The Role Of Immunoglobulin Receptors In The Pathogenesis Of Rheumatoid Arthritis

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ABSTRACT
Historically, a key immunological feature of rheumatoid arthritis is the presence of small circulating immunoglobulin G (IgG) rheumatoid factor-based immune complexes. More recently, tumour necrosis factor alpha (TNFα) has been shown to be an important mediator of inflammation in rheumatoid arthritis. The aim of this thesis was to test a hypothetical model outlining an effector mechanism for inflammation in rheumatoid arthritis. This model predicts that small immune complexes comprising self-associating IgG rheumatoid factors, owing to their small size, evade complement-mediated clearance from the circulation and access tissue macrophages. These complexes may initiate inflammation within synovium by binding to one of three immunoglobulin receptors for IgG (FcγR). Unlike FcγRI and FcγRII, FcγRIIIa expression by macrophages correlates with the location of synovitis and extra-articular features in patients with rheumatoid arthritis. Selective FcγRIIIa crosslinking by IgG rheumatoid factor complexes may trigger macrophage activation and the subsequent production of proinflammatory mediators such as TNFα.

Murine IgG anti-FcγR monoclonal antibodies (mAb) to each FcγR were used as surrogates for small immune complexes. Of the three anti-FcγR mAb, only that directed against FcγRIIIa triggered adhered monocytes to release TNFα, IL-1α and reactive oxygen species. This confirmed previous evidence that for signalling, FcγRI and FcγRII require multiple receptor aggregation. Further findings indicated anti-FcγRIII mAb-induced TNFα release to be dependent on crosslinking of either three FcγRIIIa receptors, or two receptors where one FcγRIIIa bound an IgG Fc fragment. Small, soluble human IgG anti-NIP mAb immune complexes were also able to trigger adhered monocytes to release TNFα in a subclass dependent manner. Moreover, although this TNFα response was found to be dependent upon both FcγRII and FcγRIIIa, FcγRIIIa appeared to play a dominant role.

The findings of this thesis support the hypothesis that small IgG rheumatoid factor based immune complexes may trigger the production of TNFα and other proinflammatory mediators from macrophages in rheumatoid arthritis, and implicate an important role for FcγRIIIa.
For Mum and Dad

In memory of Nana
THANKS
To Jo Edwards and Jo Cambridge. You’ve both inspired, encouraged and supported me over the past few years. I cannot thank you enough.

Thanks go to Celltech Therapeutics Ltd for their financial support.

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DECLARATION
The work presented in this thesis was carried out by the author unless otherwise stated.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADCC</td>
<td>Antibody dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BES</td>
<td>N, N-bis [2-Hydroxyethyl]-2-aminoethanesulfonic acid</td>
</tr>
<tr>
<td>BIC</td>
<td>Bicarbonate buffer</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CH</td>
<td>Heavy chain constant domain</td>
</tr>
<tr>
<td>CGM</td>
<td>Complete growth medium</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>cps</td>
<td>Counts per second</td>
</tr>
<tr>
<td>CR1</td>
<td>Complement receptor 1</td>
</tr>
<tr>
<td>CR2</td>
<td>Complement receptor 2</td>
</tr>
<tr>
<td>DAF</td>
<td>Decay accelerating factor</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>F</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Fab</td>
<td>Monovalent antigen binding fragment of immunoglobulin</td>
</tr>
<tr>
<td>F(ab’)₂</td>
<td>Divalent antigen binding fragment of immunoglobulin</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorter</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallisable</td>
</tr>
<tr>
<td>FcαR</td>
<td>Fc receptor for IgA</td>
</tr>
<tr>
<td>FcεR</td>
<td>Fc receptor for IgE</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc receptor for IgG/Fc gamma receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidylinositol</td>
</tr>
<tr>
<td>H</td>
<td>Histidine</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution without phenol red</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N’-2-ethane sulphonic acid</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leucocyte antigen</td>
</tr>
</tbody>
</table>
H₂O₂  Hydrogen peroxide
HOCl  Hypochlorous acid
HPLC  High performance liquid chromatography
HRP  Horse radish peroxidase
I  Iodine
IgA  Immunoglobulin A
IgE  Immunoglobulin E
IgG  Immunoglobulin G
IgM  Immunoglobulin M
IL-1  Interleukin-1
IL-4  Interleukin-4
IL-6  Interleukin-6
IL-8  Interleukin-8
IL-10  Interleukin-10
IL-1R  Interleukin-1 receptor
IL-1Ra  Interleukin-1 receptor antagonist
IFNγ  Gamma interferon
ITAM  Immunoreceptor tyrosine-based activation motif
ITIM  Immunoreceptor tyrosine-based inhibitory motif
κ  Kappa
λ  Lambda
L  Leucine
LGL  Large granular lymphocyte
LPS  Lipopolysaccharide
mAb  Monoclonal antibody
MCP-1  Monocyte chemoattractant protein-1
M-CSF  Macrophage colony-stimulating factor
MFI  Mean fluorescent intensity
MgCl  Magnesium chloride
MHC  Major histocompatibility complex
MM  Modified medium
MMP  Matrix metalloproteinase
MPO  Myeloperoxidase
mRNA  Messenger ribonucleic acid
MW  Molecular weight
NA  Neutrophil antigen
NaCl  Sodium chloride
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
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<tbody>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NIP</td>
<td>5-iodo-4-hydrox-3-nitrophenacetyl</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide</td>
</tr>
<tr>
<td>'OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBT</td>
<td>Phosphate buffered saline with 0.1% tween</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>pH</td>
<td>Log of free proton concentration</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12 myristate 13-acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear cell</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>R</td>
<td>Arginine</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute essential media</td>
</tr>
<tr>
<td>rsc</td>
<td>Receptor sites per cell</td>
</tr>
<tr>
<td>S.D.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SPA</td>
<td>Staphylococcal protein A</td>
</tr>
<tr>
<td>SPG</td>
<td>Streptococcal protein G</td>
</tr>
<tr>
<td>TACE</td>
<td>TNFα converting enzyme</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethylbenzidine (Dihydrochloride)</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNF-R</td>
<td>Tumour necrosis factor receptor</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris [hydroxymethyl] aminoethane</td>
</tr>
<tr>
<td>V</td>
<td>Valine</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VH</td>
<td>Variable light chain</td>
</tr>
<tr>
<td>VL</td>
<td>Variable heavy chain</td>
</tr>
<tr>
<td>ζ</td>
<td>Zeta</td>
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CHAPTER 1

INTRODUCTION
1.1. History of rheumatoid arthritis

Indian medical literature from 2000 years ago (Sturrock et al., 1977) and writings by Sydenham from 1676 may both have been describing patients with rheumatoid arthritis. However, while studies in human palaeopathology have shown the existence of both osteoarthritis and ankylosing spondylitis in the ancient and medieval worlds, convincing evidence for the existence of rheumatoid arthritis as a disease entity prior to the nineteenth century is lacking (Short, 1974; Thould & Thould, 1983). The first detailed account of a patient with all the clinical features of rheumatoid arthritis was not reported until the early 1800's by the French medical student, Augustin-Jacob Landré-Beauvais (Snorrason, 1952). This student wrote a case report on a patient presenting with what was thought to be a previously undefined form of gout (Short, 1974). The term rheumatoid arthritis was first used by the London physician Sir Alfred Baring Garrod in 1859 (Garrod, 1859).

Notwithstanding the uncertainty over its antiquity, rheumatoid arthritis has been known about for at least 200 years and yet its precise aetiology and mechanism of pathogenesis remain undetermined.

1.2. Clinical and pathological features of rheumatoid arthritis

Rheumatoid arthritis is a debilitating inflammatory joint disease which primarily targets the synovial membrane. Approximately 1-3% of the world's population is affected by rheumatoid arthritis (Harris, 1990), with a female:male ratio of 3:1 (da Silva & Hall, 1992). The onset of symptomatic disease often appears between 35 and 55 but can affect individuals of any age. Rheumatoid arthritis often begins as a swelling within the joints of the hands (metacarpophalangeal and proximal interphalangeal) wrists or feet (metatarsophalangeal), initially in a symmetrical pattern. It may then progress to any other synovial joint, the knee being a common target. Patients present with a raised erythrocyte sedimentation rate, indicative of a systemic process and obvious swellings of the soft tissue over joints, due to inflammation of the synovium. Around 80% of patients with rheumatoid arthritis are seropositive for rheumatoid factor (see 1.6. Rheumatoid factors and rheumatoid arthritis).
The normal synovium consists of an intimal lining layer of fibroblast-like synoviocytes and bone marrow derived macrophages (Edwards et al., 1982). Macrophages are also present within the subintima and deeper into the synovial tissue. Lymphocytes can be seen within the normal synovium, but at low levels. In contrast, the inflamed rheumatoid synovium is characterised by the infiltration of macrophages, lymphocytes, plasma cells and some polymorphonuclear (PMN) cells. Most notably, intimal macrophages increase in both number and size resulting in "lining hyperplasia" (Henderson et al., 1988). Many lymphocytes appear scattered throughout the tissue, however lymphoid aggregates often occur. In approximately 10% of patients, secondary follicles of B cells with germinal centres form (Edwards & Wilkinson, 1995).

Cartilage destruction and pannus formation follows. At this stage, joints are obviously swollen and muscle wasting may become apparent, particularly in the hands. Eventually, inflammation becomes less prominent and the joint erosion ultimately results in permanent distortion.

1.2.1. Extra-articular features of rheumatoid arthritis
In addition to synovitis, about 20% of seropositive patients display extra-articular features such as rheumatoid nodules, lung and cardiac involvement (Maini & Zvaifler, 1998). Seronegative patients with rheumatoid arthritis rarely have these extra-articular features.

Rheumatoid nodules occurring at points of pressure, such as over the bony prominences around the elbow, are generally associated with more severe and erosive arthritis. They can also be found in the connective tissue of the lung or heart (Ziff, 1990). The rheumatoid nodule consists of a central necrotic core of fibrin which is surrounded by a palisade of mostly macrophages and fibroblasts (Hedfors et al., 1983). The connective tissue layer surrounding this palisade consists of infiltrating monocytes, some T cells and immunoglobulin-secreting plasma cells (Ziff, 1990).

The most common form of lung involvement is pleurisy, however, pulmonary fibrosis and alveolitis are also found in patients with
rheumatoid arthritis (Anaya et al., 1995). Other systemic features include pericarditis and a rise in liver enzymes (Maini & Zvaifler, 1998).

The occurrence of systemic disease is often neglected when attempting to describe the pathogenesis of rheumatoid arthritis. In order to fully elucidate a disease mechanism, both intra- and extra-articular features need to be explained.

1.3. Aetiological features of rheumatoid arthritis

The prevalence of rheumatoid arthritis has familial links. Compared with more distant relatives, first degree relatives are more likely to develop rheumatoid arthritis. Additionally, the frequency of this disease in monozygotic twins is greater than in dizygotic twins (Reveille, 1998). However, there is only a 15% concordance rate in monozygotic twins (Jarvinen & Aho, 1994), suggesting other contributing factors. Hence, rheumatoid arthritis is a multifactorial disease.

The genetics of rheumatoid arthritis is partly determined by gender, with more women that men being affected. Furthermore, hormonal changes appear to effect disease onset and severity (da Silva & Hall, 1992).

Infectious agents are an attractive explanation for the initiation of rheumatoid arthritis. A large number of patients with rheumatoid arthritis have higher antibody titres to Epstein-Barr virus (EBV) antigens compared with controls (Alspaugh et al., 1981). However, some patients with rheumatoid arthritis lack anti-EBV antibodies (Venables et al., 1988). Other viral infections such as retroviruses and parvoviruses (Krause et al., 1996) have been associated with rheumatoid arthritis, as have a number of bacterial infections such as Mycobacterium tuberculosis and Proteus mirabilis (Holoshitz et al., 1986, Senior et al., 1995). Nonetheless, there is no evidence to suggest a causative role for any bacterial or viral agents in rheumatoid arthritis.

Rheumatoid arthritis is genetically associated with the class II major histocompatibility complex (MHC). Major histocompatibility complex molecules are encoded by human leucocyte antigen (HLA) genes which are located on chromosome 6 and are highly polymorphic. The genes for
Class II MHC are found in region D which can be further subdivided into regions R, Q and P. These genes are therefore referred to as HLA-DR, -DQ or -DP (van Jaarsveld et al., 1998a). The HLA-DR β chain gene, DRB1, encodes for HLA-DR alleles 1-4, now termed 01-04 (Gregersen et al., 1987).

Rheumatoid arthritis is linked with specific HLA-DR allotypes. Approximately 60-70% of Caucasian patients with rheumatoid arthritis are HLA-DR4 positive and these patients are more likely to have severe erosive disease with extra-articular features (Winchester, 1994; van Jaarsveld et al., 1998b; Meyer et al., 1999). Stastny (1978) was the first to report this link and found the DR4 subtype, Dw4 (HLA-DRB1*0401), to be associated with susceptibility to rheumatoid arthritis. Since this finding, other DR4 alleles have also been associated with rheumatoid arthritis, such as Dw14 (HLA-DRB1*0404/0408) and Dw15 (HLA-DRB1*0405) (Nepom et al., 1989; Yelamos et al., 1993; Meyer et al., 1999). However, a number of Caucasian patients and individuals from other racial backgrounds with rheumatoid arthritis are DR4 negative. In these cases different HLA-DR types have been connected with disease susceptibility and severity. HLA-DR1 (HLA-DRB1*0102) has been found in DR4 negative Caucasians as well as in Israeli Jews with rheumatoid arthritis (de Vries et al., 1993), while possession of HLA-DR10 has a high frequency in Spanish patients (Yelamos et al., 1993).

In 1987 Gregersen et al. proposed a hypothesis to explain the link between HLA-DR and rheumatoid arthritis. It was found that certain HLA-DR alleles shared amino acid sequences around position 70 in the third hypervariable region, encoding for the first MHC molecule domain. It was suggested that this "shared epitope" promoted disease susceptibility by influencing the interaction between MHC class II and T cell receptor (TCR) at the level of antigen presentation. Additionally the "shared epitope" may influence TCR selection in the thymus resulting in a genetically predetermined immune repertoire (Gregersen et al., 1987; Kohsaka et al., 1998). However, a link between the genetics of MHC molecules and either the susceptibility to, or the severity of rheumatoid arthritis may simply imply that an adaptive immune response is involved since antigen presentation is common to all adaptive
responses. This leaves open the question of how the genetics of MHC molecules can contribute to rheumatoid arthritis in conjunction with other genetic and non-genetic factors.

