mediated intracellular signalling on monocyte phenotype.

- Investigate the effect of antioxidant supplementation on monocyte phenotype, monocyte adhesion to endothelial cells, and serum CRP levels.

2.0 General Methods

2.1 Preface:

This chapter describes the general methods used within this thesis. Analysis of monocytic adhesion molecule expression by flow cytometry, utilising colour compensation, is described. Experiments to determine the purity of C-reactive protein used for these investigations are also described. This section also characterises the adhesion assay designed to study monocyte-endothelial interactions.

2.2 Materials:

All gases were from BOC Ltd (Guildford, UK). The human monocyte cell lines U937 and THP-1 were purchased from ECACC (Wiltshire, UK). Fetal bovine serum (FBS), RPMI 1640 (GlutaMax), hanks balanced salt solution (HBSS), trypsin (0.05%, 0.53mM EDTA), penicillin (10,000µg/ml) and streptomycin (1000U/ml; P/S) were obtained from GibcoBRL (Paisley, UK). Lipopolysaccharide (*Esherichia.Coli* serotype 026:B5), low endotoxin fraction V bovine serum albumin (BSA), 2', 7'-bis-2-carboxyethyl-5-(6)-carboxyfluorescein-acetoxymethylester (BCECF-AM), Triton-X, medium 199 (M199), gentamycin, trypan blue, sodium citrate, SigmaCote and collagenase were purchased from Sigma (Poole, UK). Human and recombinant CRP were purchased from Calbiochem (Nottingham, UK). All anti human fluorescently tagged mouse antibodies (see table 2.1) were from Serotec (Oxford, UK). Optilyse C and Isoton were purchased from Beckman Coulter (Miami, USA). Cell culture plastics and plasticware were from Orange Scientific (Braine-l'Allued, Belguim). Needles and surgical thread were obtained from Beckton & Dickenson (Oxford, UK). Endothelial growth medium (EGM; 10% FBS, 0.5ml hEGF, 0.5ml hydrocortisone, 2ml BBE) was from BioWhittaker (Wokingham, UK).

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Antigen	Source	Species	Clone	Fluorescent Tag
CD11b	Mouse	Human IgG1	ICRF-44	PE
CD18	Mouse	Human IgG1	MEM-18	FITC
CD31	Mouse	Human IgG1	B-B38	FITC
CD14	Mouse	Human IgG1	Tük-4	RPE-Cy5
CD32	Mouse	Human IgG1	AT10	FITC

Table 2.1 Characteristics of the fluorescent conjugated antibodies used to determine the membrane expression of antigens involved in cell-cell adhesion by flow cytometry. Abbreviations are fluorescein isothiocyanate (FITC), phycoerythrin (PE) and phycoerythrin-Cy5 (RPE-Cy5).

2.3 Methods:

2.3.1 Cell Culture:

The monocytes used were the leukemic cell lines, THP-1 and U937. The cell lines have previously been shown to have human primary monocytic properties, including morphological characteristics, expression of membrane antigens and receptors, and production of several secretory products (as reviewed in; Auwerx, 1991). Cells were maintained in RPMI - 1640 with 10% fetal bovine serum and 50µg/ml streptomycin and 5U/ml penicillin at 37°C, 5% CO_2, 95% humidified atmosphere. Cell lines were maintained at a concentration of $2x10^6$/ml for no more than 20 passages. The viability of all cell lines was determined by trypan blue exclusion using a haemocytometer (Neubauer; Weber Scientific Ltd., Teddington, UK).

2.3.2 Cell Activation:

Peripheral whole blood (PWB), primary isolated monocytes, or THP-1 cells were stimulated with recombinant CRP (reCRP) or serum isolated human CRP (2.5 -150µg/ml) or vehicle control (control; PBS; phosphate buffer saline 0.01M, KCl 2.7mM, NaCl 0.137M (pH 7.4)) for a 0-24hrs at 37°C. THP-1 cells were stimulated at a concentration of $2x10^6$/ml, unless otherwise stated. Resting primary human monocytes were isolated from PWB (see below) and stimulated at a concentration of $2x10^6$/ml unless otherwise stated.

PWB was stimulated with CRP using a volume of 50µl for flow cytometry analysis or 1ml for ELISA analysis, unless otherwise stated.

2.3.3 Preparation of mononuclear cells from peripheral whole blood:

Peripheral blood mononuclear cells (MNC) were isolated by density gradient centrifugation using Lymphoprep (Nycomed Pharma AS, Oslo, Norway). Venous blood (40mls) was obtained from consenting healthy, non-smoking adults and added to 10% sodium citrate (4% w/v) to prevent coagulation. Further manipulation of the blood was conducted under aseptic conditions. All containers and pipettes in contact with samples were coated with SigmaCote and allowed to dry at least 30 mins before use, in order to prevent artefactual activation and adherence of monocytes. Blood was diluted with PBS containing 0.1% BSA (w/v) in the ratio 2:5 into 50ml conical tubes. Diluted blood was gently layered onto the top of 15mls of lymphoprep. Tubes were centrifuged at 160xg (Sigma benchtop centrifuge Type 1-13, rotor # 12027, Osterode am Harz, Germany) for 15 mins at $20^{\circ}C$. The top 15mls of the supernatant was removed by aspiration to eliminate platelets. Tubes were then re-centrifuged at 350xg for 20 mins at $20^{\circ}C$. MNC appeared as a 'fluffy' band between the plasma lymphoprep interfaces. The MNC were collected by suction using a pasteur pipette and transferred to 15ml conical tubes. The cell suspensions were diluted 1:1 with PBS/0.1%BSA and washed three times with PBS/0.1%BSA by centrifugation at 225xg for 8 mins at $4^{\circ}C$. MNC were either used in experiments or resuspended to a concentration of $1 \times 10^7/150µl$ of PBS/0.1%BSA for further application.

2.3.4 Monocyte purification by negative isolation:

Monocytes were purified from washed MNC utilising Dynal negative isolation kit (Dynal A.S., Oslo, Norway). Monocytes were negatively isolated from the washed MNC sample by magnetic beads which captures B/T-lymphocytes, natural killer cells and granulocytes.

For each 1×10^7 MNC, $20 \mu l$ of gamma globulin blocking antibody was added to block FcR on monocytes to inhibit non-specific antibody binding. Following blocking, $20 \mu l$ of antibody mix was added (containing the mouse monoclonal antibodies for CD2, CD7, CD16 [specific for CD16a and CD16b], CD19 and CD56) for 10 mins with rotation at 2-$8^O C$. Cells were washed by adding 1ml PBS/0.1%BSA per 1×10^7 MNC and centrifuged for 8 mins at 500xg. Supernatant was removed and resuspended in 0.9ml of PBS/0.1%BSA per 1×10^7 MNC. The cells with bound antibody were then captured by Depletion Dynabeads (Dynal A.S. Oslo, Norway) and removed by magnetism. Depletion Dynabeads were resuspended prior to the transferral of $100 \mu l$ of beads per 1×10^7 MNC into microfuge tubes. Depletion Dynabeads are uniform, supramagnetic, polystyrene beads coated with an Fc specific human IgG4 antibody against mouse IgG. Microfuge tubes were then placed into a Dynal magnetic particle collector (MPC; Dynal A.S. Oslo, Norway) for one minute to allow beads to migrate to the surface of the tube in contact with the magnet. The solution was allowed to clear, the fluid removed and discarded without disturbing the beads. The beads were washed in 1ml of PBS/0.1%BSA and resuspended in their original volume of PBS/0.1%BSA.

Washed Dynabeads were added to the MNC suspension ($100 \mu l/10^7$ cells) and incubated at room temperature with rotation for 15 minutes. Rosettes of MNC-Dynabeads were resuspended by aspiration and the volume increased by adding 1ml of PBS/0.1%BSA. Tubes were placed in the MPC for 2 mins and supernatant (negatively isolated monocytes) transferred to a fresh tube and cell concentration determined using an improved Neubauer haemocytometer. Monocyte purity and artifactual activation throughout MNC and

monocyte isolation, was evaluated by analysing CD14 and CD11b expression by flow cytometry as described in section 2.3.9.

2.3.5 Human umbilical vein endothelial cell (HUVEC) culture:

Endothelial cells were isolated from freshly obtained human umbilical cords of greater than 7" in length from consenting patients. Ethical approval was granted from the Birmingham Women's Hospital, Edgbaston, Birmingham, UK (see appendix). The cord was stored in a sterile container containing HBSS supplemented with gentamicin (10µg/ml) at $4^{\circ}C$ until manipulation.

Handling of the umbilical cord was performed under sterile conditions. The cord vein was canulated with a blunt, hubless 16-gauge needle and secured with surgical silk thread. Blood was washed from the interior of the umbilical vein by slow perfusion with 20mls of HBSS supplemented with gentamycin (10µg/ml) and the cord allowed to drain. The other end of the umbilical cord vein was then canulated with a second blunt, hubless 16 gauge needle and secured in place as described. HBSS (20mls) was infused into the vein to wash out any gentamycin and once again, the HBSS was allowed to drain from the cord. The vein of the cord was filled with 0.1% collagenase in PBS to remove endothelial cells and the canulas closed. The cord was wrapped firstly in cellophane and then foil and incubated at 37 $^{\circ}C$ for a maximum of 20 mins so to prevent undesirable disruption of underlying structures. Subsequently, collagenase/endothelial cell suspension was eluted from the umbilical cord vein by perfusion with a further 20mls of HBSS. The eluate was collected into a 30ml universal and centrifuged at 100xg for 5 mins to pellet the endothelial cells. The supernatant was removed and discarded and the cell pellet resuspended in 5mls of M199 containing 10mM HEPES, 20% FCS and 1% P/S (Penicillin/Streptomycin). The

cell suspension was transferred to a T25 culture flask and incubated in a 37°C, 95% air, 5% CO_2 humidified atmosphere.

After 24hrs, media was removed from the culture flask to eliminate any dead contaminating suspended cells. Cells were washed gently with EGM and re-cultured under the described incubator conditions in the presence of 5mls of EGM until confluence. The cell cultures were fed every 2-3 days with a complete change of media. Morphologies were examined routinely by light microscopy (CK2-TR, Olympus, Tokoyo, Japan).

When confluent, medium was aspirated from each flask and cells were washed with HBSS to remove FCS. HUVEC were passaged by trypsin isolation. Briefly, 3mls of trypsin was added to HUVEC and incubated at 37°C for no more than 5 minutes. Any loosely adherent cells were removed by agitation. EGM (5mls) was added to each flask to neutralise trypsin and the cell suspension transferred to 30ml universals. Flasks were washed with 5mls of EGM and the remaining cell suspension was added to the universal. Cells were sedimented at 100xg for 5mins. The supernatant was removed and HUVEC resuspended in EGM. HUVEC were counted by light microscopy using an improved Neubauer haemocytometer. HUVEC were subsequently used for either further passage, adhesion molecule expression analysis by flow cytometry, migration assays or adhesion assays. For adhesion assay, HUVEC were seeded in 24 well plates at a concentration of 1×10^5/ml, 1ml per well and incubated under the described incubator conditions until confluent, with complete media changes every 2-3 days.

2.3.6 Adhesion Assay:

The adhesion of monocytes (treated, control or resting) to endothelial cells in a static system was measured adapted from the method of Weber *et al.,* (1996) by fluorescence

determination of the dye, BCECF, as described by De Clerck *et al.*, (1994). BCECF-AM is a non-fluorescent and membrane permeable dye permitting non-invasive loading of cells. Once contained within the intracellular compartment, BCECF-AM is converted by the action of intracellular esterases to the fluorescent BCECF, which is retained within cells due to its four negative charges at pH 7 (De Clerck *et al.*,1994).

Confluent, homogenous HUVEC monolayers in 24 well plates, were washed and cultured in 1ml/well of EGM and LPS (1µg/ml) added for 0, 5 or 24 hrs at 37°C, in a 5% CO_2, 95% air humidified atmosphere. At the end of the LPS incubation period, media was removed from each well and adherent cells washed three times with M199 prior to the addition of 1ml of monocyte suspension in M199.

Control, treated or resting monocytes were transferred to 15ml conical tubes and washed in PBS and resuspended in M199. Cell number was adjusted to $5x10^6$/ml using an improved Neubauer haemocytometer and labelled with BCECF-AM (10µg/ml) for 30 mins in the dark at room temperature (RT). Dye loading was terminated by ten-fold dilution with M199 and monocytes centrifuged at 100xg for 5 mins. Monocytes were resuspended in M199 to a concentration of $0.5x10^6$/ml. Monocytes (500µl) were added with or without treatment to individual HUVEC monolayers in triplicate and incubated for 30 mins, under the described culture conditions, with no change in HUVEC morphology, as analysed by light microscopy.

Non-adherent cells were removed by centrifugation of inverted plates for 5 mins at 40xg. Adhered cells were lysed with 1ml lysis buffer (0.1% Triton-X; 0.1M Tris, pH 8.8) for 30mins in the dark at RT. Lysed cells were then pipetted into 96 well plates and

fluorescence measured at an excitation of 485nm and emission of 535nm on a microplate spectrofluorometer (Spectramax GeminiXS, Molecular Devices, Sunnyvale, USA), utilising the cut off filter at 520nm.

Samples were calibrated against a standard curve of control monocytes, at concentrations of $5x10^4$, $1.25x10^5$, $2.5x10^5$ and $5x10^5$, which had been dye loaded with BCECF-AM, lysed and fluorescence analysed as described above.

Figure 2.1 shows the light microscopy photograph of a confluent HUVEC monolayer, showing the classic 'cobblestone' morphology following 24hrs activation with LPS (1µg/ml), with no change in HUVEC morphorlogy or loss of confluence. BCECF-AM loaded monocytes effectively adhered to HUVEC monolayers (see figure 2.2), within 30mins.

Scanning electron microscopy (SEM; figure 2.3), confocal and light microscopy results demonstrate that the adhesion assay can be used to examine monocyte-endothelial cell adhesion interactions.
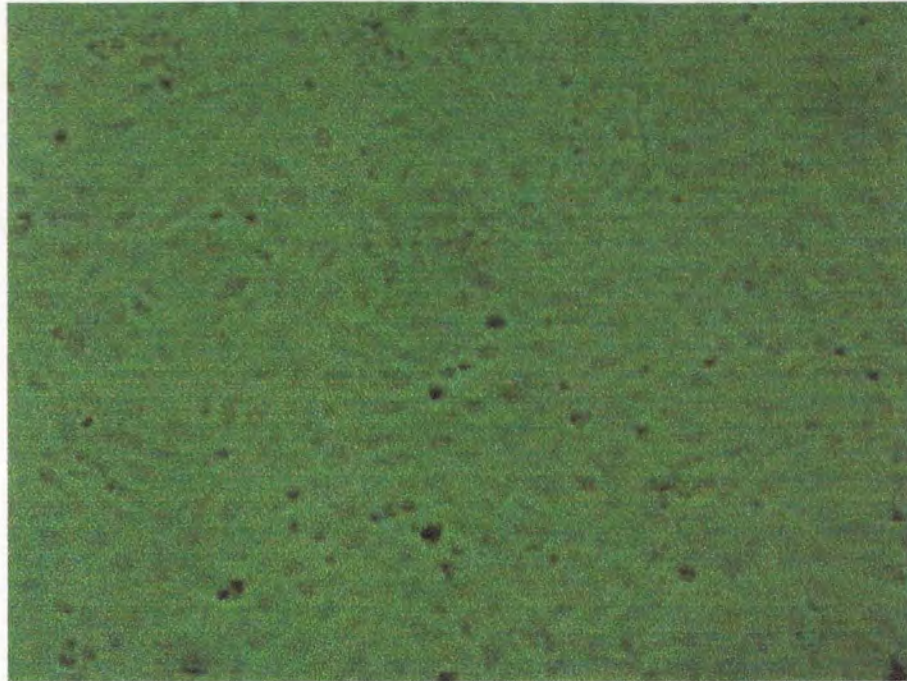
Figure 2.1 Light microscopy picture of isolated, cultured HUVEC. HUVEC were isolated from umbilical cords and cultured in 6 well plates, according to section 2.3.5.
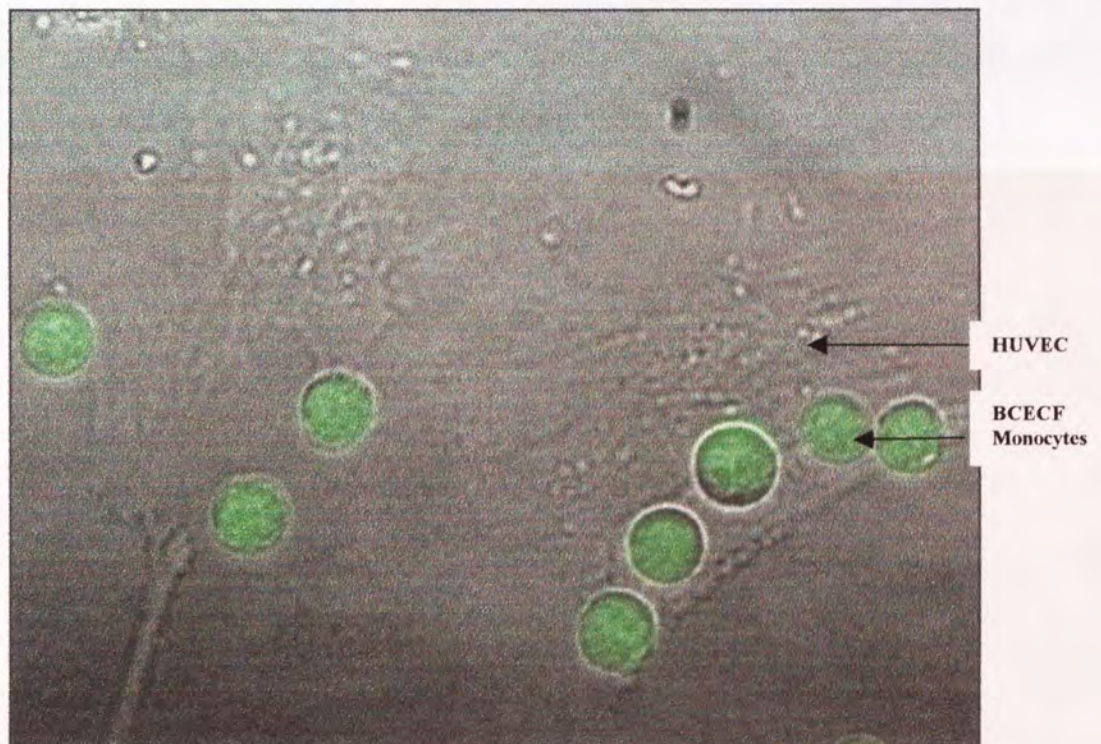
Figure 2.2 Confocal microscopy picture showing BCECF-AM loaded monocytes adhered to confluent HUVEC. HUVEC were isolated from umbilical cords and cultured in 6 well plates, according to section 2.3.5. Monocytes were loaded with BCECF-AM and mixed with HUVEC, according to section 2.3.6 and photographed under fluorescent confocal microscopy before lysis with Triton-X.

2.3.7 Scanning electron microscope (SEM) analysis of monocyte adhesion to HUVEC:

To further examine isolated monocyte adhesion to HUVEC, SEM photographs were taken using a Cambridge Stereoscan 90 (Cambridge, UK). Monocytes were isolated according to section 2.3.2 and HUVEC isolated and cultured onto 10mm by 10mm sterile slides (Orange Scientific) inside 12 well plates according to section 2.3.3. Isolated monocytes resuspended in M199 were incubated with confluent washed HUVEC for 30 mins at $37^{\circ}C$, in a 5% CO_2, 95% air humidified atmosphere. The media was removed and the cells rinsed briefly with PBS. Glutaraldehyde (2.5%; Sigma) in sodium cacodylate buffer (0.1M) was added for 30 minutes and then removed. The cells were then washed with sodium cacodylate buffer (0.1M) and dehydrated in a series of ethanol dilutions (20-100%) for 10 minutes per dilution. The cells were then dried over night in hexamethyldisilazane (1ml; Sigma). Following the drying step, the samples were attached to SEM stubs (10mm diameter) using carbon tabs (Argar Scientific, Stanstead, UK) and sputter coated with gold (Emscope SC500) prior to examination under the SEM.

A.
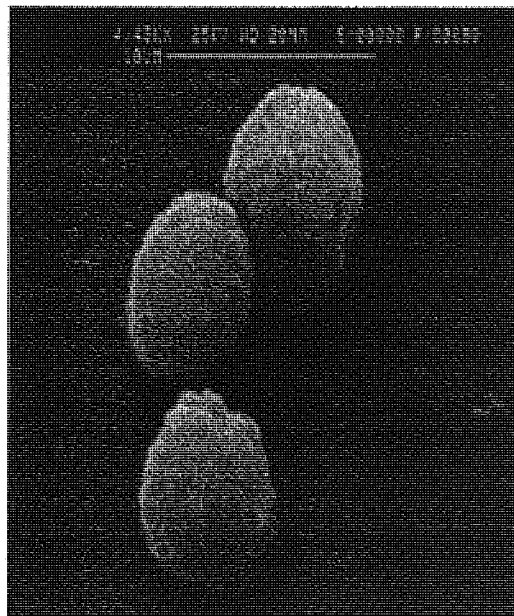
B.
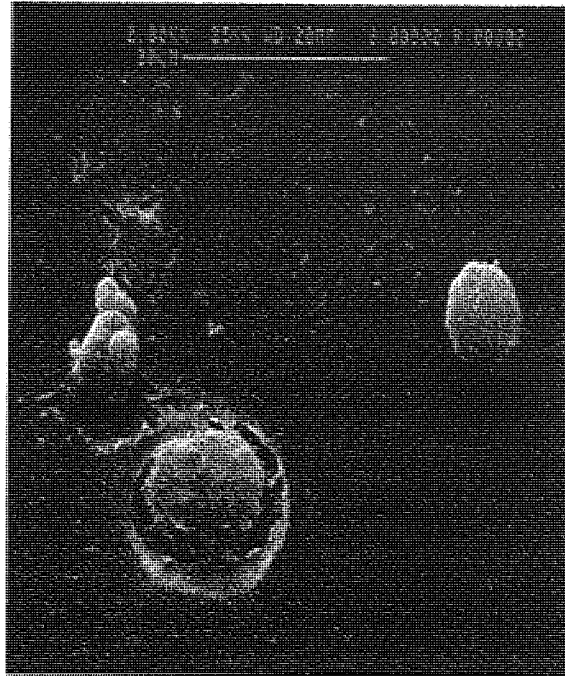
C.
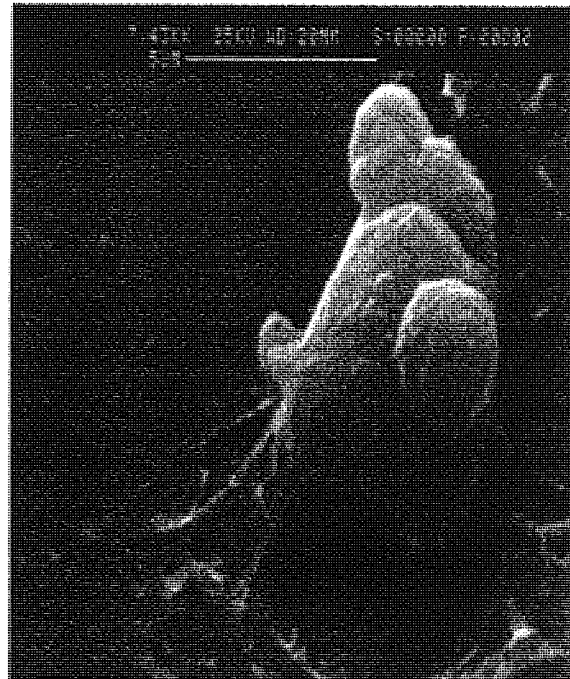
Figure 2.3 SEM pictures of adhered isolated monocytes to HUVEC monolayers.
HUVEC were isolated from umbilical cords and cultured on 6 well plates, as described in
section 2.3.5. Monocytes were added and cell populations were fixed and analysed via
SEM as described in section 2.3.7. Figure A and B demonstrates monocytes binding and
migrating through the HUVEC monolayer and figure C is a close up of a single cell from
B, demonstrating monocyte processes adhering to the HUVEC monolayer.

2.3.8 Colour compensation for multiple fluorescence analysis by flow cytometry:

The Coulter Epics XL-MCL flow cytometer is equipped with 4 fluorescent channels, which collect light within the specific wavelengths of 505-545nm (FL1), 560-590nm (FL2), 605-635nm (FL3) and 660-690nm (FL4). This allows analysis of several fluorescent probes simultaneously. However, fluorescent probes and fluorescently tagged antibodies emission spectra cover a range of wavelengths that may be wider than the bandwidth covered by a specific fluorescent detector. Consequently, fluorescence overspills into neighbouring channels (see figure 2.4). When several fluorescent probes are used, channel overspill produces elevated levels of fluorescence in neighbouring channels and hence false data is obtained. Spectral overlap may be minimised by electronic colour compensation. Regions of background, negative fluorescence of cells are established utilising the corresponding monoclonal isotype negative controls. For 3-way colour compensation, combinations of isotype monoclonal negative controls for 2 of the FL detectors with one positive fluorescently tagged antibodies are systematically analysed. Positive fluorescence over-spilling into neighbouring FL detector is electronically corrected to fall in the negative region previously established.
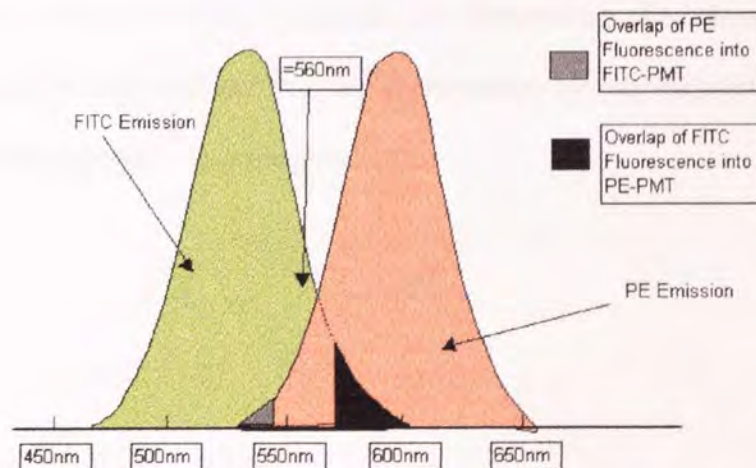
Figure 2.4 A close up view of FITC and PE Fluorescence overlap.

3-way colour compensation for the detectors FL1, FL2 and FL4 was established on the monocytic line U937 using the following antibodies; mouse monoclonal anti-human CD32 antigen FITC conjugated (clone AT10), mouse monoclonal anti-human CD11b antigen PE conjugated (clone ICRF-44) and mouse monoclonal anti-human CD14 antigen RPE-Cy5 conjugated (clone TuK4).

In brief, untreated PBS washed cells were incubated in the dark on ice for 30 mins with a saturating concentration ($>10\mu l/10^6$ cells) of antibodies and/or isotype negative controls in the combinations described in Table 2.2. Cells were then fixed by the addition of 250μl of 4% formaldehyde, vortexed and incubated in the dark for 15 mins at RT. Samples were diluted by the addition of 200μl of isoton, brief mixing and a further incubation of 10 mins in the dark at RT. For 3-way colour compensation, a flow cytometry protocol containing the dual parameter histograms of forward scatter (FS) versus side scatter (SS), log integral FL1 versus log integral FL2, log integral FL1 versus log integral FL4 and log integral FL2 versus log integral FL4 was established. Background florescence was established using cell suspensions labelled with isotype negative controls (Tube 1; see table 2.2) with 1% positive analysis selected on the histograms of fluorescence. The remaining tubes were then systematically analysed and positive fluorescence over-spilling into the established negative regions electronically corrected.

Conjugated fluorescent probe	Tube Number				
	1	2	3	4	5
Ab-FITC (FL1)		✓	✓	✓	✓
Ab-PE (FL2)		✓	✓		✓
Ab-Cy5 (FL4)		✓	✓	✓	✓
-ve-FITC (FL1)	✓	✓			✓
-ve-PE (FL2)	✓			✓	✓
-ve-Cy5 (FL4)	✓	✓		✓	✓

Table 2.2 Combinations of fluorescently tagged probes required for the determination of three-way colour compensation. Saturating concentrations (>10μl/10^6 cells) of the indicated (✓) antibodies (Ab) or isotype negative controls (-ve) were added to PBS washed cells and incubated in the dark at RT for 30 mins. Samples were then fixed with 4% formaldehyde and then diluted by the addition of isoton prior to flow cytometric adjustment for overlapping emission spectra.

2.3.9 Immunofluoresence of peripheral blood monocytes:

The percentage and hence the purity of CD14$^+$ peripheral blood monocytes and their activation status by the appearance of CD11b was evaluated by flow cytometry, following the negative isolation procedure.

Purified monocytes, MNC or PWB ex vivo were treated with 10μl of antibody per 10^6 cells or 100μl PWB. Samples were incubated at RT in the dark for 30 mins. The antibodies used were anti-CD11b PE conjugated and anti-CD14 RPE-Cy5 conjugated. For each sample, isotype negative controls of the monoclonal antibodies were used to establish background fluorescence. These were mouse monoclonal negative control IgG1 PE conjugated (clone B-Z1) and the mouse monoclonal negative control IgG1 RPE-Cy5 conjugated (for further experiments throughout this thesis, the mouse monoclonal negative control IgG1 FITC conjugated was also used) from Serotec. Optilyse C (250μl) was added to samples to fix and lyse red blood cells. Samples were then vortexed and incubated in the dark at RT for 10 mins followed by the addition of 250μl of Isoton. Samples were then analysed by flow cytometry utilising 3-way colour compensation as previously described and corrected for background fluorescence with isotype negative controls for the primary

monoclonal antibodies. A minimum of 10000 monocytes were analysed per sample using the following gating strategy: monocytes were gated according to FS (size) and SS (granularity) properties and the percentage $CD14^+$ cells evaluated on a single parameter histogram of log FL4 (CD14 RPE-Cy5) versus count. The CD11b activation of monocytes was evaluated on a dual parameter histogram of log FL2 (CD11b PE) versus log FL4. The purity of monocytes extracted was assessed as the percentage of $CD14^+$ cells on an un-gated histogram of SS versus log FL4. For all other protein expression profiles examined throughout this thesis, the same gating strategy was used (as above), where the results used were the medianX (MdX) expression (arbitrary units; a.u.) within each histogram (unless otherwise stated).

In order to determine any artefactual activation through monocyte isolation through negative selection, the integrin CD11b membrane expression levels were analysed by flow cytometry. Figure 2.5 describes SS versus FS histograms of the negative isolation of monocytes from PWB and MNC and purity assessed to be >90%. Throughout the isolation method there was no significant change in CD11b expression from PWB to isolated monocytes, with a mean±SD change in MdX expression of 0.6±0.23 (n=25) from PWB to isolated monocytes (figure 2.6).

The αM integrin chain CD11b, has been used as a marker of monocyte activation as it is rapidly expressed on the membrane surface (Buyon et al., 1997; Carlos & Harlan, 1994). Dynal isolation of primary monocytes from PWB showed no variation of CD11b expression, therefore negative isolation successfully isolated resting monocytes from PWB.
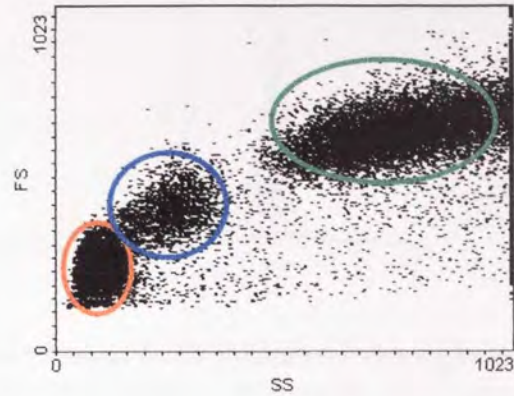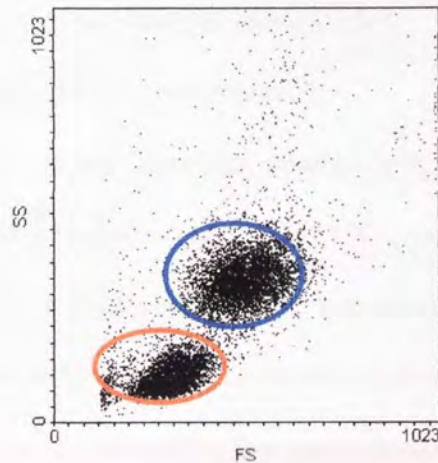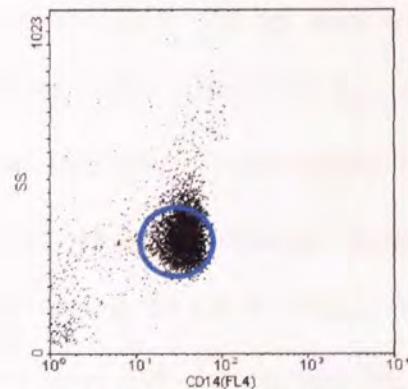
Figure 2.5 Flow cytometry histograms describing negative isolation of monocytes.
PWB (**A**), MNC (**B**) and Dynal negative isolated monocyte (**C**) samples were fixed and lysed in Optilyse (250μl) and subsequently analysed via flow cytometry, utilising FS vrs SS parameters. Figure A shows lymphocyte, monocyte, and neutrophil/granulocyte subset populations in PWB. Figure B shows lymphocyte and monocyte populations after density centrifugation with Lymphoprep, illustrating loss of the neutrophil/granulocyte subpopulation. Figure C shows isolated monocytes stained with saturating concentrations of antibody against monocyte marker, anti-human CD14-RPE-Cy5 (FL4). A monocyte population is shown with >90% purity, with loss of the lymphocyte subpopulation.

A   B   C
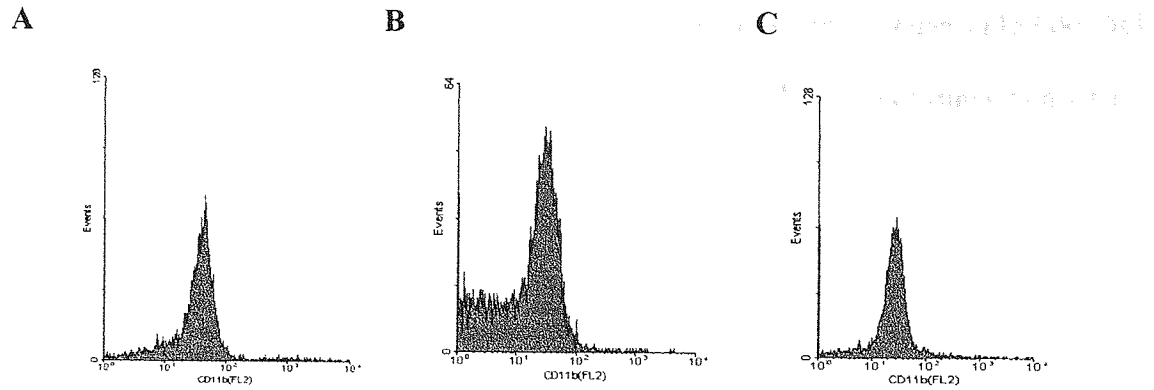
Figure 2.6 CD11b expression from monocytes throughout negative isolation method. PWB (A), MNC (B) and isolated monocytes (C) were incubated with saturating concentrations of anti human CD11b-PE (FL2) and analysed via flow cytometry gated against FS and SS properties, according to section 2.3.9.

2.3.10 C-reactive protein (CRP) characterisation:

In order to confirm the purity and determine whether both forms of purchased CRP, either isolated CRP from human serum and recombinant CRP, are non-aggregated, samples from both protein preparations were separated on a non-denaturing polyacrylamide gel. Briefly 7.5% acrylamide gel was formed by mixing monomer solution (100mls; 30% acrylamide solution, 1.5M Tris-HCl) with 10% APS (10μl) and TEMED (5μl) and poured into a gel kit (Flowgen, Ashby de la Zouch, UK). The gel stack was placed inside a running tank (Flowgen) containing running buffer (25mMTris-base and 192mM Glycine). CRP samples (10μg) were mixed with sample buffer (0.5M Tris-HCl, 192mM glycerol and 0.5%w/v bromophenol blue) and loaded onto gel along with rainbow markers (128-7.5kDa; BioRad). Samples were run for 1hr at 100mA. The gel was washed and stained with coomassie (0.1% coomassie, 50% MeOH and 7% acetic acid) overnight at RT, followed by destain washes (50% MeOH and 7% acetic acid) until bands appeared.

Figure 2.7 shows both serum isolated human CRP and recombinant CRP samples (10μg). There is no contamination with other proteins in either CRP sample and CRP is in its

native pentameric state, un-aggregated according to its molecular weight (115,000 Da). Protein samples from both CRP sources were assayed for endotoxin contamination via the E-toxate limulus assay. Both serum isolated human CRP and recombinant CRP samples had ≤0.03EU/ml contamination (n=6).

The CRP sources used within this thesis are endotoxin free or below the level of 0.03EU/ml (as analysed by E-Toxate Limulus Amebocyte Lysate assay, according to manufacturers instructions; Sigma) which is below the level of endotoxin needed to activate monocytes in-vitro (Ballauo et al., 1992) and free from contamination. Previous workers have questioned the effects of CRP have been through the contamination of endotoxin or IgG (Hundt et al., 2001; Sander et al., 2001), however the responses shown herein are due to CRP and not other contaminants. Also all preparations of CRP are stored in azide free solutions, therefore excluding possible artefactual monocytic activation through azide interactions. Other workers (Tanaka et al., 2002) have also shown that recombinant CRP (reCRP) was indistinguishable from native CRP, with respect to Ca^{2+} - dependent binding ability to bind phosphorylcholine, electrophoretic behaviour, N-terminal amino acid analysis and immunochemical properties. Therefore it is concluded that recombinant CRP can be confidently used within these investigations as a way of studying CRP interaction with monocytes.
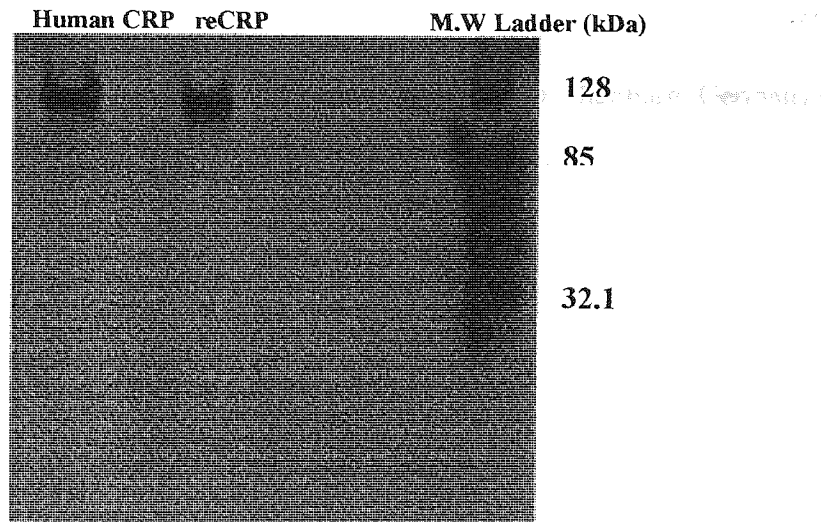
Figure 2.7 PAGE analysis of CRP sources. Serum isolated human CRP and recombinant CRP samples (10µg) were separated via PAGE and protein bands stained using coomassie stain, according to method 2.3.10

As incubations of CRP with monocytes may be toxic to the cells and therefore may interfere with CRP mediated responses, the cell viability indicator, propidium iodide (PI) was used to determine effects on cell viability via flow cytometry. To establish the contribution of necrosis in addition to that of apoptosis in the induction of cell death, flow cytometry may be used to analyse the cellular exclusion of fluorescent dyes. Cells undergoing necrosis lose the integrity of their plasma membrane permitting the release of intracellular material (as reviewed in; Cohen, *et al.*, 1993). However, this also permits the entry of cationic, polar dyes into the cell such as trypan blue, PI and ethidium bromide. Consequently, necrotic cells in the presence of a polar dye for a short period of time, display high dye uptake. Early apoptotic cells show an uptake of dye which is much lower than that observed with necrotic cells, early apoptotic cells also shrink, and appear as smaller cells, whereas healthy, viable cells show no dye uptake. When this dye is fluorescent, such as PI, it is possible to distinguish via flow cytometry cells that are healthy (PI negative), apoptotic (PI weak) and necrotic (PI strong) on the basis of the fluorescence contained with individual cells (Yeh *et al.*, 1981).