1.4. Mediators of inflammation and tissue damage in rheumatoid arthritis

1.4.1. Cytokines and rheumatoid arthritis
Cytokines are soluble proteins which mediate signals between cells, either in an autocrine or paracrine manner. Cytokines are an important component of host defence when produced locally. However, excess, sustained or systemic cytokine production can be harmful to the host and may even contribute to disease. Two cytokines, tumour necrosis factor (TNF) and interleukin-1 (IL-1) have been particularly implicated in the pathogenesis of rheumatoid arthritis.

1.4.2. Tumour necrosis factor
The proinflammatory cytokine, TNF, acquired its name from the observation that its production in response to bacterial endotoxin caused the necrosis of tumours in vivo (Carswell et al., 1975; Balkwill, 1989). TNF exists as two isoforms, TNFα and lymphotoxin (formally TNFβ) (Balkwill, 1989). Lymphotoxin (TNFβ) is now known as lymphotoxin-α following the identification of an additional member of the TNF family, lymphotoxin-β (Bronwing et al., 1993). TNFα and lymphotoxin-α exert similar effects on target cells. Lymphotoxin is only produced by T lymphocytes while TNFα can also be produced by macrophages and mast cells (Balkwill, 1989). The genes for TNFα and lymphotoxin-α have been mapped to the MHC region on chromosome 6 and their protein products exhibit 30% homology (Pennica et al., 1984; Nedwin et al., 1985; Spies et al., 1986). Lymphotoxin-β has also been mapped to the same region on chromosome 6 and exhibits 21% homology with TNFα and 24% homology with lymphotoxin-α (Bronwing et al., 1993).

TNFα is produced intracellularly as a 26-kDa polypeptide. TNFα is also found in the plasma membrane in this form and at this site has cytotoxic activity (Kriegler et al., 1988). Surface bound TNFα is enzymatically cleaved to release a trimer (of 17-kDa subunits) of biologically active,
extracellular TNFα (Wingfield et al., 1987). Membrane-associated pro-
TNFα is processed by the enzyme, TNFα converting enzyme (TACE),
which is membrane-bound and a member of the a disintegrin and
metalloprotease (ADAM) family (Black et al., 1997; Moss et al., 1997) More
recently, work has revealed that TACE also plays a role in the shedding of
other surface molecules including TNF receptors (Peschon et al., 1998).

1.4.3. Tumour necrosis factor receptors
Two receptors for TNFα and lymphotoxin-α have been identified and
their genes cloned (Brockhaus et al., 1990; Gray et al., 1990; Loetscher et
al., 1990; Schall et al., 1990). The type I TNF receptor (TNF-RI) has a
molecular mass of 55-kDa while the type II TNF receptor (TNF-RII) has a
molecular mass of 75-kDa (Brockhaus et al., 1990). Both receptors have
similar extracellular domains containing four cysteine-rich domains and
are homologous to the cell surface molecules, nerve growth factor
receptor, B cell activating CD40, the human T cell activation marker
CD27 and the rat T cell activation marker OX40 which all belong to the
same receptor family (Dembic et al., 1990; de Jong et al., 1991). Both
receptors can bind TNFα and lymphotoxin-α (Gray et al., 1990; Tartaglia
& Goeddel, 1992). Furthermore, the extracellular domains of both
receptors can exist as functional soluble receptors and therefore natural
inhibitors of TNF (Nophar et al., 1990). The cytoplasmic domains of TNF-
RI and TNF-RII are unrelated and, as a result, trigger distinct pathways
(Dembic et al., 1990). TNF-RI mediates most TNF related effects including
necrosis of tumours and cytotoxicity, while the functions of TNF-RII are
more limited. Although both TNF-R can activate nuclear factor κB, the
cytoplasmic domain of TNF-RI contains TNF-RI-associated death
do mains which allow this receptor to also trigger apoptosis (Yuan, 1997).
Lymphotoxin-β cannot bind either TNF-RI or TNF-RII but instead when
complexed with lymphotoxin-α to form a heterotrimer, binds the specific
lymphotoxin-β receptor which is expressed by activated lymphocytes
(Force et al., 1995).

TNFα, as many cytokines, is pleiotrophic and can therefore affect many
different cell types. This is made possible by the wide expression of TNF
receptors. Monocytes, macrophages and natural killer cells express both
TNF-RI and TNF-RII (Dembic et al., 1990; Naume et al., 1991). Resting T
and B lymphocytes express little, if any TNF receptors. However, following stimulation both CD4 and CD8 positive T cells and B cells express predominantly the type II TNF receptor (Erikstein et al., 1991; Ware et al., 1991), while fibroblasts and epithelial cells express mainly the type I TNF receptor (Brennan et al., 1992a).

1.4.4. Interleukin-1

There are three members of the IL-1 gene family, located on the long arm of chromosome 2 at position 2q13 - 2q21 (Webb et al., 1986). These genes encode for IL-1α, IL-β and IL-1 receptor antagonist (IL-1Ra). All three genes have been cloned (Auron et al., 1984; Lomedico et al., 1984; Eisenberg et al., 1990). IL-1 is produced by a wide range of cells, most notably monocytes, macrophages and B lymphocytes, and acts on many cell types, mediating multiple biological effects including fever, anorexia, inflammation, leucocyte activation and the induction of gene expression (reviewed in Dinarello, 1991). Both IL-1α and IL-1β proteins are initially synthesised as 31-kDa precursors (pro-IL-1) and proteolytic processing results in 17-kDa peptides (Hazuda et al., 1988). IL-1α and IL-1β 17-kDa peptides and the phosphorylated precursor form of IL-1α are all biologically active (Dinarello, 1991). IL-1α tends to remain on the cell surface and exert its effects locally. IL-1β is only fully active following intracellular cleavage into the 17-kDa peptide, following which it is readily released (Lonnemann et al., 1989; Dinarello, 1996), however, pro-IL-1β may have some biological activity (Jobbling et al., 1988).

1.4.5. Interleukin-1 receptors

Two IL-1 receptors have been identified. IL-1 receptor type I (IL-1RI) is an 80-kDa protein and the type II receptor (IL-1RII) is a 68-kDa protein (Dinarello, 1991). Both receptors belong to the immunoglobulin-gene family and their genes have been mapped to chromosome 2 (Sims et al., 1995). Both receptors are transmembrane glycoproteins with an extracellular region that contains three immunoglobulin-like domains and both can exist in soluble form. However, only IL-1RI can transduce signals and this is reflected in the short cytoplasmic tail of IL-1RII. Although both receptor types bind IL-1α, pro-IL-1α and IL-1β, IL-1α appears to bind to IL-1RI better, while IL-1RII has a higher affinity for IL-1β. Since IL-1RII cannot transduce a signal, it acts as an IL-1 antagonist.
Another natural antagonist for IL-1 is the IL-1Ra. This protein has a molecular weight of 22-kDa and competes with IL-1 for receptor occupancy (Arend et al., 1990). IL-1RI is expressed by monocytes, T cells, fibroblasts, keratinocytes, endothelial cells, hepatocytes and chondrocytes while IL-1RII expression is more limited being expressed by B cells, neutrophils and bone marrow cells (Dinarello, 1991).

1.4.6. Tumour necrosis factor, interleukin-1 and rheumatoid arthritis

IL-1α is present at high levels in rheumatoid synovial fluids (Fontana et al., 1982) and was the first pro-inflammatory cytokine to be implicated in rheumatoid synovitis and joint destruction (Saklatvala, 1986). TNFα is, however, now thought to be the dominant mediator of inflammation and tissue injury in rheumatoid synovitis (reviewed in Brennan et al., 1992a). TNFα has been detected in the synovial fluid (Hopkins & Meager, 1988), synovial tissue (Chu et al., 1991a) and serum from patients with rheumatoid arthritis (Saxne et al., 1988). Immunohistochemical studies have shown the majority of TNFα-secreting cells in rheumatoid synovia to be macrophages within the synovial lining layer and deeper into the synovium (Wilkinson & Edwards, 1991).

TNFα and IL-1 may be acting alone, additively or synergistically. In vitro studies have provided evidence supporting a role for TNFα and IL-1 in mediating tissue injury and inflammation in rheumatoid arthritis. Both TNFα and IL-1 have been shown to stimulate human synovial cells, dermal fibroblasts (Mizel et al., 1981; Dayer et al., 1985) and synovial fibroblasts (Saklatvala et al., 1985) to produce prostaglandin E₂ (PGE₂) and collagenase. Human chondrocytes have also been shown to produce PGE₂ following stimulation with IL-1 and TNFα (Campbell et al., 1990). Proteoglycan is an essential component of cartilage and proteoglycan loss is seen in patients with rheumatoid arthritis. TNFα has been shown to not only induce osteoclastic bone resorption but also inhibit proteoglycan and collagen synthesis and induce proteoglycan degradation by chondrocytes (Bertolini et al., 1986; Saklatvala, 1986; Thompson et al., 1987). IL-1 also causes chondrocytes to degrade and inhibit the synthesis of new proteoglycan (Saklatvala et al., 1984; Tyler, 1985; Benton & Tyler, 1988) and to bring about cartilage destruction and bone resorption (Gowan et al., 1983; Saklatvala et al., 1985).
TNFα and IL-1 have both been shown to act as chemoattractants for monocytes and polymorphonuclear cells (Luger et al., 1983; Gamble et al., 1985; Ming et al., 1987; Richter et al., 1989; Richter et al., 1995) and to promote leucocyte adhesion to endothelial cells (Bevilacqua et al., 1985). IL-1 and TNFα can also induce the release of chemotactic factors, such as monocyte chemotactic activating factor from fibroblasts (Larsen et al., 1989), and neutrophil chemotactic factor from endothelial cells (Strieter et al., 1989).

Studies by Buchan et al. (1988) showed that IL-1α, IL-1β, TNFα and TNFβ messenger RNA (mRNA) could all be detected in stimulated joint cell cultures isolated from patients with rheumatoid arthritis. Further studies demonstrated that mononuclear cells from rheumatoid synovium or synovial fluids would spontaneously secrete TNFα and IL-1 in the absence of exogenous stimuli. Furthermore, the utilisation of an anti-TNFα monoclonal antibody (mAb) showed this IL-1 production to be dependant upon the presence of TNFα (Brennan et al., 1989). A separate study showed that spontaneous IL-1 production from peripheral blood monocytes in vitro was greater from patients with rheumatoid arthritis than controls or patients with rheumatoid arthritis receiving gold treatment (Danis et al., 1987).

The potential for TNFα and IL-1 to contribute to the pathogenesis of rheumatoid arthritis has also been demonstrated in vivo in a number of experimental models. For example, transgenic mice expressing a 3' modified human TNFα gene developed arthritis (Keffer et al., 1991). By backcrossing this transgenic model with DBA/1 mice, which are susceptible to developing arthritis, disease onset was accelerated and more aggressive (Butler et al., 1997). Administration of a neutralising anti-TNFα/β monoclonal antibody (mAb) to mice with collagen type II-induced arthritis, either prior to the onset of disease or following disease manifestation, significantly reduced disease severity (Williams RO et al., 1992). However, the most convincing evidence for a role for TNFα in rheumatoid arthritis has been from human clinical trials. Following treatment with a chimeric anti-human TNFα monoclonal antibody, patients with rheumatoid arthritis showed a marked improvement (Elliott et al., 1993; Charles et al., 1999).
Following injection of IL-1 into the knee joints of rabbits, both proteoglycan loss and inflammatory cell infiltration were observed (Pettipher et al., 1986). The administration of both anti-IL-1α and anti-IL-1β antibodies to mice before the onset of collagen-induced arthritis completely prevented disease, while treatment after disease manifestation significantly reduced inflammation and cartilage destruction. The anti-IL-1β antibody given alone also significantly reduced disease (van den Berg et al., 1994). Local administration of murine IL-1RI into rats with antigen-induced arthritis reduced joint swelling and tissue injury (Dauer et al., 1994). Therapeutic blockade of IL-1 with recombinant human IL-1Ra in humans is effective, but less striking than for TNFα (Bresnihan et al., 1998).

The evidence discussed so far strongly supports a role for both TNFα and IL-1 in the pathogenesis of rheumatoid arthritis. The suggestion is that both cytokines, but particularly TNFα, act locally either alone or synergistically to induce an inflammatory response, the degradation of surrounding tissues and the recruitment of leucocytes to the site of inflammation.

A host of pro-inflammatory mediators and other cytokines have also been associated with rheumatoid arthritis. Although there is little evidence to support a primary role in disease pathogenesis, these additional agents may arise as a result of the inflammatory response initiated by TNFα and IL-1, and may contribute to inflammation, tissue injury and the generation of a localised immune network in the rheumatoid synovium. Some of the more important mediators of inflammation and tissue injury associated with rheumatoid arthritis will be discussed.

1.4.7. Interleukin-6 and other cytokines
Interleukin-6 (IL-6) is a B cell activator but also induces the production of acute phase proteins, an important systemic feature of rheumatoid arthritis. Elevated levels of IL-6, have been detected in the synovial tissue, synovial fluid and serum from patients with rheumatoid arthritis (Houssiau et al., 1988; Waage et al., 1989; Sack et al., 1994). Synovial macrophages appear to be the main source of IL-6 in the rheumatoid
synovium. Immunohistochemical studies have shown IL-6-containing macrophages to be located close to immunoglobulin-producing plasma cells. Since IL-6 stimulates antibody production this may be significant in the generation of rheumatoid factors within the synovium (Field et al., 1991), as will be discussed later. Interestingly, the treatment of rheumatoid arthritis patients with an anti-TNFα monoclonal antibody significantly reduced IL-6 levels (Elliott et al., 1993). This has suggested that although IL-6 might have a role to play in the perpetuation of inflammation in rheumatoid arthritis, its contribution may be secondary to the production of TNFα.

Other cytokines such as interleukin-11 (Mino et al., 1998) and interleukin-15 (McInnes & Liew, 1998) have been associated with rheumatoid arthritis, however, there is little evidence to support a primary role for these cytokines in disease pathogenesis. Instead, their presence is likely to be a consequence of the inflammatory process within the rheumatoid synovium.