THP-1 cells (2×10^6) were centrifuged (Eppendorf centrifuge 5415D, Hamburg, Germany) at 1000xg for 5mins and resuspended in serum free RPMI-1640 (1ml) and stimulated with reCRP (0-150μg/ml) for 0-1hr at 37°C. Samples were again centrifuged at 1000xg for 5mins and resuspended in serum free RPMI-1640 (1ml) containing PI (50μg; Sigma). Samples were incubated at room temperature in the dark, for 15mins and then analysed immediately by flow cytometry. The PI fluorescence of individual cells were analysed on an ungated dual parameter histogram of FS against log integral FL3 (PI, red fluorescence; 560-590nm) and the percentage of apoptotic or necrotic cells in a given sample quantified. The incubation of THP-1 cells with CRP of up to 150μg/ml had no effect on cell viability (Fig 2.8A). This was also true for the incubation period of CRP with THP-1 cells, where incubation periods of up to 60mins, had no significant ($P>0.05$) effect on cell viability (Fig 2.8B). These results indicate that CRP concentrations used throughout this thesis are not toxic to the monocytic cells analysed.

As reCRP has previously been shown to have the same biological characteristics as human CRP and as reCRP is far less likely to suffer from contamination problems (i.e., possible IgG contamination in serum isolated CRP), the response of monocytic (THP-1) cells to CRP mediated CD11b expression was tested using both sources of CRP, according to section 2.3.2. Figure 2.9 shows there was no significant difference in CRP mediated increase in monocytic CD11b expression (as discussed in chapter 4) from either source of CRP. Therefore as the source of CRP is not a factor contributing to CRP mediated phenotypic changes in monocytes, the responses seen throughout this thesis where CRP is described, result from reCRP.

A)
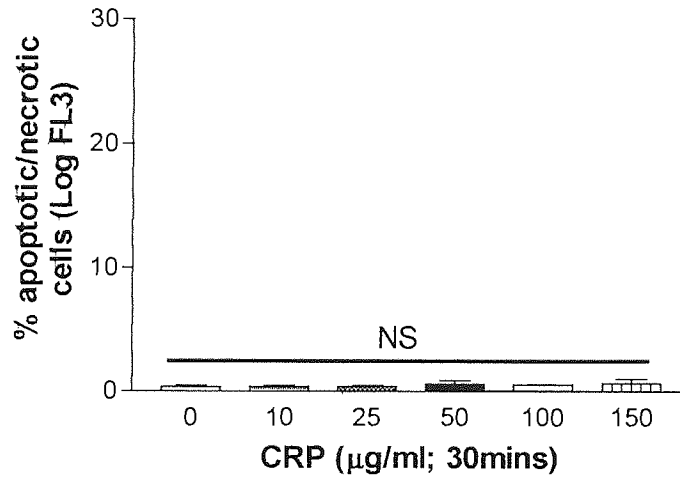
B)
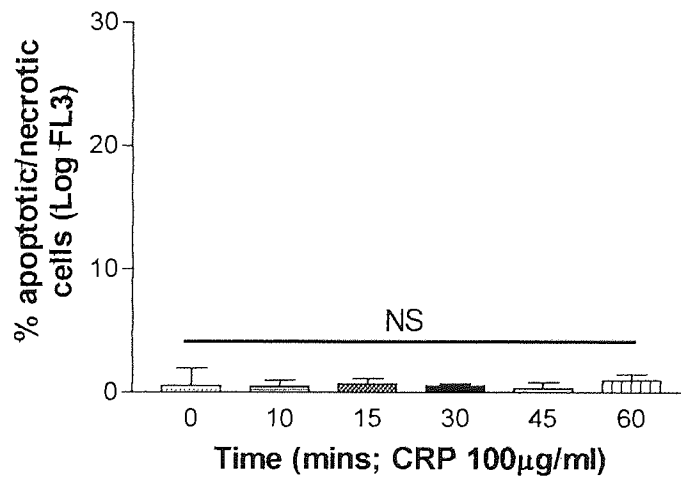
Figure 2.8 Effect of CRP concentration and incubation time on THP-1 viability.
THP-1 cells (2×10^6/ml) were stimulated with A) CRP (0-150μg/ml) for B) 0-60mins at
$37^{\circ}C$ and then dye loaded with PI according to section 2.3.10. The percentage of apoptotic
or necrotic cells was analysed via flow cytometry (FL3). Data was analysed using
ANOVA followed by tukey's post test (n=4).
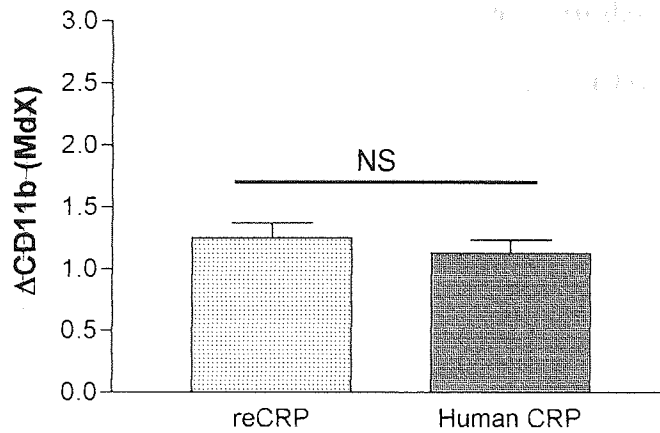
Figure 2.9 Change in monocytic CD11b expression with either reCRP or human CRP. THP-1 $(2 \times 10^6/ml)$ were stimulated with either reCRP $(100\mu g/ml)$ or human serum isolated CRP $(100\mu g/ml)$ for 30mins at $37^\circ C$ and CD11b expression measured by flow cytometry, according to sections 2.3.2 and 2.3.9. Data is presented as change (Δ) in CD11b MdX expression from vehicle control treated cells. Date was analysed via t-test (n=4).

All subjects used for PWB donation throughout subsequent chapters, were deemed healthy and as having a serum CRP concentration of $<5\mu g/ml$, analysed according to section 6.3.9. All subjects were initially screened for a CD11b response as discussed in chapters 4 and 5 and only CRP responders were used for further analysis. THP-1 monocytic cells were discovered to have a heterozygote (as analysed according to section 5.3.3) allotype at codon 131 on the FcγRIIa gene and therefore it was concluded that they would be able to bind CRP, as discussed in chapter 5.

In order to further evaluate whether reCRP mediated responses such as CD11b expression (as discussed in chapter 4 and 5), were due to endotoxin contamination (as discussed above), further experiments were carried using an anti-CD14 antibody to block endotoxin (LPS) mediated interactions in monocyte signalling through toll-like receptors (TLRs; as reviewed in; Guha & Mackman, 2001). PWB $(50\mu l)$ was pre-incubated with and without purified anti-human CD14 mouse monoclonal antibody (Serotec) for 30mins, before the addition of 0 and $100\mu g/ml$ of CRP for 30mins at $37^\circ C$ and then analysed for CD14+

CD11b expression according to section 2.3.9. Figure 2.10 describes that there was no significant difference in CRP mediated change in monocytic CD11b expression with prior blocking of the LPS receptor, CD14, nor was there any change in CD11b expression, with antibody alone. Therefore these data show that CRP mediated changes in monocyte phenotype are not signalling via endotoxin pathways and hence not contaminated with endotoxin.
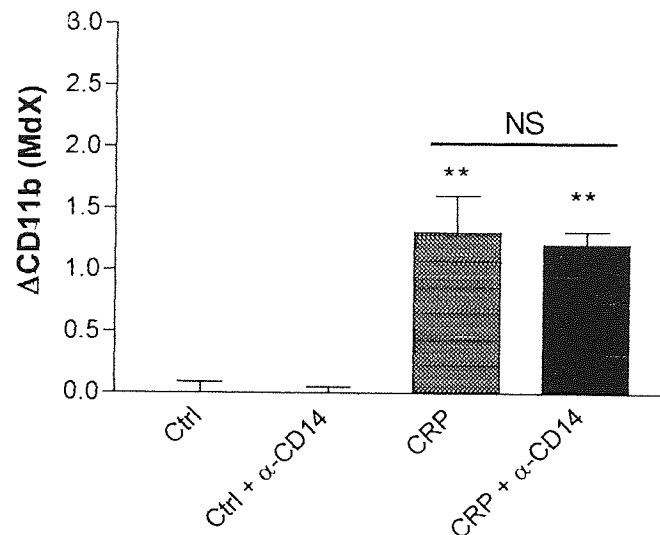
Figure 2.10 Change in CRP mediated monocytic CD11b expression with anti-CD14. PWB (50µl) was pre-incubated with and without purified anti-human CD14 mouse monoclonal antibody (α-CD14; 5µl) for 30mins, before the addition of 0 (Ctrl) and 100µg/ml of CRP for 30mins at 37°C and then analysed for CD14+ CD11b expression according to section 2.3.9. Data is presented as change (Δ) in CD11b MdX expression from vehicle control treated cells. Date was analysed via ANOVA with Tukey's post test, where ** represents P<0.001 from control treated cells (n=4).

2.3.11 Statistics:

All Data is shown as a representative result or as the arithmetic mean ± standard deviation (SD), where all experiments were a minimum of n=3 in triplicate, unless otherwise stated. Groups of data were evaluated statistically by ANOVA, followed by Tukey's post test for multiple comparisons or Wilcoxon signed ranks test for matched pair data or Mann-

Whitney test for grouped data and Spearman's rank test for identifying correlations. All significance tests were two tailed, where P<0.05 was considered significantly different from control treated samples (unless otherwise stated). All analysis was done using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California USA).

3.0 Cytokines

3.1 Preface:

This chapter examines and characterises the effects of C-reactive protein on cytokine production, expression and secretion by monocytic cells. The modulation by CRP of cytokine expression within monocytes and the likely consequences within the immune system are discussed.

3.2 Introduction:

Cytokines are proteins produced by many different cells of the immune system, which act upon other cells (as reviewed in; Nicod, 1993; Haddad *et al.*, 2001; Holloway *et al.*, 2002; Tyson *et al.*, 2001). They attach to receptors on the outside portion of cell membranes causing the target cell to produce a certain reaction, depending on the cell and the cytokine (as reviewed in; Feldman *et al.*, 1996; Parkin & Cohen, 2001). Often the target cell produces other cytokines in response to the initial cytokine. This complicated relationship is called the cytokine network and it is one of the most important ways the immune system communicates and orchestrates appropriate responses to various challenges, including viruses, bacteria, fungi and even tumours (as reviewed in; Joyce, 2001; Haddad *et al.*, 2002).

Monocytes and macrophages play a pivotal role in intiating and regulating the immunological response against pathogens by releasing various cytokines. Gram negative bacterial endotoxin (LPS) is a potent cytokine initiator in severe gram-negative infection and has been widely used as a monocyte stimulator to investigate the mechanisms underlying cytokine production in vitro. Monocytes orchestrate this innate immune response to LPS by expressing a variety of inflammatory cytokines that include TNF-α and IL-6.

In the early 1980s, proinflammatory cytokines were demonstrated to directly stimulate increased leukocyte adhesion in cultured endothelial cells (Pober *et al.*, 1986; Bevilacqua *et al.*, 1985), the consequences of which are discussed in the next chapter. Their stimulatory effect was greater in amplitude than that of other previously studied leukocyte activators, such as leukotrienes, activated complement components and chemotactic peptides, and has been shown to result in firm attachment to and transmigration across endothelial cell layers in many in vitro model systems. It has been further shown that cytokine induced enhanced adhesiveness of the endothelium requires de novo protein synthesis and increased cellular adhesion molecule expression (as reviewed in; Springer, 1994; Carlos & Harlan, 1994; Imhof & Dunon, 1995). Collectively this demonstrates that monocyte activation inducing proinflammatory cytokine expression, can modify monocytic interactions within the immune system

A possible effect of CRP on the production of inflammatory cytokines has been previously explored. In preliminary reports, Terada *et al.*, (1988) observed a 2-3 fold increase in monocyte IL-1 synthesis in response to CRP and Barna *et al.*, (1989) found modest induction of both TNF and IL-1. The preliminary studies of Suthun *et al.*, (1990) suggested that increased monocyte TNF synthesis following exposure to CRP was dose dependent and maximal at 4 hours. In another report, monocytes cultured with heat-killed fibroblasts that had been pre-incubated with acute phase sera containing high concentrations of CRP, produced substantially greater levels of TNF-α than monocytes cultured with fibroblasts pre-incubated with normal sera (Yamada *et al.*, 1990). Balluo *et al.*, (1992) published a more definitive study, showing that CRP dose dependently induced the release of IL-1β, IL-6 and TNF-α from peripheral blood monocytes. Significantly elevated levels of each cytokine were detectable in monocyte supernatants following

culture with 5μg/ml CRP, and increasing cytokine levels following culture with increasing concentrations of CRP up to 50μg/ml for TNF and up to 125μg/ml for IL-6 and IL-1. However the major discrepancy within the majority of these reports are the examination of cytokine expression from primary monocytes isolated from whole blood and cultured in vitro for up to 24 hours. This method of analysis may activate monocytes independently of CRP incubations, as monocytes are sensitive to any cell culture techniques once taken out of their natural environment. Furthermore, previous studies have not examined whether increased secretion of cytokines is a result of CRP elicited increases in transcription. One way round this problem is through the investigation of cytokine secretion after direct stimulation in PWB, or examining intracellular cytokine production in peripheral blood monocytes using the secretion inhibitor Brefeldin A (BFA).

BFA is a metabolite of the fungus *Eupenicillium brefeldinauim* and specifically and reversibly blocks protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus. BFA acts by inhibiting the activation of ARF1, a raslike GTPase whose GTP-bound form is required to initiate COP I binding to the membrane (Donaldson *et al.*, 1992). Membrane association of these cytosolic coat proteins (COP I) regulates sorting and recycling within pre-Golgi and Golgi intermediates (as reviewed in; Klausner *et al.*, 1992). In the absence of such binding in BFA-treated cells, membrane trafficking is deregulated, with anterograde, ER-to-Golgi traffic inhibited and retrograde, Golgi-to-ER traffic accelerated, essentially leading to accumulatiation of intracellular protein (see figure 3.1).
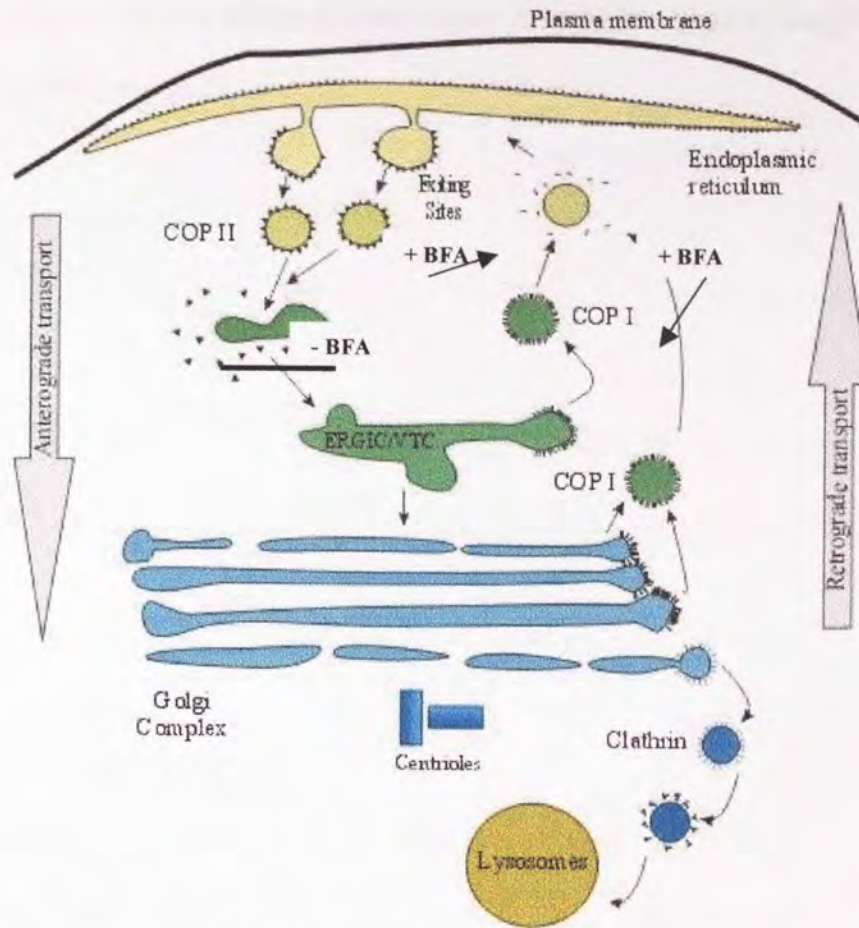
Figure. 3.1 Diagram of BFA inhibition of intracellular transport in the endoplasmic reticulum and the Golgi apparatus. The latter is surrounded by the ER and is organised around the centrioles. Between the Golgi and the ER, several tubular and vesicular structures form a pleiomorphic and dynamic compartment termed as ERGIC (endoplasmic reticulum-Golgi intermediate compartment) or VTC (vesicular-tubular transport complex).

Along with the aforementioned reports, CRP has also been shown to induce the expression

of the inflammatory agonist, soluble IL-6 receptor (sIL-6R) from primary neutrophils in

vitro (Dowdall *et al.*, 2002; Jones *et al.*, 1999), therefore enabling IL-6 to act on cells that

do not express its receptor, as sIL-6R can bind to the virtually ubiquitous gp130 protein on

the surface of cells (as reviewed in; Economides *et al.*, 1995).

However, the actions of CRP on the proinflammatory cytokine expression in monocytes

has not been fully elucidated. Therefore the effects of CRP on the expression and secretion

of TNF- α and IL-6 from human primary monocytes was investigated using RT-PCR, BFA

(flow cytometry) and ELISA.

3.3 Materials & Methods:

3.3.1 Materials:

All materials used in mRNA isolation were purchased from Dynal (Oslo, Norway). All products used within RT-PCR reactions were from Promega (Southampton, UK) unless otherwise stated. 321bp ladder, saponin and brefeldin A were obtained from Sigma (Dorset, UK). All synthesised primers were made by GibcoBRL (Paisley, UK) and IL-6 primers including positive control were from R&D systems (Abington, UK). Mouse anti-human TNF-α and IL-6 antibodies attached to FITC and PE fluorochromes respectively and TNF-α and IL-6 ELISAs were purchased from IDS (Tyne & Wear, UK). Mouse anti-human CD14-RPECy5 was from Serotec (Oxford, UK).

3.3.2 mRNA extraction and Reverse Transcription:

Peripheral whole blood mononuclear cells (MNC) and monocytes were isolated by differential centrifugation and Dynal negative isolation respectively, as previously described in sections 2.3.3 and 2.3.4. Monocytes (2×10^6/ml) were stimulated with CRP (100μg/ml) or LPS (1μg/ml) for 2hrs at 37°C. mRNA extraction was carried out using Dynal mRNA direct kit. Cells were centrifuged at 13,000xg and resuspended in lysis buffer (200μl; 100mM Tris-HCL, pH7.5, 500μM LiCl, 10mM EDTA, pH8.0, 1% LiDS and 5mM DTT). The solution was aspirated 5x using sterile 21 gauge needles and repeated using 25 gauge needles, in order to shear DNA. The solution was centrifuged at 13,000xg for 1min and washed Oligo (dT)$_{25}$ beads (30μl) in lysis buffer, were added and the solution with added beads mixed for 5mins at 25°C. The solution was magnetized (Dynal MPC-E) and supernatant removed. The pellet was resuspended x2 in Buffer A (200μl; 10mM Tris-HCL, pH7.5, 0.15M LiCl, 1mM EDTA and 0.1% LiDS) and solution magnetized and pellet washed 3x in Buffer B (200μl; 10mM Tris-HCL, pH7.5, 0.15M LiCl

and 1mM EDTA). Finally the beads were resuspended in 20μl diethyl pyrocarbonate (DEPC) treated water. For reverse transcription, mRNA beads were incubated at 37 °C for 1 hour in Expand RT buffer (10mM DTT, 1mM dNTP's, 25U RNAsin, 1U RQ1 Rnase-free DNase). DNase was heat inactivated at 70 °C for 10mins and the samples were incubated with or without 1μl of Expand RT (50U) and incubated at 42 °C for 1 hour. cDNA samples were then analysed by PCR or stored at -20 °C until analysis.

3.3.3 Polymerase Chain Reaction (PCR):

IL-6 and TNF-α transcripts were detected by using primers (Table 3.1) synthesised from GibcoBRL or purchased from R&D systems. GADPH primers were used in order to standardize transcription levels (see results). PCR reactions were performed in PCR buffer (200mM dNTP's, 10pmol of each primer, 2.5U Taq DNA polymerase) according to table 3.1.

Primer	Forward Primer (5'-3')	Reverse Primer (5'-3')	Conditions	Cycles
GADPH	ACAACATGATCCCTGCCTC	GCCAAATTCGTTGTCATACC	98°C 3mins *Taq* 60°C 2mins 72°C 2mins 94°C 30secs 60°C 30secs 72°C 30secs 94°C 30secs 60°C 30secs 72°C 4mins	26 1
TNF-α	GTGACAAGCCTGTAGCCCA	ACTCGGCAAAGTCGAGATAG	98°C 3mins *Taq* 55°C 2mins 72°C 2mins 94°C 30secs 55°C 30secs 72°C 30secs 94°C 30secs 55°C 30secs 72°C 4mins	1 35 1
IL-6	R&D Systems	R&D Systems	98°C 3mins *Taq* 55°C 2mins 72°C 2mins 94°C 30secs 55°C 30secs 72°C 30secs 94°C 30secs 55°C 30secs 72°C 4mins	1 35 1

Table 3.1 Primers and conditions used within RT-PCR reactions.

3.3.4 Agarose gel electrophoresis of PCR products:

PCR products were analysed by gel electrophoresis. PCR reaction products (10µl) and 123bp DNA ladder were mixed with gel loading solution (2.5µl; 0.05% w/v bromophenol blue, 40% w/v sucrose, 0.1M EDTA pH8.0) and separated on a 1.5% agarose gel made up in 1xTBE (89mM Tris, 89mM boric acid and 2.5mM EDTA pH 8), containing ethidium bromide (10µg/ml) at 90V for 2hrs. PCR products were photographed on a UV

transilluminator table at 312nm (Syngene, USA). Expression levels were quantified using Scion imaging software (Scion Corp., USA) and normalised to GADPH expression levels.

3.3.5 TNF-α and IL-6 protein expression analysis:

PWB (1ml) was incubated with or without BFA (10µg/ml) and stimulated with CRP (100µg/ml), LPS (1µg/ml) or vehicle control for 4hrs at 37°C. Activated or unstimulated blood (100µl) was incubated with anti-CD14 conjugated to RPE-Cy5 at room temperature (RT) in the dark for 30mins. Samples were fixed and red blood cells lysed in 250µl Optilyse (Becton Coulter, USA), mixed and incubated at RT in the dark for 10mins. Permeabilisation buffer (0.5% saponin in PBS; Sigma) was added to samples which were and incubated for 10mins at RT. Cytokine specific MAbs (TNF-α - PE and IL-6 – FITC) added for 30mins at RT. CD14+ monocytes were gated according to FS and SS properties and analysed for TNF-α and IL-6 expression via flow cytometry. Isotype matched control antibodies were included to detect non-specific binding to cells. To determine CRP effects on secretory cytokine levels, PWB (50µl) or isolated MNC samples (2x10^6/ml) were stimulated with CRP (10-100µg/ml) or LPS (1µg/ml) for 0, 16 and 24hrs at 37°C. Plasma was isolated by centrifugation at 1000xg for 10mins and 100µl samples were added to TNF-α and IL-6 ELISA plates according to manufacturers instructions (IDS).

3.3.6 Statistical Analysis:

Data are shown as a representative result or as the arithmetic mean ± SD. Groups of data were evaluated statistically by ANOVA, followed by Tukey's test for multiple

comparisons. ** represents P<0.001 and * P<0.05 and was considered significantly from controls.

3.4 Results:

3.4.1 TNF-α and IL-6 mRNA expression

Tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) cytokines are important inflammatory mediators are strongly implicated in promoting inflammation. Therefore the effect of CRP on TNF-α and IL-6 mRNA expression was examined. CRP (100µg/ml) or LPS (1µg/ml), as a positive control, were incubated with isolated primary monocytes for 2hrs at 37°C. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA expression levels were used as a housekeeping gene. GAPDH is widely used as an internal standard because this protein is an important glycolytic pathway enzyme and is essential for the maintenance of cell function and is therefore expressed at similar levels in all cell types and expression levels have been reported to be unchanged with inflammatory mediators (as reviewed in; Thellin *et. al.*, 1999).

CRP caused an increase in TNF-α and IL-6 mRNA (Fig 3.2B&C) as compared with GADPH expression (Fig 3.2A). There was some evidence of IL-6 mRNA expression in control treated samples. This may be due to the method of monocyte isolation, artefactually activating monocytic samples before IL-6 expression could be assessed. LPS was used as a positive control and showed a substantial increase in TNF-α and IL-6 mRNA expression (Fig 3.2B&C). DNA contamination was not present following the mRNA isolation procedure, as there was no detectable band within the samples incubated without reverse transcriptase; negative (RT-ve; Fig 3.2A-C). In order to quantify expression levels, GAPDH, TNF-α and IL-6 products were scanned and product (bands) density analysed using Scion Image software. The ratio of each stimulation expression over GAPDH expression was quantitatively calculated and presented as the mean ratio±SD from 3 different subjects. CRP incubation induced significant (P<0.001) increases in both

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IL-6 (Fig 3.3A) and TNF-α (Fig3.3B) expression, with a mean ratio±SD of 1.2±0.06 and

0.8±0.02 respectively, relative to GAPDH expression, after 2 hours. Induction of IL-6 (Fig

3.3A) and TNF-α (Fig 3.3B) expression was significantly increased in monocytes

incubated with LPS. However, there was no significant difference between CRP and LPS

induced expression levels of both TNF-α (Fig 3.3A) and IL-6 (Fig 3.3B).
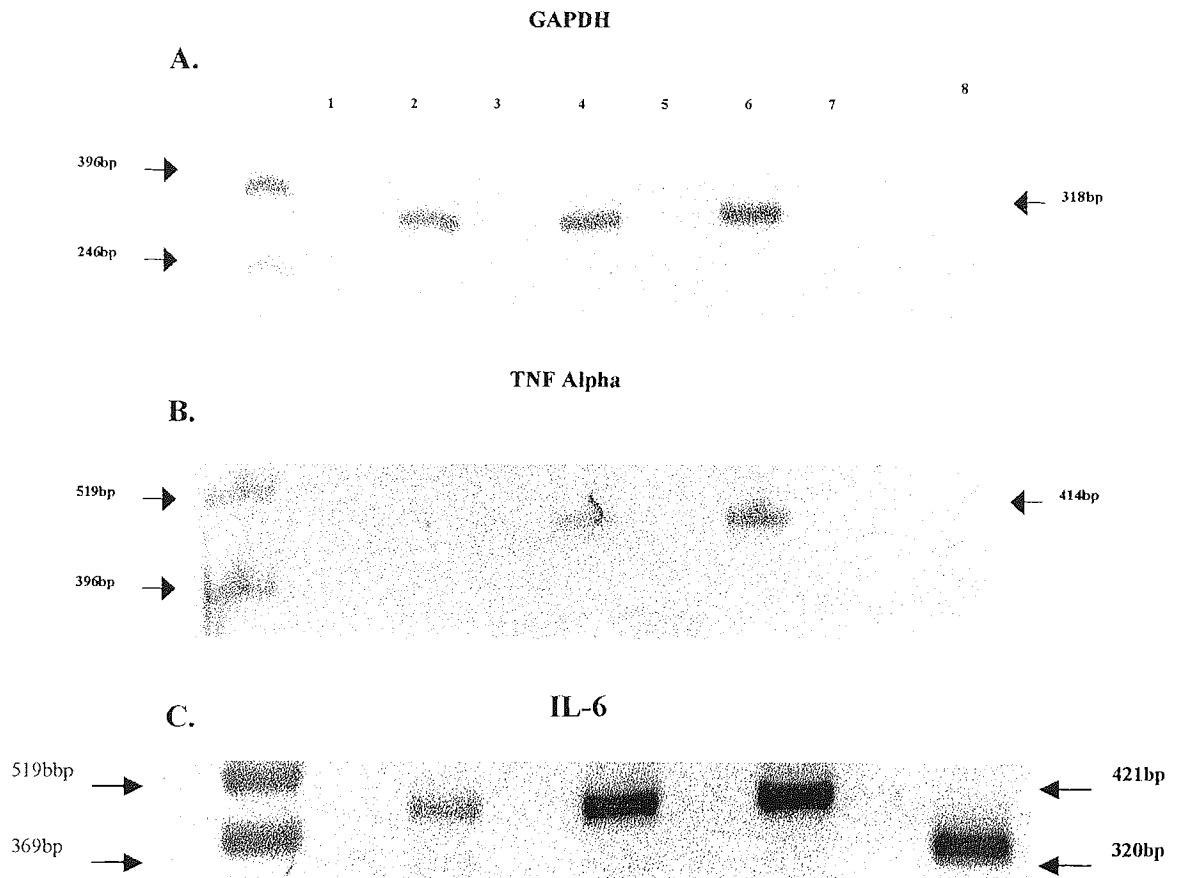
Figure 3.2 TNF-α and IL-6 mRNA expression in isolated primary human monocytes. Peripheral whole blood monocytes (2×10^6/ml) were isolated by differential centrifugation and Dynal negative isolation and exposed to CRP (100µg/ml) or LPS (1µg/ml) for 2 hours at 37°C. Isolated mRNA from stimulated cells underwent RT-PCR with GADPH (A), TNF-α (B) and IL-6 (C) primers (see methods). Products were seperated on agarose gels and analyzed following ethidium bromide staining under a UV transilluminator. Lanes (from 1-8): RT-ve Ctrl; RT+ve Ctrl; RT-ve CRP; RT+ve CRP; RT-ve LPS; RT+ve LPS; PCR Control; (IL6 +ve Ctrl). A representation of 3 experiments is shown.

A)

IL-6 mRNA

B)

TNF-α mRNA
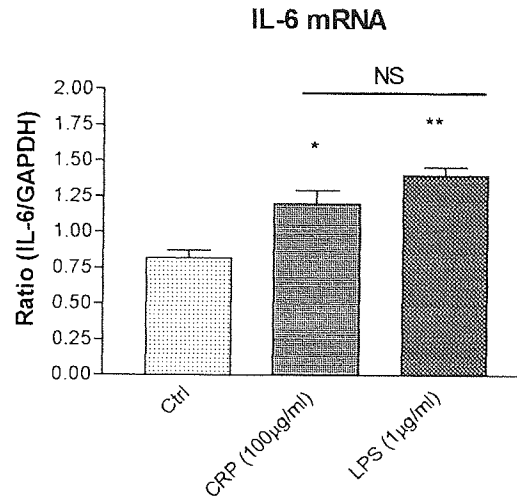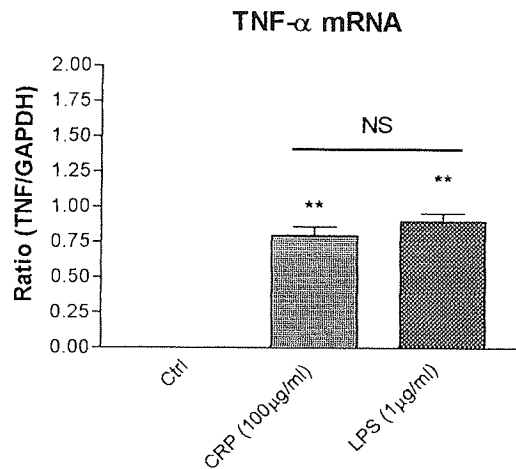
Figure 3.3 Quantitative IL-6 (A) and TNF-α (B) mRNA expression in isolated primary human monocytes. Peripheral whole blood monocytes (2×10^6/ml) were isolated by differential centrifugation and Dynal negative isolation and exposed to CRP (100μg/ml) or LPS (1μg/ml) for 2 hours at 37°C. Isolated mRNA from stimulated cells underwent RT-PCR with GADPH, TNF-α and IL-6 primers. Products were separated on agarose gels and analysed following ethidium bromide staining under a UV transilluminator and density of each band calculated using Scion Image software. The expression ratio of each stimulation over GAPDH expression was calculated from 3 different subjects. * represents P<0.01 and ** represents significantly (P<0.001) different from controls.

3.4.2 TNF-α and IL-6 protein expression

As described previously, monocytes incubated with CRP show an increase in TNF-α and IL-6 mRNA expression, however, this does not automatically imply altered secretion of cytokines. Therefore TNF-α and IL-6 protein expression was examined using intracellular cytokine staining in the presence of the secretion inhibitor brefeldin A (BFA) and secreted cytokines were also determined by ELISA.

After 6 hrs, CRP (100μg/ml) caused a significant (P<0.001) increase in intracellular TNF-α and IL-6 expression in CD14+ PWB monocytes (fig 3.4A&B; fig 3.5A&B), from a medianX±SD (MdX) expression of 2.9±0.6 (TNF-α) and 3.4±0.76 (IL-6) to 5.1±0.9 and 5.6±0.87 respectively (n=4; Fig 3.5A&B). LPS (1μg/ml) activation (fig 3.5A&B) increased MdX to 5±0.4 and 6.8±0.42 for TNF-α and IL-6 respectively (n=4; 3.5A&B), where these data were not significantly (P>0.05) different from CRP induced cytokine expression. LPS activated PWB incubated without BFA lacked any significant increase in cytokine expression, compared to LPS activated PWB incubated with BFA (Fig 3.6A). Control-treated PWB incubated with BFA showed no changes in cytokine expression, demonstrating that BFA alone lacked any activating response in resting monocyte populations (Fig 3.6B).

A)
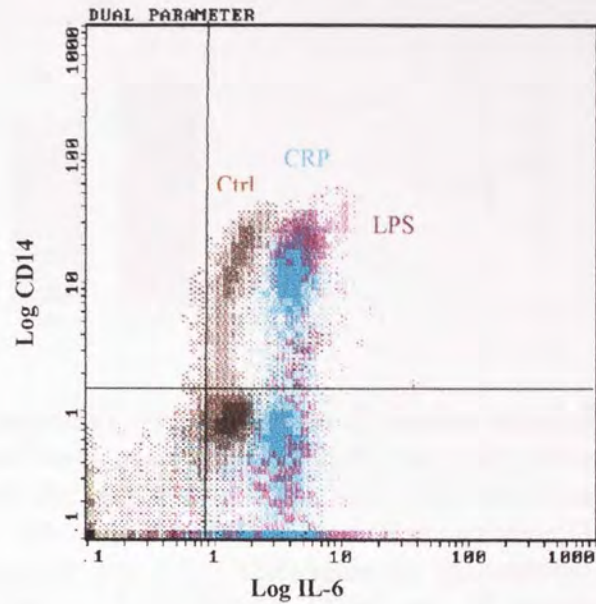
B)
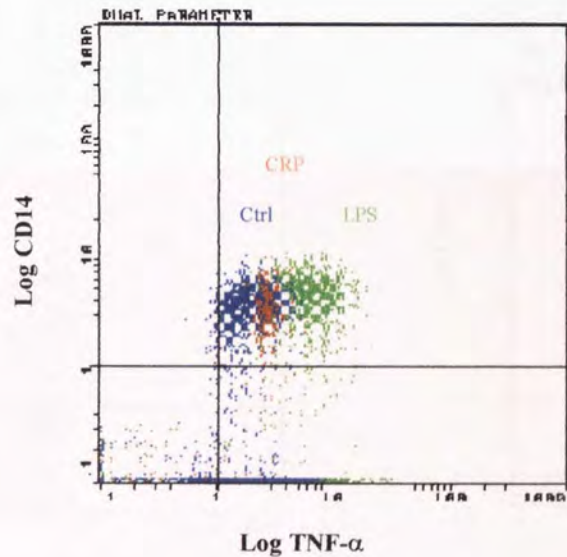
Figure 3.4 Intracellular TNF-α and IL-6 protein expression in PWB monocytes.
Whole blood (1ml) was stimulated with vehicle Ctrl, CRP (100μg/ml) or LPS (1μg/ml) for
6hrs at 37°C with the secretion inhibitor BFA (10μg/ml), prior to a fixing and
permeabilisation with saponin (0.5%). CD14+ gated intracellular IL-6 – FITC (A) and
TNF-α - PE (B) dot plot fluorescence histograms are shown. A representation of four
experiments is depicted. The lines depicted on each histogram represents the value to
which the isotype negative control Abs (PE and FITC) were set from.

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A)

TNF-α

B)

IL-6
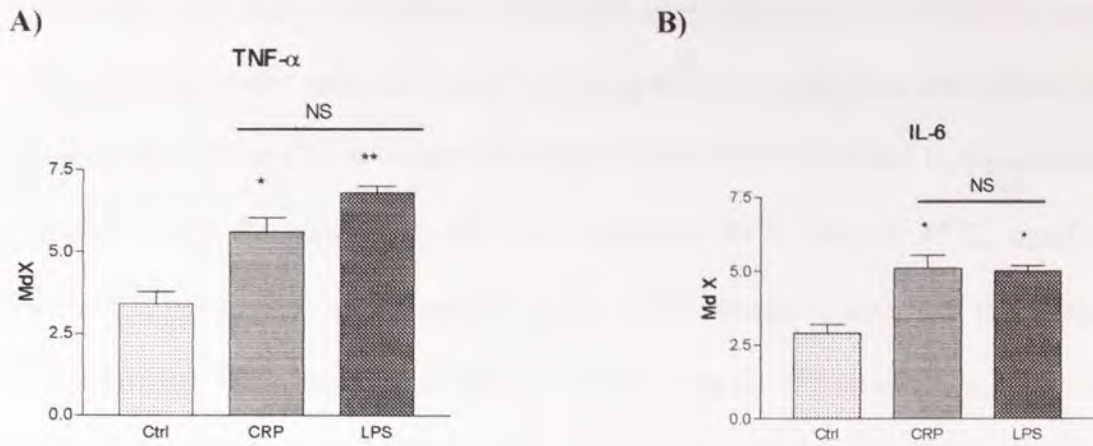
Figure 3.5 Intracellular TNF-α and IL-6 protein expression in PWB monocytes.
Whole blood (1ml) was stimulated with vehicle Ctrl, CRP (100µg/ml) or LPS (1µg/ml) for 6hrs at 37°C with the secretion inhibitor BFA (10µg/ml), fixed and permeabilised with saponin (0.5%). The mean MdX CD14+ gated intracellular TNF-α - PE (A) and IL-6 – FITC (B) fluorescence is shown. Values represent the mean±SD from 4 different normal subjects in triplicates. * represents $P<0.01$ and ** represents $P<0.001$, significant difference from controls.

A.

B.
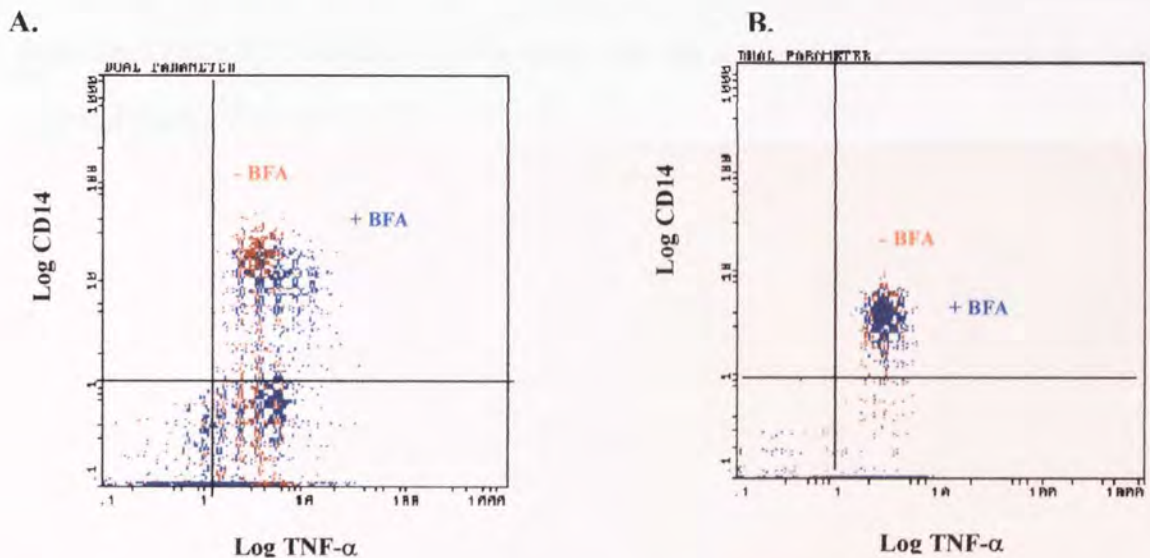
Figure 3.6 Intracellular TNF-α protein expression in PWB monocytes. Whole blood (1ml) was stimulated with vehicle Ctrl or LPS (1µg/ml) for 6hrs at 37°C with or without the secretion inhibitor BFA (10µg/ml), fixed and permeabilised with saponin (0.5%). CD14+ gated intracellular TNF-α - PE with and without pre-incubation with saponin incubated with LPS (A) or vehicle control (B) is shown. A representation of four experiments is depicted. The line depicted on each histogram represents the value to which the isotype negative control Ab (PE) was set.