1.4.8. Chemokines and growth factors
Chemokines and growth factors are instrumental in the recruitment of leucocytes to sites of inflammation. The chemokines, monocyte chemotactant protein-1 (MCP-1) and interleukin-8 (IL-8) are potent chemotactic factors for both neutrophils and monocytes (Gerszten et al., 1999). Elevated levels of IL-8 have been detected in the synovial fluids and tissues from patients with rheumatoid arthritis (Brennan et al., 1990a; Deleuran et al., 1994), while chondrocytes stimulated with IL-1 have been shown to release MCP-1 in vitro (Villiger et al., 1992).

A number of growth factors have also been detected at raised levels in patients with rheumatoid arthritis. Granulocyte macrophage colony-stimulating factor (GM-CSF) has been found at elevated levels in rheumatoid synovial fluids (Xu et al., 1989). Moreover, isolated synovial macrophages from patients with rheumatoid arthritis spontaneously secreted GM-CSF in culture and this appeared to be TNFα dependent (Harworth et al., 1991). Additionally, articular chondrocytes have been shown to produce both GM-CSF (Alsalamen et al., 1994) and macrophage colony-stimulating factor (M-CSF) (Campbell et al., 1993) following
stimulation with IL-1 and TNFα. Transforming growth factor beta (TGFβ), particularly TGFβ1, as well as abundant latent TGFβ1 binding protein have also been detected in the synovial tissue and fluid from patients with rheumatoid arthritis (Miossec et al., 1990; Brennan et al., 1990b; Chu et al., 1991b; Taketazu et al., 1994).

1.4.9. Reactive oxygen species
Possibly the most potent means of phagocytic defence against microorganisms is the generation of free radicals. Following cell stimulation, a rapid respiratory burst occurs and it is the conversion of its products into oxidising radicals that mediates cell damage (Babior, 1984a). The metabolic pathway begins with the conversion of oxygen into superoxide (O₂⁻), catalysed by the membrane bound enzyme, NADPH-oxidase (Babior, 1984b). Dismutation of O₂⁻ results in the formation of hydrogen peroxide (H₂O₂). From H₂O₂, the powerful hydroxyl radical (·OH) is generated (Edwards, 1994). Additionally, H₂O₂ is the substrate for the enzyme myeloperoxidase (MPO) (Babior, 1984a). Myeloperoxidase is found in the azurophilic granules of neutrophils and the lysosomes of monocytes and some macrophages (Edwards, 1994). Mature macrophages lack MPO activity (Fleit et al., 1982). The oxidation of H₂O₂ in the presence of chloride ions generates the highly toxic hypochlorous acid (HOCI).

While low levels of reactive oxygen species are thought to be involved in signalling by acting as second messengers, when produced in excess these molecules can cause oxidative stress which may result in cell and tissue injury (Finkel, 1998).

Reactive oxygen species, although short-lived, have been detected in the synovium of patients with rheumatoid arthritis by electron spin resonance spectroscopy (Mapp et al., 1995). However, studies on the effects of free radicals in vitro have provided probably the best evidence that oxidative stress may mediate tissue injury, most notably cartilage damage, in rheumatoid arthritis. Hypochlorous acid and ·OH have been shown to facilitate the fragmentation of collagen by the degradation of proteoglycans (Halliwell, 1978; Kauanko et al., 1989; Dayer et al., 1993), while both HOCl and H₂O₂ can inhibit proteoglycan synthesis (Kauanko
et al., 1989). Furthermore, TNFα has been shown to activate the enzyme NADPH oxidase (Dusi et al., 1996) and to cause oxidative stress in cell lines (Zimmerman et al., 1989). This has again suggested that TNFα is a major mediator of pathology in rheumatoid arthritis, by acting both directly and indirectly on surrounding tissue.

1.4.10. Matrix metalloproteinases

Matrix metalloproteinases (MMP) are proteolytic enzymes that specifically act upon matrix components. MMPs are important in the remodelling of tissues and in facilitating cell migration. There are three major classes of MMP; stromelysins, collagenases and gelatinases (Woessner, 1991). These enzymes are initially produced in latent form and are activated following enzymatic cleavage (Kleiner & Stetler-Stevenson, 1993). Tissue inhibitors of metalloproteinases are naturally occurring MMP inhibitors (Nagase & Woessner, 1999). The stromelysins, for example MMP-3, act upon a wide range of matrices including proteoglycans, fibronectin, laminin and collagen type IV. Collagenases, like neutrophil collagenase (MMP-8) and interstitial collagenase (MMP-1), are specific for collagen types I, II and III (Woessner, 1991). The gelatinases degrade collagen type IV and denatured collagen and are produced by a wide range of cell types. Gelatinase A (MMP-2) is probably the most abundant and is produced by chondrocytes, fibroblasts, osteoblasts and monocytes, while Gelatinase B (MMP-9) is expressed by monocytes and alveolar macrophages and is found in the specific granules of neutrophils (Birkedal-Hansen et al., 1993).

Matrix metalloproteinases have been implicated in the cartilage damage seen in rheumatoid arthritis. Increased levels of collagenases and stromelysins have been detected in the synovial fluid and synovial tissues from patients with rheumatoid arthritis (Clark et al., 1993; Beekman et al., 1997). Moreover, MMP production appears to be inducible by both IL-1 and TNFα (Hanemaaijer et al., 1997; Vincenti et al., 1998). This has been supported by the observation that the treatment of rheumatoid arthritis patients with an anti-TNFα monoclonal antibody resulted in the reduction of serum stromelysin levels (Brennan et al., 1997).
1.4.11. Complement

Complement is an important mediator of inflammation and there is evidence for localised complement activation in patients with rheumatoid arthritis. Complement consumption (Pekin & Zvaifler, 1964; Hedberg, 1967; Ruddy et al., 1969), as well the presence of the membrane attack complex (Morgan et al., 1988) have been detected in the rheumatoid synovium. However, as will be discussed later, it is likely that the role complement may be secondary to the initial disease mechanism in rheumatoid arthritis.

1.5. The immunopathogenesis of rheumatoid arthritis

As already discussed, TNFα and IL-1 both appear to mediate local inflammation and tissue injury in the rheumatoid synovium, as well as triggering the production of other degradative compounds and pro-inflammatory mediators in rheumatoid arthritis. Histological studies indicate that the synovial macrophage is the major source of TNFα in the rheumatoid synovium. The initial trigger for macrophage activation and subsequent TNFα production in rheumatoid arthritis is unknown and has been the focus of research for many years.

In 1981 the hypothesis that T cells played a principle role in the initiation of inflammation in the rheumatoid synovium was proposed (Janossy et al., 1981). It was postulated that self antigens were presented to CD4 positive T lymphocytes by antigen presenting cells, such as dendritic cells (Thomas & Lipsky, 1996). It was thought that this interaction was the initiating process in rheumatoid arthritis. Subsequent macrophage activation would then generate proinflammatory mediators such as TNFα and IL-1 which would in turn act on synovial chondrocytes and fibroblasts to result in the cartilage and bone destruction associated with rheumatoid arthritis.

The activation of autoreactive T cells has been attributed to either molecular cross-reactivity with foreign antigen (Albani & Carson, 1996), or the availability of cryptic epitopes previously hidden or at low concentrations (Warnock & Goodacre, 1997). These views have been supported by experimental models such as adjuvant (Pearson, 1956), streptococcal cell wall (Cromartie et al., 1977) and collagen-induced
arthritis (Trentham et al., 1977). However, despite the search for T cell responses to microbial or articular antigens, there still remains no solid evidence of an antigen specific-T cell response in rheumatoid arthritis (Fox, 1997; McInnes & Liew, 1998). Moreover, the reason for and timing of failure of T cell tolerance has not been adequately addressed.

The T cell hypothesis of Janossy et al. (1981) was also based upon the observation that the majority of lymphocytes within the rheumatoid synovium were found to be CD4 positive T cells (Duke et al., 1982). This was supported by the protection of animals against collagen type II and streptococcal cell wall-induced arthritis by pre-treatment with anti-CD4 monoclonal antibodies (Goldschmidt et al., 1992; van den Broek et al., 1992). Although, most animal models for rheumatoid arthritis are T cell-dependent, many show features more representative of the seronegative arthropathies than seropositive rheumatoid arthritis (Oliver & Brahn, 1996). Rheumatoid factor positivity in experimental models is rare. Although the MRL/lpr mouse has high serum titres of both immunoglobulin M (IgM) and immunoglobulin G (IgG) rheumatoid factor, this model is more characteristic of SLE rather than of rheumatoid arthritis (O'sullivan et al., 1995). Furthermore, the extra-articular features associated with rheumatoid arthritis are absent in experimental models, for example streptococcal cell wall-induced arthritis lacks rheumatoid nodules (Oliver & Brahn, 1996).

Although T cell depleting treatments, such as anti-CD4 therapy are effective at T lymphocyte ablation, human trials have not demonstrated a significant clinical improvement (Moreland et al., 1995; Tak et al., 1995; van der Lubbe et al., 1995; Bresnihan et al., 1998), unlike therapies directed towards macrophage-derived cytokines (Elliott et al., 1993). This implies that macrophage activation and subsequent cytokine production in rheumatoid arthritis is not a T cell driven mechanism. This is further supported by histological studies showing synovial intimal macrophage activation to be the initial event in rheumatoid arthritis, proceeding T cell accumulation (Kraan et al., 1998). Furthermore, macrophage activation in rheumatoid arthritis appears to be spatially distinct from infiltrating T cells (Kurosaka & Ziff, 1983). This therefore raises the question of, in the absence of direct T cell involvement, what is
triggering macrophage activation and cytokine production in rheumatoid arthritis.

The initial trigger for macrophage activation in the rheumatoid synovium still remains unsolved. However, its identity may have already been described and all that is required in an effector mechanism. A key feature of rheumatoid arthritis is its association with an autoantibody known as the rheumatoid factor (RF). Others have suggested that rheumatoid factor-based immune complexes could evoke inflammation by interacting with monocytes (Nardella et al., 1983; Mannik & Nardella, 1995).

1.6. Rheumatoid factors and rheumatoid arthritis

60 years ago, Dr E. Waaler in Bregen, Norway, described a component in the serum from patients with rheumatoid arthritis which caused the agglutination of sheep erythrocytes (Waaler, 1939; Waaler, 1940). In fact, the experiment in which Waaler titrated sera from patients with rheumatoid arthritis against sheep erythrocytes sensitised with rabbit IgG, dates from 11th December 1937 (Milgrom, 1988). Waaler's finding was rediscovered in 1948 by Rose et al. (1948) at the Columbia-Presbyterian Medical Centre in New York and the active component was later named the rheumatoid factor (Pike et al., 1949).

In 1957 it was found that rheumatoid factors were serum gamma-globulins with a sedimentation rate of 19S (Franklin et al., 1957). Physical studies by analytical ultracentrifugation demonstrated that this 19S component, later found to be an IgM (Kunkel et al., 1959), complexed with gamma-globulins that sedimented at a rate of 7S, to form 22S aggregates (Franklin et al., 1957). These aggregates of an IgM rheumatoid factor complexed with autologous IgG were shown to associate and dissociate under different conditions of pH and IgG concentration (Kunkel et al., 1959).

Rheumatoid factors are classically defined as polyclonal antibodies of the class IgM that recognise and bind the Fc (fragment crystallisable) region of IgG (Moore & Dorner, 1993). However, rheumatoid factors of class IgG, immunoglobulin A (IgA) and immunoglobulin E (IgE) have also been
described (Mannik, 1992). Little is known about IgE rheumatoid factors and most research has been directed at rheumatoid factors of the classes IgM and IgG. Some autoantibodies with rheumatoid factor activity also have the capacity to crossreact with other antigens such as nuclear components, viral proteins (Williams, 1992a; Moore & Dorner, 1993) or β2-microglobulin (Williams et al., 1992b). Autoantibodies to the Fab rather than the Fc portion of IgG may also occur and can contribute to rheumatoid factor activity as measured by standard assays (Milgrom, 1988). These latter autoantibodies are generally not considered to be true rheumatoid factors, although this has been a matter of controversy. Rheumatoid factors have become the basis of standard diagnostic tests for rheumatoid arthritis. However, their role in the pathogenesis of rheumatoid arthritis has remained a matter of debate.

1.6.1. Prevalence of rheumatoid factors and methods of detection
Elevated levels of IgM rheumatoid factor, detected by conventional agglutination techniques, are found in the serum of 70-80% of patients with rheumatoid arthritis (Harris, 1990). However, circulating rheumatoid factors are not confined to patients with rheumatoid arthritis. Rheumatoid factors have been detected in the serum from patients with other rheumatic diseases such as systemic lupus erythematosus (SLE), systemic sclerosis and primary Sjögren's syndrome (Shmerling & Delbanco, 1991) and in patients with chronic infections such as leprosy, tuberculosis and malaria (Williams, 1974; Shmerling & Delbanco, 1991). Low levels of rheumatoid factors are also found in the serum from many normal individuals as part of a normal immune response to foreign antigen (Levine & Axelrod, 1985) and levels are seen to rise with age (Hallgren et al., 1973; van Schaardenburg et al., 1993). A small proportion of patients with rheumatoid arthritis test negative for circulating rheumatoid factor. These seronegative patients tend to develop less erosive disease and rarely have extra-articular features, but their disease is otherwise indistinguishable (Conway et al., 1994).

Many attempts have been made to use the detection of rheumatoid factors in sera as a diagnostic test for rheumatoid arthritis. The first method was based on the original techniques of Waaler and Rose and has become known as the Rose-Waaler agglutination test (Waaler, 1940;
Rose et al., 1948). This test detects IgM rheumatoid factor by measuring the agglutination of sheep red blood cells sensitised with rabbit IgG. In 1956 the latex fixation test was developed in which the sheep red blood cells were replaced by latex particles coated with human IgG (Singer & Plotz, 1956). This method is sensitive and often used as a screen but is limited by its low specificity (Shmerling & Delbanco, 1991). Although a number of other tests, including nephelometric techniques (Roberts-Thompson et al., 1985) have been developed in an attempt to optimise sensitivity, specificity and reproducibility, the agglutination of IgG-coated particles remains the basis for routine screening tests. Solid phase assays have also been developed to detect rheumatoid factor of specific isotypes by determining the binding of rheumatoid factor to either human (Carson et al., 1977; Powell et al., 1985), rabbit (Swedler et al., 1997) or goat IgG Fc fragments coated onto a plastic substrate (Tuomi, 1989). The rheumatoid factors are detected using a radiolabelled or enzyme-conjugated anti-human IgG, IgA or IgM. An important consideration in relation to these types of assay is whether they measure the same populations of autoantibodies. Conformational changes may occur in the Fc fragment of IgG when bound either to a particle such as a sheep red cell, or a plastic surface. In solid-phase assays further conformational changes may be associated with the cleavage of IgG substrate to yield isolated Fc fragments, leading to significant changes in avidity. There are, therefore, technical reasons why rheumatoid factor activity measured by these assays may not give a direct measure of their potential biological properties.