To further characterize the cytokine expression after stimulation with CRP and to ensure that cytokine protein expression also led to cytokine secretion from intracellular stores, isolated serum from CRP stimulated PWB was analysed for TNF-α and IL-6 production by ELISA. PWB stimulated with CRP (10-100µg/ml) for 0-24hrs at 37°C, significantly (P<0.001) increased IL-6 secretion (fig 3.7A). PWB stimulated with CRP (10-100µg/ml) for 0-24hrs at 37°C dose dependently (P<0.001) increased TNF-α secretion, with a slight reduction in secretion levels after 24hrs. The secretion levels were near to that of LPS (1µg/ml) stimulated PWB (Fig 3.7A&B), which was used a positive control.

As the treatment of PWB with CRP would also include the production of cytokines by neutrophils, isolated MNC by differential centrifugation were incubated with CRP (100µg/ml). MNC treatment with CRP, showed a significant (P<0.05) increase in IL-6 secretion (Table 3.2), establishing that monocytes are at least in part responsible for IL-6 expression after CRP activation.

A.

IL6 ELISA

B.

TNF-α ELISA
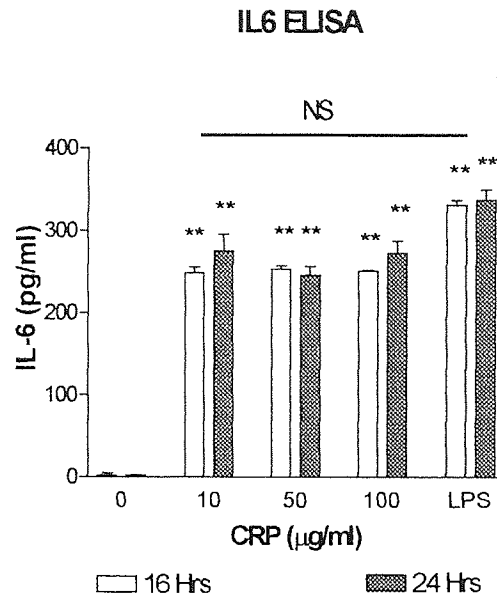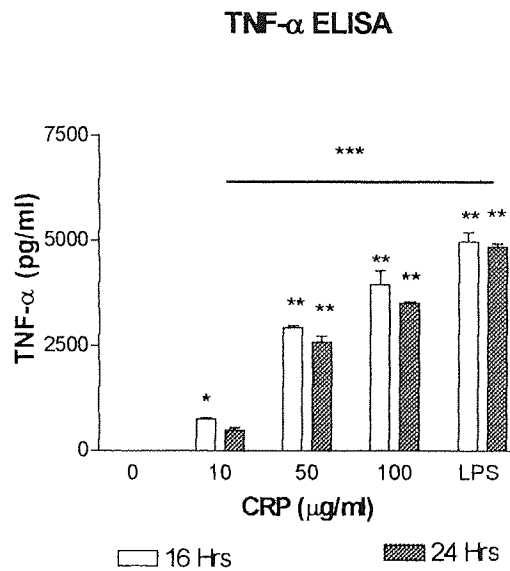
Figure 3.7 TNF-α and IL-6 protein secretion in PWB. PWB (1ml) was incubated with CRP (10, 50 and 100µg/ml) or LPS (1µg/ml) for 0 to 24hrs at 37°C. The serum was isolated by centrifugation and analysed for IL-6 (A) and TNF-α (B) ELISAs, according to manufactures instructions (IDS). Values represent the mean±SD from 3 different normal subjects in triplicate. * represents P<0.05 and ** represents significantly (P<0.001) different from controls.

Stimulus	IL-6 (pg/ml) mean±SD
Ctrl MNC	0±35.7
CRP MNC	199.1±117.76*

Table 3.2 IL-6 protein secretion from MNC. MNC ($2x10^6$/ml) were isolated from PWB by differential centrifugation and challenged with CRP ($50\mu g$/ml) for 16hrs at $37^{O}C$ and analysed for IL-6 expression by ELISA as in fig 3.8. Values represent the mean±SD from 3 different normal subjects in triplicate. * represents P<0.05 from Ctrl.

3.5 Discussion:

The pro-inflammatory cytokines TNF-α and IL-6 are known to have multiple effects in human pathophysiology, particularly inflammation, where IL-6 and TNF-α production in monocytes is triggered by FcγR crosslinking (Krutman et al., 1990; Debets et al., 1990) and CRP has been reported to bind Fcγ receptors (FcγR crosslinking). Therefore it was pertinent to examine the effect of CRP on cytokine expression and secretion in monocytes and PWB. Previous workers have demonstrated that IL-6 levels are markers of disease and disease activity in inflammatory disorders, where levels of CRP are strongly correlated with serum levels of IL-6 (Ridker et al., 2000; Harris et al., 1999). This suggests that CRP and IL-6 levels are not just separate markers, but are in fact linked together in production and activation within the immune system.

CRP dose-dependently induced the expression and secretion of TNF-α and IL-6 from 10μg/ml CRP, which corresponds to the plasma levels seen in many vascular disorders such as angina and cardiovascular disease (Berk et al., 1990; Ferreiros et al., 1999). The level of IL-6 and TNF-α production and intracellular cytokine expression induced by CRP was not significantly different from LPS activation, which has been previously been reported in numerous studies (as reviewed in; Guha & Mackman, 2001) to be a potent proinflammatory cytokine activator within monocytes. The method of using BFA as a secretion inhibitor in order to examine IL-6 and TNF-α expression in monocytes within peripheral whole blood was shown to be successful. This was supported by the fact that both LPS stimulated monocytes without BFA showed a decrease in fluorescence (cytokine expression) from BFA incubated monocytes and that resting monocytes incubated with BFA showed no significant increase in fluorescence from control resting monocytes.

The pattern of TNF-α expression after CRP treatment was also time dependent, increasing for both cytokines up to 16 hours of treatment, where the expression levels decreased after 24hrs. Previous workers (Ballou & Lozanski, 1992) have reported this pattern of elevated monocytic cytokine (TNF-α and IL-6) expression after CRP incubation, using isolated primary monocytes. Moreover CRP has shown to increase IL-6 secretion in human saphenous vein endothelial cells, thereby demonstrating the ability of CRP to induce a vascular pro-inflammatory phenotype (Verma et al., 2002). More recently CRP has been shown to increase the expression IL-6 within vascular smooth muscle cells in vitro, which could be through the activation of the redox responsive transcription factor NF-kappa B (NFκB; Hattori et al., 2003). However in both cases it remains to be elucidated whether the response is due to direct interaction between CRP and cell or through associated interactions (e.g., complement).

The activation of CRP to induce IL-6 secretion in monocytes creates somewhat of a paradox, as there appears to be a self-perpetuating process, whereby CRP can stimulate IL-6 expression that in turn stimulates CRP expression in hepatocytes (Toniatti et al., 1990; Ganapathi et al., 1991; Szialai et al., 2000). Therefore the CRP induced cytokine expression profile of anti-inflammatory cytokines needs to be examined, such as IL-10 which has been shown to be capable of inhibiting the production of most cytokines derived from monocytes/macrophages (de Waal Malefyt et al., 1991; Fiorentino et al., 1991).

IL-10 is produced by activated CD8^{+} peripheral blood T-cells, by Th0-, Th1- and Th2-like CD4^{+} T-cell clones after both antigen-specific and polyclonal activation, by B-cell lymphomas and by LPS-activated monocytes and mast cells (as reviewed in; Strle et al., 2001; Haddad et al., 2001). The synthesis of IL-10 by monocytes is primarily and

effectively inhibited by IL-4 and IL-10 (as reviewed in; St. Clair *et al.*, 2000). IL-10 is a homodimeric protein with subunits having a length of 160 amino acids that binds cytokine receptor class II. In monocytes and macrophages stimulated by bacterial lipopolysaccharide-endotoxin (LPS), IL-10 inhibits the synthesis of IL-6 and TNF-α by promoting the degradation of cytokine mRNA (as reviewed in; Quesniaux, 1992; Saito, 2000). Analysis of the IL-10 effects shows that it completely inhibits the LPS triggered p56lyn tyrosine kinase activation and all subsequent events in this cascade including Ras and Raf activation, thereby leading to inhibition of phosphorylation of the MAPK (mitogen activated protein kinases) components (Geng *et al.*, 1994).

Previous work also suggests that IL-10 causes selective inhibition of NFκB activation in LPS stimulated human monocytes and that this NFκB inhibition may be a mechanism by which IL-10 causes the suppression of the cytokine production. (Wang *et al.*, 1995). Therefore, although CRP stimulates monocytes to express and secrete proinflammatory cytokines, which creates a positive feedback to CRP production, this maybe tightly regulated by the actions of anti-inflammatory cytokines, such as IL-10, by abrogating the production of IL-6 or TNF-α within monocytes.

In wild type mice, CRP treatment resulted in an increase in IL-10 at 1 and 4 hours (Mold *et al.*, 2002) and investigators reported that transgenic mice expressing human CRP again showed an increase in serum levels of IL-10 (Szalai *et al.*, 2002). This could demonstrate that although short incubations with acute phase levels of CRP may activate monocytes to secrete proinflammatory cytokines, it may also induce anti-inflammatory cytokines that would abrogate the activating signal from CRP to produce IL-6 or TNF-α. Therefore in diseases such as rheumatoid arthritis where continuous high levels of CRP are apparent

this regulatory process may be absent. However, it must be noted that when investigating CRP responses in rat or mice models the ITIM motif situated below the Fc gamma receptor, in contrast to the activating motif expressed in human monocytes (ITAM) must be taken into account (see general discussion).

Another pathway whereby the proinflammatory effects of CRP described hitherto, maybe attenuated or regulated is through vagus nerve stimulation. Through the vagus nerve, the nervous system can significantly and rapidly inhibit the release of macrophage TNF, and attenuate systemic inflammatory responses (as reviewed in; Tracey, 2002). This physiological mechanism, termed the 'cholinergic anti-inflammatory pathway' (Borovikova et al., 2000) signals via acetylcholine-mediated (cholinergic) receptors on macrophages. Recently the nicotinic acetylcholine receptor α7 subunit has been shown to be important for acetylcholine inhibition of macrophage TNF release (Wang et al., 2003). Electrical stimulation of the Vagas nerve was shown to inhibit TNF synthesis in wild type mice, but failed to inhibit TNF synthesis in α7-defecient mice. Thus the nicotinic acetylcholine receptor α7 subunit appears to be essential for inhibiting cytokine synthesis by the cholinergic anti-inflammatory pathway.

Interleukin 1 (IL-1) receptors are expressed on sensory vagus nerve endings, which when bound with IL-1, are able to induce the cholinergic anti-inflammatory pathway. Therefore it could be hypothesised that CRP receptors may be present on sensory vagus nerves and their occupation may induce the cholinergic anti-inflammatory pathway thereby regulating cytokine release from α7 receptor expressing macrophages or monocytes.

4.0 Adhesion

4.1 Preface:

This chapter examines and identifies the consequences of C-reactive on monocytic adhesion molecule expression and their subsequent interaction with the endothelium.

4.2 Introduction:

During inflammation, the transendothelial migration of monocytes is controlled by chemotactic and activating signals, together with the sequential interaction between the immunoglobulin-like domain of the immunoglobulin superfamily of adhesion molecules and their corresponding ligands. Neutrophil and monocyte recruitment in acute inflammation is mediated in part by the β2-integrin family of receptors, of which Mac-1 ($\alpha M\beta2$, CD11b/CD18) is one of them (as reviewed in; Springer, 1990). CD11b, a component of the heterodimeric integrin that mediates monocyte adhesion via endothelial ICAM-1, has been shown to be upregulated on the surface of monocytes when activated (Springer, 1994; Muller & Randolph, 1999; Torsteinsdottir, 1999). CD31, (PECAM-1) which is a highly abundant cell surface glycoprotein expressed on haemopoietic and endothelial cells, mediates homophilic (PECAM-1/PECAM-1) adhesion of monocytes to the endothelium where it is critical in regulating monocyte passage through the endothelial junction in cytokine-activated transmigration (Nakada et al., 2000). In atherosclerosis, monocyte recruitment into the vascular wall is crucial for the induction and advance of atherosclerotic lesions (Dong & Wagner, 1998). After emigration, monocytes mature into macrophages, where secretion of matrix metalloproteinases may trigger plaque instability or the expression of tissue factor may promote thrombosis (Herman et al., 2001).

Engagement of β2-integrins by a broad repertoire of ligands generates outside-in signals leading to inflammatory cell activation. In the case of Mac-1, this activation induces the expression of genes encoding for cytokines (eg, IL-1β and TNF-α; Rezzonico et al., 2000;

Fan & Edington, 1993) via modulating activation of the transcription factor AP-1 and NF-kB (Sitrin *et al.*, 1998). CD31 supports homophillic binding on HUVEC with similar characteristics reported for leukocytes (Nakada *et al.*, 2000).

A variety of inflammatory disease states have been reported where an increase in CD11b is evident and a plethora of molecules have been described as being able to up-regulate the membrane expression of CD11b on monocytes. These include homocysteine and LPS. Homocysteine (up to concentrations of 1mMol/L) increases CD11b expression on PMN (Guo & Dudman, 2001). This effect of homocysteine is of particular importance, as in recent years homocysteine and CRP, have been proposed as risk factors for the development and progression of atherosclerosis and atherothrombotic cardiovascular disease (Guo & Dudman, 2001). Exposure to endotoxin (LPS) during Gram-negative sepsis results in the release of numerous inflammatory mediators. Two proteins are of principal importance in this stimulation of monocytic cells: LPS-binding protein (LBP), a plasma protein (Tobias *et al.*, 1986; Schumann *et al.*, 1990) and CD14, a monocyte/macrophage and granulocyte membrane protein (Wright *et al.*, 1989). LBP is a 60-kDa glycoprotein found in normal human serum at $\approx 10\mu g/ml$ (Tobias *et al.*, 1992). It binds to LPS via the lipid A moiety with an affinity of $\approx 10^9 M^{-1}$ (Tobias *et al.*, 1989). CD14 is a 55-kDa glycoprotein anchored to the monocytic membrane via a glycerophosphatidylinositol anchor (Hazoit *et al.*, 1988). The membrane bound (mCD14) serves as a receptor for complexes of LPS and LBP (Wright *et al.*, 1990). Thus current evidence supports the hypothesis that the LBP/CD14-dependent pathway contributes to monocyte stimulation and activation increasing membrane expression of CD11b (see figure 4.1).

LPS + LBP

LPS-LBP → LPS—LBP Mφ → Cell Activation

mCD14
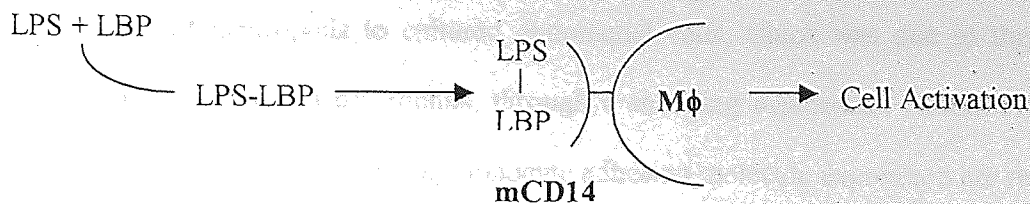
Figure 4.1. Schematic diagram of monocyte activation by LPS. Diagram of the LPS- (lipopolysaccharide), LBP- (LPS binding protein) and CD14-dependent activation of monocytes (Mφ).

A number of studies have described Mac-1 (CD11b/CD18) cooperation with different receptors on MNC, indicating Mac-1 to be a signalling partner for other receptors. These include the LPS/LBP receptors (CD14), but also, FMLP (formyl-methionine-leucine-phenylalanine) receptors, plasminogen activator receptor (CD87) and Fc receptors (Annenkov et al., 1996; Brown, 1998). The Fc receptor relationship is of particular importance as the high affinity receptor for CRP. FcγRIIa (CD32) interacts with integrins both physically and functionally. A genetically engineered phagocytosis-defective form of CD32 was used to show that CD32 is in close proximity to Mac-1 in transfectant membranes and that Mac-1 rescues IgG-dependent phagocytosis mediated by the mutant receptor (Petty & Todd, 1996). Additional studies have suggested that Mac-1 and FcγRIIIB co-operate with CD32 both in the respiratory burst and in signalling mediated by tyrosine phosphorylation (Zhou & Brown, 1994). Evidence for functional co-operation between CD32 and Mac-1 in eosinophils has been provided by studies using different receptor ligation patterns (van der Bruggen et al., 1994). Annenkov et al., (1996) have demonstrated functional interactions in K562 cells that express CD32 and Mac-1, essentially demonstrating the relationship between CD32 receptor engagement and CD11b expression and signalling.

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Previous work has shown that CRP at clinically relevant concentrations strongly inhibited adhesion of neutrophils to cultured endothelial cells, which was due to the loss of L-selectin expression in neutrophils, through a shedding mechanism (Zouki *et al.*, 1997). However, the effects of CRP on monocyte adhesion molecule expression are relatively un-reported. Therefore this chapter has investigated the hypothesis that CRP may be an important mediator of monocyte behaviour in the vasculature, through the modulation of monocyte surface antigens and their associated interaction with the endothelium.
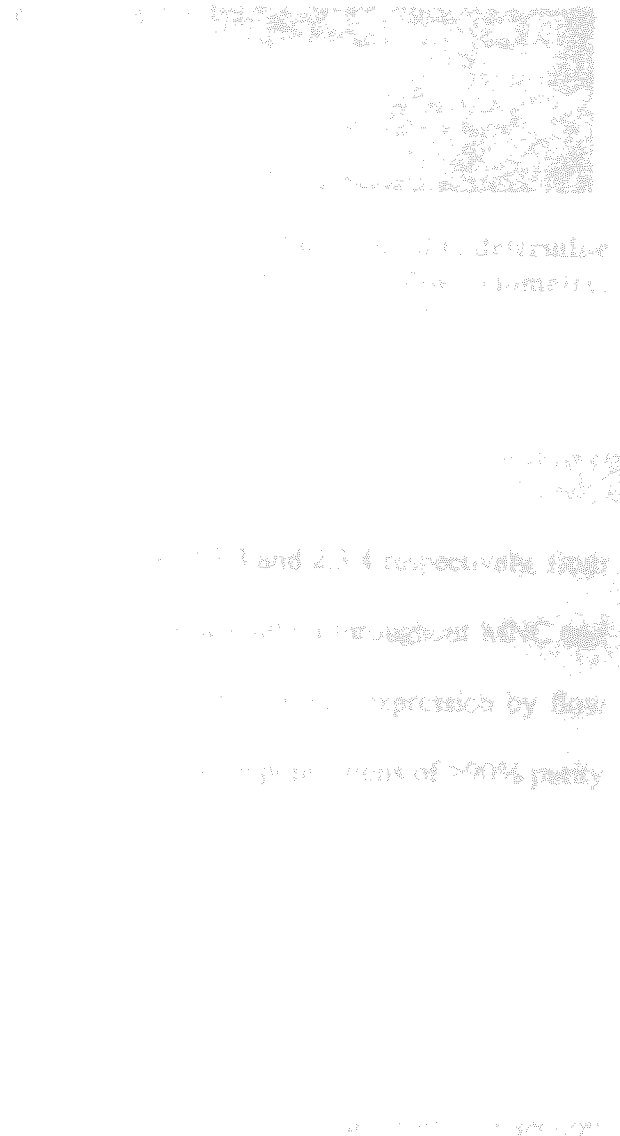
4.3 Materials & Methods:

4.3.1 Materials:

The majority of materials are described in section 2.2. All anti-human fluorescently tagged mouse antibodies used for HUVEC characterisation (see table 4.1) were from Serotec (Oxford, UK). Transwells were purchased from Gibco (Paisley, UK) and collagen was from Sigma (Dorset, UK). Cell scrapers were from Orange Scientific (Braine-l'Allued, Belgium).

Antigen	Source	Species	Clone	Fluorescent Tag
ICAM-1	Mouse	Human IgG1	15.2	FITC
CD14	Mouse	Human IgG1	Tuk-4	RPE-Cy5
CD32	Mouse	Human IgG1	AT10	FITC
CD31	Mouse	Human IgG1	B-B38	PE

Table 4.1. Characteristics of the fluorescent conjugated antibodies used to determine the membrane expression of antigens involved in cell-cell adhesion by flow cytometry. Abbreviations are fluorescein isothiocyanate (FITC), phycoerythrin (PE) and phycoerythrin-Cy5 (RPE-Cy5).

4.3.2 Preparation of mononuclear cells and monocytes from peripheral whole blood:

MNC and monocytes were isolated according to section 2.3.3 and 2.3.4 respectively, from 40mls of whole blood. Monocyte purity and artefactual activation throughout MNC and monocyte isolation, were evaluated by analysing CD14 and CD11b expression by flow cytometry as described in section 2.3.9, where only monocyte populations of >90% purity were used.

4.3.3 Cell Culture:

Endothelial cells were isolated and cultured from umbilical cords according to section 2.3.5. THP-1 cells were cultured according to section 2.3.1.

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4.3.4 Cell Stimulation:

PWB (50μl), MNC, isolated monocytes or THP-1 (2x10^6/ml) were stimulated with recombinant CRP (0-100μg/ml) for 0-30mins at 37°C according to section 2.3.2.

4.3.5 Adhesion molecule expression on monocytes:

Adhesion molecule expression on monocytes were analysed via flow cytometry according to section 2.3.9, with appropriate colour compensation and isotype negative controls according to section 2.3.8. In order to examine the effect of blocking CD32 on CD11b expression in monocytes, MNC (2x10^6/ml) were incubated with purified anti-CD32 Mab (5μl) for 30mins prior to CRP exposure and analysed as above.

4.3.6 Adhesion Assay:

The adhesion of monocytes to endothelial cells in a static system was measured according to section 2.3.6. In order to examine the effect of blocking ICAM-1 and CD31 expression on monocyte adhesion, HUVEC were pre-treated with purified anti-CD31 (5μl) and anti-ICAM-1 MAb (5μl) for 2hrs prior to CRP exposure and analysed as above.

4.3.7 Monocyte migration through transendothelial monolayers:

In order to determine the relative contributions of adhesion or diapedesis of monocytes to the total recorded monocyte binding to HUVEC, the proportion of monocytes that had

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transmigrated through the endothelial monolayer was investigated. HUVEC (1×10^5/ml) were grown in transwells (500µl; 8µm pore) coated with collagen (0.1%) for 2 days at 37°C, 5% CO_2, 95% air humidity and stimulated with LPS (1µg/ml) for 5 hours at 37°C, 5% CO_2, 95% air humidity. EGM media (500µl; 10% FCS) was replaced and 0.25×10^6 isolated monocytes added to each transwell with CRP (100ug\ml), LPS (1ug/ml) or vehicle control for 30mins at 37°C, 5% CO_2, 95% air humidity. Monocytes that had migrated below the transwell were counted using flow cytometry according to FS and SS characteristics and utilising a volume stop (1ml). Tight junction visualisation was confirmed by confocal microscopy according to section 4.3.8, to ensure HUVEC confluence.

4.3.8 Analysis of transwell HUVEC monolayers:

In order to determine HUVEC confluence for the migration assay, HUVEC were grown in transwells according to section 4.3.7 and stained with anti-human mouse CD31 (10µl) conjugated to FITC for 30mins at RT in the dark and analysed for FITC fluorescence using confocal microscopy (Ex. 488nm, Em. 540nm; Olympus, Japan). Resulting image fluorescence levels were examined using PhylumV4.0 (Improvision, Coventry, UK).

4.3.9 Adhesion molecule expression on HUVEC:

The membrane expression of ICAM-1 and CD31 was examined by flow cytometry. HUVEC were grown to confluence in 6 well plates according to section 2.3.5 and incubated with LPS (1µg/ml) for 0, 5 and 24 hours at 37°C, 5% CO_2, 95% air humidity. At each appropriate time point, HUVEC were washed in EGM and cells scraped off from

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tissue culture plastic, using cell scrapers. Cells were centrifuged at 1100rpm for 5 mins and resuspended in 1ml PBS. Cells were incubated with anti-ICAM-1 and anti-CD31 (10μl) conjugated to FITC and PE respectively for 30mins at RT in the dark. Cells were centrifuged at 1100 rpm for 5 mins and resuspended in PBS containing 4% formaldehyde (Sigma, Dorset, UK) to fix samples. Samples were then analysed for FL1 and FL2 fluorescence by flow cytometry according to section 2.3.9, using appropriate isotype negative controls and colour compensation according to section 2.3.8.

4.3.10 Statistical analysis:

Data is presented as the arithmetic mean ± SD. Groups of data were evaluated statistically by T-test or ANOVA, followed by Tukeys test for multiple comparisons or Wilcoxon signed ranks test for matched pair data, with a minimum of n=3 in triplicate, unless otherwise stated.

4.4 Results:

4.4.1 Effects of CRP on THP-1 phenotype:

Activation of CD11b enhances the inflammatory response through monocyte phagocytic activity, complement receptor expression, TNF sensitivity and adhesion to endothelial cells. Consequently, the effects of CRP on this antigen have been evaluated. Figure 4.2 illustrates the effect of CRP (50 and 100μg/ml) treatment on THP-1 CD11b expression. CRP caused a dose dependent elevation in CD11b expression within the THP-1 population, with an increase from control (mean±SD of 4.35±0.021 a.u to 5.34±0.25 a.u at 100μg/ml CRP, P<0.001, n=4). Treatment of THP-1 with 50 or 100μg/ml CRP for 30 minutes did not affect expression of the high affinity CRP receptor, CD32 (10.37±0.07 a.u to 10.64±0.06 a.u at 100μg/ml CRP, P>0.05, n=4; Fig 4.3).
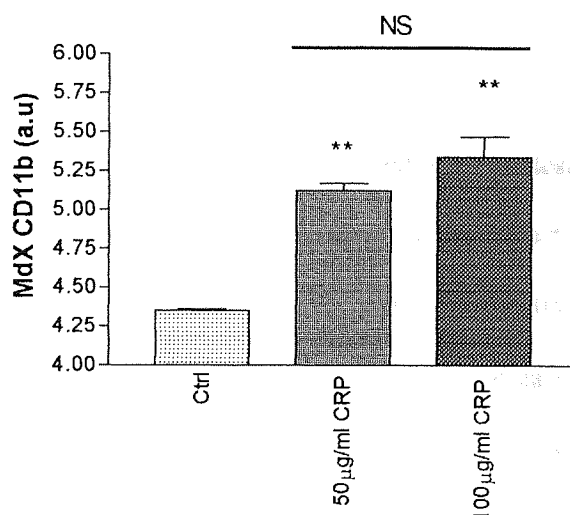
Figure 4.2. The effects of CRP on CD11b expression on THP-1 monocytes. THP-1 (2x10^6/ml) were exposed to 50 or 100μg/ml CRP for 30mins at 37 °C and THP-1 population analysed for CD11b expression via flow cytometry, according to section 2.3.9. ** represents significant difference (P<0.001) from control, using ANOVA with Tukeys post-test analysis (n=4).

SINGLE PARAMETER

Count

Log CD32
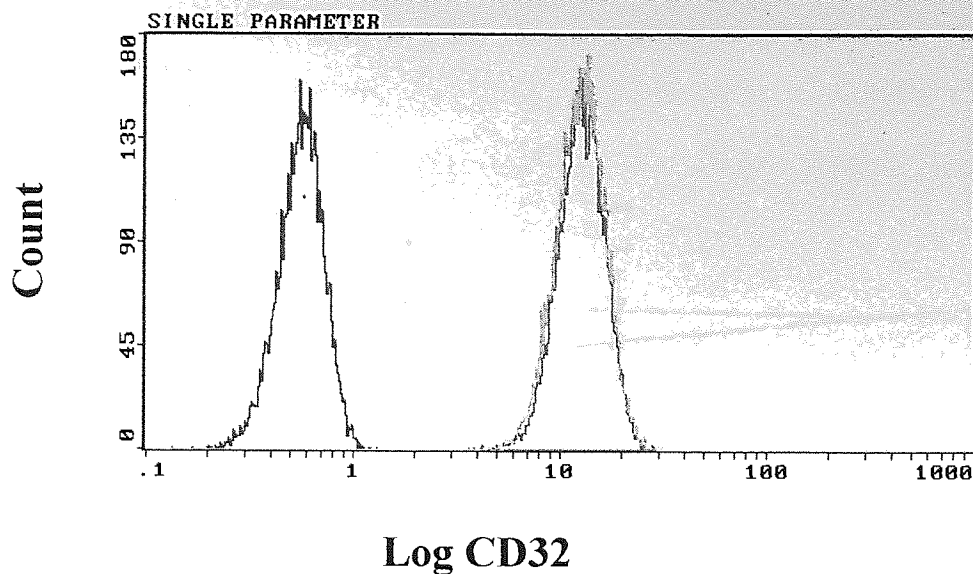
Figure 4.3. Representative flow cytometry histogram showing CRP has no effect on CD32 expression in THP-1 cells. THP-1 $(v \times 10^6)$ were exposed to 0 (▨) and 100µg/ml (▨) CRP for 30mins at 37°C or isotype matched negative control (■) and THP-1 population analysed for CD32 expression via flow cytometry, according to section 2.3.9. The histogram is a representative of four experiments.

4.4.2 Effects of CRP on primary monocyte phenotype:

As CRP was shown to affect the phenotype of the monocytic cell line THP-1, the effects of CRP on adhesion molecule expression with primary human monocytes was investigated.

CD31 is expressed on monocytes where it facilitates diapedesis and migration via homophilic interactions with the endothelium. Therefore, the effect of CRP on expression of CD31 receptor on monocytes was investigated by flow cytometry. Exposure of PWB to CRP (10 to 100µg/ml) in a healthy normal caused a decrease in monocyte CD31 expression. (Fig 4.4). CRP induced the greatest decrease in MdX CD31 expression, at 100µg/ml CRP from a control mean±SD of 9.18±0.0495 to 7.91±0.3, P<0.001 (n=6).
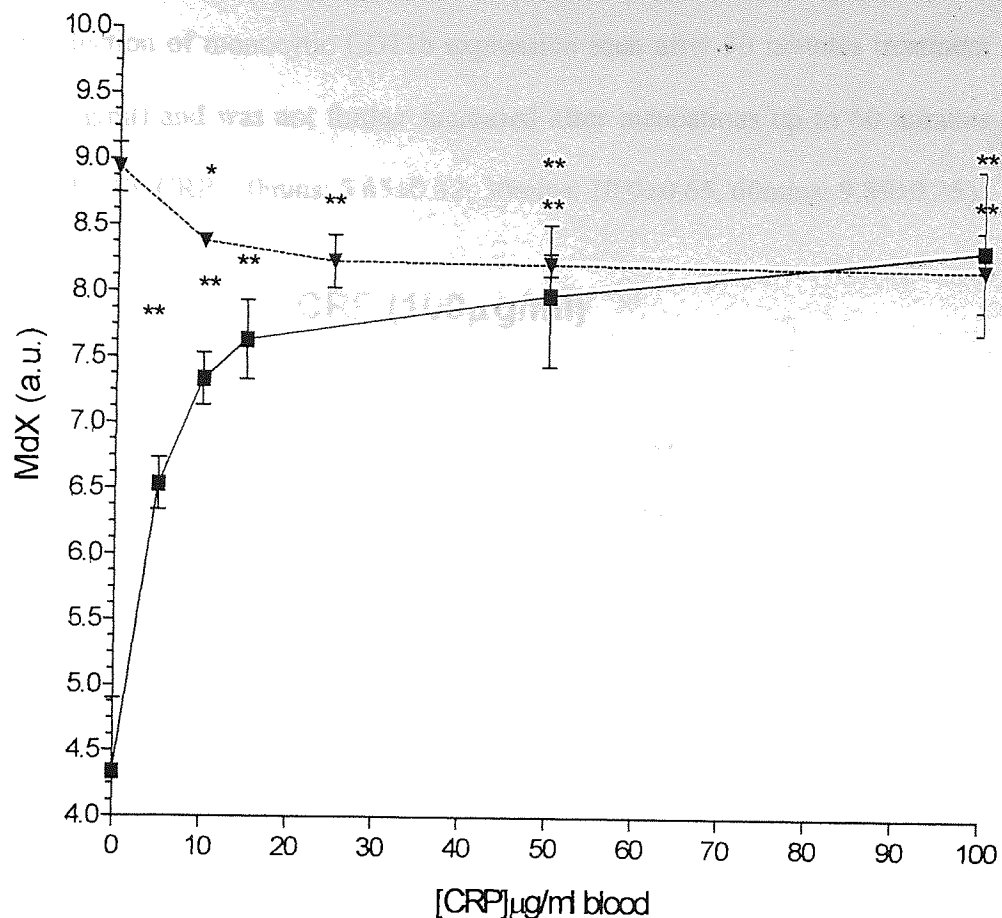
Figure 4.4. The effects of CRP on CD11b and CD31 expression on monocytes, within a healthy normal. PWB (50µl) from the same individual was exposed to 0 – 100µg/ml CRP for 30mins at 37°C and the CD14+ve monocyte population analysed for CD11b (■) and CD31 (▼) expression via flow cytometry, according to section 2.3.9. * represents P<0.05 and ** represents significant difference from control (p<0.001, n=6; where n = number of individual experiments carried out for each CRP concentration on PWB from the same individual) using ANOVA with Tukey's post-test analysis.

Figure 4.4 illustrates the effect of CRP (0-100µg/ml) treatment on monocyte CD11b expression within normal PWB. CRP caused a dose dependent elevation in CD11b expression within the CD14+ve monocyte population, with a two-fold increase from control (mean±SD of 4.34±0.18 to 8.35±0.44 at 15µg/ml CRP, P<0.001, n=6). Increasing the concentration of CRP up to 100µg/ml did not induce greater monocytic CD11b membrane expression (P>0.05, n=6; Fig 4.4).

The maximal induction of monocytic CD11b expression seen after 30 minutes treatment with CRP (100 μg/ml) and was not further increased after incubations up to 60 minutes (Fig 4.5; MdX CD11b: CRP = 0mins: 5.65±0.32, 30mins: 10.9±0.65, 60mins: 9.89±0.26).

CRP (100μg/ml)
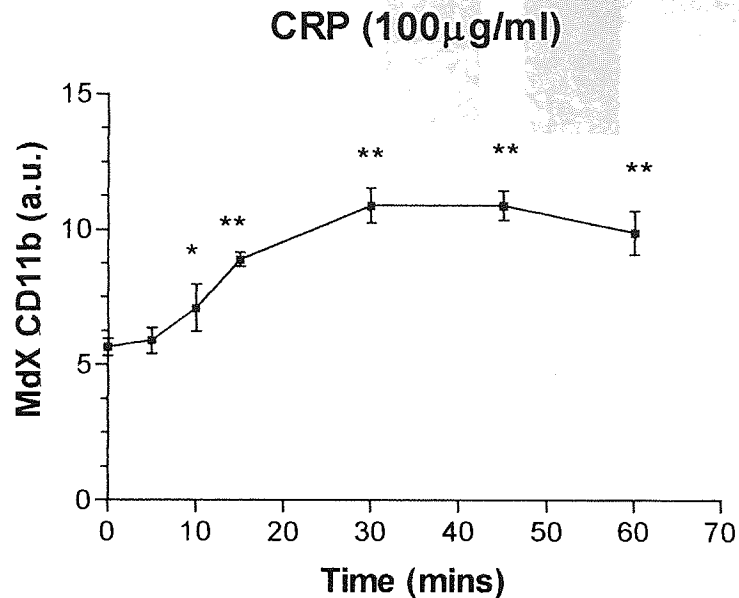
Figure 4.5. Kinetics of CRP mediated monocytic CD11b expression. PWB (50μl) was exposed to 100μg/ml CRP for 0-60mins at 37 °C and CD14+ monocyte population analysed for CD11b expression via flow cytometry, according to section 2.3.9. * represents $P<0.05$ and ** represents significant difference ($P<0.001$) from control, using ANOVA with Tukeys post-test analysis.

In order to examine whether the heterodimeric protein Mac-1 was being expressed a functional ligand and not just the alpha subunit, the β2 subunit CD18 expression on CD14+ monocytes was assessed after CRP challenge. CRP at 50 and 100 μg/ml significantly ($P<0.001$) increased CD18 expression from a control mean±SD MdX of 3.35±0.02 to 4.12±0.091 and 4.34±0.25 at 50 and 100 μg/ml CRP respectively (fig 4.6).
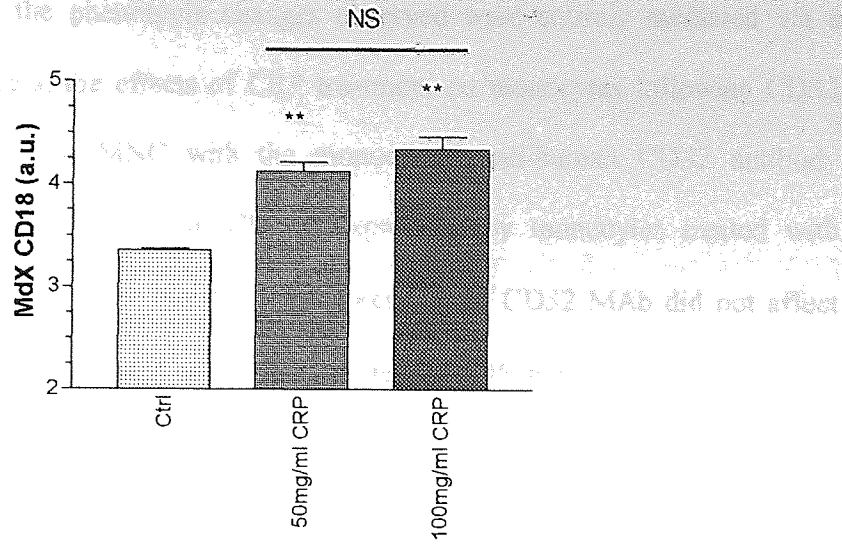
Figure 4.6. The effects of CRP on CD18 expression by monocytes. PWB (50μl) were exposed to 50 or 100μg/ml CRP for 30mins at 37 °C and CD14+ monocyte population analysed for CD18 expression via flow cytometry, according to section 2.3.9. ** represents significant difference (P<0.001) from control, using ANOVA with Tukeys post-test analysis.

LPS (1μg/ml) was used as a positive control for monocyte activation and showed a significant increase in CD11b expression following treatment at 37°C for 30 minutes, P<0.001 (MdX of 4.34±0.18 to 7.99±0.67; Fig 4.7).
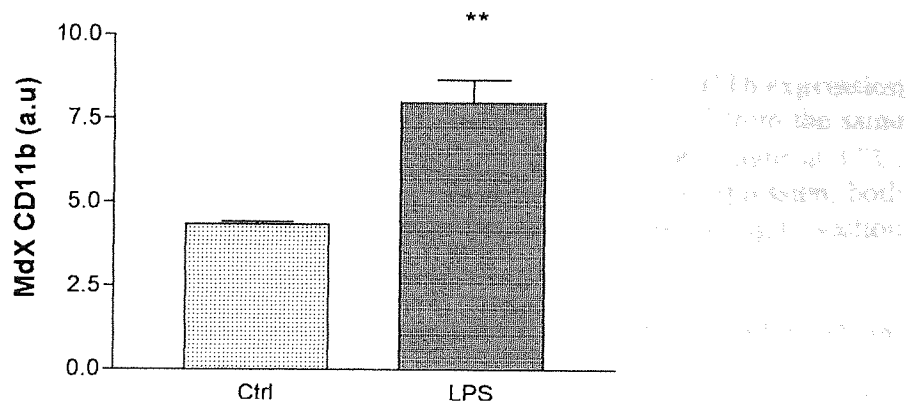
Figure 4.7. The effects of LPS on CD11b expression by monocytes. PWB (50μl) was exposed to vehicle control or 1μg/ml LPS for 30mins at 37 °C and CD14+ monocyte population analysed for CD11b expression via flow cytometry, according to section 2.3.9. ** represents significant difference (P<0.001) from control, using students T-test (n=6).