Despite the development of many different tests, the specificity and sensitivity of rheumatoid factor assays for rheumatoid arthritis remain too low to be of major clinical value. The diagnosis of rheumatoid arthritis is still largely based on clinical criteria. This implies that if rheumatoid factors are pathogenic, they act through a property which overlaps with, but is not identical to, rheumatoid factor activity as identified by IgG Fc binding assays.
1.6.2. *Rheumatoid factor isotype*

One possible reason for the divide between rheumatoid factor positivity and the development of rheumatoid arthritis, is that pathogenicity may be dependent on the isotype of the rheumatoid factor. The predominant class of rheumatoid factor in both normal individuals and patients with rheumatoid arthritis is IgM. However, there is no clear relationship between IgM rheumatoid factor levels and the severity of inflammation (Jacoby et al., 1973; Aho et al., 1997). Patients with high serum titres of IgG rheumatoid factor tend to have more severe disease and display extra-articular manifestations (Hay et al., 1979; Robbins et al., 1986). More recently, a study in Finland has shown the early detection of raised serum IgG levels in normal individuals to be indicative for the development of seropositive rheumatoid arthritis later in life (Aho et al., 1997). A number of studies have also found the presence of IgA rheumatoid factor in early synovitis to be a marker for the development of more severe disease and to be associated with the manifestation of extra-articular features in patients with rheumatoid arthritis (Teitsson et al., 1984; Moore et al., 1994; Jónsson et al., 1995; Aho et al., 1997). Additionally, Rudge et al. (1985) observed a fall in disease activity and IgG rheumatoid factor and IgA rheumatoid factor levels but no change in IgM rheumatoid factor levels following treatment with gold. While evidence for the importance of individual rheumatoid factor isotypes is inconclusive, there remains a suggestion that IgG and IgA rheumatoid factor may be more relevant to pathogenesis than IgM rheumatoid factor in rheumatoid arthritis. This is emphasised by the occurrence of IgM rheumatoid factors in normal individuals and as will be discussed later, the differentiation between IgM rheumatoid factor and IgG rheumatoid factor based immune complexes.

1.6.3. *Fine specificity of rheumatoid factors*

The fine specificity of rheumatoid factors may provide important information equating to their pathogenicity in rheumatoid arthritis. Epitopes on the Fc region of IgG that are recognised by rheumatoid factors may be influential in determining the type of rheumatoid factor-based immune complex formed as well as the relationship between affinity, avidity and dissociation kinetics for interactions between the rheumatoid
factor and the IgG Fc fragment. These factors may differentiate between a potentially pathogenic and a non-pathogenic rheumatoid factor.

Rheumatoid factors from patients with rheumatoid arthritis are polyclonal and may react with a variety of epitopes expressed by the Fc fragment of IgG (Kunkel & Tan, 1964). A number of studies have focused upon the characterisation of the fine specificity of rheumatoid factors in an attempt to reveal the function of these autoantibodies. Most have studied IgM rheumatoid factors from patients with rheumatoid arthritis. Owing to the polyclonality of these antibodies, a number of groups have chosen to utilise monoclonal IgM rheumatoid factors found in patients with Waldenström's macroglobulinemia and essential mixed cryoglobulinemia (Sasso et al., 1988). However, it has been found that significant differences exist between these paraproteins and rheumatoid factors derived from patients with rheumatoid arthritis, in both their specificity and avidity for IgG (Bonagura et al., 1993; Robbins et al., 1993). A direct comparison with paraproteins from patients with Waldenström's macroglobulinemia was achieved by using monoclonal IgM rheumatoid factors derived from patients with rheumatoid arthritis. These monoclonal rheumatoid factors were generated by the EBV transformation of either synovial or peripheral blood B cells from patients with rheumatoid arthritis. Furthermore, this process did not affect the specificity of the rheumatoid factors produced for genetically engineered IgG (Bonagura et al., 1999). In one study it was shown that monoclonal IgM rheumatoid factors derived from patients with rheumatoid arthritis reacted with epitopes within the second heavy chain constant domain (CH₂, also referred to as Cy2) of IgG, that were identical to those recognised by the majority of polyclonal rheumatoid factors examined (Williams & Malone, 1994).

The dominant binding site on the Fc fragment of IgG for both polyclonal (IgG and IgM) and monoclonal (IgM) rheumatoid factors, has been located to the CH₂-CH₃ interface (Nardella et al., 1985; Sasso et al., 1988). This was determined by measuring the levels of binding of rheumatoid factors to proteolytically cleaved fragments of IgG. The binding site on IgG for Staphylococcal Protein A (SPA) is also located at the CH₂-CH₃ interface. Both IgG and IgM rheumatoid factors and this microbial
protein recognise some of the same amino acid residues (Nardella et al., 1985; Sasso et al., 1988). In one study IgG and IgM rheumatoid factors were found to carry the internal image of SPA (Oppligner et al., 1987). Streptococcal Protein G (SPG) was also found to bind IgG at the CH$_2$-CH$_3$ interface (Nardella et al., 1981) and to inhibit the binding of both polyclonal and monoclonal IgM rheumatoid factors to IgG (Stone et al., 1989). Although not identical, the binding sites for SPA and SPG are similar. By genetically engineering IgG using site directed mutagenesis and exon exchange, it was shown that at least three regions of the CH$_2$ domain and one region of the CH$_3$ domain contributed to the epitope recognised by a monoclonal IgM rheumatoid factor derived from a patient with Waldenström's macroglobulinemia (Artandi et al., 1992). This work supported previous observations for the specificity of IgM rheumatoid factors from patients with rheumatoid arthritis for IgG myeloma proteins (Natvig et al., 1972).

The CH$_2$-CH$_3$ interface also includes the antigen, designated Ga, which is recognised by both polyclonal and monoclonal IgM rheumatoid factors. Ga is found on IgG1, IgG2 and IgG4, but not IgG3 (Allen & Kunkel, 1966). The isotype distribution of the Ga antigen and the binding site for SPA follows the same pattern, while SPG binds all four IgG subclasses (Stone et al., 1989). In keeping with the Ga antigen distribution, serum derived IgM rheumatoid factors from patients with rheumatoid arthritis were found to bind IgG1, IgG2 and IgG4, but not IgG3. However, rheumatoid factors derived from rheumatoid synovium exhibited greater specificity and affinity for IgG3 relative to the binding of other subclasses by serum rheumatoid factors (Robbins & Wistar, 1985, Robbins et al., 1987, Robbins et al., 1993). Artandi et al. (1992) showed the non-binding to IgG3 to be dependent upon a single amino acid residue. At amino acid position 435, IgG1, IgG2, and IgG4 express a Histidine, while IgG3 expresses an Arginine. These findings have suggested that rheumatoid factors derived from the synovium may have different fine specificities compared with circulating rheumatoid factors. This may be relevant to the type of immune complex formed in the circulation and the rheumatoid synovium and is an area that will be discussed later in this chapter.
1.6.4. IgG as an immunogen for rheumatoid factor production

In terms of traditional immunological theory, the existence of rheumatoid factors is a puzzle. It is difficult to understand how an immune response to a plasma protein such as IgG, present in high concentration, can arise. Lang et al. (1999) have recently reported circulating IgG Fab fragment specific T cells clones in patients with rheumatoid arthritis, supporting previous work by Radoiu et al. (1982). These findings raise the possibility of a true failure of T cell tolerance to IgG. However, the consensus view has been that T cell responses to IgG are insignificant in rheumatoid arthritis.

Another group has reported high levels of a protein designated p205 in rheumatoid synovial fluid. This antigen was found to have amino acid sequences that matched those in the IgG CH2 domain, the area where rheumatoid factors bind (Bläss et al., 1999). This may indicate a cross-reactivity between anti-P205 T cells and IgG Fc peptides. However, the question of how tolerance to such a ubiquitous protein can occur remains the same.

An alternative explanation for the occurrence of rheumatoid factor production, is that the immunogen may be IgG which has been altered in some way, resulting in a structural change. Stimulation of an immune response to altered IgG might generate rheumatoid factors which recognise altered IgG specifically, but may also extend to the production of rheumatoid factors which bind native IgG. Some support for this idea comes from studies showing that rheumatoid factors from patient sera react with IgG from species other than human (Jones et al., 1990). Initially, Robbins & Wistar (1985) found that serum IgM rheumatoid factor had a greater specificity for rabbit, than for human IgG. However, in a subsequent study serum rheumatoid factors from patients with rheumatoid arthritis were found to react equally with rabbit and human IgG, while rheumatoid factor produced by cells extracted from rheumatoid synovium had a higher avidity for human IgG (Robbins et al., 1987).

It has been suggested that the glycosylation status of IgG is somehow related to the production of rheumatoid factor. The glycosylation state of
IgG varies with age (Parekh et al., 1988), however, it has been shown that patients with rheumatoid arthritis have an increased expression of agalactosyl IgG glycoforms compared with age-matched controls (Parekh et al., 1985). Nevertheless, this loss of galactose on the Fc region of IgG may not be specific for rheumatoid arthritis (Rademacher et al., 1988; Tomana et al., 1988). Moreover, it has been shown that the binding of IgM rheumatoid factors to IgG is not dependent upon the nature of the carbohydrates, but instead the amino acid sequence of the IgG Fc region (Newkirk et al., 1990). However, the binding of a monoclonal IgG rheumatoid factor derived from a patient with rheumatoid arthritis to IgG Fc did appear to be influenced by carbohydrate (Newkirk & Rauch, 1993).

As will be discussed later, recent analysis of the possible mechanisms of rheumatoid factor production makes the need for an altered IgG immunogen less relevant. However, the detailed steric and glycosylation states of IgG may still be highly relevant in the context of rheumatoid factor immune complex formation.

### 1.6.5. Origins of rheumatoid factors

Rheumatoid factors, as all immunoglobulins, are generated by cells from the B lymphocyte lineage (Male & Roitt, 1993). B lymphocytes exist as three distinct populations termed B-1a, B-1b and B-2 (Kantor, 1991). Cells of the B-1a population express the surface antigen CD5 and were originally referred to as Leu-1 B cells (Kipps, 1989). The progenitors of B-1a cells are B-1b cells which are surface CD5- but CD5 mRNA+ (Mantovani et al., 1993). In the adult, CD5+ B cells constitute up to 30% of circulating and splenic B lymphocytes, while in the fetal spleen and newborn cord blood, CD5+ B cells are the dominant subpopulation. B-2 or "conventional" B cells, arising from the bone marrow, constitute the majority of B lymphocytes in the adult. Unlike B-2 lymphocytes, CD5+ B cells maintain their numbers by self-repopulation (Casali & Notkins, 1989a; Kipps, 1989). Previous work by Casali and colleagues demonstrated that the majority of B cells generating polyreactive IgM autoantibodies with rheumatoid factor activity were B-1a cells (reviewed in Casali & Notkins, 1989b). In contrast to B-2 cells which produce high affinity, monospecific antibodies following an antigen-driven immune response,
B-1a derived autoantibodies arise from an antigen-independent process and remain close to germline configuration (Bona, 1988; Casali & Notkins, 1989a). Rheumatoid factors such as these can be found in normal individuals following infection or immunisation (Carson et al., 1987) and these so called "natural" autoantibodies may have some physiological function relating to their low affinity and polyreactivity (Cohen & Cooke, 1986). Natural autoantibodies may play a protective role against autoimmune disease by binding to self antigens which present epitopes also expressed by pathogenic antigens (Cohen & Cooke, 1986). Alternatively, the low affinity of natural autoantibodies may allow them to act as opsonins for the silent clearance of breakdown products which may be potentially immunogenic (Avrameas, 1991). Furthermore, natural autoantibodies may enhance normal immune responses by promoting complement fixation and immune complex clearance, increasing the avidity of low affinity IgG antibodies or enhancing antigen presentation (Carson et al., 1987; Kipps, 1989).

Increased circulating levels of CD5+ B cells have been reported in patients with rheumatoid arthritis (Plater-Zyberk et al., 1985; Hardy et al., 1987). However, a study by Jones et al. (1993) found patients with rheumatoid arthritis and IgA nephropathy to have decreased levels of circulating CD5+ B cells and increased levels of CD5- B cells, compared with normal individuals and patients with Graves' disease where circulating rheumatoid factors are thought to be non-pathogenic. This study would therefore support a protective role for rheumatoid factors derived from CD5+ B cells and may suggest a distinct origin for potentially pathogenic rheumatoid factors. This concept is supported by the observation that in patients with rheumatoid arthritis, IgG rheumatoid factor secreting B cells were predominately CD5- whereas in controls and Graves disease they were mainly CD5+ (Jones et al., 1993). The contribution made by B-1a cells to rheumatoid factor levels in vivo is far from clear but may provide some clue as to the origin of rheumatoid factors from patients with rheumatoid arthritis and from normal individuals.

Studies on IgM rheumatoid factors from normal individuals and patients with rheumatoid arthritis suggest that potentially pathogenic rheumatoid factors from rheumatoid synovium and those from normal
subjects differ significantly in a way that reflects their origin. Thompson et al. (1994) found that following immunisation with foreign antigen, the majority of IgM rheumatoid factors in normal individuals had their variable light chain (V\textsubscript{L}) derived from the V\textsubscript{\kappa}3 gene segments, while the variable heavy chain (V\textsubscript{H}) gene segments utilised families V\textsubscript{H}1, V\textsubscript{H}3 and V\textsubscript{H}4. Rheumatoid synovium derived rheumatoid factors predominantly used the heavy chain gene segments of the V\textsubscript{H}3 family. Interestingly, similar studies by other groups have shown an over-representation of V\textsubscript{H}4 gene segments by rheumatoid synovium derived rheumatoid factors (Brown et al., 1992; Pascual & Capra, 1992; Williams et al., 1999). Thompson et al. (1995a) showed that rheumatoid factors from normal individuals had undergone some somatic hypermutation, but little, if any affinity maturation. In contrast, the synovial rheumatoid factors from patients with rheumatoid arthritis exhibited a pattern of hypermutation distinct from that in normals, as well as both affinity maturation and class switching. Peripheral blood rheumatoid factors from patients with rheumatoid arthritis displayed a pattern of V-gene usage intermediate of that seen in rheumatoid synovium and normal individuals (Thompson et al., 1995b). These findings suggested that rheumatoid factors in patients with rheumatoid arthritis may arise, not only from the subset of B cells responsible for natural rheumatoid factor production but also from conventional B cells, as a result of a gene mutation in the variable region coding for immunoglobulins of unrelated specificities.

Thompson et al. (1995a) demonstrated that rheumatoid factors from both normal immunised individuals and patients with rheumatoid arthritis bear evidence of somatic hypermutation. It is believed that in order to undergo somatic hypermutation, B cells must receive T cell help. B cells with rheumatoid factor specificity should be unable to obtain this help, since T cells specific for IgG Fc are deleted or made anergic (Bikoff, 1983). However, B cells with rheumatoid factor specificity may obtain T cell help through one of two mechanisms, both of which bypass the need for Fc specific T cells. They may obtain T cell help by presenting peptides derived from an irrelevant foreign antigen taken up in the form of IgG containing immune complexes via the B cell surface receptor specific for IgG Fc (Roosnek & Lanzavecchia, 1991). Alternatively, they may present
peptides from their own immunoglobulin V regions which might be shared by a common foreign antigen to which T cells were responsive (Thompson et al., 1994). The first mechanism requires the presence of foreign antigen and might be expected to operate during an immune response to infection or vaccination (Roosnek & Lanzavecchia, 1991). The second mechanism does not require the presence of foreign antigen and might be more important in the long term production of rheumatoid factors seen in association with rheumatic disease. Although in theory, the sharing of a peptide between an rheumatoid factor V region and a foreign antigen, such as SPA, could allow the second mechanism to operate, little or no information is available about the help received by rheumatoid factor specific clones.

Rheumatoid factors from patients with rheumatoid arthritis exhibit both affinity maturation and class switching to IgG and IgA, as expected from an antigen-driven response (Ermel et al., 1993). Rheumatoid factors from normal individuals, however, do not display affinity maturation or class switching, although Mantovani et al. (1993) have shown that B-1a cells from one patient with rheumatoid arthritis generated high affinity IgM rheumatoid factors which had undergone somatic hypermutation, without class switching. Nevertheless, in most cases rheumatoid factors from normal individuals may be prevented from undergoing affinity maturation and therefore remain non-pathogenic, while disease-associated rheumatoid factors may escape this control mechanism (Thompson et al., 1994; Thompson et al., 1995a). IgG rheumatoid factors are commonly found in rheumatoid arthritis patients with a high IgM rheumatoid factor titre, suggesting that class switching may be determined by the level of rheumatoid factor response. It may therefore be that IgG rheumatoid factors are derived from B cells previously producing IgM rheumatoid factor as a result of class switching. Therefore, a proportion of rheumatoid factor in rheumatoid arthritis may arise, as in normal individuals, as a physiological "spillover" from an antigen specific immune response. Alternatively, IgG rheumatoid factor may arise from B cell clones of unrelated specificities. In support of this concept Williams et al. (1999) have shown rheumatoid synovium derived IgA and IgM rheumatoid factors to be clonally related, whilst no clonal relationship was observed between rheumatoid synovial IgG and
IgM rheumatoid factors. However, there is growing evidence to suggest that a proportion of rheumatoid factor in patients with rheumatoid arthritis arise and persist through quite different mechanisms, which may relate in some way to their potential pathogenicity (Edwards et al., 1999).

1.6.6. Rheumatoid factors and the formation of immune complexes
A rheumatoid factor of any class will bind to the Fc region of IgG and since IgG is present in most body fluids, including normal synovial fluids (Hrncir et al., 1972), rheumatoid factor based immune complexes exist in vivo (Mannik, 1992). Immune complexes of rheumatoid factors may play a primary role in the initiation of inflammation and the pathogenesis of rheumatoid arthritis. Rheumatoid factor isotype may strongly influence the size, stereochemistry and stability of the immune complex formed, and therefore may be important in determining which complexes may have pathogenic properties.

1.6.7. IgM rheumatoid factor immune complexes
IgM exists as a pentameric structure. Therefore, one IgM rheumatoid factor molecule has the potential to bind up to five IgG molecules (Figure 1a). Steric hindrance prevents the binding of more IgG molecules (Mannik, 1992). This structure is, however, unstable and may be in a constant state of association and dissociation (Mannik & Nardella, 1988). The avidity of an IgM rheumatoid factor molecule for aggregated IgG is far greater, forming a more stable complex (Mannik, 1992). An IgM molecule will only activate the complement component C1q once its structure has been altered to form a "five legged, table-like" configuration (Feinstein & Munn, 1969; Perkins et al., 1991). This conformation will not occur following the binding of monomeric IgG as shown in Figure 1a. Recently, crystal structure studies have shown the "table-like" structure to form if either side of the Fc region of monomeric IgG is symmetrically bound by two IgM rheumatoid factor Fab fragments (Corper et al., 1997) (Figure 1b). The formation of circulating IgM rheumatoid factor complexes are unlikely to play a pathogenic role in rheumatoid arthritis since those stable enough to form may be rapidly cleared following activation of C1q.
Figure 1. Schematic showing the formation of IgM rheumatoid factor immune complexes with a) monomeric IgG as described by Mannik (1992) and b) monomeric IgG as described by Corper et al. (1997).

1.6.8. IgG rheumatoid factor immune complexes
Kunkel et al. (1961) were the first to describe "intermediate" immune complexes in the serum of patients with rheumatoid arthritis that had a sedimentation rate lying between 9S and 17S. These complexes were found to consist of IgG (Kunkel et al., 1961; Schrohenloher, 1966) and possess rheumatoid factor activity (Schrohenloher, 1966). Similar complexes were also detected in the synovial fluids of patients with rheumatoid arthritis (Hannestad, 1967; Winchester et al., 1970) and rheumatoid synovium (Munthe & Natvig, 1971; Munthe & Natvig,
1972). Characterisation by Pope et al. (1974) revealed these intermediate complexes to consist of two IgG rheumatoid factors that had self-associated to form a dimer. A model was proposed by Mannik and colleagues (Pope et al., 1974) wherein the Fab portion of one IgG rheumatoid factor was bound to the Fc fragment of the other and *visa versa*, so that each IgG rheumatoid factor molecule was simultaneously acting as both antibody and antigen. Hence, a cyclic ring structure was formed, stabilised by the two antigen-antibody bonds (Figure 2).

**Figure 2.** Schematic showing the proposed cyclic structure formed during the self-association of two IgG rheumatoid factor molecules. Taken from Pope et al. (1974).

IgG rheumatoid factor self-association enables the formation of immune complexes in the absence of any other antigen. From the estimation of dissociation constants, it was predicted that IgG rheumatoid factors would preferentially self-associate in the presence of normal monomeric IgG. The interaction of an IgG rheumatoid factor molecule with monomeric IgG will have a dissociation constant of approximately $10^{-5}$ L/mol (Pope et al., 1974). However, when two IgG rheumatoid factors self-associate the stabilising effect of two interactions may reduce the dissociation constant by an order of magnitude or more, thus resulting in an increased avidity. Although this interaction may have a longer on-rate than the binding of an IgG rheumatoid factor with a normal IgG molecule, the off-rate will also be slower making the self-associating IgG rheumatoid factor interaction more stable.
It was proposed that once self-association of the IgG rheumatoid factors had occurred, these complexes then had the potential to undergo concentration-dependent aggregation to form tetramers, octamers and 12-mers (Pope et al., 1974), since one antigen binding site would still be available on the Fc fragment of each IgG rheumatoid factor molecule (Pope et al., 1975). Full exposure of these antigenic determinants would arise during the conformation changes that occurred with ring closure (Mannik, 1992). However, it was predicted that when a normal monomeric IgG molecule bound to an IgG rheumatoid factor, this second antigenic determinant became unavailable, owing to steric hindrance (Nardella et al., 1981). Normal IgG molecules may be constantly associating and dissociating with these available sites at a rate such that in excess normal IgG, further polymerisation of the IgG rheumatoid factor dimer may be inhibited (Figure 3). The binding of two normal monomeric IgG molecules to an IgG rheumatoid factor dimer may explain why circulating complexes are small while the synovium houses larger polymers (Hannestad, 1967; Winchester et al., 1970; Nardella et al., 1981). As will be discussed later, this may be important with respect to rheumatoid arthritis as these small complexes containing self-associating IgG rheumatoid factor may be crucial to the initiating disease mechanism and the development of systemic manifestations.

Not all IgG rheumatoid factor may have the potential to self-associate. Self-association may only occur if the two IgG rheumatoid factors bind to the same or complementary epitopes (Pope et al., 1975). Differences in epitope pairing may result in differences in pathogenic potentiality. Additionally, IgG rheumatoid factor dimers may differ in pathogenic potential when compared to IgG rheumatoid factors complexed with non-rheumatoid factor IgG. These considerations further emphasise the concept that only a proportion of IgG rheumatoid factors may generate pathogenic immune complexes and may explain why rheumatoid factor levels as measured at present, do not correlate precisely with the presence of disease.
Figure 3. Schematic diagram of the termination of IgG rheumatoid factor polymerisation by excess normal IgG. Taken from: Nardella et al. (1981).

1.6.9. Rheumatoid factor complexes and Waldenström’s hypergammaglobulinemic purpura
In 1943, Waldenström (1943) described the syndrome of hypergammaglobulinemic purpura, characterised by a recurrent purpura of the lower extremities, mild anaemia and hypergammaglobulinemia. These features may occur in patients with rheumatoid arthritis and especially Felty’s syndrome, however, they may also occur in the absence of rheumatoid arthritis (Clark et al., 1974). Moreover, intermediate complexes of IgG rheumatoid factor have been detected in the sera of patients with Waldenström’s hypergammaglobulinemic purpura in the absence of synovitis (Kunkel et al., 1961; Capra et al., 1971; Kyle et al., 1971). Clark et al. (1974) performed a direct comparison between the rheumatoid factor complexes found in the sera of patients with
rheumatoid arthritis and the complexes in the sera of patients with only Waldenström's hypergammaglobulinemic purpura. It was found that sera from patients with Waldenström's hypergammaglobulinemic purpura contained a higher proportion of IgG and IgA rheumatoid factor complexes than from patients with rheumatoid arthritis. Furthermore, the IgG rheumatoid factor complexes from patients with Waldenström's hypergammaglobulinemic purpura were predominantly intermediate complexes, while the sera from patients with rheumatoid arthritis contained mostly large, IgM rheumatoid factor based complexes. Analysis of these intermediate IgG rheumatoid factor complexes by Capra et al. (1971) indicated the prevalence of the subclass IgG1. However, the radial immunodiffusion technique used to identify IgG subclass was relatively unsophisticated. Two separate studies further analysed the self associating IgG rheumatoid factor dimers from patients with Waldenström's hypergammaglobulinemic purpura and found the predominant subclass in these complexes to be IgG3 (Bignold et al., 1980; Serino et al., 1983). In comparison, a study on rheumatoid factor subclass in the serum from patients with rheumatoid arthritis revealed IgG1 and IgG4 as the dominant subclasses (Cohen et al., 1987). These observations further suggest that only a subgroup of IgG rheumatoid factors may be pathogenically involved in the generation of synovitis in rheumatoid arthritis.

1.6.10. Fixation and activation of complement by rheumatoid factors
A number of studies have shown complement consumption in the synovial fluids of patients with rheumatoid arthritis (Pekin & Zvaifler, 1964; Hedberg, 1967; Ruddy et al., 1969) relative to complement levels within the serum (Winchester et al., 1970). Complement consumption occurring within the synovium was thought to be a result of a localised immune response. It was therefore thought the rheumatoid factor complexes found in the synovium and synovial fluids of patients with rheumatoid arthritis were behaving pathogenically by activating the complement system. Work by Winchester and his colleagues (Winchester et al., 1970) demonstrated the ability of large IgG rheumatoid factor complexes, isolated from the synovial fluids of patients with rheumatoid arthritis, to fix and activate components of human complement. Complement fixation was further enhanced by the addition
of IgM rheumatoid factor to the IgG rheumatoid factor complexes. Smaller complexes, isolated from the synovial fluids of patients with rheumatoid arthritis, failed to precipitate with C1q. Similarly, Bianco et al. (1974) demonstrated complement fixation by IgM rheumatoid factor. As found by Bianco et al. (1974) and other groups, IgG rheumatoid factors from patients with rheumatoid arthritis did not fix complement (Tanimoto et al., 1976; Sabharwal et al., 1982). This was probably because in these studies, the polymerisation of the IgG rheumatoid factors was blocked by the presence of normal IgG and these complexes were too small to fix complement. Brown et al. (1982) demonstrated the ability of polymerised, self-associated IgG rheumatoid factor complexes from patients with rheumatoid arthritis to fix and activate the human complement system. They suggested that within the joint it is more likely to find larger IgG rheumatoid factor polymers and therefore complement activation, explaining the observations by Winchester et al. (1970) who found that high synovial fluid rheumatoid factor titres correlated with depressed complement levels.

In rheumatoid arthritis, rheumatoid factors may be generated both in the synovium and in lymphoid organs. From either source they may gain access to the circulation either in isolation or in the form of complexes. There is a large body of evidence indicating the activation of complement in the joint in rheumatoid arthritis, but relatively little evidence of systemic complement activation. In approximately 2-5% of patients with high rheumatoid factor titres, microvascular injury may occur. Vasculitis in rheumatoid arthritis usually manifests as nail fold infarcts and ulcers of the lower leg (Maini & Zvaifler, 1998). SLE patients with glomerulonephritis and other forms of microvascular damage exhibit immune complex deposition in basement membranes and systemic complement activation (Schur, 1993). However, in rheumatoid arthritis there is evidence of neither (Conn et al., 1993), suggesting that complement activation is not the primary mechanism of rheumatoid vasculitis. Instead, vasculitic lesions in rheumatoid arthritis may occur as a result of platelet activity within the microvasculature (Cunningham et al., 1986). High circulating rheumatoid factor levels may create a sludging effect at the sites of venular injury common to rheumatoid arthritis, Waldenström's macroglobulinaemia and Waldenström's
hypergammaglobulinemic purpura. Platelet aggregation and activity in response to aggregated immunoglobulin at these sites (Fink et al., 1979) may therefore be the cause of vasculitis in these disorders, rather that immune complex deposition and complement activation.