To determine whether the phenotypic changes observed were entirely mediated via a receptor mediated process, the effects of CRP treatment on monocytes following CD32 blockade. Pre-incubation of MNC with the monoclonal anti-human CD32 antibody significantly reduced the elevation in CD11b expression by monocytes treated with 100µg/ml of CRP (p<0.001, n=6; Fig 4.8). The presence of CD32 MAb did not affect - CD11b expression on vehicle treated control monocytes (p>0.05, n=6)
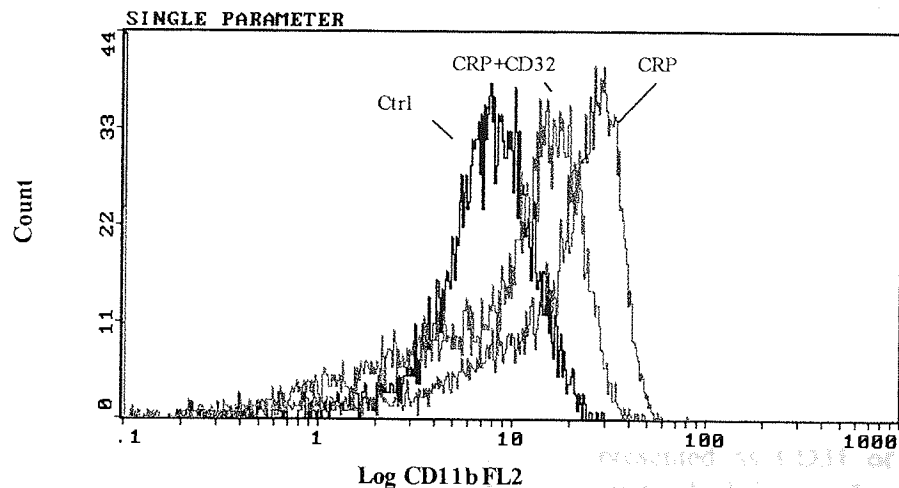
Figure 4.8. The effects of anti-CD32 treatment on the CRP induced CD11b expression of monocytes from one normal subject. MNC (2×10^6/ml) were isolated from the same individual on 6 separate occasions and exposed to 100µg/ml of CRP for 30mins at 37°C. A representative flow cytometry histogram is depicted, showing CD11b expression, both with and without prior exposure to anti-CD32Ab for 30mins at 4°C, according to section 4.3.5.

In order to establish the responses to CRP on monocytic CD31 expression in the general population, PWB was collected from 14 healthy normals and the MNC isolated and treated with vehicle control or CRP (100µg/ml) for 30 minutes. In a similar way to the response observed in a single individual (Fig 4.4), the CD31 expression on CD14+ve monocytes within this sample population was significantly reduced by CRP treatment (Fig 4.9A). In contrast, incubation of MNC with CRP (100µg/ml) caused a significant (p<0.001)

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elevation in monocyte CD11b expression, with an average 2-fold increase in MdX (Fig

4.9B). However 3 out of the 14 subjects did not show any significant change in either

CD11b or CD31 expression after CRP exposure (Fig 4.9A&B)

A.

B.
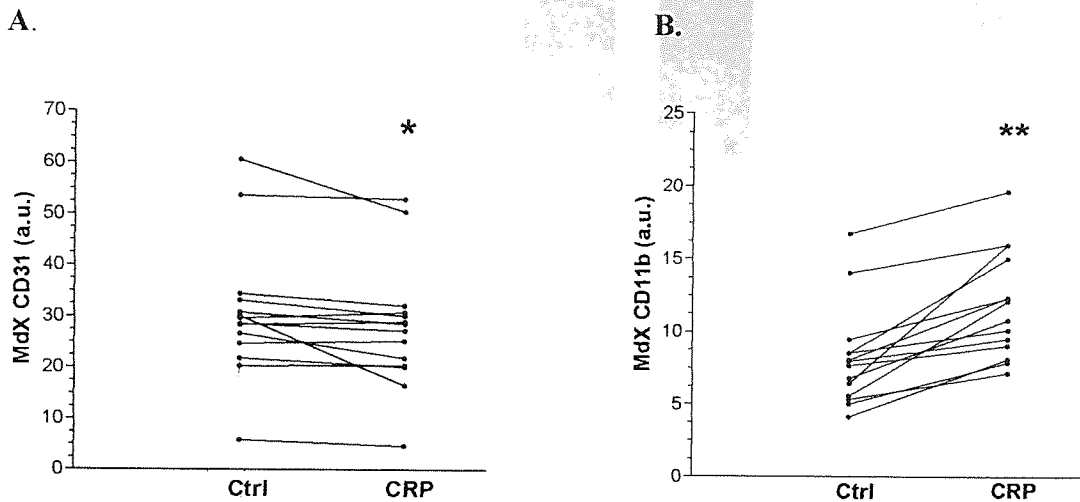
Figure 4.9. The effects of CRP on human monocyte CD31 and CD11b expression.
MNC (2×10^6/ml) from 14 individuals were treated with vehicle controls or 100μg/ml CRP
for 30mins at 37°C, and analysed for monocytic CD31 (A) and CD11b (B) expression via
flow cytometry, according to section 2.3.9. Data is presented as CD31 or CD11b
fluorescence intensity of CD14+ve monocytes from each individual donor. * represents
$P<0.05$ and ** indicates $P<0.0001$ compared to vehicle treatment using Wilcoxon matched
paired test.

The increase in CD11b expression of monocytes exposed to CRP (100μg/ml) for 30

minutes did not differ significantly (p=0.98; n=6) when examined in PWB, MNC or

isolated monocytes, within the same individual. Indeed CD14+ve monocyte expression of

CD11b within PWB, MNC and isolated monocytes incubated with CRP elevated by 2.23,

2.50 and 2.35 respectively (Fig 4.10).

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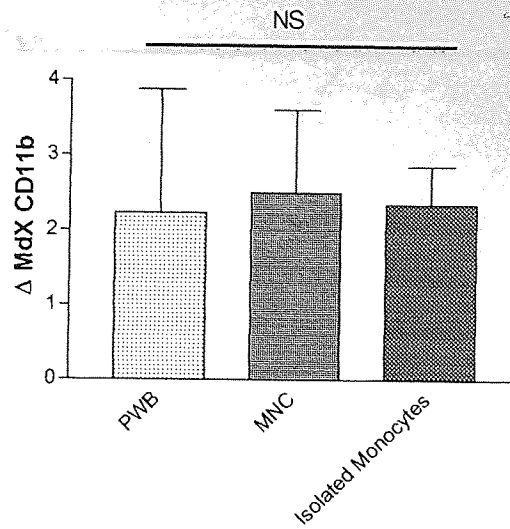
Figure 4.10. The mean change (Δ) in CD11b expression in CD14+ populations in PWB, MNC and isolated monocytes. PWB (50μl), MNC (2×10^6/ml) or isolated monocytes (2×10^6/ml) from the same individual monocytes were exposed to 0 and 100μg/ml CRP for 30mins at 37°C and the CD14+ve monocyte population analysed for CD11b expression via flow cytometry, according to section 2.3.9. Data was analysed using ANOVA with Tukey's post-test analysis

4.4.3 Effects of CRP on primary neutrophil phenotype:

As the treatment of PWB with CRP may also modulate the expression of CD11b by neutrophils, the neutrophil population, as analysed by FS and SS properties, was examined for CD11b expression. Figure 4.11 shows that incubation of CRP (100μg/ml) for 30 minutes increased netrophil CD11b expression. LPS (1μg/ml) was used as a positive control and significantly increased CD11b expression by neutrophils.
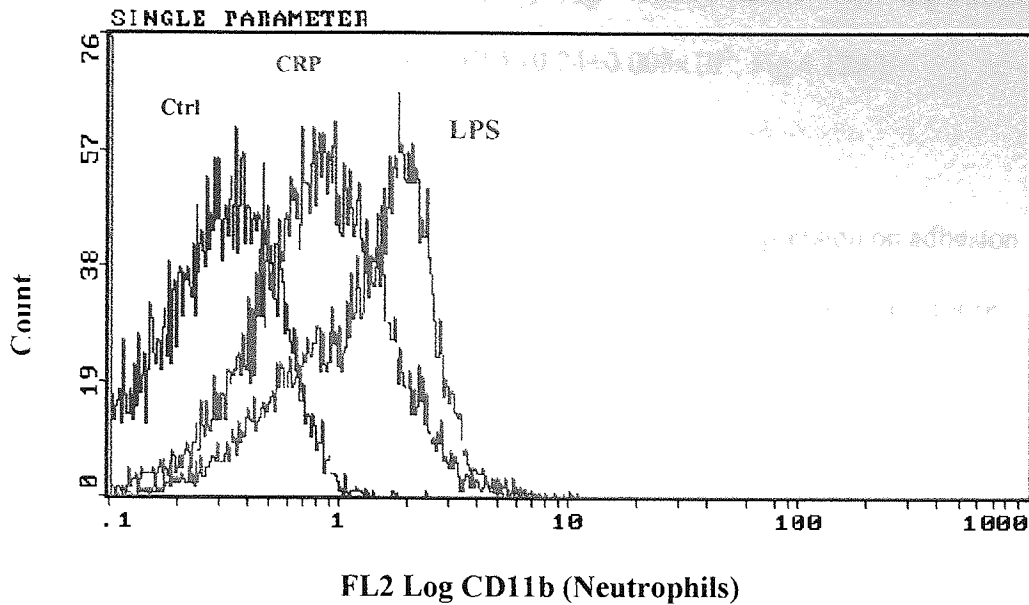
Figure 4.11. The effects of CRP treatment on neutrophil CD11b expression from one normal subject. PWB (50μl) was exposed to vehicle control, LPS (1μg/ml) or 100μg/ml of CRP for 30mins at 37°C on 6 separate occasions. A representative flow cytometry histogram is depicted, showing neutrophil CD11b expression, after vehicle control, CRP or LPS exposure.

4.4.4 Effects of CRP on THP-1 adhesion to HUVEC:

The functional consequences of CRP induced alterations in monocytic phenotype were investigated using a HUVEC model. Resting THP-1 show significantly (p<0.001) increased adherence to HUVEC that had been activated for 5 and 24 hours with LPS compared with resting HUVEC (Fig 4.12A). Resting THP-1 adhesion to HUVEC that had been activated for 5 hours was significantly greater than THP-1 adhesion to HUVEC that had been activated for 24 hours with LPS (Fig 4.12A).

Using resting HUVEC, the adhesion of CRP (100μg/ml) treated THP-1 was significantly increased compared with adhesion of untreated THP-1 as shown in figure 4.12A (from $0.19\pm0.003\times10^6$ total number of cells, to $0.21\pm0.002\times10^6$; p<0.001; n=4). Moreover, THP-1 incubated with CRP (100μg/ml for 30 minutes), showed lower adhesion to 5 hours

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activated HUVEC $(0.21\pm0.007\times10^6$; Fig 4.12B), which was significant $(p<0.001)$ compared to non CRP-treated THP-1 $(0.24\pm0.003\times10^6$; Fig 4.12A).

In order to investigate the role of CRP induced CD11b expression on adhesion of THP-1 to endothelial cells, the interaction between THP-1 and the ICAM-1 receptor on HUVEC was examined, following 24-hour stimulation of HUVEC with LPS. Adhesion to 24 hour LPS-activated endothelial cells was significantly $(P<0.001)$ elevated, where 100 µg/ml CRP treated THP-1 showed $0.23\pm0.002\times10^6$ adherent cells (Fig 4.12B), compared to control THP-1 cells $(0.21\pm0.008\times10^6$; Fig 4.12A). In summary CRP treated THP-1 cells showed an increase in adhesion to 24 hour activated HUVEC

A.

B.
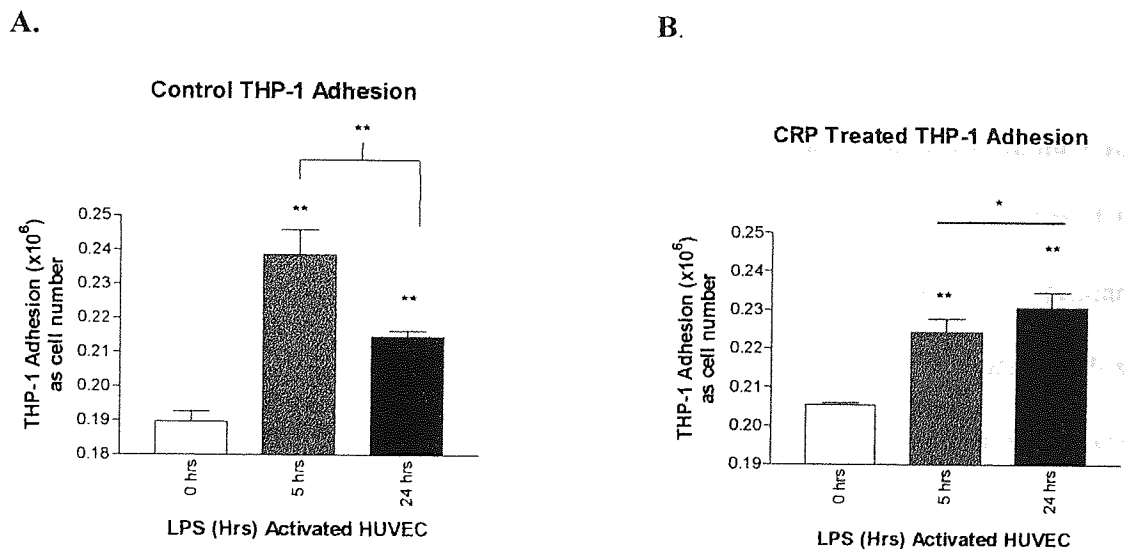
Figure 4.12. Effects of CRP on THP-1 adhesion to 0, 5 and 24 hour LPS-activated HUVEC. HUVEC were treated with LPS (1µg/ml) for 0 hours (□), 5 hours (▨) and 24 hours (■) prior to the addition of THP-1 monocytes to study adhesion. THP-1 cells (0.5 x 10^6/ml) were treated with (**A**) vehicle control or (**B**) 100 µg/ml CRP for 30mins. Treated THP-1 cells were then incubated with HUVEC for 30 minutes and their adhesion quantified. All results are expressed as the total number of cells adhered (x10^6). Data is expressed as the mean±SD of 4 individual experiments where $P<0.05$ (*) and $P<0.001$ (**) was considered significantly different by ANOVA followed by Tukeys post-test analysis.

4.4.5 Effects of CRP on primary monocyte adhesion to HUVEC:

As CRP was shown to alter THP-1 adhesion to HUVEC monolayers, primary isolated monocytes were examined to investigate CRP:monocytic interactions with the endothelium. HUVEC were pre-treated with LPS for either 5 hours (to increase CD31 expression) or for 24 hours (to increase ICAM-1 expression), prior to monocyte addition. Resting monocytes show significantly ($p<0.001$, $n=6$) increased adherence to HUVEC that had been activated for 5 hours with LPS compared with resting HUVEC (Fig 4.13A). However there was no significant change ($p>0.05$, $n=6$) in the adhesion of resting monocytes to HUVEC that had been activated for 24 hr with LPS compared with resting HUVEC (Fig 4.13A). LPS ($1\mu g/ml$) stimulated monocytes were used as a positive control and showed a similar pattern of adhesion as resting monocytes (Fig 4.13A).

Using resting HUVEC, the adhesion of CRP (10 or $100\mu g/ml$) treated monocytes decreased dose dependently compared with adhesion of resting monocytes as shown in Figure 4.13B (from $100\%\pm1.4$ to $95\%\pm0.9$ and $92.5\%\pm1.1$ at 10 and $100\mu g/ml$ CRP respectively $p<0.01$; $n=6$). On average half of the total resting monocytes adhered to resting HUVEC after 30-minute incubations ($1.24\times10^5\pm0.002$). Moreover, primary monocytes incubated with CRP (10 or $100\mu g/ml$ for 30 minutes), exhibited a dose dependent reduction in adhesion to activated HUVEC ($1\mu g/ml$ LPS, 5 hours), which was significant ($p<0.001$, $n=6$) compared to non-CRP treated monocytes (Fig 4.13C). Within a given population ($n=25$) the average adhesion of resting monocytes to 5 hours activated endothelium was $120\%\pm1.44$, however, following CRP ($100\mu g/ml$) treatment, adhesion reduced to $115\%\pm1.56$, $p<0.001$.

Again, in order to investigate the role of CRP induced primary monocyte CD11b expression on adhesion to endothelial cells, the interaction between primary monocytes and the ICAM-1 receptor on HUVEC were examined, following 24-hour stimulation of HUVEC with LPS. Following incubation of monocytes with CRP (10 or 100µg/ml) for 30 minutes, adhesion to 24 hour LPS-activated endothelial cells was significantly elevated, where 10-100 µg/ml CRP treated monocytes showed 115%±2.7 and 120%±2.9 adhesion, compared to control monocytes (104%±3.01; Fig 4.13D).
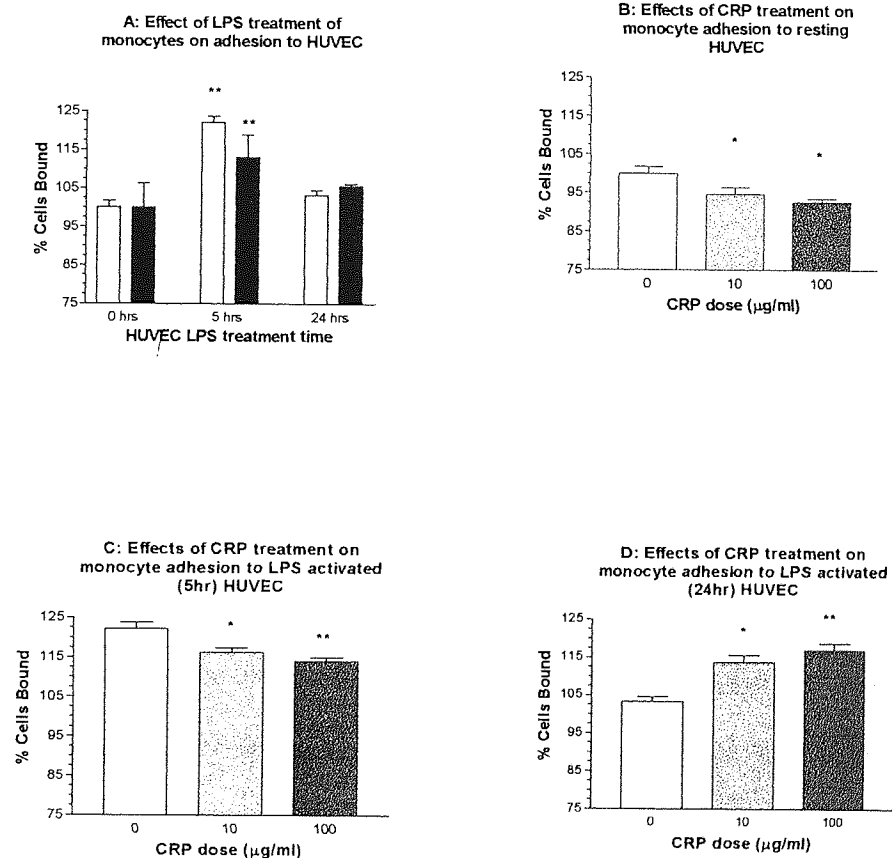
Figure 4.13. Effects of CRP on monocyte adhesion to 0, 5 and 24 hour LPS activated HUVEC. Isolated human monocytes (0.5 x 10⁶/ml) were treated with vehicle control (□), 1µg/ml LPS (■), 10 µg/ml (▨) or 100 µg/ml (▨) CRP for 30mins, washed and resuspended in M199. Treated monocytes were then incubated with HUVEC for 30 minutes and their adhesion quantified. Control resting monocytes and LPS treated monocytes were incubated with 0, 5 and 24 hour LPS activated HUVEC (A). HUVEC were treated with LPS (1µg/ml) for 0 hours (B), 5 hours (C) and 24 hours (D) prior to monocyte incubations. All results are expressed relative to resting monocyte adhesion to resting HUVEC, which is given as 100% adhesion. Data are expressed as the mean ± SD of 6 individual experiments where P<0.05 (*) and P<0.01 (**) was considered significantly different by ANOVA followed by Tukey's post-test analysis.

Pre-treatment of HUVEC with CRP (100μg/ml) had no effect on subsequent binding of resting monocytes (p>0.05, n=4: Fig 4.14), nor was CD32 expressed by HUVEC (as analysed by flow cytometry using anti-CD32 conjugated to FITC). Furthermore, there was no change in HUVEC morphology with 30 minutes serum free incubations (see chapter 2).
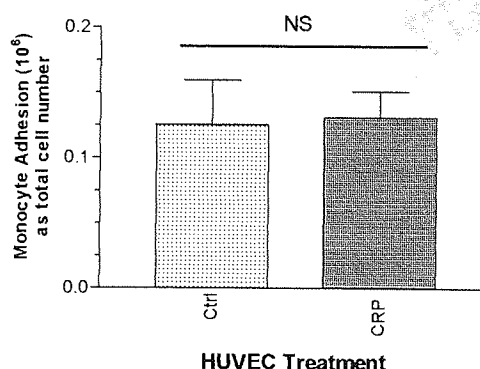
Figure 4.14. CRP treatment of HUVEC does not alter resting monocyte adhesion. Confluent resting HUVEC were washed in serum free M199 and treated with vehicle control or CRP (100μg/ml) for 30mins at $37^{\circ}C$, 5% CO_2, 95% air humidity. Resting isolated monocytes (0.25×10^6) were than added for 30mins at $37^{\circ}C$, 5% CO_2, 95% air humidity and adhesion quantified, according to section 2.3.6. Data are expressed as the mean±SD of total cells adhered and analysed by T-test.

4.4.6 HUVEC characterisation:

In order to examine the expression of the endothelial receptors for the monocytic ligands CD11b and CD31, the levels of ICAM-1 (CD54) and CD31 were explored by flow cytometry, after 5-24 hrs incubation with LPS (1μg/ml). Figure 4.15 shows the expression of ICAM-1 over time with LPS. LPS (1μg/ml) significantly (P<0.05, n=4) increased ICAM-1 expression on HUVEC over 24 hrs from a mean±SD of MdX expression from 6.75±0.54 to 8.13±0.67 and 8.25±0.71 at 5 and 24 hrs activation respectively.
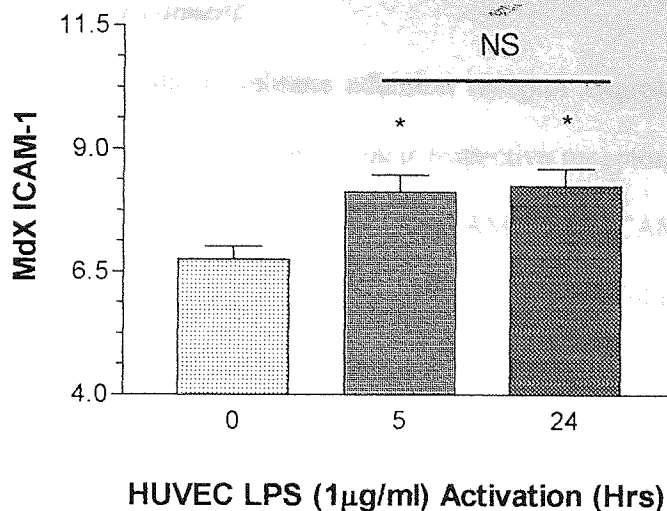
Figure 4.15. The effects of LPS treatment on HUVEC ICAM-1 expression with time. HUVEC monolayers were exposed to LPS (1μg/ml) for 0-24 hours at 37°C 5% CO_2, 95% air humidity on 4 separate occasions and analysed for ICAM-1 expression via flow cytometry, according to section 4.3.9. A representative flow cytometry histogram is depicted, showing HUVEC ICAM-1 expression, after LPS exposure. Data was analysed using ANOVA with Tukey's post-test analysis, where * represents P<0.05.

In contrast, LPS stimulation (1μg/ml) significantly increased CD31 expression over 5 hours, which reverted back to near resting CD31 expression levels, after 24hrs (figure 4.16).
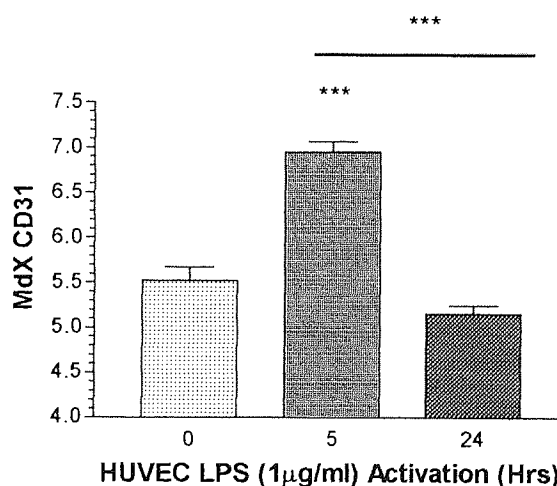
Figure 4.16. The effects of LPS treatment on HUVEC CD31 expression with time. HUVEC monolayers were exposed to LPS (1μg/ml) for 0-24 hours at 37°C 5% CO_2, 95% air humidity on 4 separate occasions and analysed for CD31 expression via flow cytometry, according to section 4.3.9. Data was analysed using ANOVA with Tukey's post-test analysis, where *** represents P<0.001.

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4.4.7 Blocking antibody treatment:

Following the analysis of the membrane adhesion receptor expression by HUVEC, the interaction of CD11b and CD31 ligands or their respective receptors was examined. The use of specific monoclonal antibodies to block PECAM-1 and ICAM-1 receptors on LPS activated HUVEC at 5 and 24 hours respectively, significantly inhibited the adhesion of monocytes (Fig 4.17). Pre-incubation of anti-CD31 at 5 hours LPS activated HUVEC significantly ($p<0.001$; n=6) reduced the adhesion of control monocytes to the same extent as CRP ($100\mu g/ml$) treatment of monocytes ($P>0.05$, n=6). No further significant reduction in adhesion of CRP ($100\mu g/ml$) treated monocytes to 5 hour LPS ($1\mu g/ml$) activated HUVEC pre-treated with anti-CD31 was observed ($P>0.05$, n=6; Fig 4.17). Pre-incubation with anti-ICAM-1 led to a 3.1% reduction in adhesion, however this reduction in adhesion was not reduced to control monocyte levels or anti-ICAM-1 pre-treatment levels (Fig 4.17).
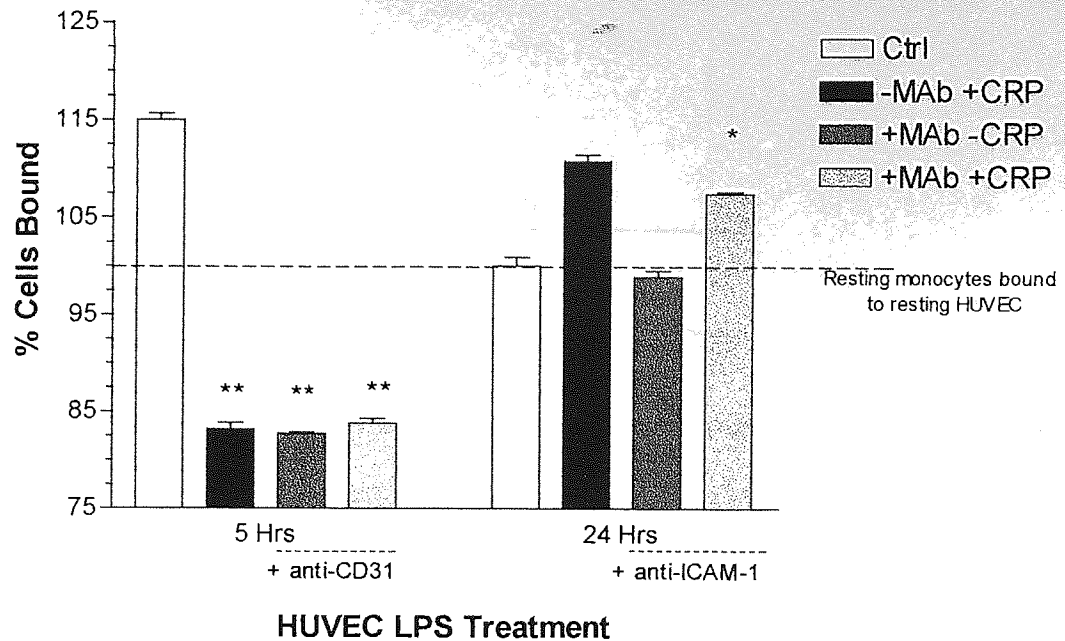
Figure 4.17. CD31 or ICAM-1 inhibition of monocyte adhesion to 5 hour or 24 hour LPS activated HUVEC respectively. HUVEC were exposed to anti-CD31 or anti-ICAM-1 (5μl) for 2hrs at 37°C and then incubated with 0.5×10^6/ml of isolated monocytes for 30mins at 37°C. Monocytes were treated with vehicle control or CRP (100μg/ml) for 30mins at 37°C and adhesion assessed according to section 4.3.6. All results are expressed relative to resting monocyte adhesion to resting HUVEC, which is given as 100% adhesion. Data are expressed as the mean ± SD of 6 individual experiments where $P<0.01$ (**) was considered significant vrs control monocytes 5hrs activated HUVEC and $P<0.05$ (*), was considered significant vrs -Ab+CRP/24hrs LPS activated HUVEC, using ANOVA and Tukey's post test to analyse data.

4.4.8 Effects of CRP on trans-endothelial migration:

To determine the degree of monocyte migration through the HUVEC monolayer following CRP treatment, the effect of CRP was investigated on monocyte trans-endothelial migration after 30 mins. Less than 1% of monocytes migrated through the transwell under these conditions, where 1750±5 isolated resting monocytes were detected after 30 mins (fig 4.18). In contrast, 2450±60 monocytes migrated through the transwell and the presence of LPS, compared with 1250±6 following 30 min treatment with CRP ($P>0.05$, n=4; fig 4.18).

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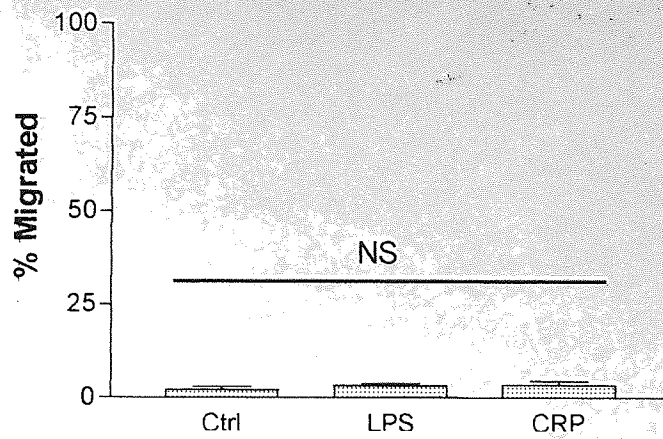
Figure 4.18. The effect of CRP and LPS on monocyte transendothelial migration. HUVEC were grown to confluence on transwells at 37°C 5% CO_2, 95% air humidity and activated for 5hrs with LPS (1μg/ml). Wells were washed (3x) and serum free M199 added. Isolated monocytes ($1.25x10^5$) were added and stimulated with vehicle control, LPS (1μg/ml) or CRP (100μg/ml) for 30mins at 37°C 5% CO_2, 95% air humidity. Transmigrated monocytes that had migrated through the transwell monolayer were counted using flow cytometry, according to section 4.3.7. Data is expressed as the percentage (mean±SD) migrated of total number of monocytes added and analysed by ANOVA followed by Tukey's post test ($P>0.05$, n=4).

The confluence of HUVEC growing on transwell surfaces is critical in order to study the migration of monocytes through the endothelial cell junctions; therefore the confluence of HUVEC was assessed by confocal microscopy. The expression of CD31 was examined, which is only expressed at adjoining endothelial cells when tight junctions are formed (Springer, 1994). A random set of HUVEC cultured transwells stained with CD31 conjugated to PE was analysed. Figure 4.19 shows the expression of CD31 round each adjoining endothelial cell, forming a confluent monolayer.
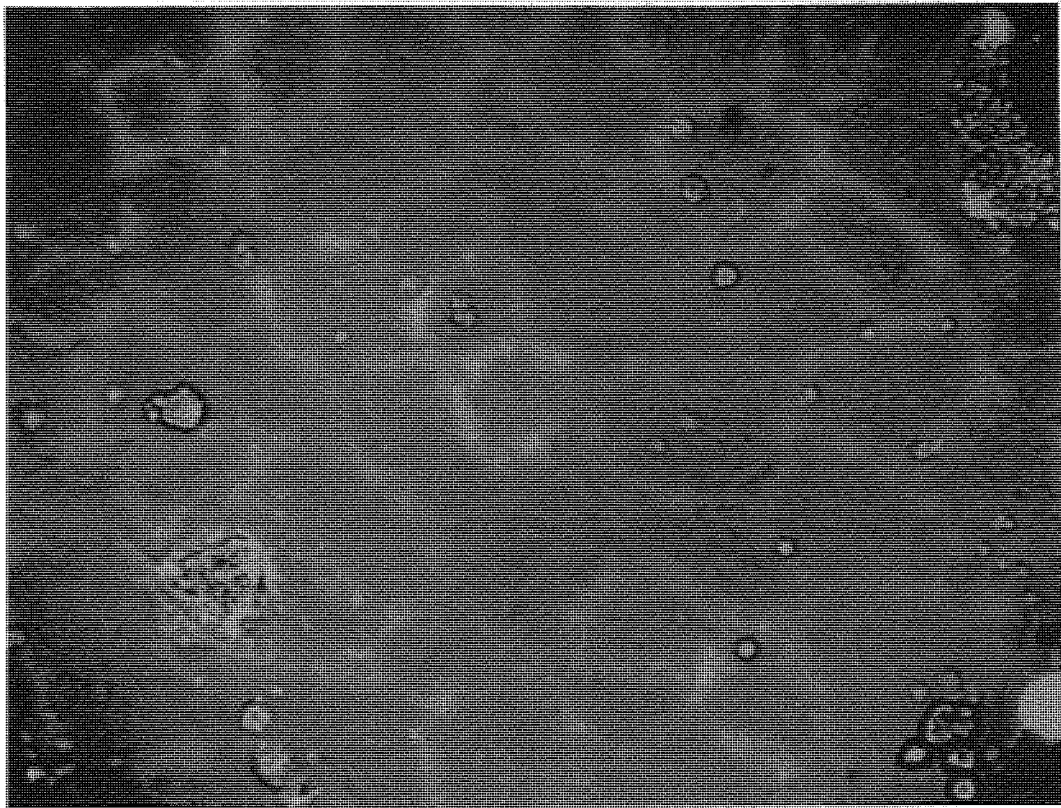
Figure 4.19. Examination of HUVEC monolayers grown in transwells. HUVEC were cultured in transwells as above and incubated with anti-human mouse CD31 conjugated to PE (10μl) for 30mins at RT in the dark. Fluorescence of CD31 expression was assessed by confocal microscopy, according to section 4.3.8. A representative image is shown photographed on PhylumV4.0.

4.5 Discussion:

The hypothesis that C-reactive protein (CRP), the prototypic acute phase serum protein and the strongest link for risk prediction and disease activity in many acute inflammatory pathologies (as reviewed in; Albert, 2000), can exert effects on monocyte phenotype, has been investigated. The role of CRP in the complement system has been well documented and studied, but the effects of CRP on specific cell types involved within the inflammatory process has been little studied.

Herein, this investigation has demonstrated that CRP exerted differential effects on monocyte phenotype, where an increase in the monocytic surface integrin CD11b was observed, in association with a reduction in CD31. Indeed pathologies associated with elevated CRP such as RA and atherosclerosis show a significantly increased PWB monocyte surface expression of CD11b (Torsteinsdottir *et al.*, 1999; Kassirer *et al.*, 1999).

Examining the change in expression of both CD31 and CD11b expression within a larger cohort of 14 subjects confirmed the observations of changes in monocytic antigen expression modulation by CRP. Interestingly there was a large variation in basal expression of both CD11b and CD31 within the group, with a variation of MdX expression from 4-62 and 4-17 for CD31 and CD11b respectively. The consequences of such a differing basal expression of adhesion molecules is questionable, however, it might relate to inflammatory outcomes (e.g., monocyte adhesion in the vasculature) and requires further investigation.

The incubation of CRP with PWB would include the activation of other cell types expressing Fcγ receptors (as described in chapter 5); therefore the expression of CD11b on

neutrophils after CRP challenge was investigated. As shown CRP was able to significantly increase CD11b expression on neutrophils, therefore demonstrating that CRP is able to interact with and activate other important cells involved in inflammation within the immune system.

In the normal population, CD32 receptor specificity of CRP effects was confirmed by blocking CRP-induced CD11b expression with anti-CD32, but not anti-CD14 (see general methods for results), therefore demonstrating effects of CRP were not due to endotoxin contamination.

In contrast to the observations of CRP effects on CD31, elevated CD31 expression has been associated with cardiovascular disease (Masuda *et al.*, 1998). Such an effect is likely to have downstream consequences via the capacity of activated, phosphorylated CD31 to transduce intracellular signals and up-regulate other molecules involved in endothelial cell interaction. However, other workers have described the propensity for CD31 down regulation on endothelial cells following IFN gamma treatment, and suggest that this process may be important in the down regulation of the inflammatory response by this antigen (Bujan *et al.*, 1999). In this context, CRP may have an important role in down regulating the early stages of monocyte recruitment and diapedesis.

In order to evaluate whether cell crosstalk is important in the ability of monocytes to respond to CRP, the effects of treatment of PWB, MNC or isolated monocytes on phenotype within the same individual were compared. Monocytes exhibited the same enhancement of CD11b expression as PWB or MNC. This supports the hypothesis that

CRP acts directly on monocytes and that CRP-induced phenotypic changes were not due to interaction and signalling between receptors of other cell types.

As CRP affected expression of CD antigens involved in monocyte: endothelial cell adhesion, the consequences for cell adhesion were determined. CRP treatment for 30 minutes dose dependently decreased the adhesion of monocytes to resting and early-activated endothelial cells (5 hours LPS treatment). Indeed there was a reduction in adhesion at 10μg/ml of CRP, which corresponds to the plasma levels noted in many vascular diseases, including angina and CVD (Berk *et al.*, 1990; Liuzzo *et al.*, 1994; Ferreiros *et. al.*, 1999). This decrease in adhesion was not due to CRP effects on endothelial cells, as there was no change in adhesion if CRP was incubated with endothelial cells, prior to monocyte exposure under our experimental conditions of 30 minutes incubation in serum free medium.

The reduced adhesion of CRP treated monocytes compared with resting monocytes may be related to the significant reduction in expression of CD31 after exposure to CRP for 30 minutes, or may reflect in an alteration in the affinity of PECAM-1 for its homophilic ligand. This phenotypic change in adhesion molecule expression shown by CRP in vitro, may be important in reducing monocyte recruitment in early inflammation.

In contrast, CRP increased the adhesion of monocytes to late activated HUVEC (24 hours LPS). Whilst this may be partly due to the increased expression of CD11b, LPS activated monocytes showed an elevation in CD11b in the absence of increased adhesion to late activated HUVEC. Other studies (Pasceri *et al.*, 2000) have shown that CRP is able to directly induce the expression of adhesion molecules (ICAM-1 & VCAM-1) upon

endothelial cells. However this observation was only noted with the presence of serum and these workers noted that, when serum free media was used (as in this study), there was no significant change in expression, suggesting that complement may be involved in this process.

It is interesting to speculate the mechanism adapted by LPS to activate HUVEC monolayers. Previous work has shown that HUVEC express CD14 (Jersmann *et al.,* 2001), however this has been not been confirmed within these studies as analysed by flow cytometry. Therefore the activation response seen by LPS may be through the interaction with LPS-binding protein (LBP), similar to that reported of LPS-LBP binding via membrane bound CD14 contributing to monocyte activation. Previous workers (Bazil *et al.,* 1986) have reported a soluble form of CD14 (sCD14) lacking the glycerophosphatidylinositol anchor is also present in serum. This LPS-sCD14 complex represents a possible mechanism by which LPS is able to activate endothelial cells.

A specific receptor for LPS-sCD14 complexes has been reported (as reviewed in; Pugin *et al.,* 1993). It has also been suggested that endothelial cells express very low levels of mCD14 (membrane CD14) and that the mCD14 acts as the cellular receptor for sCD14-LPS complexes. If this is correct the mCD14 on endothelial cells and monocytes have different properties with regard to LBP-LPS complexes because LBP opsonizes LPS-coated particles for monocytes but not for HUVEC (Wright *et al.,* 1989). This evidence therefore suggests that the endothelial receptor(s) for CD14-LPS complexes are distinct from receptor(s) for LPS-LBP complexes.