Complement activation may contribute to synovitis once the tissue has become populated with rheumatoid factor secreting plasma cells. When an immune complex interacts with complement it may either generate inflammation if this occurs within tissues or it may be cleared if this occurs within the circulation. It may be that the rheumatoid factor containing complexes responsible for disseminating the disease to joints are those which do not interact with complement i.e. small self-associating IgG rheumatoid factor complexes. These small IgG rheumatoid factor immune complexes, (unlike IgM rheumatoid factor complexes or IgG rheumatoid factor multimers) may escape clearance in the circulation, but may still be able to generate inflammation in the extravascular space through a mechanism independent of complement. Such a mechanism may involve the direct activation of synovial macrophages through the binding of small IgG rheumatoid factor complexes to surface immunoglobulin receptors.

1.7. Human immunoglobulin receptors

Human immunoglobulin receptors have been identified and characterised for IgG (FcγR), IgA (FcaR) and IgE (FceR) (reviewed in: Ravetch & Kinet, 1991; van de Winkel & Anderson, 1991; Morton et al., 1996). Fc receptors function as a link between the antibody response and effector cell function. The three human FcγR, which will be discussed in detail, are glycoproteins belonging to a subgroup of the immunoglobulin supergene family (Williams & Barclay, 1988). FcγR have the ability to mediate the phagocytosis of particles, such as microbes, the internalisation of immune complexes, antibody dependent cellular cytotoxicity (ADCC) and the release of pro-inflammatory factors, such as cytokines or free radicals (reviewed in: Fanger et al., 1989; van de Winkel & Capel, 1993; Gergely & Sarmay, 1994). Fc receptors for IgG were first described in the late 1960's (Berken & Benacerraf, 1967). They were identified as receptors on human monocytes and macrophages that bound and phagocytosed red blood cells coated with human IgG, in the
absence of complement (Huber & Fudenberg, 1968a; Huber et al., 1968b; Huber et al., 1969). Three murine FcγR have also been identified and characterised. Although some homology exists, murine FcγR differ from human FcγR in their signalling capacities, structure and cell distribution (reviewed in: Mellman et al., 1988; Unkeless et al., 1988).

Fcγ receptors may play a dominant role in the pathogenesis of rheumatoid arthritis by binding immune complexes of IgG rheumatoid factor and mediating macrophage activation. Several studies in vivo have elegantly demonstrated IgG immune complex-induced inflammation and tissue injury to be FcγR-mediated (Sylvestre & Ravetch, 1994; Sylvestre et al., 1996; Clynes et al., 1998; Clynes et al., 1999). However, equating the role of individual Fcγ receptors in these experimental models to human disease is dangerous since murine and human FcγR differ, particularly in terms of signalling capacities.

The possibility of Fc receptor involvement in the inflammatory process in rheumatoid arthritis emphasises the pathogenic potential of IgG rheumatoid factor and the possible non-pathogenicity of IgM rheumatoid factor. There is no substantial evidence for the existence of an IgM-specific Fc receptor. Although IgM rheumatoid factor may bind and activate macrophages through complement receptors (Walport, 1993), as will be discussed later, it seems unlikely that circulating IgM rheumatoid factor would be able to access tissue macrophages in order to initiate inflammation in rheumatoid arthritis. In contrast, small immune complexes of self-associating IgG rheumatoid factor may be the subpopulation of rheumatoid factor based immune complexes that can selectively initiate inflammation in rheumatoid arthritis.

As discussed previously, the presence of circulating IgA rheumatoid factor in patients with rheumatoid arthritis also appears to correlate with disease activity. FcαR (CD89) is expressed by granulocytes and monocytes and can bind both monomeric and polymeric IgA1 and IgA2 (Monteiro et al., 1990; Monteiro et al., 1992). The binding of IgA rheumatoid factor complexes to FcαR may also activate macrophages in rheumatoid arthritis and this will be expanded upon in the discussion (see: 4.5. Rheumatoid factor, Fcγ receptors and rheumatoid arthritis).
1.7.1. *FcγRI (CD64)*

FcγRI is a heavily glycosylated 72-kDa molecule that was originally isolated from human monocytes and U937 cells by affinity chromatography (Anderson, 1982). FcγRI is the only Fc receptor with a high affinity (Ka = 10^8 - 10^9 M^-1) for monomeric IgG (Anderson & Abraham, 1980). FcγRI is constitutively expressed by monocytes and macrophages (Looney et al., 1986a), and its expression can be increased 5-to 10-fold by treatment with gamma interferon (IFN-γ) (Guyre et al., 1983). It is also expressed by dendritic cells and CD34+ myeloid progenitors (Deo et al., 1997). FcγRI expression by neutrophils can also be induced following treatment with IFN-γ or granulocyte colony stimulating factor (G-CSF) (Perussia et al., 1983; Kerst et al., 1993). FcγRI binds human IgG subclasses in the order of magnitude: IgG1 > IgG3 > IgG4 >> IgG2 (Anderson & Abraham, 1980), while the receptor subclass specificity for murine IgG is: 2a = 3 >> 1, 2b (Jones et al., 1985).

The three FcγRI genes; FcγRIA, FcγRIB and FcγRIC have been mapped to the long arm of chromosome 1 (1q21.1) (Ernst et al., 1992). FcγRIA encodes for a receptor (FcγRIa) that consists of a 292 amino acid extracellular region which contains three immunoglobulin-like domains, a 21 amino acid transmembrane region and a highly charged cytoplasmic domain of 61 amino acids (Allen & Seed, 1989). FcγRIB encodes for two transcripts, FcγRIB1 and FcγRIB2, while FcγRIC encodes for the receptor FcγRlc (van de Winkel et al., 1991). All three transcripts have an extracellular region containing two immunoglobulin-like domains (Ernst et al., 1992). Both FcγRIB1 and FcγRlc are soluble receptors which lack membrane anchorage, as a result of a translation stop codon in exon EC3. Cells expressing FcγRI all contain transcripts for FcγRIa, which binds monomeric IgG with high affinity, and FcγRIB2, which only binds complexed IgG (Porges et al., 1992).

1.7.2. *FcγRII (CD32)*

FcγRII is a 40-kDa protein which was first purified from human peripheral blood monocytes and U937 cells by affinity chromatography (Anderson & Abraham, 1980). It is a low affinity FcγR and cannot efficiently bind monomeric IgG (Ka < 10^7 M^-1). It can, however, bind
complexed human IgG and murine IgG1 and IgG2b (Jones et al., 1985; Looney et al., 1986a). Of all Fcγ receptors, FcγRII has the widest distribution. It is expressed by monocytes, macrophages, neutrophils, eosinophils (Looney et al., 1986b), basophils, dendritic cells (Deo et al., 1997), platelets (Rosenfeld et al., 1985), B cells (Cohen et al., 1983; Looney et al., 1986a), Langerhans cells (Schmitt et al., 1990) and placental endothelial cells (Sedmak et al., 1991).

The cloning and sequencing of complementary DNA (cDNA) has shown that FcγRII has an extracellular region of 180 amino acids which contains two immunoglobulin-like domains, an transmembrane region of 27-29 amino acids and a cytoplasmic domain that varies from 44 to 76 amino acids (Stuart et al., 1987; Hibbs et al., 1988; Stengelin et al., 1988). The three genes for FcγRII (FcγRIIA, FcγRIIB, FcγRIIC) have been mapped to 1q23-24 of chromosome 1 and encode the six isoforms: FcγRIIa1, FcγRIIa2, FcγRIIb1, FcγRIIb2, FcγRIIb3 and FcγRIIc (Qui et al., 1990). All transcripts have identical extracellular domains but are heterogeneous in their cytoplasmic domains. FcγRIIa2 is a soluble isoform as a result of alternate RNA splicing (Warderdam et al., 1990). FcγRIIa1 and FcγRIIc are expressed by monocytes, macrophages and neutrophils, while the FcγRIIb isoform is expressed by monocytes, macrophages and B cells (Brooks et al., 1989). FcγRIIb binds human IgG subclasses in the order of 3>1>4>>2 and murine IgG in the order of 2b>2a>1>>3 (Ravetch & Kinet, 1991). The subclass specificity for FcγRIIa varies with allotype (see 1.7.5. FcγR polymorphisms).

1.7.3. FcγRIII (CD16)

FcγRIII is an extensively glycosylated protein with a molecular weight ranging from 50 to 80 kDa. It was first identified as a receptor on neutrophils that differed from the previously identified monocyte Fcγ receptors in the binding of both human IgG1 and a murine monoclonal antibody (3G8) (Fliet et al., 1982). Two FcγRIII genes have been identified and mapped to 1q23-24 on the long arm of chromosome 1 (Ravetch & Perussia, 1989). FcγRIIIA encodes the transcript, FcγRIIIa, and FcγRIIIB encodes for FcγRIIb. Both receptors possess an extracellular region of approximately 190 amino acids which contains two immunoglobulin-like domains. FcγRIIIa has a transmembrane region and a 25 amino acid
cytoplasmic tail (Simmons & Seed, 1988), whereas FcγRIIIb is attached to
the outer side of the plasma membrane by a glycoposphatidylinositol
(GPI) anchor (Huizinga et al., 1988; Selvaraj et al., 1988). This variance
between FcγRIIIa and FcγRIIIb is the result of a single amino acid
substitution at position 203. At this amino acid position, FcγRIIIA
expresses a phenylalanine which results in a transmembrane and
cytoplasmic region, while a serine at position 203 results in the GPI
moiety (Lanier et al., 1989a; Ravetch & Kurosaki, 1989). Both FcγRIIIa and
FcγRIIIb can exist as soluble receptors, generated by proteolytic cleavage
(Huizinga et al., 1990a; Harrison et al., 1991).

FcγRIIIa is expressed by macrophages (Fleit et al., 1982), a subpopulation
of monocytes (Ziegler-Heitbrock et al., 1988), natural killer (NK) cells and
large granular lymphocytes (LGL) (Lanier et al., 1983; Perussia &
Trinchier, 1984). Modulation of FcγRIIIa expression by monocytes will be
discussed later in this section. FcγRIIIa is a moderate-affinity receptor
since in addition to binding complexed IgG, it can also bind monomeric
IgG, albeit with a lower affinity than FcγRI (Ka ~ 3x10^7 M⁻¹) (van de
Winkel & Anderson, 1991; Vance et al., 1993). FcγRIIIb is expressed
constitutively by neutrophils (Fleit et al., 1982) and on IFN-γ treated
eosinophils (Hartnell et al., 1992) and has a low affinity for human IgG
(Ka < 10^7 M⁻¹). Both receptor isoforms bind human IgG subclasses in the
order of: 1=3>>>2,4 and murine IgG subclasses in the order of:
3>2a>2b>>1 (van de Winkel & Capel, 1993). The IgG binding site for both
FcγRIIIa and FcγRIIIb lies within the second immunoglobulin-like
(membrane-proximal) domain (Hibbs et al., 1994; Tamm et al., 1996;
Tamm & Schmidt, 1996).

1.7.4. Fcγ receptor subunits and signalling
Unlike FcγRII, FcγRI and FcγRIII lack a signalling motif and therefore rely
on associated subunits for signal transduction (Figure 4). FcγRIIIa (but not
FcγRIIIb) is associated with disulphide linked subunits. Macrophage
FcγRIIIa is associated with a gamma chain homodimer (γ-γ) which was
first described for the high affinity mast cell receptor, FceRI (Hibbs et al.,
1989; Ra et al., 1989), and is also associated with FcαR (Pfefferkorn &
Yeaman, 1994). FcγRIIIa expressed by NK cells may be associated with this
γ-chain dimer, however, it can also be found associated with either a
homodimer of the zeta chain (ζ-ζ) found in the TCR-CD3 complex (Lanier et al., 1989b; Anderson P. et al., 1990) or a heterodimer (ζ-γ) (Masuda & Roos, 1993). Expression of these subunits with FcγRIIIa is essential for receptor expression and the prevention of its degradation (Kurosaki & Ravetch, 1989; Kurosaki et al., 1991). Furthermore, work by Withmueller et al. (1992) has shown that the γ-chain is essential for FcγRIIIa signal transduction.

FcγRI and FcγRII have both been shown to associate with the γ-chain dimer (Ernst et al., 1993; Masuda & Roos, 1993). As for FcγRIIIa, this γ-chain subunit may be necessary for FcγRI receptor expression (van Vugt et al., 1996), however, others have demonstrated that not all membrane bound FcγRI is complexed with the γ chain (Allen & Seed, 1989; Masuda & Roos, 1993). Although FcγRI utilises the γ-chain for signalling (Scholl & Geha, 1993; Melendez et al., 1998), in some circumstances it may recruit FcγRII, which contains a signalling motif within its cytoplasmic domain (Melendez et al., 1998). Moreover, work by Miller et al. (1996) has suggested that the γ-chain may increase FcγR affinity for IgG and this may explain the high affinity of FcγRI and intermediate affinity of FcγRIIIa for monomeric IgG.