LPS activated HUVEC showed an increase in the expression of ICAM-1 and CD31 over 5 hours. On the contrary, LPS activation over 24 hours again increased ICAM-1 but decreased CD31 expression, therefore showing LPS activation altered HUVEC phenotype, providing a suitable model for endothelial dysfunction, where endothelial dysfunction is characterised by an increased expression of ICAM-1 and CD31 (Imaizumi *et al.*, 2000, Smailbegovic *et al.*, 2001).

Analysis of HUVEC antigens expressed after LPS incubation over 24hours, supported the hypothesis that CRP was able to modulate adhesion by reducing adhesion to 'early activated' endothelium through interaction of CD31 with its homophillic ligand CD31. CRP then further increases adhesion to 'late activated' endothelium through interactions of CD11b and its corresponding ligand ICAM-1 which is increasingly expressed on the endothelium following activation. Examining the role of ICAM-1 in the process, using blocking antibody treatment of the endothelium, showed little reduction of adhesion in the presence of anti-ICAM-1. This may be due to the fact ICAM-1 is not the sole receptor for CD11b and indicating the possible involvement of other adhesion molecules, such as VCAM-1 and its reciprocal ligand VLA-4. The altered adhesion of monocytes to endothelial cells after treatment with CRP is relatively modest. However, this may still have a physiological role in vivo by regulating adhesion over the entire inflammatory process and have a greater effect during chronic inflammatory conditions, where the increased adhesion of monocytes to late activated antigens on the endothelium may promote recruitment. CRP altered adhesion directly rather than altering migration, as only a small percentage of cells migrated through the endothelial monolayer after 30 minutes, with no significant decrease in migration with CRP treated monocytes.

Herein, the experiments used a static adhesion assay to investigate functional effects of CRP on monocyte adhesion, which does not take into account the effects of shear forces on adhesion to the vascular endothelium in vivo. However, the effects of CRP are related to CD11b and CD31, such that a static model is relevant as these are important molecules in the latter stages of the adhesion cascade (Springer, 1994; Springer, 1990; Masuda, 1998; Muller, 1993). It is now important to evaluate whether CRP has any effect on adhesion/migration in a flow model and furthermore, in the tethering stages of monocyte recruitment. The involvement of the rest of the monocytic adhesion molecules, such as L-selectin and the remainder of the integrins on CRP treated monocyte adhesion to activated endothelial cells, also merits study.

Further analysis of the effects of CRP on individual subjects within the cohort revealed a variation in response baseline expression of antigens and variation between subjects in respect of monocyte activation and adhesion. This may reflect different CD32 genotypes. A single nucleotide polymorphism in Fc gamma RIIa that encodes histidine or arginine at position 131 strongly influences both Ig2a and CRP binding (Stein *et al.*, 2000) and may identify individuals with functionally discrete responses to CRP. Allele specific response to CRP therefore may be important in monocyte activation during vascular disease (chapter 5).

In summary these studies provide evidence for the role of C-reactive protein in both monocyte activation and adhesion. The capacity for CRP to activate human monocytes in vitro may have a corresponding role, not only in acute inflammatory states correlated with a dramatic increase in CRP levels, but also in chronic states of lower levels of CRP elevation ($10\mu g/ml$) such as atherosclerosis and CVD. Whilst CRP did not promote

adhesion to early activated endothelium, monocyte binding to late activated endothelial cells may be important in chronic inflammation, and may play a role in the complicated events that ultimately lead to extravasation into tissue, at sites of inflammation.

5.0 Intracellular Signalling

5.1 Preface:

This chapter assesses and characterises the binding of CRP to monocytes and investigates functional consequences of CRP receptor (CRP-R) engagement on signalling events within monocytes.

5.2 Introduction:

The biological effects of CRP are considered to be receptor mediated. Initial reports described the interaction of CRP with 2 distinct receptors on the monocytic cell line, U937 (Tebo & Mortensen, 1990; Crowell *et al.*, 1991). Later work determined that the high and low affinity receptors on human leukocytes are Fc gamma RIIa (CD32) and Fc gamma RI (CD16), respectively (Stein *et al.*, 2000; Bhardwaj *et al.*, 1999). However, there has been extensive discussion as whether this theory is correct, as previous work has shown that white blood cells lack any CRP-R (Hundt *et al.*, 2001; Sander *et al.*, 2001).

A previous section in this thesis has observed that the response to CRP of monocytic adhesion molecule expression from different donors is variable (Chapter 3), which suggests heterogeneity in the level, activity or availability of receptors among donors. However, there was no difference in receptor expression level seen within the experimental conditions. Further analysis revealed there is a polymorphism that exists within the FcγRIIa gene that produces a single amino acid difference – arginine (R) or histidine (H) – at position 131 (Clark *et al.*, 1989). These FcγRIIa alleles show variability in binding of human IgG2 (Warmerdam *et al.*, 1991) and murine IgG1 (Anderson *et al.*, 1987). The H-131 allele is also the only human FcγR to bind human IgG2 efficiently. Previous work (Stein *et al*, 2000) has shown that CRP binding to monocytes and MNC was clearly evident in cells from donors with the R-131 allele of FcγRIIa with only minimal binding to cells from donors homozygous for the H-131 allele.

Human FcγRs are members of the immunoglobulin superfamily, which signal via immunoreceptor tyrosine-based activation motifs (ITAMs; as reviewed in; Reth, 1989). These motifs are located either in the cytosolic domains of the receptors themselves (FcγRIIa), or within associated γ (FcγRI and FcγRIIIA) or ξ (FcγRIIIA) subunits (Cox & Greenberg, 2001). The non-receptor type protein-tyrosine kinases (PTKs) are associated with cell surface receptors and serve to amplify receptor-activated signals intracellularly. More than 30 mammalian non-receptor type PTKs have been identified and divided into 11 distinct subfamilies based upon functional domains and sequence motifs. Among them, Syk (p72^{Syk}) has been well characterised (as reviewed in; Sada *et al.*, 2001), where Syk is critical for FcγR signalling, as Syk$^{-/-}$ macrophages are defective in phagocytosis induced by FcγR but show normal phagocytosis in response to complement (Crowley *et al.*, 1997). Furthermore, Syk$^{-/-}$ neutrophils are incapable of generating reactive oxygen intermediates, via the NADPH oxidase system, in response to FcγR engagement (Kiefer *et al.*, 1998). Additionally, FcγR-mediated activation of Syk is necessary for FcR transport from endosomes to lysosomes and MHC class II restricted antigen presentation (Bonnerot *et al.*, 1998).

Piceatannol (3, 4, 3', 5'-tetrahydroxy-trans-stilbene) has been widely used to study Syk functions in vitro and results obtained with this inhibitor have been used to predict the role of Syk in various cell-signalling events. Previous work has proven that piceatannol inhibits FcεRI-mediated IP$_3$ production, serotonin secretion, membrane ruffling and cell spreading by inhibiting Syk but not Lyn (Oliver *et al.*, 1994) indicating that piceatannol is an effective Syk antagonist.

Previous work has indicated that a leukocyte respiratory burst was greatly diminished by inhibiting tyrosine kinase phosphorylation, indicating that tyrosine phosphorylation is an early and important signal for this activity. Also, an increase in Ca^{2+} mobilisation leads to a change in intracellular peroxide concentration (Lofgren *et al.*, 1999). Intracellular peroxides are believed to modulate redox state, cell signalling and ultimately transcription factor activation (as discussed in section 1.5). Therefore the effect of CRP on monocytic peroxide changes should be investigated. One way in which intracellular peroxide levels can be effectively assessed is through the use of the dye, 2', 7'- dichlorofluoroescein diacetate (DCFH-DA). DCFH-DA is a stable non-polar, non-florescent compound, which freely diffuses across selectively permeable membranes. Intracellular DCFH-DA is activated by intracellular esterases to hydrolyse the acetate groups forming the non-fluorescent 2',7'-dichlorofluorescein (DCFH), effectively trapping the compound within the cell. In the presence of cytosolic peroxide ($[peroxide]_{cyt}$), DCFH acts as a substrate that is rapidly oxidised to the highly fluorescent 2', 7'- dichlorofluorescein (DCF). DCF is excited at 488nm and emits fluorescence within the range 505-545nm and hence can be measured by flow cytometry (Figure 5.1; Bass *et al.*, 1983).

Flow cytometric analysis has advantages over standard spectrofluorometric techniques for the evaluation of intracellular peroxide levels, allowing quantitative examination of large numbers of individual cells rather than measuring mean response of a total population (Bass *et al.*, 1983).

[DCFH-DA]$_e$

H_3CCO ... $OCCH_3$

Cl ... Cl

H

COOH [DCFH-DA]$_i$

Non-fluorescent

Alkaline hydrolysis

HO ... OH

Cl ... Cl

H

COOH

[DCFH]$_i$

Non-fluorescent

Oxidation

[peroxide]$_{cyt}$

HO ... O

Cl ... Cl

COOH

[DCF]$_i$
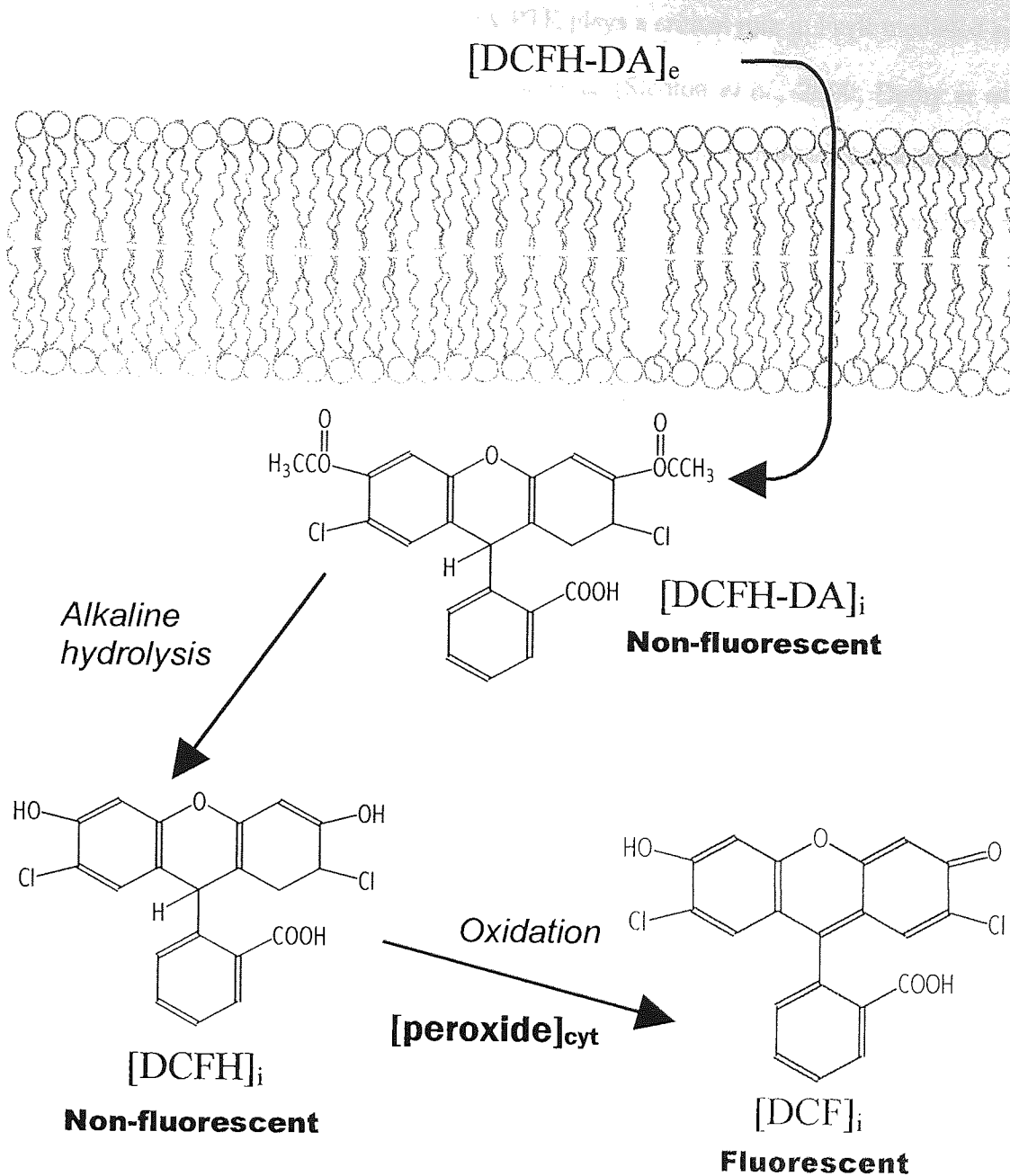
Fluorescent

Figure 5.1. Mechanism of action of the peroxide sensitive probe 2',7'-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA enters the intracellular compartment by diffusion where it is immediately hydrolysed by intracellular esterases to form the non-fluorescent pre-cursor dichlorofluoroescein (DCFH). DCFH is a polar molecule therefore processes poor membrane permeability and is essentially trapped with the cytosol. In the presence of cytosolic peroxides ([peroxide]$_{cyt}$), DCFH is oxidised to fluorescent isomer, DCF. When excited at 488nm, DCF emits green light detectable by flow cytometry within the bandwidth 505-545nm (FL1).

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Previous work has indicated that Syk PTK plays a critical role in FcγR mediated signalling and function in monocytes and macrophages (Stenton *et al.*, 2000; Darby *et al.*, 1994; Scholl *et al.*, 1992). Therefore to further evaluate the functional consequences of CRP interactions with Fcγ receptors on monocytes, this section examines the signalling response to CRP by investigating Syk phosphorylation and peroxide production using DCFH-DA in the human monocytic leukaemia cell line, THP-1, which possess abundant FcγII receptors (Auwerx, 1991). This chapter also investigates the expression and internalisation of the monocytic CRP receptor and examines the hypothesis that the variability seen within CD11b expression on primary monocytes within donors may correspond to the single nucleotide polymorphism in FcγRIIa.

5.3 Materials and Methods:

5.3.1 Materials:

Materials are described in section 2.3 or 3.3, unless specifically stated here. Fluorescein isothiocyanate (FITC), Kodak autoradiography film, Igepal lysis buffer, DCFH-DA, Brefeldin A, Na_3VO_4, Igepal, Triton-X-100, Tween20, L-ascorbic acid, α-tocopherol and the protease inhibitors (aprotinin and leupeptin) were purchased from Sigma. Piceatannol and indo-1-AM were purchased from Calbiochem. The polyclonal rabbit anti-human antibodies used to immunoprecipitate Syk were 4D10 and the HRP linked anti-human mouse Tyr-P Ab PY20 and were purchased from Santa Cruz biotechnology (California, USA). Mouse monoclonal anti-human CD14, FcγRI (CD16) and FcγRII (CD32) conjugated to RPE-Cy5, RPE and FITC respectively were from Serotec (Oxford, UK). PAGE gels, SDS sample buffer, PVDF membranes, ECL reagents and rainbow markers were obtained from BioRad (Hertfordshire, UK). Recombinant protein G-Sepharose beads and Sephadex PD-10 columns were obtained from Pharmacia Biotech (Uppsala, Sweden).

5.3.2 Cell Culture:

The monocytic cell line THP-1 was maintained in culture according to section 2.3.1.

5.3.3 CRP receptor (CRP-R) binding analysis:

CRP binding to FcγRII (CD32) was assessed by flow cytometry with competing concentrations against isolated human IgG. Human immunoglobulins (Ig) were isolated from human serum by ammonium precipitation as described previously (Johnston & Thorpe, 1987). Ig were suspended in carbonate buffer (0.25M Na_2CO_3 pH9, 0.1M NaCl) in Sephadex PD10 columns (Roche) and adjusted to a protein concentration of 1mg/ml.

Fluorescein isothiocyanate (Sigma) was mixed with protein fractions (0.05mg/mg) overnight at 4°C. The mixture was applied to Sephadex columns to remove bound from free FITC and eluted with PBS. THP-1 cells (2×10^6) were washed and resuspended in 100μl serum free media and incubated with 10μl anti-CD16 MAb for 30mins before the addition of 10μl of FITC conjugated Ig with or without increasing concentrations of CRP (x1, x10 and x50) for 30mins at 37°C and analysed for FL1 fluorescence by flow cytometry.

5.3.4 CD32 expression kinetics:

THP-1 cells were resuspended at 0.25×10^6 in 250μl serum free RPMI-1640 and pre-warmed to 37°C incubation with and without CRP (100μg/ml) for 0-120secs at 37°C. Receptor internalisation is temperature dependent, and is inhibited by incubation at 4°C (Harrison et al., 1994). Transferring the cells onto ice stopped CRP stimulation, and thereafter, cells were kept continuously on ice until analysis by flow cytometry. Cells were analysed for CD32 expression via flow cytometry, according to section 2.3.9.

5.3.5 FcγRIIa polymorphism:

Peripheral whole blood was obtained from healthy volunteers and 100μl taken for determination of CD14+ve CD11b response to CRP according to section 4.3.5. The remaining blood was used to extract genomic DNA using the QIAamp DNA Blood Mini Kit (Qiagen, Crawley, UK) according to manufacturers instructions.

Identification of the R-131 (CGT-Arg) and H-131 (CAT-His) polymorphism were by means of a technique known as Primer-Induced Restriction Analysis (PIRA). The sense strand starts from exon 2, 46 base pairs downstream to the polymorphic site located at codon 131. The forward primer for PCR amplification was modified which contains a single nucleotide substitution (shown in red below).

5'-TCAAGGTCACATTCTTCCAGAATGGAAAATCCCAGAAATTCTCGC-3'

This modification introduces a *Bsh-1* restriction enzyme site (5'-CGCG-3') in to the PCR product only if the next nucleotide is G. This unusually long primer was designed to resolve in a 2% agarose gel after the restriction digestion, where normally a shorter fragment would not be detected.

As discussed in the general introduction there are three types of FcγRII designated a, B and C. In order to distinguish the FcγRIIa from the highly homologous FcγRIIB and IIC, the antisense primer is chosen is situated in the region where there are seven mismatches and one gap when comparing IIa to IIB/C, as shown below by blue and dash respectively:

IIa: 5'-CAACAGCCTGACTACCTATTACCTGGG-3'
IIB/C: 5'-CAACAGCTGGTC-ACCTATCACCTGAG-3'

The divergence of the FcγRIIa primer from the FcγRIIB and IIC sequences results in FcγRIIa specific amplification. The GenBank/EMBL accession numbers for the full intron sequences at this location of each of the three FcγRII genes are L08107 to L08109 inclusive.

PCR reactions were performed in PCR buffer (200mM dNTP's, 10pmol of each primer, 2.5U Taq DNA polymerase), using the primers; sense: 5'-TCAAGGTCACATTCTTCCAGAATGGAAAATCCCAGAAATTCTCGC-3' and antisense: 5'- CAACAGCCTGACTACCTATTACCTGGG-3'. 35 cycles (94°C for 1min, 71°C for 2min and 72°C) of PCR amplification were performed using a Techgene Thermal cycler (Dorset,UK). PCR reaction products were mixed with gel loading solution (10µl; 0.05% w/v bromophenol blue, 40% w/v sucrose, 0.1M EDTA pH8.0) and separated on a 2% agarose gel containing ethidium bromide (10µg/ml) at 90V for 2hrs. PCR products were purified using Qiagen purification kit (Qiagen) according to manufacturers instructions. Purified products were then digested with the restriction enzyme *Bsh-1* (Helena Biosciences, UK), in a 40µl reaction mixture containing 20µl purified product, 10U/µl *Bsh-1* and corresponding reaction buffer (Buffer R+; 10mM Tris-HCl, 10mM MgCl$_2$, 100mM KCl and 0.1mg/ml BSA). The samples were incubated overnight at 60°C per manufacturers recommendations and mixed with gel loading solution prior to electrophoretic separation on 2% agarose gel as above. Products were photographed on a UV transilluminator. The specificity of the amplification of FcγRIIa polymorphism has been confirmed by DNA sequencing using all three genotypes: R/R, R/H and H/H (see appendix). The sequencing was completed by Alta Biosciences (Birmingham, UK).

5.3.6 Cell activation and determination of Syk phosphorylation:

THP-1 cells were washed twice and resuspended in serum free RPMI 1640 at a concentration of 4x10^6 cells/100 µl. Cells were warmed to 37°C for 15 min before the addition of CRP (100 µg/ml) for 0-15min at 37°C. The cell activation reactions were stopped by sonication on ice for 1 minute and centrifugation at 8000xg at 4°C for 10 min to remove nuclei and large debris. The cells were lysed in Igepal lysis buffer consisting of:

1% Igepal, 20 mM Tris at pH 7.4, 150 mM NaCl, 10 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 3 mM Na_3VO_4. Insoluble material from THP-1 cells lysed in Igepal buffer was removed by centrifugation at 16,000 x g for 10 min and the supernatant was immunoadsorbed overnight at 4^OC with 10 μg of specific IgG Syk Ab mixed with 10 μl of recombinant protein G-Sepharose. Following immunoadsorption, unbound proteins were removed with five washes in the Igepal lysis buffer containing 1 mM Na_3VO_4. Protein G-Sepharose beads and proteins bound were resuspended in SDS sample buffer (187.5mM Tris-HCL (pH 6.8), 6% w/v SDS, 30% glycerol, 150mM DTT and 0.03% w/v bromophenol blue) and separated along with rainbow M.W. markers (7.5-199 kDa) by SDS-PAGE (7%). After electrophoresis, proteins were immediately transferred to PVDF membranes at 170mA for 2 h at 4^OC. The membrane was washed with TBS (200mls; 24g NaCl, 12mg Tris-HCl) and saturated with 5% powdered milk in TBS plus 0.1% Tween20, incubated overnight at 4^OC with anti-Tyr-P conjugated to HRP. The membrane was washed 3x and the signal detected by enhanced chemiluminesence. Autoradiograms were developed on film and scanned for relative intensity (PDQuest, BioRad).

5.3.7 Cytosolic Ca^{2+} measurement:

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was assessed by loading THP-1 cells with the noncharged ester form of indo-1. Cells were washed, resuspended in medium at $5x10^6$/ml, incubated with 1 μM indo-1 AM for 30 min at 37^OC washed and resuspended to $2x10^6$/ml in a buffer of 125 mM, 5 mM KCl, 1 mM Na_2HPO_4, 0.1% glucose, 0.1% BSA, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$ and 25 mM HEPES (pH 7.4). Aliquots (500 μl) of cells were added to 24 well plates with shaking at 37^OC in a recording spectrofluorometer (Molecular Probes, UK). The excitation and emission wavelength was 331nm and 480nm

respectively, with a cut off filter at 455nm. A baseline level of $[Ca^{2+}]_i$ was recorded for 3 min before the agonist (CRP 10-100 µg/ml) and or antagonist (piceatannol; 25µg/ml) was added and the emission monitored for 20 min. The maximum emission was determined by permeabilising the cells with 1% triton-X100; the minimum emission was determined by adding 4 mM EGTA. The change in $[Ca^{2+}]_i$ was expressed as the area under the curve as calculated by SoftMaxPro software (Molecular Probes, UK).

5.3.8 Phosphatidylinositol (PI) turnover:

THP-1 cells (2×10^6/ml) were resuspended in fresh RPMI-1640 (10% FCS) containing ^{3}H-inositol (5µCi/ml; Amersham-Buchler, Braunschweig, Germany). Cells were then washed in serum free RPMI containing LiCl (10mmol/L) and unlabelled inositol (1mmol/L), followed by addition of CRP (10-100µg/ml) or vehicle control for 30mins at 37^OC. The media was then removed and perchloric acid (1ml; 3.3%) was added to extract the free inositol phosphates (mono- $[IP_1]$, bis- $[IP_2]$ and tris- $[IP_3]$). After 20 mins incubation at 4^OC, the perchloric acid extracts were collected, each mixed with 80µl KOH (10mmol/L), followed by centrifugation to remove the precipitate. The supernatants were applied to 1ml anionic Dowex columns (AG 1-X8; Bio-Rad). Samples were initially eluted with 2ml each of water and ammonium formate (0.1mol/L), in order to remove free inositol, glycerophosphoinositol and cyclic inositol monophosphate (Patel & Schrey, 1990). IP_1, IP_2 and IP_3 were then eluted together with ammonium formate (1mol/L). The radioactive content of the fractions containing free inositol phosphates was determined by liquid scintillation counting (Canberra Packard 1900TR; Pangbourne, UK) in 5mls of OptiPhase'Hisafe' scintillation fluid (Wallac Oy, Turku, Finland). Rate of PI turnover by

CRP is expressed as the amount of radioactivity (cpm) in the free inositol phosphates fraction.

5.3.9 Syk signalling in IL-6 production and CD11b expression:

PWB was pre-incubated with piceatannol (0-100nM) for 15mins at $37^{O}C$, then stimulated with CRP (0-100µg/ml) for 30mins and analysed for serum levels of IL-6 by ELISA according to section 3.3.5 or CD14+ve CD11b expression by flow cytometry according to section 4.3.5.

5.3.10 Intracellular peroxide determination:

A procedure adapted from Bass et al., (1983) was used to measure $[peroxide]_{cyt}$ levels by flow cytometry. Optimised conditions for 2', 7', dichlorofluorescein diacetate (DCFH-DA) incubations were previously calculated in the lab. Briefly, to optimise, systematic variations in DCFH-DA concentration (1-50µg/ml), DCFH-DA pre-incubation period (5-240mins), co-incubation of DCFH-DA and H_2O_2 (0-300µM; 0-60mins) and incubation system were evaluated experimentally. Immediately following agent/DCFH-DA incubation, cell treatments were analysed by flow cytometry. Measurement of forward scatter, side scatter and log FL1 fluorescence (green light, band width 505-545nm) were recorded. Cells were gated to exclude cellular debris. 10,000 cells were examined from each sample on a histogram of count versus log FL1.

THP-1 cells were maintained in the standard conditions and centrifuged at 1200 rpm for 5mins and resuspended in serum free RPMI-1640. Applying the optimised conditions for the use of DCFH-DA for the detection of $[peroxide]_{cyt}$ by flow cytometry, the standard assay was as follows. Viable THP-1 cells ($2x10^6$/ml) were incubated at 37^OC for 10mins with 50μM DCFH-DA after which cells were treated with varying concentrations (0-150μg/ml) of CRP and varying incubation periods of up to 120mins at 37^OC. The total incubation period with 50μM DCFH-DA was always 40mins. Cell treatments for longer than 40mins were incubated with 50μM DCFH-DA for the last 40mins prior to analysis. Immediately following agent/DCFH-DA incubation, cell treatments were analysed by flow cytometry. Effects of CRP on THP-1 intracellular peroxides are expressed as percentage change in MdX DCF FL1 fluorescence from controls or MdX DCF FL1 fluorescence. In order to confirm the effects of enhanced DCF fluorescence were due to an increase in cellular peroxides, the effects of the antioxidants ascorbic acid and α-tocopherol on CRP mediated peroxides was investigated. Pre-incubation of THP-1 cells (as above) was carried out with 0-150μM L-ascorbic acid or 0-100μM α-tocopherol for 4 or 8hrs respectively at 37^OC and then subjected to the same incubation conditions as above. The time points selected for antioxidant incubations were previously described as successfully inhibiting mediated increases in ROS production (Koga *et al.*, 2002; Rayment & Griffiths, 2002).

5.3.11 ROS signalling in CD11b expression:

PWB (50μl) were pre-incubated with L-ascorbic acid (0 and 50μM) for 4hrs at 37^OC before the addition of CRP (0 and 75μg/ml) for 30mins at 37^OC. CD14+ve monocytes were then analysed for CD11b expression according to section 4.3.5.

5.3.12 Statistical Analysis:

Data was calculated as the arithmetic mean ± s.d of area under the curve for Ca^{2+} mobilisation, cpm for PI turnover and percentage change from control or MdX DCF FL1 for intracellular peroxide fluorescence. Data was evaluated statistically for multi-comparison using ANOVA, followed by Tukey's post-test. *P* values less than 0.05 were considered significant, where * represents P<0.05, ** represents P<0.01, *** represents P<0.001 and all samples were at least n=3 in triplicate, unless otherwise stated.

5.4 Results:

5.4.1 FcγRIIa binding studies:

In order to demonstrate that CRP binds to CD32 (FcγRIIa) on monocytes, the characteristic that THP-1 only express two different forms of Fcγ receptors, FcγRI (CD16) and CD32 was exploited. CRP dose dependently competed out Ig binding to CD16-blocked THP-1 monocytes over 30 minutes, resulting in a reduction in FL1 (FITC) fluorescence, from a CRP concentration ranging between 1-50x concentration of Ig (Fig 5.2).
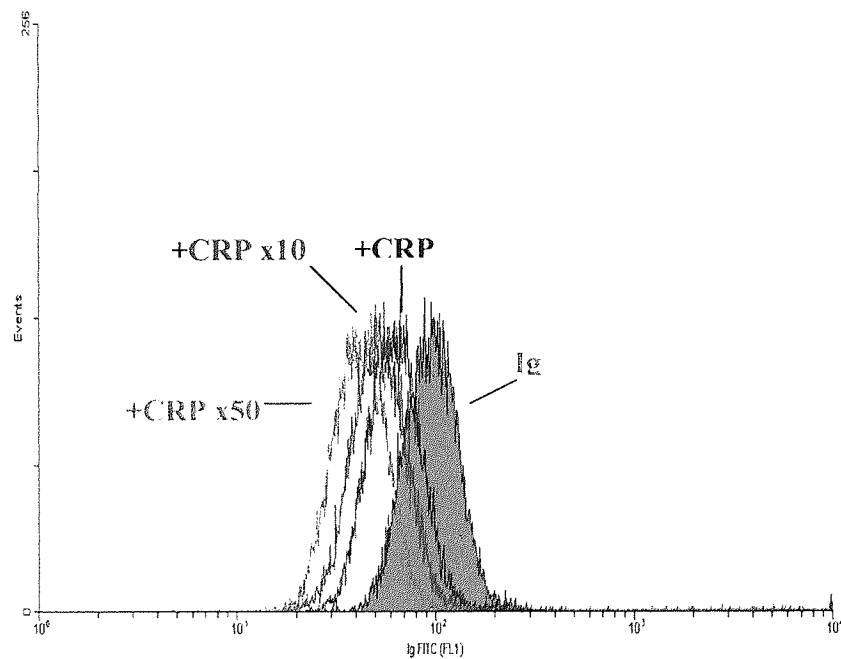
Figure 5.2. CRP binding profiles to THP-1 FcγRIIa receptors. THP-1 (2×10^6) were pre-incubated with anti-CD16 $(10\mu l)$ to block the functional FcγRI and FITC conjugated isolated IgG $(10\mu g/ml)$, with or without increasing concentrations of CRP. Ig bound to CD32 was analysed by flow cytometry, according to section 5.3.3. Ig peak fluorescence histograms are shown with increasing concentrations of CRP, from equivalence at $10\mu g/ml$, to 50x at $500\mu g/ml$. A representative of five experiments is shown.

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5.4.2 CD32 expression kinetics:

Previous studies showed CD32 cross-linking induces internalisation of ligand-receptor complexes in mouse B lymphoma cells lines (Van Den Herik-Oudijk *et al.*, 1994), human neutrophils (Barabe *et al.*, 2002), and THP-1 cells (Ghazizadezh and Fleit, 1994). Tebo and Mortensen (1991) also demonstrated internalisation of CRP-receptor complexes in U937 cells. Therefore, the effect of CRP on CD32 expression over time was examined in THP-1 cells. As it appeared there was no significant change in CD32 expression after 30min incubations with CRP (see chapter 4; Fig 4.3), shorter time points were examined for the loss of the FcγIIa receptor. Cells were stimulated with 100μg/ml CRP for 30, 60 and 120secs at 37°C. Transferring the cells onto ice stopped the stimulation. Cells not exposed to CRP were used as a control for constitutive CD32 surface expression.

Figure 5.3 shows that, at all time points examined, CRP-treated cells showed reduced CD32 surface expression compared to untreated controls with the greatest decrease seen at 30 seconds (Mn±SD CD32 MdX expression of 3.79±0.3). However, by 120secs, there was no significant difference from resting CD32 expression.

CRP (100μg/ml) treated THP-1
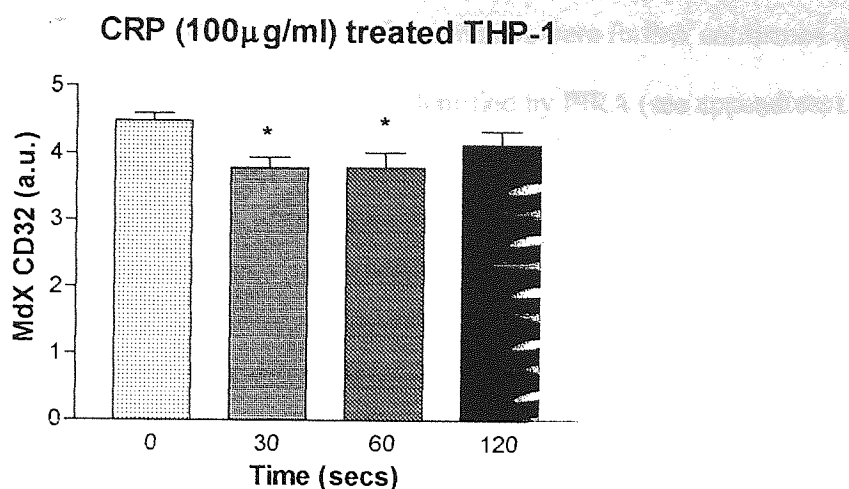
Figure 5.3. The effects of CRP on monocyte CD32 expression. THP-1 $(2 \times 10^6/\text{ml})$ monocytes in serum free media were exposed to 100μg/ml CRP for 0-120secs at 37°C, at each time point, cells were placed on ice according to section 5.3.4 and analysed for CD32 expression via flow cytometry, according to section 2.3.9. Where * represents P<0.05, using ANOVA with Tukey's post-test analysis (n=4).

5.4.3 The effects of the FcγRIIa polymorphism on CRP induced CD11b expression:

Determination of the FcγRIIa-H/R[131] genotype was undertaken using genomic DNA from individuals who had been examined previously for response to CRP (100μg/ml for 30mins) as monocytic CD11b expression. The PCR PIRA (polymorphism induced restriction analysis) strategy is summarised in figure 5.4. In the PCR amplification, a mutagenic primer introduces a new *Bsh-1* site (5'-CGCG-3') on the 5'-end, which terminates immediately adjacent to the polymorphic A/G nucleotide in codon 131. The wild-type sequences near codon 131 are H[131]: CC(CAT)TT; and R[131]: CG(CGT)TT. Thus a *Bsh-1* site is produced only when the codon is CGT, or arginine (R).

After *Bsh-1* digestion, the H/H[131] genotype produces a 383bp fragment, the R/R[131] genotype produces a 373bp and 46bp fragments and H/R[131] genotype produces 383bp, 373bp and 46bp fragments. The results of representative genotyping experiments (Fig 5.5) reveal ready discrimination of the three genotypes: R/R, H/R and H/H (46bp band of H/R

genotype was too faint for photographic analysis). These results were further confirmed by

sequence analysis, confirming all three genotypes, as identified by PIRA (see appendices).

A)

Genomic DNA
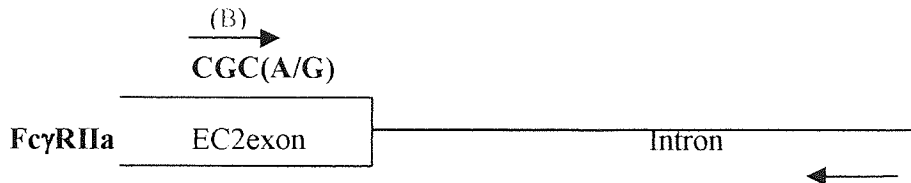
PCR Product
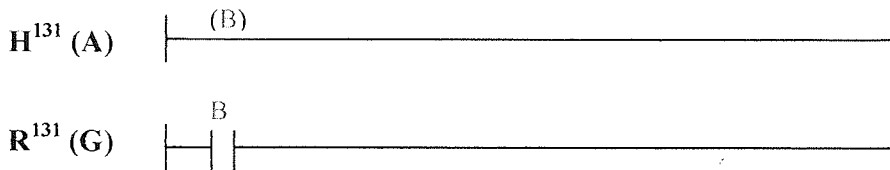
B)
Restriction digestion patterns

	H/H^{131} (A/A)	H/R^{131} (A/G)	R/R^{131} (G/G)
383bp	▬	▬	
373bp		▬	▬
46bp		▬	▬

Figure 5.4. **Schematic diagram of the allele-specific restriction enzyme digestion method for determination of the FcγRIIa-H/R^{131} genotype. A)** the FcγRIIa gene and the PCR product are shown using allele-specific primers (arrows). B indicates the location of *Bsh-1* sites. The expected results of *Bsh-1* digestion of PCR products for H^{131} (nucleotide A, no digestion at the 5' site) and R^{131} (nucleotide G, digestion) are shown. **B)** the expected agarose gel electrophoresis patterns are shown for uncut and *Bsh-1*-digested PCR products for each genotype.

	1	2	3	4	5	6	7

Ladder

← 383bp
← 373bp

← 46bp

RE:	Cut	Uncut	Cut	Uncut	Cut	Uncut
Genotype:	H/H		H/R		R/R	
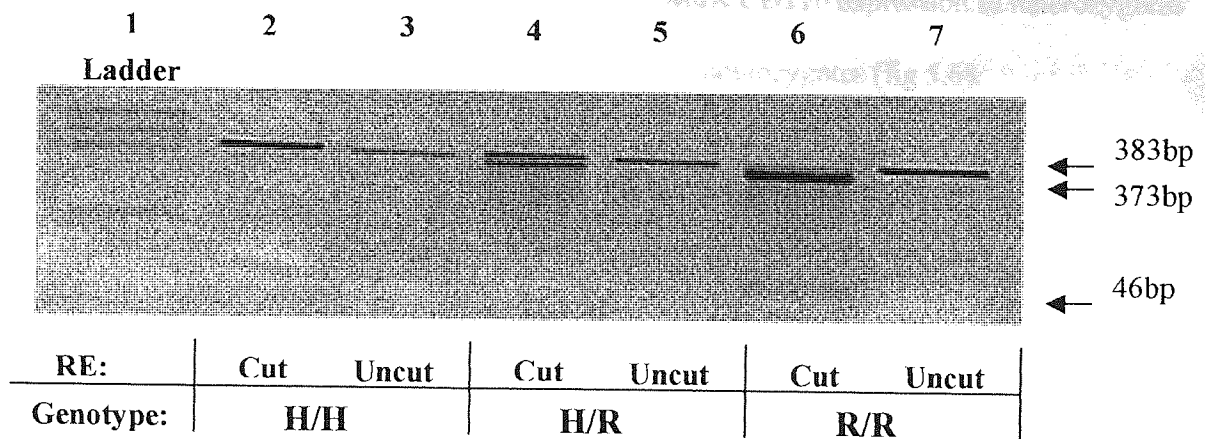
Figure 5.5. Allele-specific restriction enzyme digestion reveals each FcγRIIa genotype. The agarose gel electrophoresis picture shows PIRA analysis of 3 individuals, as investigated according to section 5.3.5. Lane 1, DNA ladder; lanes 3, 5 and 7 uncut PCR products; and lanes 2, 4 and 6: *Bsh-1* digested DNA from the 3 individuals. The R/R pattern is seen in lane 6, H/R in lane 4 and H/H in lane 2.