The cytoplasmic domains of both the γ and ζ chains contain a structural signalling motif, essential for their activating properties and common to all multichain immune recognition receptors. This structure was first described by Reth (1989) and later termed the Immunoreceptor Tyrosine-based Activation Motif (ITAM) (Cambier et al., 1995). It has the sequence YXXL-7x-YXXL where 7x denotes seven variable amino acids (Cambier et al., 1994). FcγRII also contains an ITAM within its cytoplasmic tail, however, this ITAM is unique in that the two YXXL sequences border 12 amino acids (Reth, 1989). Therefore, FcγRII can signal independently, however, recruitment of the γ-chain homodimer can modulate the signalling behaviour of this receptor (Van den Herik-Oudijk et al., 1995a). Fcγ receptor crosslinking or aggregation results in the phosphorylation of tyrosine residues within the ITAM by src-family protein tyrosine kinases (PTK). This is followed by the binding of syk-family PTK to the phosphorylated ITAM. Subsequent activation of these syk PTK results in a cascade of intracellular physiological responses.
Within the cytoplasmic tail of FcγRIIb1 lies an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) (Amigorena et al., 1992) which consists of a single YXXL sequence (Daëron, 1997) and this gives this receptor its inhibitory activity. For example, the simultaneous crosslinking of FcγRIIb1 and the B cell receptor (BCR) downregulates antibody secretion (Amigorena et al., 1992).
Figure 4. Schematic diagram of surface expressed human Fcγ receptors and their associated subunits. Adapted from Deo et al. (1997).
1.7.5. **Fcγ receptor polymorphisms**

If Fcγ receptors play a role in the pathogenesis of rheumatoid arthritis, then receptor polymorphisms may influence either an individual's susceptibility to development of disease, or may have an affect upon disease severity. However, to date, there are no reports linking any of the following FcγR polymorphisms with rheumatoid arthritis.

A rare expression polymorphism for FcγRI was identified by Ceuppens *et al.* (1988). They found that four healthy sisters from a Belgium family lacked the expression of FcγRI by their peripheral blood monocytes. Monocytes from 3000 normal individuals were tested from the same area and all expressed FcγRI (Van de Winkel *et al.*, 1995). Monocytes from the FcγRI deficient individuals were unable to bind the Fc region of mouse IgG2a or monomeric human IgG1 and were unreactive with two anti-FcγRI monoclonal antibodies. Furthermore, monocytes from these individuals failed to support mouse IgG2a anti-CD3 monoclonal antibody-induced T cell proliferation (Ceuppens *et al.*, 1985a; Ceuppens *et al.*, 1985b). Further analyses showed this expression polymorphism to be a result of a single nucleotide substitution in the open reading frame of the FcγRIα gene, causing an early stop codon in the first immunoglobulin-like extracellular domain of FcγRIα. Messenger RNA instability was observed and reverse transcription polymerase chain reaction analysis showed the FcγRIα transcript to be 15-20 fold lower than in controls. Interestingly, FcγRIβ2 transcripts were elevated in the FcγRIα deficient individuals and it was postulated that this may account for their normal phagocyte function (van de Winkel *et al.*, 1995).

FcγRIIa expressed by monocytes, macrophages, neutrophils and B cells displays a well described genetic polymorphism. This was first identified by the ability of human monocytes to bind a murine IgG1 anti-CD3 monoclonal antibody and induce T cell proliferation (Tax *et al.*, 1983). Monocytes from 70% of Caucasian individuals possessed this ability and were termed high responders, while the remaining 30% were termed non responders. This distribution was found to be reversed within the Asian population, with 15% high responders and 85% low responders (Abo *et al.*, 1984).
Molecular studies found that the polymorphic forms of FcγRIIa differed in a single amino acid at two amino acid positions. At position 27, within the first immunoglobulin-like domain (membrane-distal), FcγRIIa from high responders displayed a glutamine while low responders expressed a tryptophan (Clark et al., 1991). However, the FcγRIIa polymorphism was found to be due to the amino acid difference that occurred at position 131, within the second immunoglobulin-like domain (Warderdam et al., 1990). This membrane-proximal domain is the location of the IgG binding site (Hulett et al., 1995). At this position, FcγRIIa from high responders expressed an arginine (R) and low responders expressed a histidine (H). The allotypes were therefore termed FcγRIIa-131R (high responder) and FcγRIIa-131H (low responder). While FcγRIIa-131R binds murine IgG1 well, it was found that this allotype had only a weak affinity for human IgG2 dimers. Conversely, FcγRIIa-131H, which has a low affinity for murine IgG1, was found to bind human IgG2 dimers strongly. Both allotypes bind dimers of human IgG1 and IgG3 (Warderdam et al., 1991). Therefore the subclass specificity for human IgG is; FcγRIIa-131R: 1=3>2>4 and FcγRIIa-131H: 1=3=2>4 (Salmon et al., 1992), and for murine IgG; FcγRIIa-131R: 2a=2b=1 and FcγRIIa-131H: 2a=2b>>1 (van de Winkel & Capel, 1993). Furthermore, the murine anti-FcγRII monoclonal antibody, 41H16, binds to an epitope only expressed by the FcγRIIa-131R allotype (Gosselin et al., 1990).

The FcγRIIa polymorphism has also been found to influence neutrophil function. Neutrophils expressing FcγRIIa-131H were found to have a greater capacity for the phagocytosis of human IgG2 or IgG3-coated erythrocytes than neutrophils expressing FcγRIIa-131R (Salmon et al., 1992; Bredius et al., 1994). FcγRIIa polymorphism has also been associated with increased susceptibility for infections (Fijen et al., 1993; Sanders et al., 1994; Platonov et al., 1998) and the autoimmune disease SLE (Duits et al., 1995).

FcγRIIIb expresses a polymorphism designated neutrophil antigen 1 (NA1) and neutrophil antigen 2 (NA2). 37% of Caucasians express the FcγRIIIb-NA1 allotype, while the remaining 63% express FcγRIIIb-NA2 (van de Winkel & Capel, 1993). The two isoforms display differing levels of glycosylation and deglycosylated NA1 and NA2 have distinct
electrophoretic mobility (Ory et al., 1989a). Ravetch & Perussia (1989) found 5 nucleotide differences between NA1 and NA2. However, the polymorphism was found to be due to a two amino acid difference occurring at positions 65 and 82. This difference was found to result in two extra N-linked glycosylation sites within the membrane-distal domain of FcγRIIIb-NA2, giving it a total of six sites (Ravetch & Perussia, 1989; Ory et al., 1989b, Huizinga et al., 1990b). Furthermore, the monoclonal antibody, CBL Gran 11 only bound to FcγRIIIb-NA1, while the monoclonal antibody, GRM1 only reacted with FcγRIIIb-NA2 (van de Winkel & Capel, 1993). Functionally, neutrophils expressing the FcγRIIIb-NA1 allotype were found to have a greater capacity for the phagocytosis of both IgG1 and IgG3 coated particles than neutrophils expressing the FcγRIIIb-NA2, irrespective of the FcγRIIa polymorphism (Salmon et al., 1990; Bredius et al., 1994). However, there was no difference between the two NA allotypes in the levels of binding of opsonised erythrocytes (Salmon et al., 1992).

FcγRIIIb polymorphism has been associated with severe renal disease in systemic vasculitis (Wainstein et al., 1996) and with susceptibility to bacterial infections in combination with FcγRIIa polymorphism (Fijen et al., 1993). Recent data has suggested a link between FcγRIIIb polymorphism and multiple sclerosis (Myhr et al., 1999). FcγRIIIb deficiencies have also been observed. A patient with SLE was found to lack the expression of FcγRIIIb as a result of gene disorganisation (Clark et al., 1990), while neutrophils from two normal individuals were found by Huizinga et al. (1990c) to lack the gene, FcγRIIB.

A number of polymorphisms have been identified for FcγRIIIa. Vance et al. (1993) have described an expression polymorphism for FcγRIIIa on LGL and NK cells. By measuring the levels of human monomeric IgG1 binding to these cells it was found that some individuals expressed twice the number of FcγRIIIa than others. This was also reflected functionally since LGL/NK cells from individuals with high levels of FcγRIIIa showed a greater capacity for the ADCC of erythrocytes. The first structural polymorphism for FcγRIIIa was identified by Ravetch & Perussia (1989). They found that a nucleotide substitution at position 559 resulted in either a Valine (V) or a phenylalanine (F) at amino acid position 158 in
the membrane-proximal domain of FcγRIIIa, expressed by natural killer cells. A second structural polymorphism was described by de Haas et al. (1996). They found at amino acid position 48, NK cell FcγRIIIa possessed either a leucine (L), a histidine or an arginine, resulting in a triallelic polymorphism. Natural killer cells therefore expressed either FcγRIIIa-48L, FcγRIIIa-48H or FcγRIIIa-48R. Characterisation of this polymorphism showed that NK cells expressing FcγRIIIa-48R or FcγRIIIa-48H had a greater capacity for binding human IgG1 and IgG3 than cells expressing FcγRIIIa-48L. Furthermore, it was found that 86% of Caucasian individuals expressed the FcγRIIIa-48L allotype, while 8% expressed FcγRIIIa-48H and 6% expressed FcγRIIIa-48R. In contrast Japanese individuals were only found to express the FcγRIIIa-48L allotype. Further studies of both the -158V/F and -48L/H/R polymorphisms demonstrated that the two were linked. Individuals expressing the -158F genotype also expressed FcγRIIIa-48L, while those positive for the -158V genotype expressed either FcγRIIIa-48H or FcγRIIIa-48R (Koene et al., 1997). However, NK cells expressing FcγRIIIa-158V bound higher levels of human IgG1 and IgG3 than NK cells expressing FcγRIIIa-158F and this was independent of the -48L/H/R polymorphism (Koene et al., 1997; Wu et al., 1997). Moreover, the selective binding of the anti-FcγRII monoclonal antibody, MEM154, to the FcγRIII-158V allotype was found to be dependent upon this amino acid (Koene et al., 1997) and not the linked polymorphism at amino acid position 48 as originally thought (de Haas et al., 1996). However, the amino acid expressed at position 48 influenced the binding of the monoclonal antibody, B73.1 (Koene et al., 1997). The FcγRIIIa-158F genotype may be associated with an increased risk of developing systemic lupus erythematosus (Keone et al., 1998).

1.7.6. Modulation of Fcγ receptor expression

The levels of FcγRI expression can be enhanced by IFN-γ or G-CSF (Guyre et al., 1983; Perussia et al., 1983; Kerst et al., 1993). FcγRI and FcγRII expression can also be downregulated by interleukin-4 (IL-4) (te Velde et al., 1990). Previous studies have shown that a number of cytokines and growth factors can modulate monocyte FcγRIIIa expression. Liao and colleagues found that the treatment of monocyte-derived macrophages with TNFα or IL-1β resulted in the rapid downregulation of FcγRIIIa expression (Liao & Simone, 1994; Liao et al., 1994). FcγRIIIa levels
returned to at least that of the controls within 24 hours following initial cytokine exposure. It has also been demonstrated that the culturing of freshly isolated monocytes with either TGF-β1 or TGF-β2 induced FcγRIIIa expression (Welch et al., 1990; Wong et al., 1991). However, work generated from our laboratory suggests that TGFβ may in fact act as an inhibitor of FcγRIIIa expression by human monocytes in vitro (Bhatia et al., in preparation). More recently, it has been shown that human monocytes cultured with interleukin-10 (IL-10) induced high levels of FcγRIIIa expression (Calzada-Wach et al., 1996). FcγRIIIa expression has also been shown to be downregulated by the presence of IL-4 (te Velde et al., 1990; Wong et al., 1991).

1.7.7. Fcγ receptor expression by tissue macrophages
As discussed previously, synovial macrophages are activated in the rheumatoid synovium. It is possible that macrophage activation and the subsequent production of damaging cytokines may be occurring in response to a small IgG rheumatoid factor complex following its binding to a surface immunoglobulin receptor.

Tissue macrophages constitutively express FcγRII (van de Winkel & Capel, 1993) and some tissue macrophages express FcγRI at levels equal to, or greater than blood monocytes (Anderson CL et al., 1990). However, FcγRIIIa expression is highly restricted. Both alveolar and peritoneal macrophages from normal individuals have been shown to express FcγRIII (Anderson CL et al., 1990; Kindt et al., 1991), later confirmed to be the transmembrane isoform, FcγRIIIa (Levy et al., 1991). Bröker et al. (1990) investigated FcγR distribution in the synovium from patients with either rheumatoid arthritis or osteoarthritis. In both cases FcγRIIIa was found to be strongly expressed by synovial intimal macrophages. However, it was not clear whether this was constitutive or secondary to a local inflammatory response. More recently, studies on the expression of FcγR in normal tissues have demonstrated that high levels of FcγRIIIa are expressed by synovial intimal macrophages (Edwards et al., 1997a; Bhatia et al., 1998). Interestingly, in fetal limbs, FcγRIIIa was only expressed in the synovial intima (Edwards et al., 1997a). Furthermore, expression of FcγRIIIa by macrophages in other normal tissues (Bhatia et al., 1998) appears to correlate closely with the localisation of extra-
articular features (Maini & Zvaifler, 1998) in patients with rheumatoid arthritis (Table 1).

Table 1. The distribution of FcγRIIIa expression by normal tissue macrophages correlates with the pattern of systemic disease seen in patients with rheumatoid arthritis. Taken from Bhatia et al., 1998.

<table>
<thead>
<tr>
<th>Sites of FcγRIIIa expression</th>
<th>Systemic disease in RA</th>
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</thead>
<tbody>
<tr>
<td>Synovium (Intimal macrophages)</td>
<td>Synovitis</td>
</tr>
<tr>
<td>Lung (Alveolar macrophages)</td>
<td>Alveolitis</td>
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<tr>
<td>Pericardium</td>
<td>Pericarditis</td>
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<tr>
<td>Liver (Kupffer cells)</td>
<td>Hepatic dysfunction</td>
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<tr>
<td>(Palisading macrophages)</td>
<td>Acute phase response</td>
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<tr>
<td>Dermis under mechanical stress</td>
<td>Subcutaneous nodules</td>
</tr>
<tr>
<td>(Palisading macrophages)</td>
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<td>Bone marrow</td>
<td>Anaemia</td>
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<td>(Suppressed haematopoiesis)</td>
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These findings provide strong evidence for a role for FcγRIIIa in macrophage activation in rheumatoid arthritis. If either FcγRI or FcγRII were to be the dominant receptor involved in the binding of pathogenic IgG rheumatoid factor immune complexes in rheumatoid arthritis, then one would expect to see evidence of pathology in a less restrictive pattern. In theory, a small IgG rheumatoid factor complex would be able to activate any blood monocyte or tissue macrophage at any site. Instead, only a few specific tissues are affected in patients with rheumatoid arthritis. Furthermore, FcγRI is expressed very weakly in normal synovium, being limited to the subintima (Edwards et al., 1997a; Bhatia et al., 1998). FcγRI has a high affinity for IgG and its saturation with monomeric IgG at concentrations less than physiological levels can inhibit the binding of small IgG oligomers (Ceuppens et al., 1988; Tax & van de Winkel, 1990). Only a large immune complex would have the
ability to displace any monomeric IgG bound by FcγRI (Tax & van de Winkel, 1990). FcγRII is functionally associated with large immune complexes (Huizinga et al., 1989a). FcγRIIIa has higher affinity than FcγRII for aggregated IgG and unlike FcγRII, FcγRIIIa can bind monomeric IgG (Vance et al., 1993). Furthermore, neutrophil studies have shown FcγRIII to be the dominant receptor for the binding of dimeric immune complexes (Klaassen et al., 1988; Huizinga et al., 1989b). Therefore, it appears that FcγRIIIa may be the likely candidate for the selective binding of small IgG rheumatoid factor complexes, and owing to its restricted distribution, may explain tissue targeting in rheumatoid arthritis.