As discussed in chapter 4, the single nucleotide polymorphism in FcγRIIa that encodes histidine or arginine at position 131, has been reported to strongly influence both Ig2a and CRP binding where arginine homozygotes (R/R) have a significantly greater binding affinity to CRP than histidine homozygotes (H/H), with reported dissociation constants (K_d) of 48.97, 107.2 and 14360 for R/R, H/R and H/H genotypes respectively (Stein *et al.*, 2000). Therefore, the hypothesis that the Fc receptor phenotype may influence responsiveness to CRP on monocytes as CD11b expression was investigated. The genotypes of 17 volunteers were assessed and related to CD14+ve CD11b expression (analysed via flow cytometry as in chapter 4). Of the 17 individuals tested, 4 of the cohort were H/H and 4 R/R homozygotes, with the remaining majority of 9 being heterozygotes (fig 5.6). As shown in figure 5.6 the mean change±SD in MdX CD11b expression to CRP in H/H homozygotes (0.16±0.18) was significantly different (P=0.002) to heterozygotes with a mean change±SD in MdX CD11b of 2.59±1.62. H/H homozygotes again were significantly different (P=0.02) from R/R homozygotes with a mean change±SD in MdX

CD11b of 5.18±2.52. The mean change±SD in MdX CD11b expression in heterozygotes was also significantly (P=0.046) different from R/R homozygotes (fig 5.6).

| % Population (n=17) | 23.5 | 53 | 23.5 |
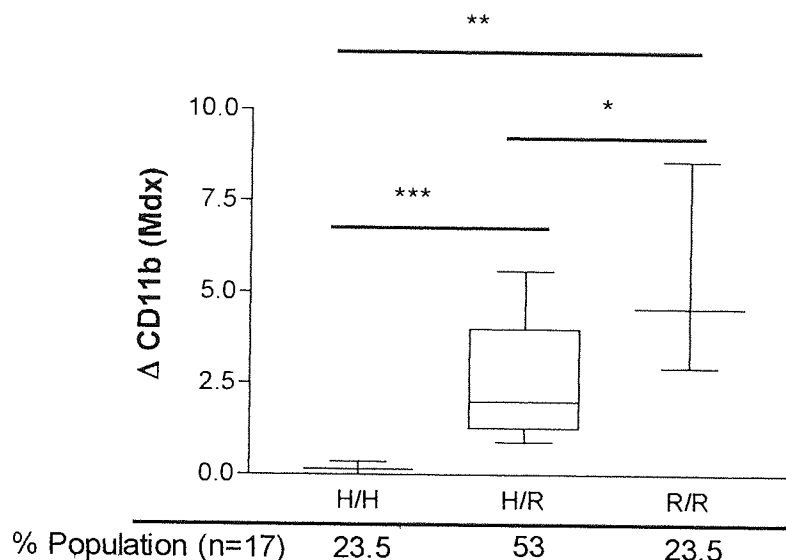
Figure 5.6. Effect of FcγRIIa polymorphism on CRP induced CD11b expression on monocytes. Genotype data was collected from volunteers (n=17) according to section 5.3.5. PWB (50µl) from each individual was incubated with or without CRP (100µg/ml) for 30mins at $37^{\circ}C$ and analysed for CD14+ve CD11b expression via flow cytometry according to section 2.3.9. Data is shown as change (Δ) in MdX CD11b from controls. Results are expressed as box and whisker plots where boxes represent 95% confidence limits, centre lines are median values and bars are ranges CRP induced increase in CD11b expression. Using Mann-Whitney t-test to compare between populations. *** represents P=0.002, ** represents P=0.02 and * represents P=0.046.

5.4.4 Effects of CRP on Syk phosphorylation:

Previous studies of human FcγRIIa have indicated that the SH2 domains of Syk directly bind the phosphorylated tyrosine within the ITAM (Chacko *et al.*, 1994; Chacko *et al.*, 1996). Other workers have also observed that in vitro treatment of human peripheral blood monocytes with Syk antisense oligonucleotides inhibited Syk mRNA and protein expression (Stenton *et al.*, 2000). This inhibition correlated with the suppression of Fcγ mediated phagocytosis and indicated that Syk PTK plays a critical role in Fcγ mediated

cell signalling in monocytes. Therefore, the effect of binding of CRP to THP-1 monocytes

effect on phosphorylation of the 72 kDa Syk kinase was investigated. The initial detection

of Tyr-P on Syk proteins was 30 seconds after stimulation with CRP (100µg/ml), with a

maximum signal observed following 1 minute activation by CRP, which decayed by 6-

10mins (Fig 5.7A). Similar amounts of Syk protein were compared (Fig 5.7B).

Time (mins)	Van	.5	1	2	4	6	10	15	0	-
CRP (100µg/ml)	-	+	+	+	+	+	+	+	-	-

anti-TyrP   A
72 kDa

anti-Syk   B
72 kDa
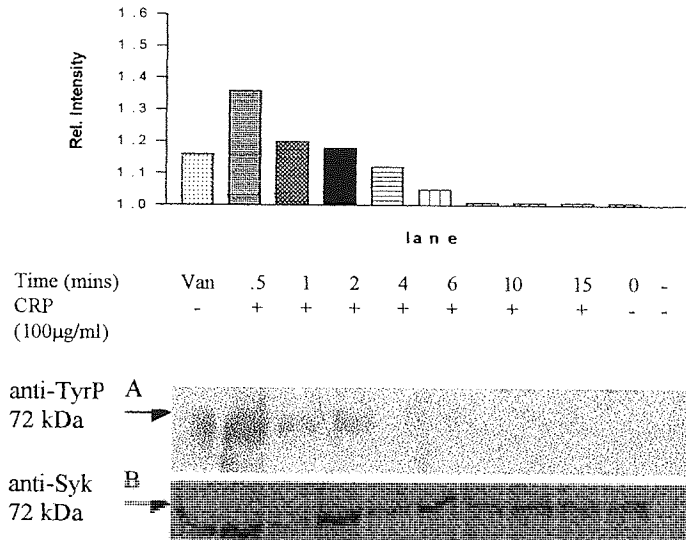
Figure 5.7. Kinetics of Tyr-P of Syk in response to CRP. The time course for Tyr-P of
Syk (72 kDa) in THP-1 cells in response to CRP (100µg/ml), was assessed, according to
section 5.3.6. Na$_3$VO$_4$ (Van) was used a positive control. **A.** Immunoblot probed with anti-
Tyr-P and the relative intensity of the 72-kDa Syk band determined and shown in the bar
chart below. **B.** The same membrane was reprobed with anti-Syk. One of five similar
experiments is shown, with the relative intensity (Rel Intensity) calculated from bands in
anti-Tyr-P immunoblot (A; SoftMAxPro).

5.4.5 Intracellular Ca^{2+} and IP$_3$ mobilisation in response to CRP:

The rapid mobilisation of intracellular stores of Ca^{2+} in response to inositol triphosphate is

characteristic of signalling by FcγRs in human neutrophils (Jaconi *et al.*, 1990; Vosselbeld

et al., 1995). Therefore the ability of CRP to elevate [Ca^{2+}]$_i$ in indo-1 loaded THP-1 was

determined. CRP induced a rapid, dose dependent increase in [Ca^{2+}]$_i$ at 10-100µg/ml (Fig

5.8), which peaked after 5mins and returned back to baseline levels before 20mins

stimulation (Fig 5.9). In order to determine that CRP binding to THP-1 was indeed causing the Tyr-P of Syk and consequently the release of $[Ca^{2+}]_i$, the Syk specific inhibitor piceatannol was used. Piceatannol (3,4,3',5'-tetrahydroxy-*trans*-stilbene) has been shown to inhibit FcεRI mediated IP_3 production, serotonin secretion, membrane ruffling and cell spreading by inhibiting Syk but not Lyn (Oliver *et al.*, 1994). The Syk specific inhibitor piceatannol (25μg/ml), completely abrogated the CRP-induced release of $[Ca^{2+}]_i$, where no increase in $[Ca^{2+}]_i$ was observed with incubations of piceatannol alone (Fig 5.8).
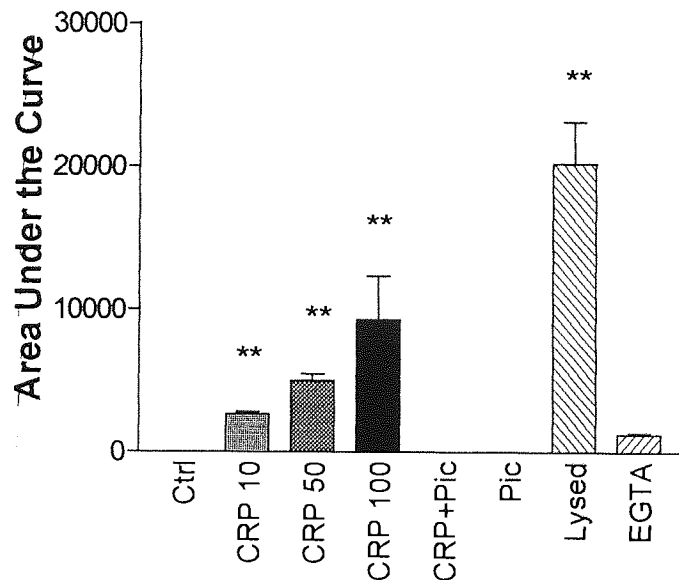
Figure 5.8. Effect of CRP on changes in $[Ca^{2+}]_i$ in THP-1 cells. Indo-1 loaded THP-1 $(2 \times 10^6/ml)$ were stimulated with CRP (10-100μg/ml) with or without piceatannol (Pic; 25μg/ml). Fluorescence was detected (Ex331nm, Em480nm) for 20mins and the area under the curve noted. THP-1 cells incubated with Triton-X-100 (1%) and EGTA (4mM) were used to determine maximum and minimum fluorescence respectively, according to section 5.3.7. Values represent the mean increase in $[Ca^{2+}]_i$ from four experiments. ** represents (P<0.001) significant difference from controls (n=4), as analysed using ANOVA and Tukey's post test.
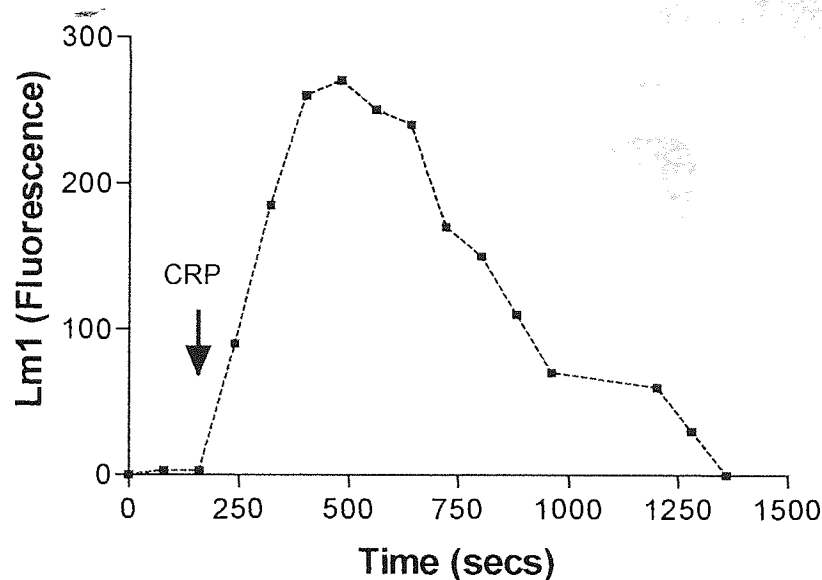
Figure 5.9. Kinetics of CRP on changes in $[Ca^{2+}]_i$ in THP-1 cells. Indo-1 loaded THP-1 (2×10^6/ml) were stimulated with CRP (50µg/ml) Fluorescence was detected (Ex331nm, Em480nm) for 20mins every 80secs after stimulation, according to section 5.3.7. A representation of the kinetics of CRP induced $[Ca^{2+}]_i$ release is shown.

FcγRIIa signalling generates lipid secondary messengers such as phosphatidylinositol 3,4,5-triphosphate (IP_3; PI turnover), which is required for basic monocytic functions such as phagocytosis (Ninomiya *et al.*, 1994), and, as previously stated, the rapid release of intracellular Ca^{2+} in response to IP_3 is characteristic of signalling by FcγRs. Therefore the effect of CRP on PI turnover was measured using tritiated inositol. CRP dose dependently (10-100µg/ml) and significantly ($P<0.001$) increased inositol phosphates production in THP-1 cells stimulated for 30mins at 37^oC (fig 5.10).

1500 —

1000 —

cpm

500 —

0 —

** **

** **

**

0   10   50   100

CRP (μg/ml)
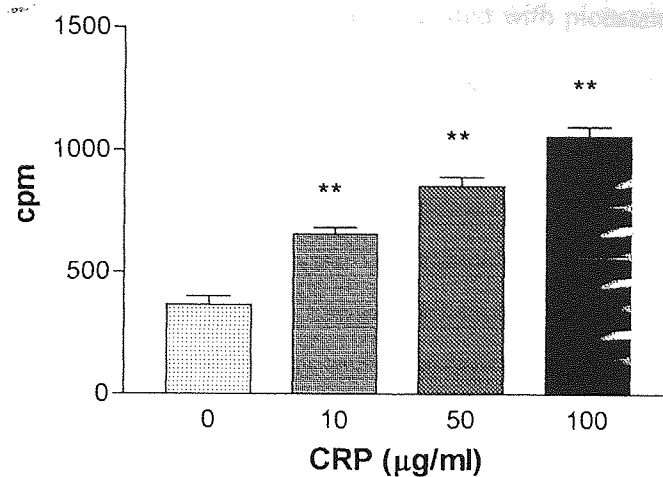
Figure 5.10. Effects of CRP on phosphotidyl inositol turnover. THP-1 $(2x10^6/ml)$ were loaded overnight with ^3H-inositol according to section 5.3.8 and stimulated with (10-100μg/ml) or without CRP for 30mins at $37^\circ C$. Free inositol phosphates were removed according to section 5.3.8 and radioactivity (cpm) determined by liquid scintillation counting. ** represents significant difference (P<0.001) from controls (0; n=4) as analysed using ANOVA and Tukey's post test.

5.4.6 Syk signaling on cytokine and CD11b expression:

In order to establish whether CRP mediated monocytic responses were indeed due to signalling through Syk, the antagonist piceatannol was incubated with MNC, prior to CRP treatment. In the presence of piceatannol, CRP treated cells showed a significant reduction (P<0.05) of IL-6 secretion, from CRP treatment alone (Fig 5.11).

In order to examine whether Syk was responsible for mediating CRP induced monocytic CD11b expression, the Syk specific antagonist piceatannol was used. Piceatannol, at concentrations greater than 1nM, significantly inhibited the CRP mediated increase in CD11b (fig 5.12). This effect was dose dependent, with no further significant reduction in CRP mediated CD11b expression after 3nM of piceatannol (fig 5.12). Complete inhibition by piceatannol was reached by 10nM, as there was no significant difference between resting CD11b expression and piceatannol>10nM (fig 5.12). There was no significant

change (P>0.05) in control treated PWB pre-incubated with piceatannol (1-100nM) alone,

with a mean±SD CD11b MdX expression of 4.4±0.45.
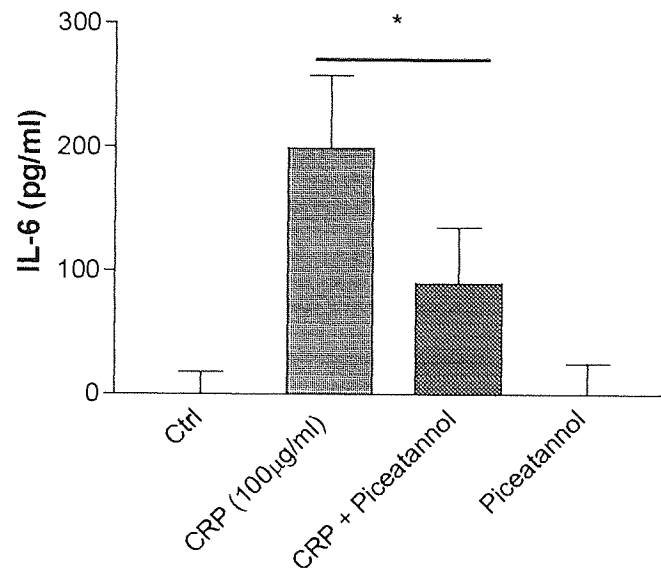
Figure 5.11. IL-6 protein secretion from MNC. MNC (2×10^6/ml) were isolated from PWB by differential centrifugation and incubated with CRP (50µg/ml) for 16hrs at 37°C, with or without prior incubation with piceatannol (25µg/ml) and analysed for IL-6 expression by ELISA as described in section 5.3.9. Values represent the mean n=3 from 3 different normal subjects. * represents P<0.05 from CRP treatment alone.
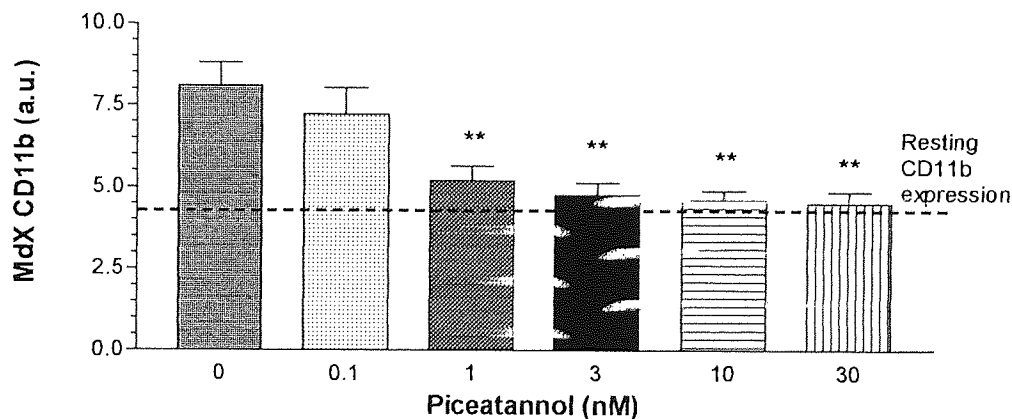
Figure 5.12. Effect of Syk antagonist piceatannol on CRP mediated monocytic CD11b expression. PWB (50µl) was pre-incubated with piceatannol (0-30nM) for 15mins at 37°C before the addition of CRP (100µg/ml) for 30mins. PWB was prepared for flow cytometry according to section 5.3.9 and analysed for CD14+ve CD11b expression according to section 4.3.5. Data is presented as the mean±SD percentage increase from resting (100%) CD11b expression, where ** represents P<0.001 from CRP control treated PWB using ANOVA followed by Tukey's post test (n=3 in triplicate).

5.4.7 Effects of CRP on monocytic peroxide production:

Previous work has indicated that protein tyrosine kinases and Ca^{2+} mobilisation are important factors in leading to changes in peroxide formation concentration, therefore the effect of CRP on THP-1 intracellular peroxide was analysed. Figure 5.13 shows the effect of CRP on peroxides in THP-1 cells. CRP dose dependently and significantly increased peroxide production from 10μg/ml of CRP. This increase in intracellular peroxide peaked by around 25μg/ml of CRP, with an average±SD percentage increase from control of 68.27%±15.89. Despite a trend towards higher basal levels of peroxide production with all concentrations of CRP treatments, the CRP mediated increase in peroxide by THP-1 cells returned back to baseline by 100μg/ml of CRP, with no significant difference in peroxide production from control treated cells.
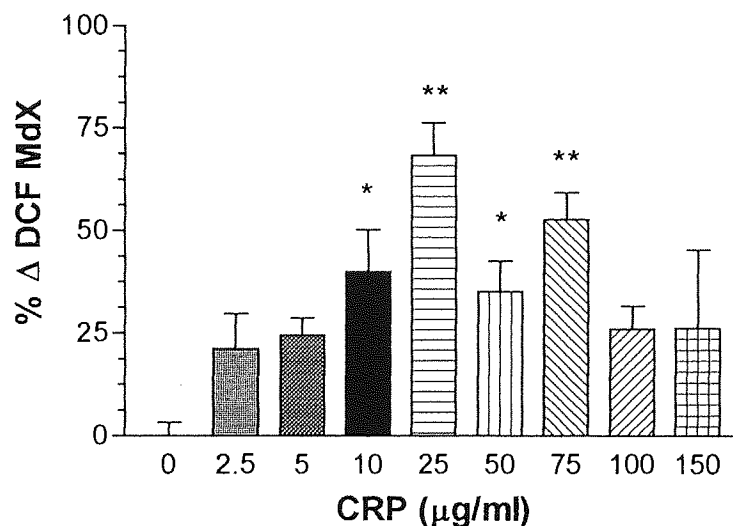
Figure. 5.13. The effects of CRP concentration on monocyte cytosolic peroxide levels. THP-1 cells ($2x10^6$/ml) were dye loaded with the peroxide indicator DCFH-DA and exposed to 0-150μg/ml CRP for 30 min at 37°C and the quantity of intracellular peroxide was analysed by flow cytometry according to section 5.3.10. Data was calculated as the arithmetic mean ± s.d of percentage change (Δ) from vehicle control of MdX DCF FL1 florescence. Data was evaluated statistically for multi-comparison using ANOVA, followed by Tukey's post-test. P values less than 0.05 were considered significant, where * represents P<0.05 and ** represents P<0.01.

In order to examine the kinetics of CRP (25μg/ml) mediated peroxide production, the peroxide production over 960mins was examined. Peroxide formation was significantly

(P<0.001) increased by 10mins of CRP incubation with a mean±SD percentage increase

from control of 54.73%±16.7 (fig 5.14). This change in peroxides over time, increased up

to 40min with incubations of CRP, with a mean±SD percentage increase from control of

60.94%±16.66 (fig 5.14). By 60mins there was no significant (P>0.05) increase in

peroxide production with CRP, where cellular peroxides had returned back to baseline

levels (fig 5.14). There was no significant (P>0.05) difference between each time point

between 10min and 40min stimulation with CRP (fig 5.14).
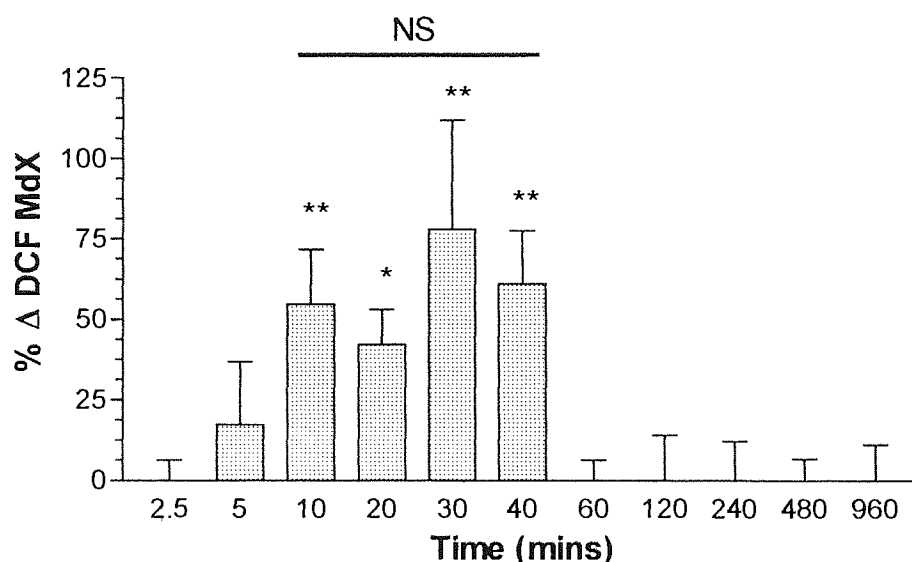
Figure 5.14. The effects of CRP on monocytic intracellular peroxide kinetics. DCFH-DA loaded THP-1 cells (2×10^6/ml) were exposed to 25µg/ml CRP for 0-960 min at 37°C and the quantity of intracellular peroxide was analysed by flow cytometry, according to section 5.3.10. Data was calculated as the arithmetic mean ± s.d of percentage change from vehicle control of MdX DCF FL1 fluorescence. Data was evaluated statistically for multi-comparison using ANOVA, followed by Tukey's post-test. *P* values less than 0.05 were considered significant, where * represents P<0.05 and ** represents P<0.01.

In order to investigate the nature of the increase in DCF fluorescence, pre-incubation with

antioxidants prior to CRP exposure was tested. Figure 5.15 shows the effects of ascorbic

acid (AA) pre-incubation on CRP mediated peroxide production. AA at concentrations

above 50µM, significantly reduced CRP mediated increase in intracellular peroxide

production from a mean±SD MdX DCF fluorescence of 97.15±4.74 in CRP stimulated

THP-1 cells to 83.13±3.27 in AA pre-incubated, CRP-treated THP-1 cells (fig 5.15). This reduction in CRP mediated peroxide flux was not further decreased with incubations of AA up to 150μM (fig 5.15). Pre-incubation of CRP treated cells with AA above 50μM resulted in no significant difference in peroxides from resting THP-1 cells with a mean±SD MdX DCF fluorescence of 79.2±21.2 (fig 5.15), therefore indicating AA incubations completely inhibited CRP mediated increases in peroxide concentration. There was a small but not significant change in intracellular peroxide from controls, in resting THP-1 cells treated with AA alone.

Figure 5.16 shows the effects of α-tocopherol (α-toc) pre-incubation on CRP intracellular peroxide levels. α-toc at concentrations above 25μM, significantly (P<0.001) reduced CRP induced increases in intracellular peroxides from a mean±SD MdX DCF fluorescence of 122±3.39 in CRP stimulated THP-1 cells to 106.47±6.6 in α-toc pre-incubated CRP treated THP-1 cells (fig 5.16). This reduction in CRP induced peroxide levels was dose dependent with incubations of α-toc up to 100μM (fig 5.16). The α-toc pre-incubated CRP treated cells (above 25μM) showed a small non-significant loss in peroxides from resting THP-1 cells with a mean±SD MdX DCF fluorescence of 94.4±10.18 (fig 5.16).
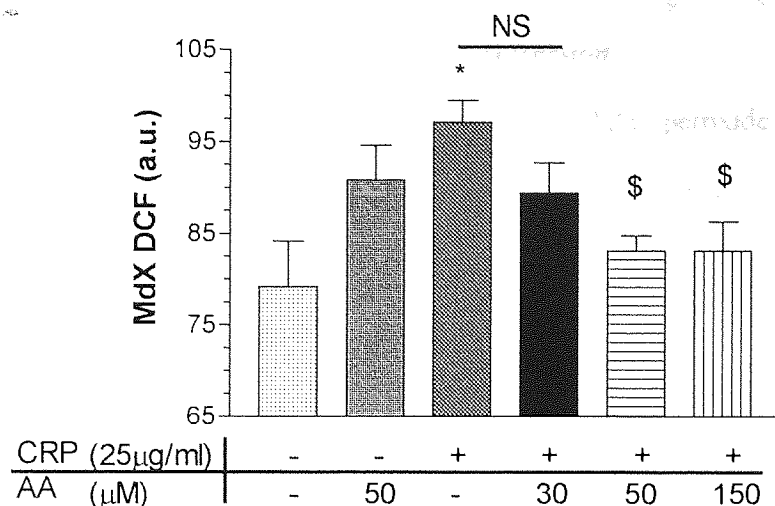
Figure. 5.15. The effects of L-ascorbic acid (AA) exposure on monocyte cytosolic peroxide levels. DCFH-DA loaded THP-1 cells (2×10^6/ml) incubated at $37^\circ C$, 5% CO_2, 95% humidified atmosphere with or without 25µg/ml CRP for 30mins and 4hrs pre-incubation with L-ascorbic acid (0-150µM) and the quantity of intracellular peroxide was analysed by flow cytometry, according to section 5.3.10. Data was calculated as the arithmetic mean (Mn) ± s.d of MdX DCF FL1 fluorescence. Data was evaluated statistically for multi-comparison using ANOVA, followed by Tukey's post-test. P values less than 0.05 were considered significant, where * represents P<0.05 from control and $ represents P<0.05 from CRP stimulated THP-1 cells.
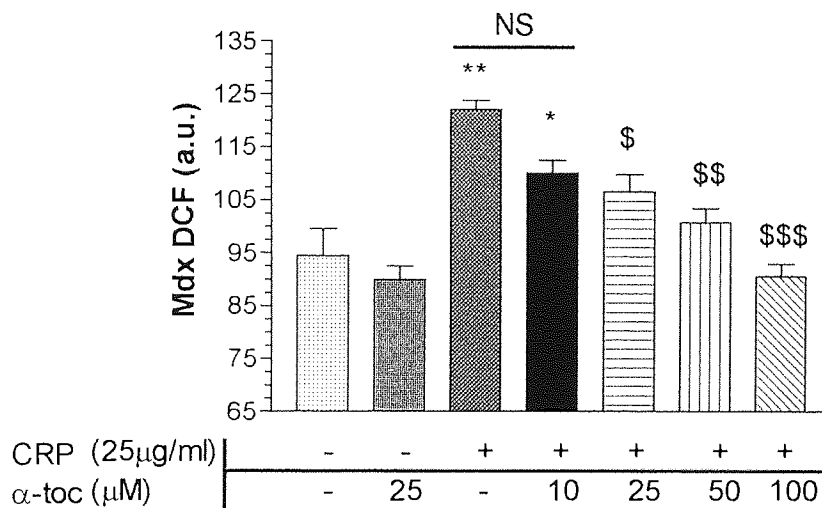
Figure. 5.16. The effects of α-tocopherol (α-toc) exposure on monocyte cytosolic peroxide levels. DCFH-DA loaded THP-1 cells (2×10^6/ml) were incubated at $37^\circ C$, 5% CO_2, 95% humidified atmosphere with or without 25µg/ml CRP for 30mins following 8hrs pre-incubation with α-tocopherol (0-100µM). The quantity of intracellular peroxide was analysed by flow cytometry, according to section 5.3.10. Data was calculated as the arithmetic mean (Mn) ± s.d of MdX DCF FL1 fluorescence. Data was evaluated statistically for multi-comparison using ANOVA, followed by Tukey's post-test. P values less than 0.05 were considered significant, where* represents P<0.05 or ** represents P<0.01 from control and $ represents P<0.05 or $$ represents P<0.01 or $$$ represents P<0.001 from CRP stimulated THP-1.

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5.4.8 Intracellular peroxide signalling and CD11b expression:

As previously shown, CRP is able to increase in intracellular peroxide production in monocytes, therefore, the consequences of intracellular peroxide signalling on monocytic CD11b expression was analysed. Figure 5.17 shows the effects of pre-incubation with ascorbic acid (AA) on CRP mediated monocytic CD11b expression. AA failed to inhibit CD11b expression after CRP incubation, as there was no significance difference in CD11b level between monocytes pre-incubated with AA prior to CRP and monocytic treatment with CRP alone, from a mean±SD MdX CD14+ve CD11b expression of 8.19±0.77 and 8.09±0.869 ($P<0.01$; n=3 in triplicate) respectively.
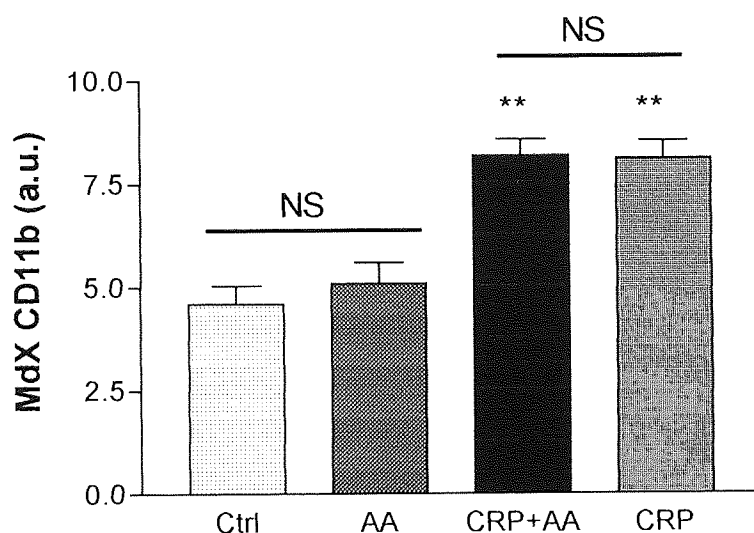
Figure 5.17. The effect of ascorbic acid (AA) on the CRP mediated increase in monocytic CD11b expression. PWB (50μl) was incubated with L-ascorbic acid (0 and 50μM) for 4 hours at 37°C and then treated with or without CRP (75μg/ml) for 30mins at 37°C. PWB was then analysed for CD14+ve CD11b expression via flow cytometry according to section 5.3.11. Data was evaluated statistically using ANOVA, followed by Tukey's post-test. P values less than 0.05 were considered significant, ** represents $P<0.01$ from vehicle control treated PWB.

5.5 Discussion:

Previous workers have demonstrated the ability of CRP to modulate neutrophil activity, showing functional changes in phagocytosis and on chemokine and chemotactic factor induced neutrophil chemotaxis (Zhong et al., 1998). Indeed, this has further supported the concept that the acute phase reactant, CRP, has an important role to play in modulating leukocyte activities at inflammatory sites. Therefore, understanding the signalling mechanism and effects of CRP on monocyte protein expression in the later phases of inflammation are important to improve our knowledge of the possible role for CRP in chronic inflammation.

As previously described, initial reports show the interaction of CRP with 2 distinct receptors on the monocytic cell line, U937 (Tebo & Mortensen, 1990; Crowell et al., 1991). Later work determined that the high and low affinity receptors for CRP on human leukocytes are CD32 (FcγRIIa) and CD16 (FcγRI), respectively (Stein et al., 2000; Bhardwaj et al., 1999). To confirm this effect in THP-1 monocytes, these results describe the ability of CRP to compete with isolated IgG for binding affinity for CD32. This further supports the identity of the CRP-R on monocytes being CD32.

Cross-linking of Fc receptors can be followed by internalisation of the receptor-ligand complexes, which is a common theme in Fc receptor cell signalling. The receptor-ligand complexes are either degraded in endosomal compartments, or the receptor dissociates from the ligand and recycles to the cell membrane. Surface level expression of the receptors may also be maintained by de novo synthesis of receptors and/or release of preformed receptors from intracellular stores. In U937 cells Tebo and Mortensen (1991) demonstrated internalisation and partial degradation of radioiodinated (^{125}I)-CRP after

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CRP-receptor ligation. Cross-linking of CD32 with aggregated Ig or F(ab')$_2$ fragments caused receptor internalisation in mouse B lymphoma cells lines (Van Den Herik-Oudijk *et al.*, 1994), human neutrophils (Barabe *et al.*, 2002), and THP-1 cells (Ghazizadezh & Fleit, 1994). These studies showed that receptor internalisation occurred over a time interval of 30 seconds to 30 mins after receptor ligation. Work in this thesis has shown stable CD32 expression at 30mins therefore only very early time points (30secs to 2mins) were focused on. The data shows that CRP treatment induced a decrease in CD32 surface expression by 30 seconds, which seemed to return to resting expression by levels by 2 minutes. However, the exact CRP-receptor internalisation mechanisms still remain to be elucidated.

As stated previously, there are reports' describing the effects of CRP on neutrophils and monocytes, however, there has been significant controversy surrounding the binding of CRP to leukocytes, where purity of CRP has been questioned and bacterial or immunoglobulin contaminants have been suggested to mediate events observed (Hundt *et al.*, 2001; Sander *et al.*, 2001). Nevertheless, the responses reported here in both primary monocytes and THP-1 cells after incubation with CRP, are CRP specific, as all reactions were undertaken with reCRP which excludes activation with IgG via the CD32 receptor. Furthermore, endotoxin contaminants can also be excluded as the recombinant CRP used in each experiment was determined to be endotoxin free (below 0.03 EU/ml), as analysed by the Limulus assay, which is below the concentration of LPS needed to activate monocytes, as determined by cytokine secretion (Ballou & Lozanski, 1992).

Previous workers have already demonstrated a difference in the extent of Ca^{2+} mobilisation levels according to Fc gamma polymorphisms (Stein *et al.*, 2000), where R/R homozygotes showed increased Ca^{2+} mobilisation after CRP challenge from H/H homozygotes.

However, there have not been any studies carried out on examining the influence of the FcγRIIa polymorphism on monocytic phenotype. The polymorphism at codon 131 on the FcγRIIa affects CRP mediated CD11b expression, where the homozygote H/H genotype, exhibited a complete lack of ability to increase CD11b expression after CRP engagement. In contrast the R/R genotype showed a significantly elevated CD11b response to CRP. This supports previous work showing the decreased efficiency of CRP binding to the H/H genotype and increased binding efficiency of CRP to R/R genotypes on monocytes (Stein et al., 2000).

Previous workers have proposed that the FcγRIIa allotype R131 represents a new predisposing factor for meningococcal septic shock (Bredius et al., 1994), showing a significant increase in the FcγRIIa-R/R131 allotype in children with a history of fulminant meningococcal septic shock. It must be noted that the retrospective study only evaluated children who survived an episode of septic shock. Consequently, the results may have been biased (i.e., children who did not survive may have all had the FcγRIIa-R/R131 or, on the contrary, the FcγRIIa-H/H131 allotype). However, whether CRP plays a role in opsonising or clearance of pathogens in this model still remains to be elucidated. In another interesting investigation Osborne et al., (1994) studied the distribution of the FcγRIIa-131 polymorphism in various ethnic groups, and showed that Chinese sample groups had a significantly higher H/H131 frequency (85%) than the Caucasian group at 21% (in agreement with the results found here). This may be related to the observation that eastern Asian populations have a much lower incidence of developing atherosclerosis than westernised populations (as reviewed in; Dwyer et al., 2003). Indeed, it is a possibility that the Asian population who express the FcγR phenotype where there is a

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lower efficiency of CRP engagement (H/H131), do not get increased susceptibility to CRP mediated monocytic inflammatory pathologies, such as atherosclerosis.

As CRP is able to bind to the functional receptor CD32, the hypothesis was investigated that concentrations of CRP associated with acute phase inflammation can signal downstream through phosphorylation of the non-receptor protein tyrosine kinase, Syk, which is associated with the ITAM motif below the CD32 receptor. The results suggest that the Tyr-P of Syk is an early, rapid event induced by CRP. This data supports the previous results of Chi *et al.*, (2002), describing the Tyr-P of Syk by CRP in HL-60 (G) cells. In further support of Chi *et al.*, (2002), this effect was achieved without the need for aggregation of CRP, where receptor clustering has been postulated to arise from each subunit of the pentraxin binding to FcR. As THP-1 cells also express CD16 (Auwerx, 1991) and that CD16 has been previously reported as a possible receptor for CRP (Marnell *et al.*, 1995), these results may indicate Syk phosphorylation through CD16 as well as CD32. However, overall it does indicate that ultimately CRP is able to engage CRP-R on monocytic cells and induce tyrosine phosphorylation within Syk.

After Syk kinase phosphorylation, the effective signal transduction pathways are incompletely understood in monocytes. However, adaptor molecules such as LAT and BLNK have shown to be important in T and B cell receptor signalling respectively (Finco *et al.*, 1998; Fu *et al.*, 1998). Further Syk kinase interacting proteins (SKIP) were identified by the yeast two-hybrid system in monocytes. The N-terminal half of c-Cbl and Vav were isolated as SKIP and Syk-SKIP interactions were found to be dependent on Syk kinase activity (Dekert *et al.*, 1998). Downstream from these adaptor proteins and enzymes, signal propagation may be achieved through PLC-γ or PI 3-K, Ca^{2+} mobilisation

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and transcription factor activation, which have all been shown to be dependent on Syk kinase activity (Beits *et al.*, 1999; Takata *et al.*, 1994).

Whilst the detection of Tyr-P within the Syk protein is qualitative, the analysis of the downstream mobilisation of intracellular calcium can be achieved semi-quantitatively, and therefore the effects of inhibition of Syk phosphorylation can be more easily examined. Herein it is shown that the CRP-mediated cell activation signal through Syk kinase in monocytes resulted in the release of intracellular calcium stores. This was inhibited by the Syk specific antagonist piceatannol, which has been proven to abolish the signal transduction response from phosphorylated Syk in other studies (Oliver *et al.*, 1994). The intracellular Ca^{2+} mobilisation response to CRP is entirely consistent with a cell activation signal and has been reported by previous workers (Jaconi *et al.*, 1990; Vossebeld *et al.*, 1995). The results describe CRP mediated PI turnover which is again representative of a cellular signal, where the rapid mobilisation of intracellular stores of Ca^{2+} in response to inositol triphosphate is characteristic of signalling by FcγRs in human leukocytes (Jaconi *et al.*, 1990; Vossebeld *et al.*, 1995). These results are in complete agreement with previous studies on CRP signalling events in HL60 granulocytes (Chi *et al.*, 2002).

In order to evaluate the hypothesis that CRP mediated signalling event in monocytes, through the non-receptor tyrosine kinase Syk, were responsible for the phenotypic changes in cytokine expression and adhesion molecule expression, the effects of the Syk specific antagonist piceatannol were examined. Monocyte pre-incubation with piceatannol inhibited both IL-6 secretion and CD11b expression. The concentrations needed for inhibition were the same concentration required for complete inhibition of Ca^{2+}. This inhibition supports the hypothesis that CRP engagement with FcγRs mediates signal

transduction though ITAM receptor associations leading to Syk phosphorylation and downstream effects on protein expression.

There have been conflicting reports on the effect of human CRP on ROS (reactive oxygen species) / peroxide generation. While there have been reports that human CRP decreases the production of ROS by activated neutrophils and monocyte/macrophages (Dobrinich & Spangnuolo, 1991; Buchta et al., 1987), there are also reports of increased O_2^- and H_2O_2 production after prolonged exposure to CRP (Tebo & Mortensen, 1991; Barna et al., 1984). CRP is indeed able to influence the production of intracellular peroxide concentration in monocytes, in the conditions described here. Peroxide levels peaked at CRP concentrations of $25\mu g/ml$, which returned to resting peroxide levels by CRP concentrations of $100\mu g/ml$.

The intracellular peroxide indicator, DCFH-DA as used here, is not directly oxidised by O_2^- (Royal & Ischiropoulos, 1993; Zhu et al., 1993; Carter et al., 1994) but may react with peroxynitrite ($ONOO^-$; Lokesh & Cunningham, 1986; Crow, 1997), as formed by the reaction between O_2^- and NO (see figure 1.6), therefore indicating that CRP may increase either NO or O_2^- production in monocytes, or elicit peroxides from an alternative source such as the mitochondrion.

The kinetics of CRP mediated monocytic peroxide production show that there is significant generation of peroxides within 10 minutes of CRP incubation. Interestingly previous workers have noticed that NADPH oxidase activities are dependent on intracellular calcium (Lofgren et al., 1999). However, the role of calcium in activating NADPH oxidase is complex, Della Bianca et al., (1990, 1993) have shown that the calcium

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requirement depends on how the ligand is presented. Erythrocyte IgG-induced respiratory burst is far more sensitive than yeast IgG for an intracellular calcium signal. This could at least in part be explained by the fact that erythrocyte IgG were less effective in activating respiratory burst than yeast IgG and also associated with less phagocytosis (Della Bianca *et al.*, 1993). Furthermore, the natural phagocytic ligands, IgG and complement fragments, trigger more than one receptor possibly activating redundant pathways, implying that distinct activation of each receptor type may further increase the calcium sensitivity of the respiratory burst. The calcium response to FcγR activation is in agreement with the results found here, as FcγR engagement with CRP induces Syk phosphorylation, which in turn mediates Ca^{2+} mobilisation via IP_3, which then activates peroxide formation through NADPH oxidase. This pattern of cell activation is in agreement with the kinetic data of Ca^{2+} mobilisation (5mins) to peroxide formation (at 10mins). Also, work by Lofgren *et al.*, (1999) and Hundt & Schmidt (1992) have shown FcγRIIa signalling via Syk is an important event enabling NADPH oxidase activation, further supporting the role of CRP in monocytic intracellular peroxide production.

The NADPH oxidase system is not the only source of ROS within monocytes. Mitochondrial ROS production is an important source of intracellular ROS production. The intracellular peroxide indicator, DCFH-DA as used here, does not permit the determination of the source of cytosolic peroxide changes and therefore can not distinguish between either CRP mediated peroxide formation within NADPH oxidase or mitochondria. However as peroxynitrite is able to oxidase DCFH-DA to its fluorescent form (as discussed previously), and NO is a precursor to peroxynitrite formation, CRP may be increasing NO production. Previous work has described how CRP-treated macrophages release NO in a time- and dose dependent manner (Ratnam & Mookerjea, 1998). Indeed,

if CRP is able to increase NO production, this has implications for the signalling events studied here, as there is a possibility that nitration of tyrosine may directly inhibit the phosphorylation of proteins (as reviewed in; Sabetkar *et al.*, 2002). As in recent studies (Low *et al.*, 2002), tyrosine phosphorylation of Syk was found to be decreased by low doses of peroxynitrite, without the nitration of this protein. Therefore the signalling mechanisms underlying NO and mitochondrial ROS release in CRP activated monocytes, still remains to be elucidated.

The antioxidant α-tocopherol was more effective than ascorbic acid at quenching or inhibiting CRP mediated increases in monocytic cytosolic peroxides. A lower concentration of α-tocopherol was needed to produce a significant reduction in peroxide generation, compared with ascorbic acid pre-incubations. The pre-incubation time needed for α-tocopherol to quench ROS is much greater, as α-tocopherol is lipophillic and requires more time for cellular uptake than the hydrophilic ascorbic acid molecule, which can move directly through anion channel or GLUT transporters (chapter 1 introduction).

The expression of integrins (CD11b) has been previously shown to be redox regulated and subject to inhibition by synthetic antioxidants (Weber *et al.*, 1995). However, this was not observed with the conditions described here, as there was no significant change in CRP mediated monocytic CD11b expression after pre-incubation with the antioxidant ascorbic acid. This suggests that CRP mediated monocytic changes in phenotype, are not as a consequence of changes in intracellular peroxide levels. However, this does not rule out the redox regulation of gene expression, where longer incubations of antioxidants may influence gene expression and hence translation and membrane protein expression. This pathway still remains to be examined.

In summary CRP is able to bind FcγRIIa. CRP binding to FcγR initiates an intracellular signalling cascade that phosphorylates the tyrosine kinase Syk and leads to PI turnover, which ultimately leads to Ca^{2+} mobilisation in monocytes. This CRP mediated Syk phosphorylation in monocytes leads to an increase in CD11b and IL-6 expression. CRP engagement with monocytes also leads to an increase in peroxide production, which can be inhibited in vitro using the antioxidants α-tocopherol and ascorbic acid. However, intracellular peroxide generation appears to be independent of CRP-induced CD11b expression. The FcγRIIa polymorphism at codon 131 effects the phenotypic driven changes described in monocytes by CRP, where R/R allotypes have a greater increase in CD11b, which may be involved in promoting the monocytic inflammatory response.

6.0 Antioxidant Supplementation Trial

6.1 Preface:

This chapter describes the effects of a supplementation study with vitamin E and C on human monocyte phenotype, monocyte adhesion to endothelial cells and circulating serum CRP levels.

6.2 Introduction:

Epidemiological studies have demonstrated an association between increased plasma antioxidants and reduced morbidity and mortality from cardiovascular disease (CVD; Marchioli, 1999; Diaz *et al.*, 1997). Specifically, the EPIC study has identified that subjects in the lowest quintile for plasma vitamin C were significantly more likely to die from CVD than the highest quintile (Khaw *et al.*, 2001). Plasma ascorbic acid levels were inversely related to mortality from cardiovascular disease and ischaemic heart disease in men and women in the UK. Khaw et al (2001) reported a 25% fall in risk of all-cause mortality (independent of other risk factors), was associated with a $20\mu M$ rise in plasma ascorbic acid. As discussed previously, early atherosclerotic lesions are characterised by increased monocyte adhesion to the overlying endothelium (Gimbrome *et al.*, 1995). It is known that oxidised LDL enhances the recruitment, retention and adhesiveness of human monocytes to endothelial cells (EC; Basta *et al.*, 1997).

Oxidants are increasingly recognised as important mediators of signal transduction via several pathways including ras/raf, protein kinase C and MAP kinase cascades (Aikawa *et al.*, 1997; Accorsi *et al.*, 2001). Several transcription factors, including NF-κB are subject to redox regulation where innate immune response genes, such as cytokines and adhesion molecules are typically upregulated under oxidative stress conditions (Janseen-Heininger *et al.*, 2000; Hirota *et al.*, 2000; Tomita *et al.*, 2000).

Oxidant induced changes in adhesion molecule expression by EC and reciprocal ligands on monocytes are implicated in CVD (Price & Loscalzo, 1999). Furthermore, lipid hydroperoxides have been shown to be effectors of both chemokine receptors and adhesion molecule expression on monocytes and endothelial cells through a pro-oxidant mechanism (Weber *et al.*, 1999). In vitro experiments have demonstrated that synthetic antioxidants can inhibit cytokine-induced changes to adhesion molecule expression and monocyte adhesion to EC following TNF-α stimulation, further implicating the importance of oxidants in signalling to adhesion molecule expression (Weber *et al.*, 1994).

Taken together these data suggest that plasma antioxidants may play an important role in modulating EC-monocyte interactions, where low plasma vitamin C or E may be an important risk factor.

A previous study has reported that monocyte-EC adhesion was increased in smokers, who had lower vitamin C status than non-smokers. Furthermore, a reduction in the level of adhesion to EC was observed following high dose vitamin C intervention (2g/day) over 10 days (Weber *et al.*, 1996). However, it is not clear from this study whether the effects observed may be due to direct scavenging of specific oxidants generated through smoking or via a direct antioxidant effect of vitamin C.

Vitamin E is the collective name for molecules that exhibit the biological activity of α-tocopherol. α-tocopherol has been shown to inhibit macrophage-mediated lipid oxidation in vitro (Jialal *et al.*, 2001). However there is limited data on the effect of α-tocopherol on monocyte function. Jialal *et al.*, (2001) have shown that supplementation with 1200IU/day of α-tocopherol significantly influenced monocyte function by decreasing lipid oxidation,

release of O_2^- and hydrogen peroxide, decreasing release of the pro-atherogenic cytokine, IL-1β, as well as decreasing monocyte-endothelial cell adhesion.

It has been suggested that the degree of pathology in a supplementation subject group may be an important issue in outcome studies (Halliwell, 1999). Indeed, several supplementation studies have recently reported no clear benefit on mortality from antioxidant intervention, however, these have attempted to redress well-established pathologies (Stephens et al., 1996).

To study the importance of low plasma vitamin C and E on monocyte adhesiveness to EC and subsequent modulation by antioxidants, this study undertook a supplementation study in 20 healthy male subjects with low vitamin C status ($<50\mu M$) and compared with 20 male subjects of normal plasma vitamin C ($>50\mu M$) as described by Mitmesser et al., (2000).

Previous workers (Devaraj & Jialal, 2001) have noted that α-tocopherol supplementation significantly decreased serum levels of CRP and monocyte IL-6. Therefore CRP levels from volunteers in this supplementation trial was examined for serum CRP levels pre- and post-vitamin C and E supplementation, to see if antioxidant supplementation is a useful tool in reducing circulating CRP levels.

6.3 Materials & Methods:

6.3.1 Materials:

All materials used within this chapter are from previously described sources.

6.3.2 Intervention study design:

Forty healthy male volunteers, between the 20 and 45 years (mean age 30), were recruited at Glenfield Hospital Leicester. Written informed consent and information about drinking, dietary habits, smoking and medication were obtained from all volunteers. Individuals, who were smokers, had a previous history of cardiovascular disease or who were currently taking vitamin supplements were excluded from the study. Volunteers for the study were split into two groups based on their vitamin C status on recruitment: low plasma ascorbate ($<50\mu M$, referred to as LOC subjects) and above average plasma ascorbate ($>$ or $= 50\mu M$, referred to as HIC subjects). Each subject received dietary supplements in a double blind manner to receive either vitamin C (250mg/day) with placebo or vitamin E (200IU/day) with placebo. Blood was collected at the beginning and end of a six-week period. After six weeks, subjects were withdrawn from supplementation for 10 weeks (washout period), prior to cross over, and received the alternative vitamin, and dose for six weeks duration of supplement. Again blood samples were taken at the start and at the end of the supplementation period. Blood collection took place at a rate of four samples per week between August 2000 and July 2001. Peripheral whole blood (PWB) was collected into citrate (10%) from the antecubital vein, placed on ice and analysed within 2hrs. Monocyte

counts were determined for each blood sample using a Coulter Counter (Beckman-Coulter, Miami, USA), according to manufacturers instructions.

6.3.3 Determination of plasma vitamin C:

After sample collection, plasma was separated by centrifugation at 1600rpm for 20mins and vigorously mixed with an equal volume of cold 10% metaphosphoric acid to precipitate protein. Following centrifugation at 1500g for 15min at $10^{\circ}C$, the supernatant was retained and samples were stored at $-80^{\circ}C$ prior to analysis within four weeks. Determination of plasma ascorbate was according to the method of Lunec and Blake (1985). In brief, opthalaldehyde was used to derivatise ascorbate, prior to separation on a LC-NH_2 column, with citrate in acetonitrile as eluent. The eluent was monitored at an absorbance of 254nm and measured against a standard curve over a range of 5-100μM ascorbic acid. All measurements were done at Leicester Royal Infirmary (Leicester, UK).

6.3.4 Determination of plasma vitamin E:

Plasma vitamin E levels were determined by HPLC detection in the Clinical Biochemistry Department, Leicester Royal Infirmary (Leicester, UK).

6.3.5 Monocyte culture:

Viable U937 monocytes were maintained in culture according to section 2.3.1. For the supplementation trial U937 monocytes (1×10^6/ml) were treated with LPS (1μg/ml) for 5hrs at $37^{\circ}C$ prior to BCECF-AM dye loading and determination of adhesion to HUVEC (see

adhesion assay below) to standardise both dye loading and HUVEC adhesion conditions between subjects and over duration of the study.

6.3.6 Primary monocyte isolation from whole blood:

All tubes and equipment in contact with PWB were Sigmacoted to minimise artefactual activation. Primary monocytes were isolated according to section 2.3.4 and CD14+ CD11b expression was analysed throughout the isolation procedure according to section 2.3.9, in order to detect any artefactual activation. Only isolated CD14+ monocytes of >90% purity were used for adhesion analysis.

6.3.7 Flow Cytometry:

Samples were analysed for CD11b, CD54 and CD14 expression by flow cytometry, according to section 2.3.9.

6.3.8 Adhesion Assay:

Monocyte adhesion to cultured Human Vein Endothelial Cells (HUVEC) was determined by dye loading isolated monocytes with the fluorescent dye BCECF-AM and analysed for monocyte binding to LPS activated ($1\mu g/ml$; 5hrs) HUVEC monolayers according to section 2.3.6.

6.3.9 C-reactive protein assay:

Fasting blood samples were collected in tubes containing citrate, and were drawn and centrifuged immediately. Serum was then aliquoted and stored at $-80^{\circ}C$ until analysis. No specimen inadvertently thawed during storage. C-reactive protein measurements were performed on the COBAS Integra (Roche Diagnosis Ltd., East Sussex, UK) using the CRP-latex assay in both the high-sensitivity application (hsCRP; analytical range, 0.2 to $12\mu g/ml$) and the normal application (analytical range, 2 to $160\mu g/ml$). Samples outside the analytical range of the hsCRP-latex assay were analysed by the CRP-latex assay in the normal application. All measurements were done at Leicester Royal Infirmary (Leicester, UK).

6.3.10 Statistical Analysis:

Monocyte adhesion is expressed as a ratio of isolated monocyte adhesion: standard U937 adhesion, to correct for inter-endothelial cell batch variation in adhesion. Data are presented as the arithmetic means ± SEM. Groups of data were evaluated statistically by Wilcoxon signed ranks test for matched paired data, two-tailed Mann-Whitney test for grouped data and Spearman's ranks test for comparing correlation between data sets. Differences were considered significant different when <0.05. Where * represents $P<0.05$, ** represents $P<0.001$ and *** represents $P<0.0001$. Adhesion results are expressed as the ratio of isolated subject monocytes bound relative to U937 monocytes bound, where cells were always presented at an equivalent density of 2.5×10^5/well. Efficiency of BCECF-AM dye loading was evaluated for each visit and standard curve of U937 fluorescence versus cell number was calibrated.

6.4 Results:

6.4.1 Effect of vitamin supplementation on plasma antioxidant concentrations, monocyte count, monocyte adhesion and adhesion molecule expression:

To determine whether antioxidant supplementation would have any effect on monocyte adhesion and phenotype in vivo and specifically, to identify whether subjects with low plasma vitamin C would receive greatest benefit, a vitamin C supplementation study was undertaken. Initially, the groups were allocated on the basis of their vitamin C status. In the total cohort, volunteers were determined to have a mean plasma ascorbate (vitamin C) level of $50.4\pm3.4\mu M$ prior to vitamin supplementation. Based on this value, volunteers were divided into two groups determined by plasma ascorbate status; lower than average (LOC) or above average (HIC). The mean plasma ascorbate level for the LOC chohort was 32.92 ± 13.98 (means$\pm$SD, n=20) and was significantly lower than for the total group (P=0.036, two tailed Mann-Whitney t-test). Figure 6.1 shows that LOC subjects showed a two-fold increase following supplementation for six weeks with 250mg vitamin C/day, compared with an increase of 50% in the HIC cohort (from $67.61\pm6.32\mu M$ before supplementation to $95.32\pm25.92\mu M$ after supplement). Furthermore, there was no significance between the groups following supplementation.
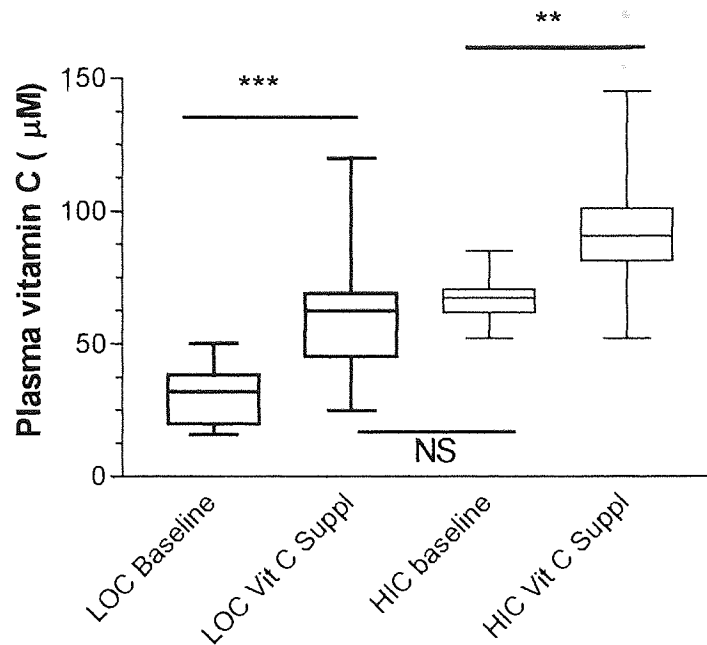
Figure 6.1. The effects of dietary vitamin C supplementation (250mg/day for 6 weeks) on plasma ascorbate concentration during the supplementation study. Bold type boxes describe the low baseline vitamin C cohort (LOC) and normal type boxes describe the above average baseline vitamin C cohort (HIC). Results are expressed as box and whisker plots where boxes represent 95% confidence limits, centre lines are median values and bars are ranges of plasma ascorbate concentrations. Using Mann-Whitney t-test to compare between populations. ** represents P<0.001 and *** represents P<0.0001.

Low vitamin C status may be less effective in recycling vitamin E and therefore only those subjects that had a normal plasma ascorbate level may receive benefit from vitamin E supplementation. Therefore to determine whether vitamin E would also have any effect on monocyte adhesion and phenotype in vivo LOC and HIC groups were supplemented with 200IU/day of vitamin E for 6 weeks. Supplementation with vitamin E caused a significant (P=0.0063, n=40) mean 38% increase in plasma levels of vitamin E in both LOC and HIC groups from a mean±SD baseline concentration of 11.51±5.51 and 11.99±7.01 in LOC and HIC groups respectively (fig 6.2). There was no significant difference in plasma vitamin E levels between LOC and HIC groups (fig 6.2).

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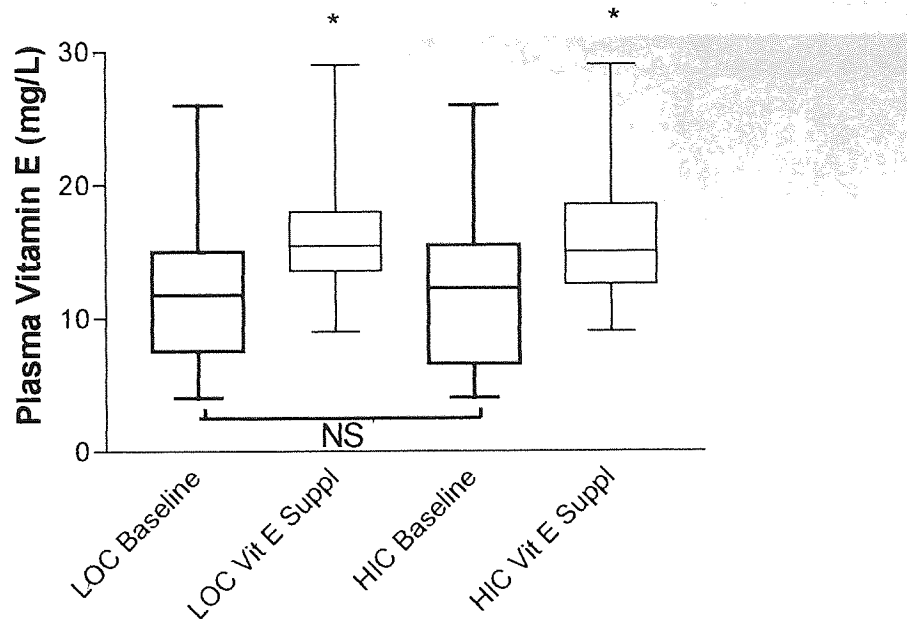
Figure 6.2. The effects of dietary vitamin E supplementation (200IU/day for 6 weeks) on plasma vitamin E concentration. Bold type boxes describe the baseline vitamin E levels and normal type boxes describe post-supplementation levels. Results are expressed as box and whisker plots where boxes represent 95% confidence limits, centre lines are median values and bars are ranges of plasma vitamin E concentrations. Using Mann-Whitney t-test to compare between populations. * represents P<0.05.

To analyse the benefits of supplementation on monocyte adhesiveness to EC it was necessary to develop a method of monocyte purification that yielded a pure resting population of peripheral blood monocytes. To this end negative isolation was adopted. Monocyte purity and activation throughout preparation was determined by flow cytometry, where Dynal negative extraction increased the purity of monocytes from 7% in whole blood to 92% without change in CD11b expression as a marker of activation (see chapter 2).

There was no significant difference in monocyte number between the two cohorts, neither were monocyte levels in blood affected by supplementation with vitamin C or E (see table 6.1).

	Monocytes $(x10^6)$
Low baseline cohort (LOC)	6.8±2.17
High baseline cohort (HIC)	7.92±2.72
Before supplementation	7.47±2.62
After supplementation	7.11±2.13

Table 6.1. The effects of dietary vitamin C supplementation (250mg/day for six weeks) on monocyte count. Results are expressed as means±SD, where n=20.

Since smoking has been previously shown to increase monocyte adhesiveness to endothelial cells and this could be ameliorated by dietary vitamin C (Weber *et al.* 1996). The hypothesis that low vitamin C in normal subjects may be associated with increased monocyte adhesiveness was investigated by examining the effects of vitamin C and E supplementation in normal subjects with low plasma ascorbate. Monocyte adhesion to HUVEC was determined in subjects pre- and post-supplementation with antioxidant vitamins for six weeks.

In vitamin C supplemented LOC subjects, figure 6.3, monocyte adhesion to EC was significantly (P<0.02) greater than that observed for monocytes from HIC subjects (>50µM ascorbate) and a significant (P<0.0001; n=20) inverse correlation between adhesion and plasma ascorbate was observed, using Spearmen's rank correlation coefficient analysis (Fig 6.4). Furthermore, a significant reduction in adhesion was observed from 0.88±0.09FU to 0.65±0.11FU after 6 weeks of supplementation with 250mg vitamin C per day, P<0.02, n=20 in LOC subjects. Following supplementation, monocyte adhesion was not significantly different between LOC and HIC subjects (Fig 6.3).
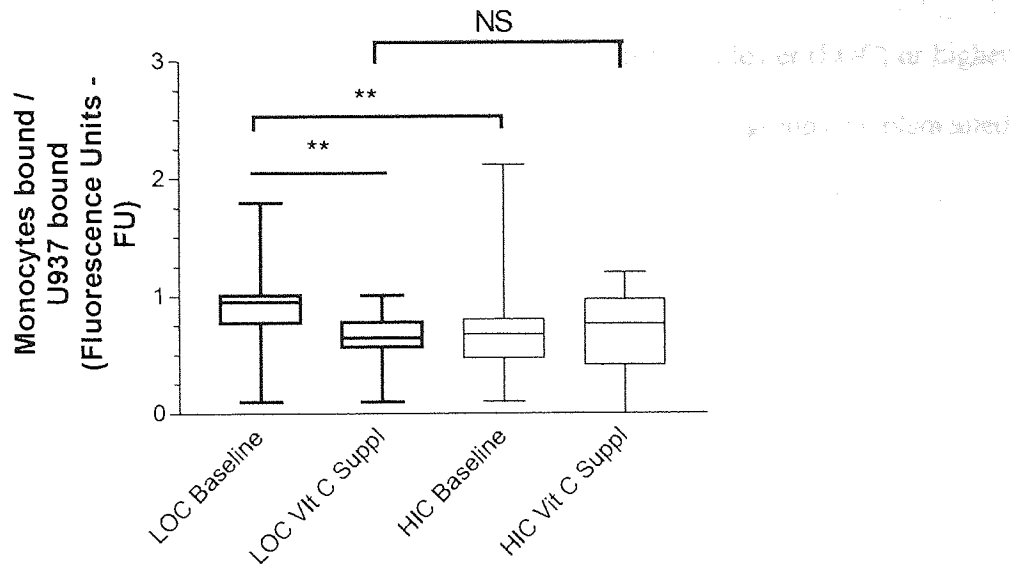
Figure 6.3.   The effects of dietary vitamin C supplementation (250mg/day for 6 weeks) on monocyte adhesion to human umbilical vein endothelial cells (EC). Bold type boxes describe monocyte adhesion in the low baseline vitamin C cohort (LOC) and normal type boxes describe monocyte adhesion in the above average baseline vitamin C cohort (HIC).   Results are expressed as primary monocyte adhesion relative to an equivalent number of standard U937 monocytes. Data are described by box and whisker plots where boxes represent 95% confidence limits, centre lines are median values and bars are ranges of plasma ascorbate concentrations. Using Wilcoxon matched pair analysis to compare between matched populations and Mann-Whitney t-test between unmatched populations. ** represents P<0.02.
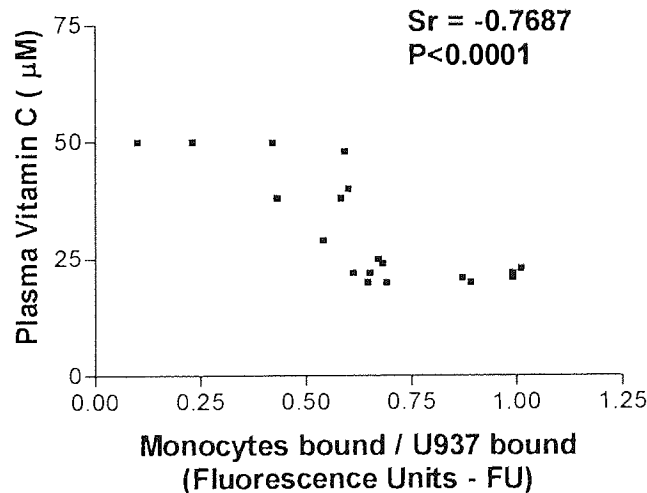
Figure 6.4. Correlation between monocyte adhesion and plasma ascorbate levels in the LOC group. Results are expressed as primary monocyte adhesion relative to an equivalent number of standard U937 monocytes (analysed according to section 6.3.8) versus results expresses as variation in plasma ascorbate concentration (analysed according to section 6.3.3). Data was analysed for correlation using two tailed Spearman's rank analysis where P<0.05 was considered significantly different. Shown in the histogram is the significance value (P) and Spearmen's rank correlation coefficient (Sr).

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There was no significant difference in monocyte adhesion between lower (LOC) or higher

(HIC) than average baseline plasma vitamin C levels (Fig 6.5), in groups supplemented

with vitamin E (200IU/day) for 6 weeks, or between LOC baseline and vitamin E

supplemented group or HIC baseline and vitamin E supplemented group.
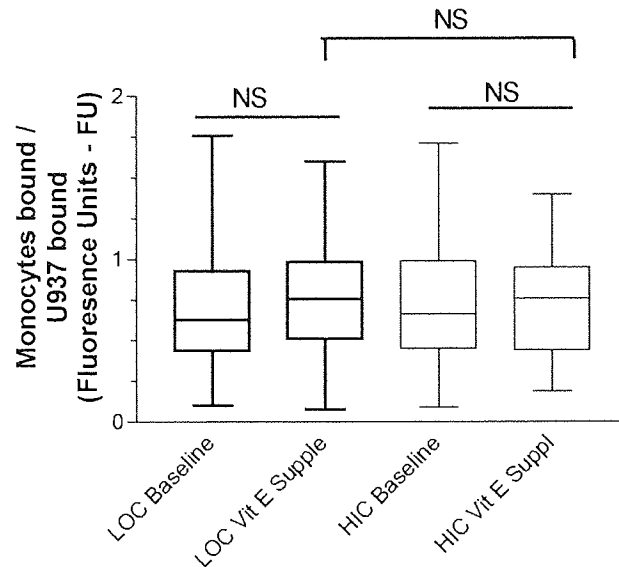
Figure 6.5. The effects of dietary vitamin E supplementation (200IU/day for 6 weeks) on monocyte adhesion to human umbilical vein endothelial cells (EC). Bold type boxes describe monocyte adhesion in the low baseline vitamin C cohort (LOC) and normal type boxes describe monocyte adhesion in the above average baseline vitamin C cohort (HIC), after supplementation with vitamin E for 6 weeks. Results are expressed as primary monocyte adhesion relative to an equivalent number of standard U937 monocytes. Data are described by box and whisker plots where boxes represent 95% confidence limits, centre lines are median values and bars are ranges of plasma vitamin E concentrations. Wilcoxon matched pair analysis was used to compare between matched populations and Mann-Whitney t-test between unmatched populations.

The integrin CD11b is involved in monocyte adhesion to endothelial cells through ICAMs

(discussed in chapters 1 & 4). As the LOC cohort produced the strongest significant

change in monocyte phenotype (i.e. LOC – monocyte adhesion), the LOC groups were first

analysed in all subsequent data for significant changes in pre- and post-supplementation

with antioxidants. Figure 6.6A shows there was no signicant change in CD11b expression

post-supplementation with vitamin E in LOC groups. Neither was there any significant

there any significant change in monocytic CD11b expression in the LOC cohort following

vitamin C supplementation (fig 6.6B). There was no significant difference in CD11b

expression in HIC supplemented cohorts.

A)
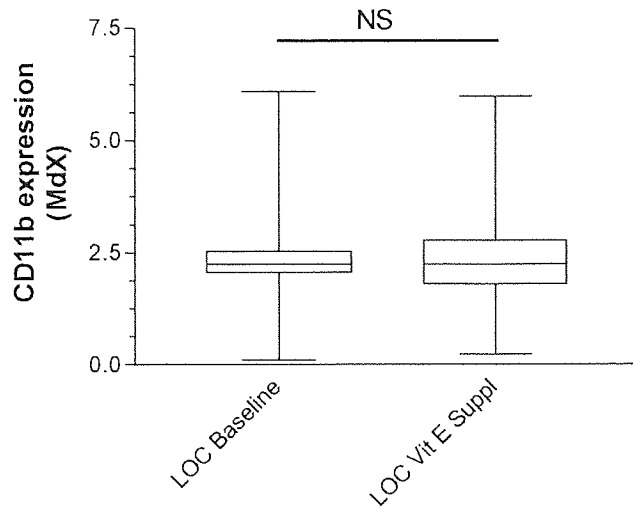
B)
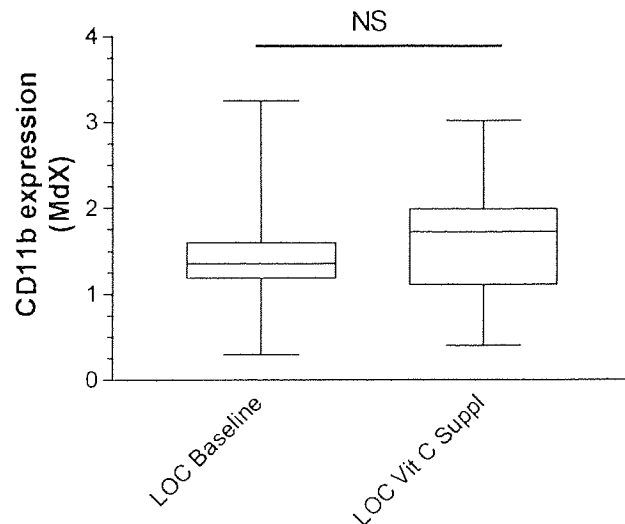
Figure 6.6. The effects of vitamin E (A) or vitamin C (B) supplementation on median X (MdX) CD11b expression on monocytes, pre- and post-supplementation with vitamin C (250mg/day) or vitamin E (200IU/day) for six weeks. PWB (100µl) was analysed for CD14+ CD11b expression via flow cytometry according to section 6.3.7. LOC groups for baseline plasma ascorbate <50µM are shown. Results are expressed as box and whisker plots as ranges in monocyte CD11b expression, where boxes represent 95% confidence limits, centre lines are median values and bars are ranges of change in CD11b expression within the cohort. Statistical significance was analysed using a Wilcoxon matched paired analysis.

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As monocytes constitutively express the immunoglobulin cellular adhesion molecule ICAM-1 and as this adhesion molecule is able to bind its ligand CD11b as previously described, this molecule is important for cell-to-cell adhesion between monocytes. Therefore ICAM-1 expression was analysed in the LOC/LOE group for both vitamin C and E supplemented groups. There was no significant change in monocytic CD14+ ICAM-1 expression levels between in the LOE cohort for vitamin E (Fig 6.7A) or LOC vitamin C (Fig 6.7B) supplemented subjects. There was no significant difference in ICAM-1 expression in HIC supplemented cohorts.

6.4.2 Effect of vitamin supplementation on serum CRP concentration:

Previous work has shown that vitamin E supplementation can significantly reduce CRP levels (Devaraj & Jialal, 2000). Therefore CRP levels were measured in all antioxidant vitamin supplemented subjects, pre- and post-supplementation. The mean±SD CRP level in all subjects (n=40) was 1.72±2.8 μg/ml, showing that all volunteers used within the supplementation were healthy normals. There was a trend towards a reduction in CRP levels in vitamin E supplemented subjects, however, this was not significantly different (P=0.77; fig 6.8). The LOC groups were analysed for change in CRP levels post-supplementation from baseline levels. CRP levels were not significantly different in the LOC group after vitamin E supplementation (Fig 6.9A). There was also no significant effect of vitamin C supplementation on CRP levels (P=0.38). Again there was a trend for a general reduction in CRP levels within the LOC vitamin C supplemented group, however this was not significantly different (Fig 6.9B). There was no significant change in CRP levels post-supplementation with vitamin C and E in the whole cohort.

A)
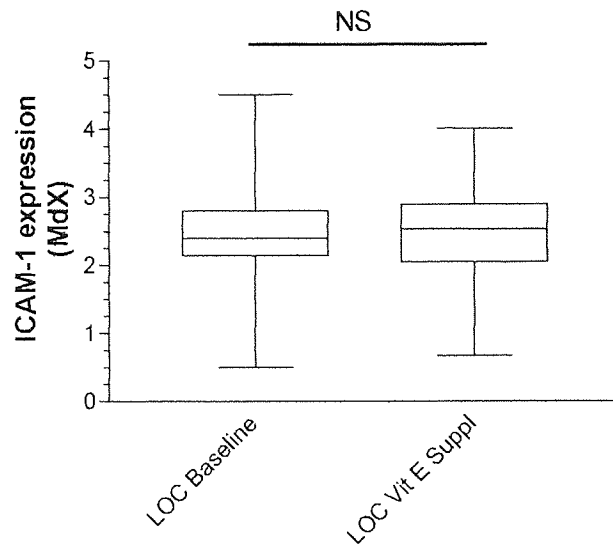
B)
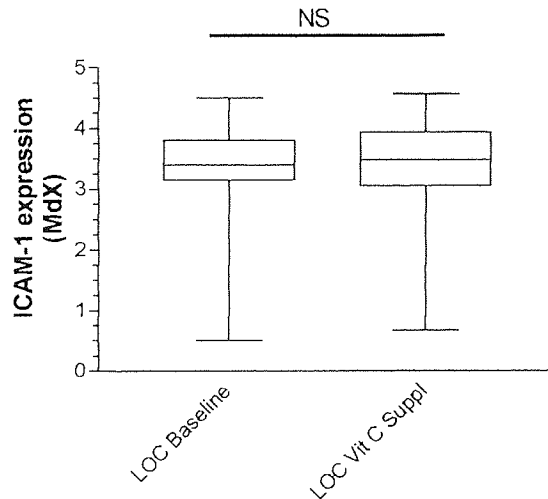
Figure 6.7. The effects of vitamin E (A) or vitamin C (B) supplementation on median X (MdX) ICAM-1 expression by monocytes, pre- and post-supplementation with vitamin C (250mg/day) or vitamin E (200IU/day) for six weeks. PWB (100µl) was analysed for CD14+ ICAM-1 expression via flow cyometry according to section 6.3.7. LOC groups for baseline plasma ascorbate <50µM are shown. Results are expressed as box and whisker plots as ranges in monocyte ICAM-1 expression, where boxes represent 95% confidence limits, centre lines are median values and bars are ranges of change in ICAM-1 expression within the cohort. Statistical significance was analysed using Wilcoxon matched paired analysis

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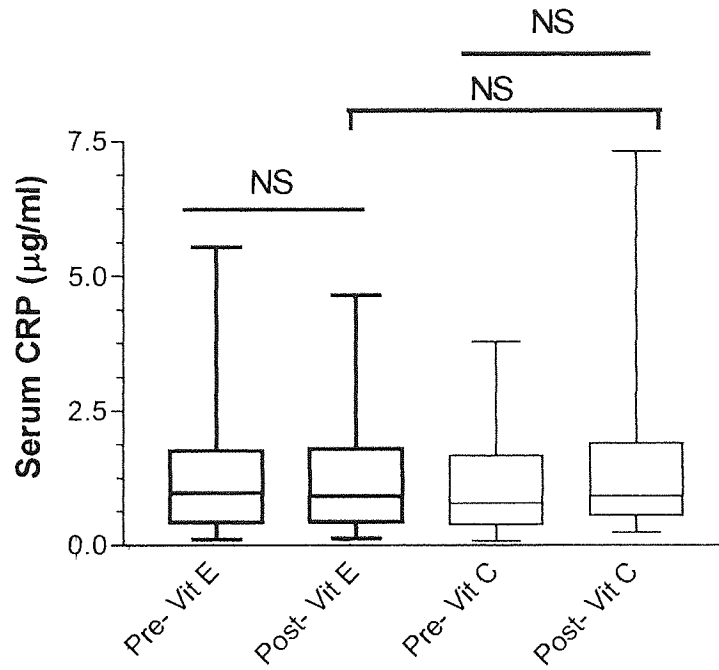
Figure 6.8. Effect of antioxidant supplementation on serum CRP levels. Volunteers (n=40) were supplemented with vitamin C (normal type boxes; 250mg/day) or vitamin E (bold type boxes; 200IU/day) for 6 weeks and serum CRP levels were measured pre- or post-supplementation. Results are expressed as box and whisker plots where boxes represent 95% confidence limits, centre lines are median values and bars are ranges of CRP concentrations within the cohort. Wilcoxon matched pair analysis was used to compare between matched populations and Mann-Whitney t-test between unmatched populations.

A)
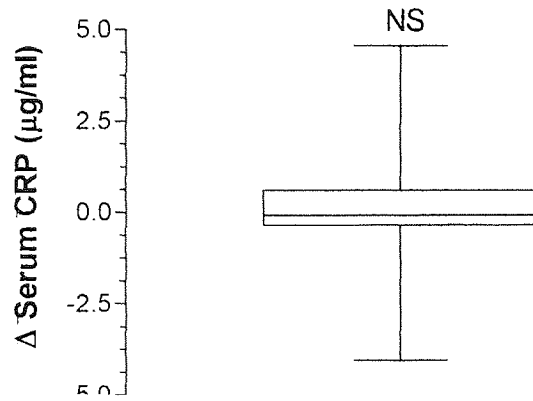
B)
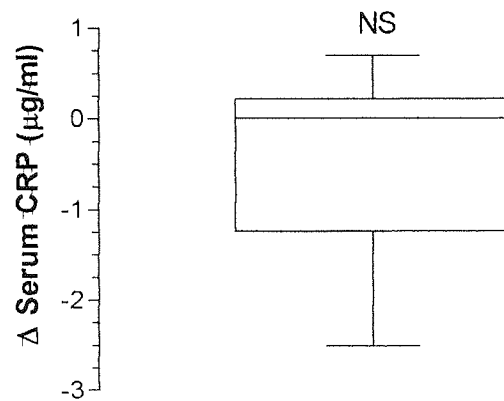
Figure 6.9. The effects of vitamin E (A) and vitamin C (B) supplementation on change in CRP concentrations within the LOC cohort. Volunteers (n=40) were supplemented with vitamin C (normal type boxes; 250mg/day) or vitamin E (bold type boxes; 200IU/day) for 6 weeks and serum CRP levels were measured pre- or post-supplementation. Results are expressed as box and whisker plots as change (Δ) in CRP levels, where boxes represent 95% confidence limits, centre lines are median values and bars are ranges of change in CRP concentrations within the cohort. Statistical significance was analysed using a one-sample t-test from the theoretical mean of 0.

6.5 Discussion:

A growing body of evidence from epidemiological studies describes an association between low plasma vitamin C concentration and death from cardiovascular disease (Marchioli, 1999; Diaz *et al.*, 1997; Khaw *et al.*, 2001). Whilst the mechanisms underlying this process are not completely clear, there appears to be significant involvement of inflammatory cells, particularly monocytes and platelets, in the atherosclerotic process (Price & Loscalzo, 1999). As previously discussed cell adhesion molecules mediate the localisation of the cellular and humoral immune responses, where cell surface expression and activation of adhesion molecules in response to pathophysiological stiumuli mediates the interaction between endothelial cells and leukocytes central to the development of atherosclerosis.

Antioxidant intervention studies have examined the roles of dietary antioxidants vitamin E and C, in amelioration of monocyte adhesion to endothelial cells (EC); Devaraj *et al.*, (1996) demonstrated benefit from vitamin E (1200IU/day over 8 weeks) in healthy subjects, resulting in a reduction of monocyte adhesion by 33%. Previously, monocytes isolated from smokers have been shown to be more adhesive to endothelial monolayers than those from non-smoking subjects (Weber *et al.*, 1996). Adams *et al.*, (1997) did not observe protection using vitamin C, however, reported benefit from supplementation with oral L-arginine, where nitric oxide may play an important role in inhibition of adhesion to EC. In contrast, high dose supplementation with vitamin C (2g/day for 10 days) was shown to reduce smokers' monocyte adhesion to normal levels (Weber *et al.*, 1996). Nevertheless, it is unclear from this study whether non-smokers who are deficient in vitamin C and who are at increased risk of CHD based on epidemiological data, can benefit

from supplementation in the context of monocyte adhesion. In addition Deveraj *et al.*, (1996) has shown benefit from vitamin E supplementation, in the context of monocyte adhesion, and the concentrations provided were 6 fold higher and intervention time longer, than used here. The 8 week supplementation period may be more advantageous for vitamin E intervention studies compared with the 6 week study (as used here), as vitamin E is not hydrophilic compared to vitamin C and therefore requires much higher doses and longer intervention to saturate cells. Thus the lack of any reduction seen with vitamin E supplementation on monocyte adhesion in the overall cohort or between LOC and HIC groups in this study may be due to the dose being too low or the period of intervention too short.

In these studies, a static adhesion assay was adopted to quantitatively assess monocyte adhesion to endothelial cells. Whilst being removed from the shear stresses naturally achieved during flow (Hsiai *et al.*, 2001), the static assay offers several advantages over the flow model; namely sensitivity (0.25×10^6 cells required) and quantitative analysis.

It has been previously reported that biomarkers of oxidation to protein and DNA are significantly higher in subjects with lower than average plasma vitamin C and that these differences can be ameliorated on supplementation (Rehman *et al.*, 1998; Carty *et al.*, 2000). Therefore this study identified two normal healthy non-smoking male subject groups that have normal plasma vitamin C status (HIC) and lower than average vitamin C status (LOC) in which to evaluate the hypothesis that dietary vitamin C and E can offer vascular benefit through reduction of monocyte adhesion in LOC subjects. The male sex is an important risk factor for CVD and McChrohan *et al.*, (1999) report increased EC adhesion of monocytes following exposure to androgens in vitro. Therefore this study was

exclusive to males. Supplementation with vitamin C (250mg/day) caused a 58% increase in plasma levels and vitamin E (200IU/day) caused a 38% increase in plasma levels, after six weeks, indicating that normal healthy subjects studied do not normally have a saturating dietary supply of antioxidant vitamin C and E. The duration of the study period and the crossover design eliminates any seasonal effects on diet and antioxidant status.

There was no significant difference in monocyte number between the two cohorts, neither were monocyte levels in blood affected by supplementation with vitamin C. Although monocyte turnover is much less than 6 weeks (as discussed in the general introduction), it does indicate there was no affect of antioxidant supplementation on bone marrow production of monocytes.

As shown previously in HUVEC, LPS induces the expression of E-selectin, ICAM-1 and VCAM-1, which are evident after 5hrs of challenge (see chapter 3). In addition, it enhances release of chemokines such as MCP-1 and proinflammatory cytokines such as TNF thereby facilitating monocyte recruitment (Shukaliak & Dorovini-Zis, 2000). This model was adopted for the adhesion assay to mimic early activation of endothelium, as reported in atherosclerotic vessels. Adhesion of freshly isolated non-activated monocytes from LOC subjects was significantly higher than adhesion of corresponding monocytes from HIC groups, within the vitamin C cohort. Furthermore, supplementation with 250mg/day of vitamin C significantly reduced LOC monocyte adhesion to HIC baseline levels. HIC monocyte adhesion was unaffected by supplementation. In both subject groups, there was wide inter-individual variation in adhesion at baseline, but following supplementation adhesion reduced, indicating that variation in dietary vitamin C intake may influence adhesion. These data support the finding of Weber et al., (1994), which

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reported benefit from vitamin C in smokers, and demonstrates that low plasma vitamin C contributes to monocyte stickiness in the absence of any mitigating oxidant such as cigarette smoke. Furthermore, the results observed an overall inverse correlation between plasma vitamin C status and monocyte adhesion to HUVEC, suggesting an association between plasma vitamin C status and adhesion.

This thesis has previously described the importance of CD11b in the adhesion of monocytes to HUVEC. In determining the control of monocyte-HUVEC interaction, most studies have focused on regulation of adhesion molecule expression on HUVEC. However it is evident that the control of the β_2 integrin CD11b, is more complex, involving not only transcriptional regulation but also trafficking and cell surface conformational activation (Noti et al., 2001). There was no significant difference in CD11b expression between either HIC or LOC subjects or between pre- and post-supplementation in either group. Nor was there any alteration in monocytic ICAM-1, involved in modulating cell-to-cell adhesion, within any supplemented group. Previous work (Phillips et al., 2003) has shown that redox regulated upregulation of integrin expression does not always associate with increased adhesion to HUVEC and therefore, that phenotype does not necessarily inform on function, where conformational activation is key determinant of activity. Adhesion to VCAM-1 on HUVEC through VLA-4 on monocytes has previously been reported (Verdegaal et al., 1993), showing VLA-4 to play a co-operative role in monocyte adhesion to IL-4 stimulated HUVEC. To date the control of VLA-4 is unclear and the possible regulation of this ligand by vitamin C or E remains to be investigated.

In summary, this data shows benefit from intake of vitamin C at 250mg/day in healthy human subjects over six weeks in the context of monocyte/endothelial interaction. In

accordance with other studies (Rehman *et al.*, 1998; Carty *et al.*, 2000), in all cases of benefit afforded by vitamin C intake, the effects were greater in subjects with lower than average plasma levels of vitamin C at the outset. It is suggested that only certain subgroups of the general population will receive benefit from vitamin C intervention and this merits further investigation using outcome measures of disease.

Neither vitamin C at 250mg/day nor vitamin E at 200IU/day over 6 weeks were able to significantly reduce serum CRP levels in either the entire cohort or between LOC and HIC groups. There was a slight trend towards vitamin E reducing CRP levels. Previous workers have reported this trend as being significant (Devaraj & Jialal, 2000), however, the dose was much higher and the intervention time longer. In summary the use of either vitamin C or E as a tool for reducing circulating CRP concentrations, as a means of health benefit by reducing endogenous CRP effects, is ineffective in the study conditions shown here and requires further study to confirm any benefit at higher antioxidant concentration or longer period of intervention. There may have also been no change in circulating serum levels of CRP after intervention with antioxidants in the conditions described here, as all volunteers used within the trial were considered healthy and lead a healthy lifestyle (i.e., non-smokers), therefore their baseline CRP levels were already $<5\mu g/ml$. It would be interesting to investigate antioxidant intervention in high-risk groups, with already higher than normal average serum CRP concentrations, such as in RA or atherosclerosis. The actual mechanism as to how antioxidants could lower serum CRP concentrations is not examined in this thesis. Antioxidants may be able to act directly on CRP mediated changes in cellular phenotype by quenching CRP mediated increases in monocytic ROS, as shown in chapter 5, or it could be through an indirect mechanism, such as lowering serum levels of IL-6 and in turn reducing CRP transcription within hepatocytes (as discussed in

the general introduction). Previous workers have developed a human cytokine array system in order to further characterise the molecule mechanisms involved in vitamin E supplementation (Lin *et al.*, 2002). Healthy human volunteers were orally supplemented with 800IU/day of vitamin E for 8 weeks. Several cytokines including, IL-1α, MCP-1, Rantes and TNF-β, were found to be significantly down-regulated after supplementation with vitamin E. Therefore supporting the hypothesis that antioxidant intervention may be able to indirectly reduce CRP levels by reducing cytokine expression.

7.0 Final Discussion

7.1 Final Discussion:

It is described here that CRP is able to bind the constitutively expressed FcγRIIa receptor on monocytes. This receptor is then rapidly internalised after CRP engagement. The ability of CRP to bind FcγRIIa is in agreement with previous studies, however, after CRP interaction the propagation of intracellular signals via the ITAM motif within monocytes has not been previously explored. Other studies have indicated that Syk PTK plays a critical role in FcγR mediated signalling and function in monocytes and macrophages (Stenton et al., 2000; Darby et al., 1994). Therefore the effect of CRP on the phosphorylation of Syk associated with ITAM motifs was examined. CRP rapidly and transiently phosphorylated tyrosines within Syk, which was entirely consistent with an intracellular signalling response as seen by both phosphatidylinositol (PI) turnover and Ca^{2+} mobilisation. After Syk kinase phosphorylation, the effective signal transduction pathways are incompletely understood in monocytes, however possible adaptor molecule interactions are discussed in chapters 1 & 5.

Some signals triggered through FcγR reach the nucleus via the Ras pathway. Ras is able to phosphorylate Raf, which in turn phosphorylates the MEK kinases, which eventually leads to the phosphorylation of MAP kinase. MAP kinase activates transcription factors such as NF-κB and then leads to gene expression. An important function of monocytes involves the expression, synthesis and secretion of polypeptide inflammatory mediators, including the cytokines interleukin 6 (IL-6) and tumour necrosis factor (TNF-α). IL-6 and TNF-α expression and secretion is regulated by the transcription factor NF-κB. Therefore the significant increases in both IL-6 and TNF-α expression and secretion induced by CRP, could be signalling via the ITAM, Syk, Ras, NF-κB pathway following CRP engagement

with Fcγ receptors expressed on monocytes. The role of the non-receptor tyrosine kinase Syk was confirmed as IL-6 secretion was significantly inhibited by the pre-incubation with the Syk specific antagonist piceatannol. This rapid Syk mediated signal, maybe being deactivated by phosphatases such as CD45, however the regulation of the monocytic proinflammatory response by CRP seems to be more complicated. CRP, at concentrations seen in many diseases states, increases IL-6 and TNF-α expression and secretion. As the proinflammatory cytokine IL-6 is able to induce secretion of CRP by the liver via activating the promoter region of CRP within hepatocytes, these results would suggest a self perpetuating response as CRP increases IL-6 and vice-versa. As not everyone within a given normal population has continued systemically raised CRP or IL-6 levels, there must be a down-regulatory mechanisms modulating both pro- and anti-inflammatory monocytic responses to CRP that remains to be elucidated. One such mechanism could be through negative coreceptors that contain ITIM (Immunoreceptor tyrosine inhibiting motifs).

FcγRIIB is understood to be a negative coreceptor of all receptors with ITAMs. Negative cooperation was recognised first between FcγRIIB and BCR. Passively administered antigen-specific IgG antibodies were shown to inhibit in vivo primary responses to that antigen (Uhr & Moller, 1968). FcγRIIB has been demonstrated to inhibit cell activation via TCR in BW5147 thymoma cells reconstituted with CD3ε and TCRζ and transfected with cDNA encoding murine FcγRIIB. IL-2 secretion, induced by anti-CD3ε, was inhibited if TCR was coaggregated to FcγRIIB (Daeron et al., 1995). More importantly FcγRIIB was shown to inhibit activation via FcR with associated ITAMs. Serotonin release, triggered by FcεRI aggregation, was inhibited when FcεRI was coaggregated to murine (Colonna & Samaridis, 1995) or human (Daeron et al., 1995b) FcγRIIB expressed in RBL cells. Serotonin release triggered by FcγRIIa was also inhibited when FcγRIIa was

coaggregated with human FcγRIIB (Daeron et al., 1995). This finding extends FcγRIIB-dependent inhibition to all FcR with ITAMs. It follows that FcγRIIB may regulate various IgE-, IgG- and IgA-induced responses of the many cells that coexpress FcγRIIB (monocytes being one of them) and corresponding Fc receptors and therefore may also regulate CRP-FcγR mediated responses. ITIM is a 13-amino acid sequence, in the intracytoplasmic domain of FcγRIIB (Amigorena et al., 1992). Phospho-ITIM has been shown to be a potential ligand of SH2 domains and previous workers have shown that peptides corresponding to phospho-ITIM, but not peptides corresponding to non-phosphorylated ITIM, precipitated the cytoplasmic phosphates SHP-1 and SHP-2, which contain two tandem SH2 domains (Yi et al., 1992). If they are indeed recruited in vivo by phospho-ITIM, the activity of these phosphatases might be enhanced, since in vitro phosphatase activity of SHP-1 increased several fold in the presence of phospho-ITIM but not phospho-ITAM (D'Amrosia et al., 1995). The SH2-containing inositol phosphate phosphatase SHIP has also been shown to bind phosphorylated ITIM in vitro and to coprecipitate with phosphorylated FcγRIIB in macrophages (Ono et al., 1996). This suggests that FcγRIIB may trigger several inhibitory pathways in different cell types.

As discussed in chapter 3 the anti-inflammatory cytokine IL-10 may also play a role in regulating the effects of CRP in monocytes. As previously mentioned, in 1994, Geng et al., (1994) reported for the first time a direct relationship between IL-10 and its anti-inflammatory actions mediated via the Ras-MAPK signalling pathway, showing that IL-10 blocks the phosphorylation of Ras, Raf and possibly p56lyn, thereby leading to inhibition of the phosphorylation of MAPK components. Previous work has shown that IL-10 synthesis is uniquely dependent on the endogenous pro-inflammatory cytokines IL-1 and TNF-α (Foey et al., 1998). Therefore whilst these data suggest that CRP is pro-

inflammatory, it may be regulating inflammation through negative co-receptor aggregation or through IL-10 secretion, which still remains to be elucidated. It is interesting to note that studies done in vivo using mouse models (as discussed in chapter 3) have shown that CRP injection increases serum levels of IL-10, suggesting that CRP had an anti-inflammatory effect (Mold et al., 2002). However, the authors of this study failed to comment on the fact that murine FcγRIIa receptors have cytoplasmic ITIM rather than ITAM associations (Mold et al., 2002) and thus maybe expressing monocytic IL-10 due to ITIM signalling.

CRP incubations and interactions with the FcγRIIa receptor increased the expression of the monocytic surface integrin CD11b. Again the signalling events leading to CD11b upregulation were associated with the ITAM and Syk phosphorylation, as pre-incubation with the Syk specific antagonist piceatannol, significantly reduced CRP-induced monocytic CD11b expression to baseline levels. The CRP mediated upregulation of CD11b was also successfully blocked, using antibodies occupying FcγRIIa binding sites. Conversely CRP incubations decreased CD31 expression on the surface of monocytes. This modulation of monocytic surface adhesion molecules by CRP was then investigated in a static adhesion assay. CRP altered the adhesion profile of monocytes to primary cultured endothelial cells (EC), by decreasing their adhesion to early activated EC and increasing adhesion to late activated EC. This modulation of adhesion may be associated with the alteration in expression pattern of monocytic adhesion ligands, however, when antibodies were used to block specific late activated EC receptors for each monocytic adhesion molecule, such as ICAM-1, the reduction in adhesion was not returned to resting levels. This would indicate that not one particular adhesion molecule is fundamentally important in monocyte-endothelial cell adhesion, moreover, it is the plethora of adhesion

molecules expressed on monocytes involved with the cell adhesion cascade (as discussed in the introduction) interacting with their associated ligand on EC that determines the extent of adhesion or structural change not recognised by the antibody. Interestingly previous workers have also noted that after CD31 interaction with its natural homophillic ligand, monocytic CD11b integrin expression is increased, which resulted in increased monocytic adhesion to cultured EC (Muller et al., 1993). Therefore this could indicate that the reduction in monocyte adhesion to early activated EC would not only result from the reduction in surface CD31 after CRP incubation, but that CD31 could directly alter CD11b expression and consequently monocyte adhesion.

The kinetics of monocytic adhesion molecule expression after CRP incubations, were relatively rapid, with peak expression seen at 30mins. This demonstrates that the modulatory effects of CRP are relative to intracellular signalling kinetics, however it does not reveal the effects of chronic incubations of CRP on monocytic adhesion molecule expression and related adhesion, as would be seen in vivo. The difficulty of examining chronic CRP effects on monocytic adhesion, is that the assay used here is undertaken in a serum free medium. When previous workers used serum containing media within a static adhesion assay, they noticed CRP was able to directly increase EC adhesion molecule expression (Pasceri et al., 2000). When serum was removed there was no noticeable modification in adhesion molecule expression, suggesting a possible role of complement in upregulation of adhesion molecules. Long term serum free incubations are not possible, as cultured EC would begin to die and lose their confluent phenotype needed for the adhesion assay. Therefore the exact mechanism of chronic CRP incubations on monocyte adhesion still remains to be clarified.

While the role(s) of cytokines in eliciting integrin activation is not well studied or understood, there is vast literature on the regulation of expression of integrin receptors by cytokines. The receptors for Mac-1 ($\alpha_M\beta_2$) and VLA-4 ($\alpha_4\beta_1$; CD49α:CD29), ICAM-1 and VCAM-1 respectively, are both strongly induced by pro-inflammatory cytokines, including TNF-α and IL-6, in a wide range of cell types (Zohlnhofer et al., 2000). Besides their inducible expression in normal EC (HUVEC), ICAM-1 and VCAM-1 have been shown to be greatly induced by pro-inflammatory cytokines in transformed endothelial cell lines and tumour – derived cell lines (Alexandroff et al., 1994; Chadwick et al., 1998). In contrast, CD31, which localises to intracellular junctions of EC and is required for transmigration of both monocytes and neutrophils, is not up regulated by TNF-α. However TNF-α does induce redistribution of CD31 on human EC and this may serve as a mechanism for transmigration of leukocytes across the vascular endothelium (Romer et al., 1995). IL-10 has also been reported to inhibit TNF-α induced ICAM-1 expression on EC (Chang et al., 1994). Therefore when discussing CRP effects on monocyte adhesion, the interactions especially in vivo of CRP-mediated monocytic effects, but also the consequences of altered monocytic phenotype on the vasculature, have to be taken into account.

The polymorphism located in codon 131 of the FcγRIIa gene, that encodes either a histidine or arginine, has previously been reported to affect CRP binding and the Ca^{2+} mobilisation response (Stein et al., 2000), however, has not identified the effects of this polymorphism on downstream monocytic responses. Herein, it is described that he FcγRIIa polymorphism affected CRP response to monocytic CD11b expression in normals, where the R/R genotype had a significantly greater CRP induced CD11b response than either heterozygotes or H/H homozygotes, where homozygotes had little or no significant

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response (CD11b expression) to CRP. The consequences of this polymorphism within the general population have been previously discussed in chapter 5, however the result of this phenotype-genotype relationship is intriguing within inflammatory disease. Previous workers have shown there is not a significant relationship between $Fc\gamma RIIa$ H/H or R/R frequency when comparing rheumatoid arthritis (RA) patients with normals (Brun *et al.*, 2002). However, they did notice a significant relationship between disease severity and $Fc\gamma RIIa$ polymorphism frequency, with R/R homozygotes, having a much higher probability of increased severity or aggressiveness of disease (RA). Therefore, the fact that CRP mediated responses in R/R homozygotes are much greater, could contribute to disease severity or disease aggressiveness. There also could be a relationship between $Fc\gamma RIIa$ polymorphisms and end points of disease. As the $Fc\gamma RIIa$ polymorphism will determine CRP mediated monocytic phenotype, and in inflammatory diseases such as RA, there is a direct relationship between RA and atherosclerosis (as reviewed in; Odeh, 1991), the two could be related through CRP mediated induction of pro-inflammatory monocyte phenotype. However this hypothesis still remains to be elucidated.

The effect of CRP on monocyte intracellular peroxide formation was examined as ROS are believed to play an important role in transducing pro-inflammatory signals to gene expression. CRP incubations significantly increased cytosolic peroxides in monocytes. This CRP mediated increase in monocytic peroxides was quenched by pre-incubation with antioxidants such as vitamin C or E. Therefore, it would appear that CRP engagement with $Fc\gamma R$ increases peroxide levels via signalling through Syk. It can be theoretically assumed that after $Fc\gamma RIIa$ engagement Syk phosphorylation, signalling through various adapter proteins containing SH2 domains (as previously described), may lead to cellular peroxide generation (see figure 7.1), via NADPH oxidase.

As previously discussed (chapters 1&5) another important source of ROS within monocytes is the mitochondria. However, the method used herein, utilising the fluorescent dye DCFH-DA, does not identify the source of CRP mediated changes in peroxide production, nor the exact molecules involved. Therefore the role of each intracellular peroxide generating system in response to CRP still remains to be elucidated. Mitochondrial ROS production can be analysed utilising the mitiochondrial specific properties of the brightly fluorescent probe rhodamine 123. Its precursor, dihydrorhodamine 123 (DHR-123) is uncharged and non-fluorescent, passively diffusing across membranes where it is converted to rhodamine 123 by intracellular esterases. This cationic cyanine dye accumulates in the electrically negative compartments such as the mitochondria. The large surface area of the mitochondrial matrix binds large amounts of dye. Like DCFH-DA, rhodamine 123 reacts with peroxide to produce a fluorescent compound (Rothe *et al.*, 1991). To further examine the contribution of the respiratory burst to change in ROS, specific NADPH oxidase inhibitor can also be used, such as diphenylene iodonium, which has been shown to prevent ROS generation in CD95 activated cells (Suzuki *et al.*, 1998; Suzuki & Ono, 1999).

Previous work has demonstrated that the non-receptor tyrosine kinase Syk can be activated by oxidative stress following treatment of cells with H_2O_2 (Qin *et al.*, 1996). Although the receptor for oxidative stress was not identified, oxidative stress-induced tyrosine phosphorylation of Syk was dependent on the presence of Src-family PTK Lyn in B cells (Qin *et al.*, 1996). Also H_2O_2 treatment of MNC has been previously shown to induce elevation of CD11b that was allied to enhanced adhesion to resting or TNF-α activated

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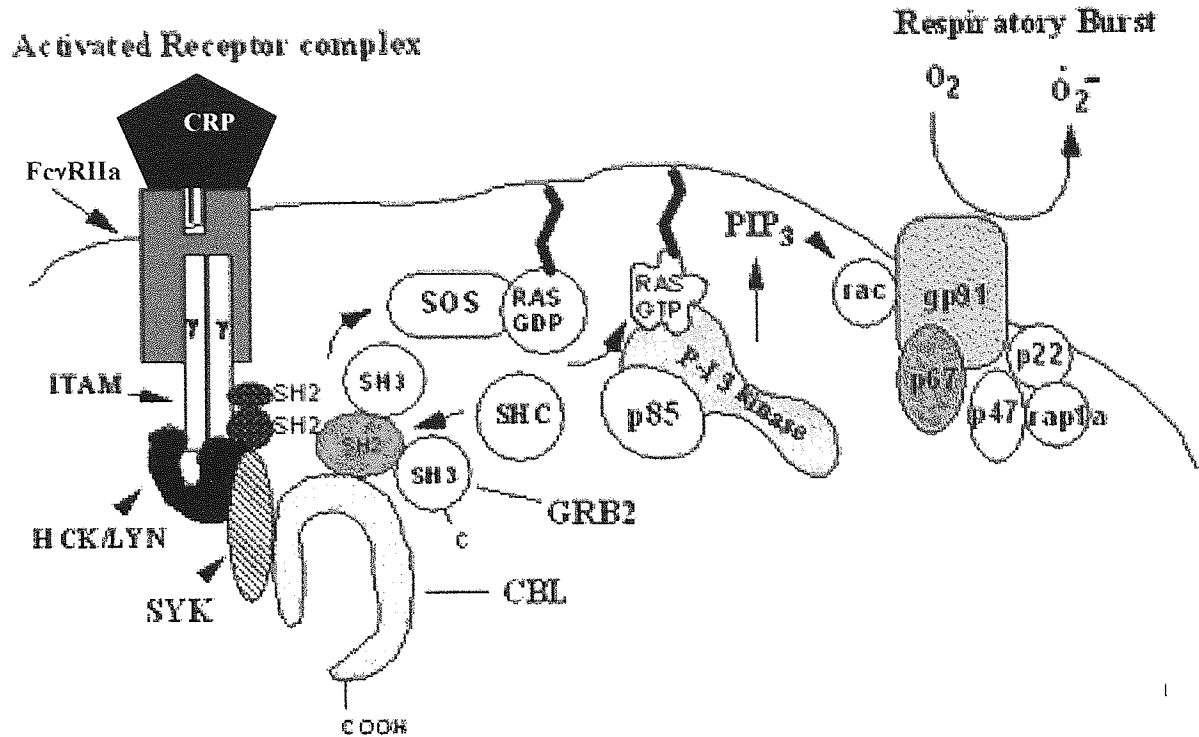
Figure 7.1. Theoretical model for CRP mediated FcγRIIa-induced ROS generation in monocytes. CRP binds to the FcγRIIa subunit resulting in a conformational change in the homodimeric ITAM subunits. This change induces the activation of HCK kinase activity that results in the tyrosine phosphorylation of the ITAM motif of FcγRIIa. Phosphorylation of FcγRIIa subunit recruits the binding and activation of the HCK, LYN and SYK kinases. Other proteins are tyrosine phosphorylated including the CBL and SHC adapter protein. The tyrosine phosphorylation of SHC is noted to bind to GRB2 (not shown) and the SOS nucleotide exchange protein, thus activating small GTPases in the cell through the conversion of GDPras to GTPras. GTPras activates downstream cascades including PI-3 kinase that generates PIP₃ and activates other pathways. The role of CBL phosphorylation is to recruit to the receptor complex the PI-3 kinase p85 subunit. Downstream targets for FcγRIIa stimulation are the small GTPases, RAS, RAP1A, and RAC, which control the myeloid respiratory burst. The respiratory burst response involves the macromolecular assembly of p47phox, p67phox, p40phox, p91phox and p22phox along with RAP1A and RAC resulting in the generation of superoxide anions. (SH2, src homology 2 domain; SH3, src homology 3 domain; SOS, "son of sevenless" protein.)

HAEC (Human Artery Endothelial Cells). Similar results were obtained following MNC exposure to xanthine and xanthine oxidase to mediate free radical production. However, treatment of MNC with H_2O_2 and desferroxamine to chelate Fe^{2+} and prevent the formation of $OH^.$, did not alter the elevation in the adhesion of monocytes treated with only H_2O_2. It was reasoned that $OH^.$ radicals do not contribute peroxide mediated monocyte adhesion (Fraticelli *et al.*, 1996). Therefore this paper suggests that the exposure of leukocytes to oxidants may promote their interaction with endothelial cells via a peroxide sensitive up-regulation in the membrane expression of monocyte adhesion molecules. Thus essentially indicating that the monocytic changes seen after CRP incubations may be a result of the CRP mediated change in peroxide formation. However, in the context of this study where the determination of CRP mediated oxidant elevation and integrin expression which was limited to CD11b, antioxidant monocytic pre-incubations had no effect on CRP mediated CD11b expression, showing that this CRP mediated phenotypic change, was not a result of cellular peroxide changes. This points towards the activation of separate signalling pathways by CRP-FcγRIIa interactions via Syk, peroxide production and integrin expression which, at a protein mobilisation stage, may not be connected.

Whilst epidemiological evidence strongly supports a role for antioxidants in prevention of cardiovascular disease, there has been much conjecture in the literature regarding the benefits of dietary antioxidants; antioxidant supplementation studies using biomarkers of oxidation as endpoints have shown benefit in some cases and adverse effects in others (as reviewed in; Griffiths *et al.*, 2002). Furthermore, large intervention trials examining disease outcome have shown no clear benefit (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardio, 1999; Stephens *et al.*, 1996). However, to date

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interest has focused on the use of lipophillic antioxidants particularly vitamin E because of their direct association with LDL. Whilst LDL deposition is a clear pathological hallmark of CVD, the adhesion of monocytes to endothelium and their subsequent extravastion into the vessel walls is one of the prominent features of atherosclerosis. In the absence of any benefit through antioxidant effects on LDL peroxidation, regulation of monocyte recruitment remains an attractive therapeutic target. Previous work has also shown that the expression of several chemokine receptors (CCR2) and integrins (CD11b) are regulated by ROS and subject to inhibition by synthetic antioxidants (Weber *et al.*, 1999; Weber *et al.*, 1994), where uptake of vitamin C as dehydroascorbate into monocytes occurs rapidly in a non-saturating manner via GLUT receptors (McCrohan *et al.*, 1999). In vivo, an elevation of intracellular ascorbate may again reduce both responsiveness to chemokines through down-regulation of CCR2 and down-regulation of integrins, with an associated reduction in adhesion/transmigration to the endothelium. Further evidence in support of this hypothesis is provided by the observation that vitamin C uptake is reduced in diabetes mellitus, and is associated with increased integrin expression on monocytes and elevated adhesion to endothelial cells (Price *et al.*, 2001). Therefore the hypothesis that long-term intervention of vitamin C and E supplementation would benefit through reducing monocytic integrin expression and subsequent adhesion was tested, in normal subjects. Only those that had a lower than average baseline serum concentration of ascorbic acid received any benefit from vitamin C supplementation for 6 weeks, with regards to monocyte adhesion to EC, where no benefit from taking vitamin E was noted. The hypothesis that this reduction in adhesion through CD11b expression following vitamin C supplementation was not supported. However as discussed in chapter 6 this may be related to previous observations that phenotype does not always inform on function, but where conformational activation is the key determinant of activity.

As previous workers have noted that antioxidant supplementation may reduce serum CRP concentration (Devaraj & Jialal, 2000) due to the inhibition of the systemic inflammatory response, CRP concentrations were measured pre- and post-supplementation with vitamin C or E for 6 weeks. There was no significant reduction in serum CRP concentration noted after supplementation with either vitamin C or E, however, there was a slight trend towards a reduction with vitamin E supplementation. This may reach significance if larger doses of vitamin E over longer periods are examined due to the longer time required to saturate tissue supplies. However the lack of any significant change may be due to studying normals as a test group, where CRP concentrations may not be able to be reduced through intervention. However, groups with slightly raised serum CRP concentrations, such as in smokers (Rohde *et al.*, 1999) or obesity where there is a strong correlation between BMI (body mass index) and CRP levels (Rohde *et al.*, 1999), may show a reduction on CRP in response to antioxidant intervention.

In men participating in the Physicians' Health Study, taking aspirin, supplementation for 7 days, with 81 or 325mg aspirin per day did not lower pre- or post-exercise CRP levels (Feng *et al.*, 2000). However, a 6-week course (300mg/day) lowered CRP levels in patients with chronic stable angina (Ikonomidis *et al.*, 1999), although the net reduction was relatively small. The CARE (Cholesterol Recurrent Event Investigation) trial of secondary prevention randomised post-MI patients to receive 40mg/day pravastatin or placebo. In a retrospective analysis of this trial, there was an association between recurrent MI and high baseline levels of CRP. The proportion of recurrent coronary events prevented by pravastatin was 54% in the subgroup with inflammation (those above the 90th percentile of CRP) and 25% in the subgroup without inflammation (Ridker *et al.*, 1998).

In another analysis of the CARE data, the same group found that median CRP levels decreased by 17% in those allocated to pravastatin. At the 5yr analysis, the difference in median CRP levels (21.6%) and the absolute mean change in CRP (0.137μg/ml) between placebo and pravastatin groups were significant, but still relatively minor (Ridker *et al.*, 1999). Therefore, an effective intervention strategy for lowering resting serum CRP levels or interfering with its biological activities still remains to be elucidated.

7.2 Conclusion:

Overall these results show that CRP is able to bind FcγRIIa. CRP binding FcγR initiates an intracellular signalling cascade that phosphorylates the non-receptor tyrosine kinse, Syk, associated with intracellular tyrosine activating motifs on the cytoplasmic tail of Fcγ receptors. Syk phosphorylation leads to PI turnover, which ultimately leads to Ca^{2+} mobilisation in monocytes. This CRP mediated Syk phosphorylation in monocytes leads to an increase in CD11b and IL-6 expression. CRP engagement with monocytes also leads to an increase in intracellular peroxide, which can be inhibited in vitro using the antioxidants α-tocopherol and ascorbic acid. Monocyte adhesion to endothelial cells was inhibited by dietary vitamin C but not E for LOC subjects. CRP mediated CD11b expression is not redox regulated by CRP mediated changes in cytosolic peroxides. Nor were antioxidant supplements able to modulate serum CRP levels or adhesion molecule expression in vivo. The FcγRIIa polymorphism at codon 131 affects the phenotypic driven changes induced in monocytes by CRP, where R/R allotypes have a greater increase in CD11b, which may be involved in promoting the monocytic inflammatory response. CRP leads to an alteration in the balance of pro- (and maybe anti-) inflammatory cytokines, which alters the immune phenotype of circulating monocytes.

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In conclusion CRP appears to be much more than just a marker of ongoing inflammation or associated inflammatory disease and disease activity. At pathophysiological concentrations, CRP may be able to directly modulate inflammation through interacting with monocytes and alter the inflammatory response associated with vascular inflammatory diseases.

8.0 References

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9.0 Appendix
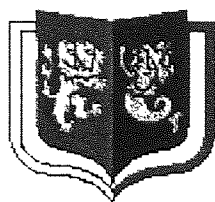
UNIVERSITY OF BIRMINGHAM
School of Medicine
Edgbaston, Birmingham

ASTON UNIVERSITY

DONOR INFORMATION SHEET

Title: RESEARCH INTO THE IMPORTANCE OF ENDOTHELIAL CELLS IN VASCULAR PATHOLOGY

Principal Investigator: **Professor Gerard Nash**, Dept. Physiology, University of Birmingham
Co-applicant **Professor Caroline Savage**, Dept. of Medicine, University of Birmingham
Co-applicant **Professor David Adams**, Dept. of Medicine, University of Birmingham
Co-applicant **Professor Asif Ahmed**, Div. Reproductive and Child Health, University of Birmingham
Co-applicant **Dr. Helen Griffiths**, Pharmaceutical Sciences, Aston University

WHAT IS THE STUDY ABOUT?
We are investigating how some blood cells stick to the wall of blood vessels and cause damage, and how new blood vessels grow in healthy and damaged tissue. The damage contributes to diseases in several organs. We aim to develop new methods of modifying these processes, so that we can protect people against diseases affecting blood vessels.

WHAT DO WE NEED?
To carry out these studies, we need to obtain samples of human blood vessels. We can grow the cells which line the blood vessels (endothelial cells) in the laboratory and study how blood cells stick to them, and how the endothelial cells help form new vessels.

WHY USE THE UMBILICAL CORD AND WHAT WILL HAPPEN TO IT?
The umbilical cord supplies blood from the mother to the baby before birth. It contains blood vessels that have been studied in research all over the world. It is the only widely available source of human endothelial cells and is very important for medical research.
Both the cells that we extract from the cord vessels and the cords are destroyed after use. The cells will not be used for genetic studies. We will not hold any information on the donors. The scientific information that we obtain will be made freely available to other doctors and scientists, so that maximum benefit can be obtained

WHAT WILL I HAVE TO DO?
We are asking you to agree to let us have a section of the umbilical cord, which would otherwise be destroyed after your child's birth.

WHAT ARE THE BENEFITS?
We aim to get a better understanding of how blood vessels become damaged and to develop new methods for avoiding such damage.

WHAT ARE THE RISKS?
There are no risks to you or your baby.

WHAT IF I DO NOT WANT TO TAKE PART?
You are not obliged to consent. This decision will have no effect on your treatment.
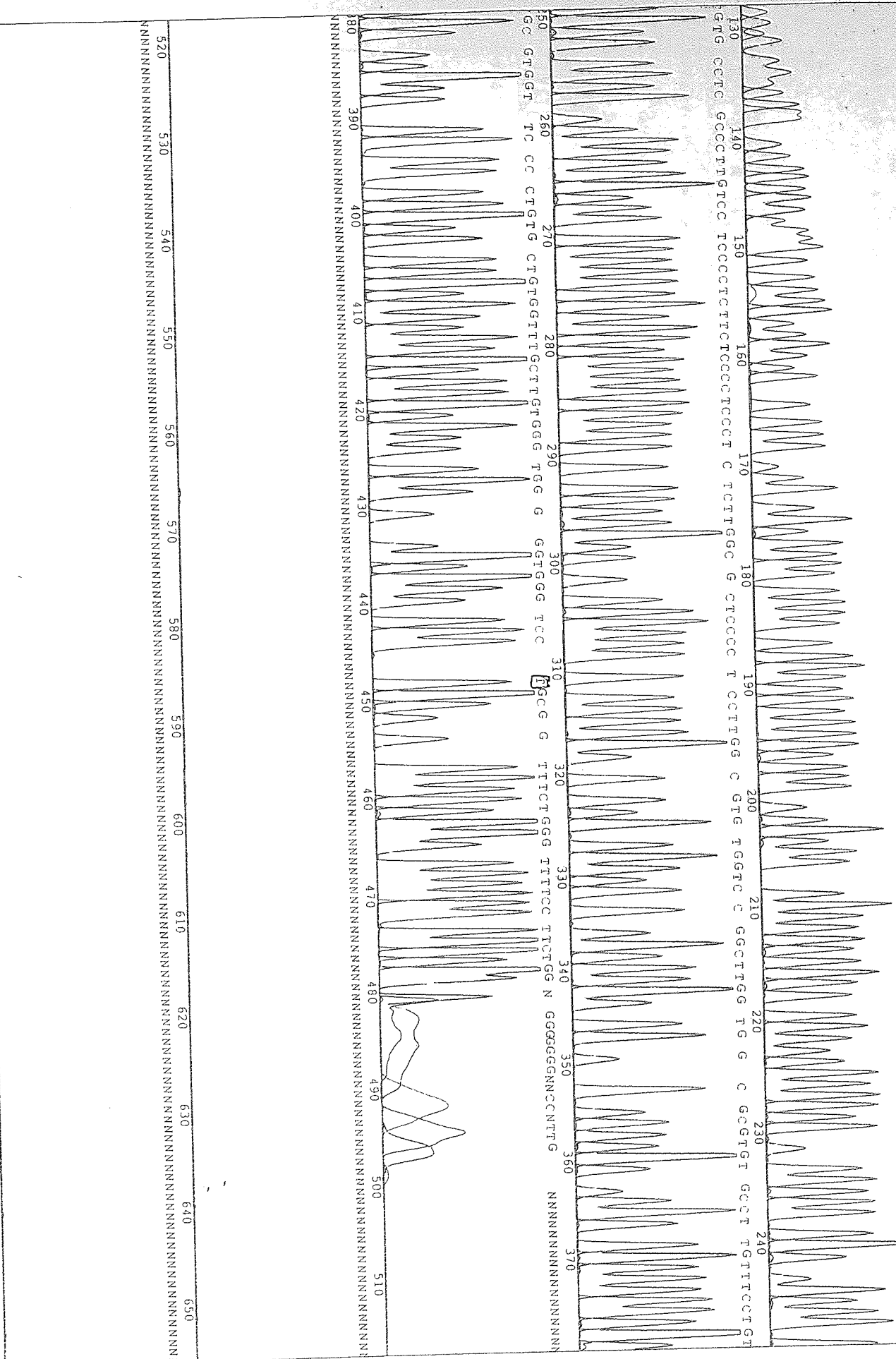
WHAT HAPPENS NOW IF I GIVE CONSENT?
We will cut off a portion of the umbilical cord, after any necessary medical tests have been carried out. This will be done after it has been sent for disposal.

CONTACT NAME AND NUMBER: Prof.. Gerard Nash.

Aston University

ATTGGA TCCC TTCTCCA TCCCACACAG CAAACCACAG TCCCC GTGGTG TTCC CTGCC C GGG C T GGCT C CGCTGTTCTC TCC GCCTGTG CC TC CTGTCC GGT TGG

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2/4 (A/G)

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R/R (G/G) Forward