1.8. **A novel hypothesis for the pathogenesis of rheumatoid arthritis**

From the evidence discussed so far and a number of other observations which will also be discussed, a hypothetical model for the pathogenesis of rheumatoid arthritis was constructed (Edwards & Cambridge, 1998).

1.8.1. **Effector mechanism for the initiation of inflammation in rheumatoid arthritis**

The initiation of an inflammatory response within the synovium may occur as follows (Figure 5). Intermediate IgG rheumatoid factor complexes, owing to their small size, may evade clearance through complement receptor 1 (CR1) on erythrocytes (Tausk & Gigli, 1990; Wong, 1990), and therefore may persist within the circulation. In contrast, IgM-rheumatoid factor immune complexes may be rapidly cleared from the circulation by anti-phlogistic complement fixation (Walport et al., 1997). The small IgG rheumatoid factor complexes may then, again owing to their size, leave the circulation by crossing endothelium to enter the extravascular space, where they may access tissue macrophages. In the case of the synovium, intimal macrophages express the immunoglobulin receptor, FcγRIIIa. The small IgG rheumatoid factor complexes may selectively ligate FcγRIIIa. Crosslinking of this receptor may trigger macrophage activation, resulting in the production of pro-inflammatory cytokines and the generation of an intense respiratory burst. The release of both TNFα and reactive oxygen species could then cause local inflammation and tissue injury (reviewed by Moulton, 1996).
1.8.2. *IgG rheumatoid factor subclass*

The subclass of the IgG rheumatoid factor may be a major influence on the pathogenicity of these immune complexes, in addition to their size. Recent work has highlighted the importance of both IgG subclass and size on interactions between immune complexes and Fcγ receptors and complement receptors (Voice & Lachmann, 1997). Complexes of the subclasses IgG2 and IgG4 have been shown not to bind to neutrophils or monocytes, while IgG1 and IgG3 dimers bind neutrophils preferentially through FcγRIII (Klaassen et al., 1988; Huizinga et al., 1989b; Voice & Lachmann, 1997). However, IgG1 and IgG3 may differ in the way they interact with this receptor (Morgan et al., 1995). Furthermore, the Fc binding sites on an IgG3 dimer are approximately 200Å apart (with the hinges extended), while the Fc binding sites on an IgG1 dimer are around 75 Å apart (Edwards & Sutton, 1998). Therefore, IgG3 may only dimerise with another IgG3 molecule and although IgG3 binds FcγRIII very well, these steric properties may prevent FcγRIIIa signalling as effectively as with IgG1 dimers. This may explain the high levels of IgG3 rheumatoid factor dimers in patients with Waldenström’s hypergammaglobulinemic purpura, with no evidence of any synovitis (Bignold et al., 1980; Serino et al., 1983). Therefore the proposed mechanism outlined above may be triggered by small rheumatoid factor complexes of the subclass IgG1.

1.8.3. *Chronicity of the initial inflammatory event in rheumatoid arthritis*

The mechanism outlined in section 1.8.1. describes an effector mechanism for the initiation of inflammation within the synovium. However, for clinical synovitis and eventually joint degradation to manifest symptomatically, this inflammatory process must be a persistent one. In addition to causing local inflammation, TNFα has been shown to act upon synovial stromal cells. Unlike fibroblasts within most other tissues, synovial fibroblasts are particularly responsive to TNFα. As for bone marrow stromal cells, TNFα induces synovial fibroblasts to express vascular cell adhesion molecule-1 (VCAM-1), decay accelerating factor (DAF) and complement receptor 2 (CR2) (Marlor et al., 1992; Edwards et al., 1997b). Expression of these molecules is necessary for the survival and differentiation of B lymphocytes. Within germinal centres in normal lymphoid tissues, VCAM-1, DAF and CR2 are co-expressed by
follicular dendritic cells (Lampert et al., 1993; Koopman et al., 1994; Lui et al., 1997; Fang et al., 1998). The expression of these molecules by fibroblasts within the rheumatoid synovium (Wilkinson et al., 1993; Edwards et al., 1997b) could create a microenvironment capable of supporting the accumulation of B lymphocytes and allow their differentiation into plasma cells. This is seen in most rheumatoid synovia (Dechanet et al., 1995), however, true ectopic germinal centres form in the synovium of only 10 - 20% of patients with rheumatoid arthritis (Gardner, 1992). Some of these accumulating plasma cells within the synovium may produce IgM or IgG rheumatoid factors (Youinou et al., 1984; Carson, 1993). IgG rheumatoid factors generated locally may form large polymers since non-rheumatoid factor IgG levels are low compared with those in the circulation. These IgG rheumatoid factor multimers as well as IgM-rheumatoid factor complexes will have the ability to fix and activate complement in the environment of the synovium, and this may amplify the inflammatory process, originally initiated by the small circulating IgG rheumatoid factor complexes. The formation of IgG rheumatoid factors within the synovium may also augment macrophage activation and the subsequent release of proinflammatory factors by crosslinking FcγRI and FcγRII. Additionally, FcγRIIIa crosslinking results in the release of the chemokine, MCP-1 (Marsh et al., 1997) which may recruit monocytes from the peripheral blood into the synovium.

The question of why there should be a continuous production of IgG rheumatoid factor has not yet been addressed. A recent extension of the hypothesis proposes that IgG rheumatoid factors may have the potential to perpetuate their own existence by bypassing control mechanisms at both the T cell help and germinal centre levels (Edwards et al., 1999). This aspect of the hypothesis is largely outside the context of this dissertation but will be returned to briefly in the discussion.
Legend for Figure 5. Schematic diagram outlining the initiating effector mechanism for inflammation in rheumatoid arthritis.

1. The mechanism begins when small IgG rheumatoid factor complexes leave the circulation by crossing endothelium and entering tissues such as the synovium.

2. Here the small IgG rheumatoid factor complexes may access tissue macrophages which are expressing FcγRIIIa in addition to FcγRII and low levels of FcγRI. These complexes may selectively ligate FcγRIIIa.

3. Crosslinking of FcγRIIIa by the small IgG rheumatoid factor complex may activate the macrophage to produce proinflammatory cytokines like TNFα and to generate an intense respiratory burst, causing local tissue injury and inflammation.

4. Additionally, the TNFα may upregulate the expression of certain surface molecules by synovial stromal cells. TNFα induced-expression of VCAM-1, CR2 and DAF by synovial fibroblasts could create a microenvironment that can support B lymphocyte survival.

5. B lymphocytes may accumulate within the synovium and differentiate into plasma cells. Some of these plasma cells may then generate local rheumatoid factor production.

6. Production of both IgM and IgG rheumatoid factors within the synovium may generate large complement fixing polymers which may amplify the inflammatory response. Large IgG rheumatoid factor complexes may also crosslink FcγRI and FcγRII and perpetuate macrophage activation.
1.9. Hypothesis and aims of this thesis

1.9.1. Hypothesis and previous work by other groups

The hypothesis of this study is that FcγRIIIa may preferentially ligate small IgG rheumatoid factor immune complexes and mediate the activation of macrophages to produce proinflammatory mediators such as TNFα in patients with rheumatoid arthritis.

The aim of this study was to test this hypothesis in vitro by investigating the ability of human monocyte-derived macrophages to produce pro-inflammatory mediators, such as TNFα, following ligation of each of the three human Fc receptors for IgG (FcγR) by small IgG immune complexes.

FcγR mediated TNFα production by human monocytes in vitro has been previously investigated (Debets et al., 1988; Polat et al., 1993). However, few studies have focused upon the roles of individual Fcγ receptors. Work by Debets et al. (1990) studied the ability of FcγRI and FcγRII crosslinking to induce TNFα release from human peripheral blood monocytes. TNFα production through FcγRI was only observed in response to murine IgG2a in solid phase, while FcγRII only evoked a response to murine IgG1 in solid phase, following receptor treatment with proteolytic enzymes. Human FcγRIIIa has also been shown to mediate TNFα production following receptor crosslinking with either immune complexes or murine monoclonal antibodies. However, data is only available for natural killer cells (Anegón et al., 1988; Hendrich et al., 1991).
1.9.2. Aims

1. TNFα production by human monocytes or macrophages following FcγRIIIa ligation had not been previously demonstrated. Therefore, the initial aim of this thesis was to determine whether specific ligation of each of the three FcγR could mediate TNFα production by adherent human monocytes in vitro. This was achieved using murine monoclonal antibodies to each FcγR.

2. To demonstrate the production of other pro-inflammatory mediators by adherent human monocytes following ligation of each of the three Fcγ receptors using murine monoclonal antibodies. To examine any differential effects following ligation of each Fcγ receptor type.

3. To determine whether small IgG rheumatoid factor immune complexes could induce TNFα production by adherent human monocytes in vitro and to establish which FcγR were involved. For this purpose, IgG immune complexes of various size and subclass composition, as well as human IgG rheumatoid factor monoclonal antibodies were also used to ligate FcγR.

1.9.3. Experimental approaches

Peripheral blood monocytes were used in this project instead of synovial derived macrophages. Synovial macrophages from patients with rheumatoid arthritis spontaneously produce TNFα in culture due to their activation in vivo (Brennan et al., 1989) and normal synovial macrophages were not readily available. To create an in vitro system that was representative of the environment within the synovium, it was decided to use adherent monocytes. Many previous in vitro studies have used non-adherent monocytes and it was felt that this was not an accurate representation of in vivo cell conditions.

Within the synovium and other tissues targeted in patients with rheumatoid arthritis, tissue macrophages express all three FcγR, although within synovium FcγRI expression is often low. Therefore, the in vitro system used in these studies required expression of all three Fcγ receptors by the adherent human monocytes. Peripheral blood monocytes constitutively express FcγRI and FcγRII, however, FcγRIIIa expression is
low if at all evident (Fleit et al., 1982). A number of studies report that approximately 10% of peripheral blood monocytes express FcγRIIIa (Ziegler-Heitbrock et al., 1988; Passlick et al., 1989). Consequently, there have been studies investigating the upregulation of FcγRIIIa expression by peripheral blood monocytes in vitro. Fleit et al. (1982) found that after 7 days of culture in Teflon beakers, 15% of peripheral blood monocytes were strongly FcγRIIIa positive. Clarkson et al. (1987) confirmed these observations and showed that by day 10 of culturing in Teflon plates, 100% of monocytes expressed FcγRIIIa. Work by Klaassen et al. (1990) demonstrated that when peripheral blood monocytes were adhered to plastic and cultured, FcγRIIIa expression was also upregulated with time and that levels of expression plateaued after only 3 days in culture. This later system was chosen for the studies in this thesis since FcγRIIIa expression could be induced quickly on adherent peripheral blood monocytes, without the need to use exogenous cytokines.
CHAPTER 2

MATERIALS AND METHODS
2.1. **Primary cells and hybridomas used in these studies**

2.1.1 **Primary cells**
The only primary cell cultures used in these studies were of human peripheral blood monocytes, isolated from whole blood.

2.1.2. **Mouse cell lines**
The mouse cell line 3G8 which was a kind gift from Professor MW Fanger (Department of Immunology, Dartmouth College, Lebanon, NH) produced a murine IgG1 anti-human FcγRIII mAb.

2.1.3. **Human cell lines**
The two hybridomas, C1 and C2 were generous gifts from Professor PP Chen (Department of Medicine, University College of Los Angeles, Los Angeles, CA) and Professor DA Carson (Department of Medicine, University of California, San Diego, CA). Both cell lines produced human IgG rheumatoid factor mAb. The C1 clone produced a mAb of the isotype IgG2, while C2 produced an IgG1 rheumatoid factor mAb.

2.2. **Culturing of primary cells and continuous cell lines**
All primary cells and hybridomas were cultured in complete growth medium (CGM) which constituted modified medium (MM) (RPMI-1640 with 25mM HEPES; Gibco BRL, Paisley, Scotland) supplemented with 20μg/ml gentamycin, 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 2% non-essential amino acids, 1mM sodium pyruvate (all from Gibco) and 10% heat inactivated (incubated at 56°C for 30 minutes) fetal calf serum (FCS) (Sigma Chemical Co, Poole, Dorset). All cells were cultured in a humidified incubator set at 37°C in an atmosphere of 5% carbon dioxide in air.

2.3. **Murine monoclonal antibodies used in these studies**

2.3.1. **Anti-FcγRI monoclonal antibodies**
The anti-FcγRI mAb, designated 10.1, was a kind gift from Dr N Hogg (Imperial Cancer Research Fund, London). This murine mAb was originally derived from a fusion of Sp2/0-Ag14 cells with spleen cells from a BALB/c mouse previously immunised with rheumatoid synovial
fluid cells and purified human monocytes (Dougherty et al., 1987). The mAb 10.1, was found to be a murine IgG1 by double immunodiffusion in agar using murine class and subclass specific antisera.

The specificity of this monoclonal antibody for the high affinity receptor FcγRI, was determined following the immunoprecipitation of a 71-kDa protein by 10.1 from 125Iodine (I) labelled U937 cells (a human monocytic cell line). This was supported by indirect immunofluorescent staining and flow cytometry of human leucocytes and cell lines. It was found that 10.1 reacted with a surface molecule expressed by human monocytes, U937 cells and HL60 cells (a human promyelocyte cell line) and a small number of human neutrophil samples.

Studies of the inhibition of rosette formation between U937 cells and opsonised human red blood cells by 10.1 suggested that this monoclonal antibody bound to an epitope located near to, or within, the ligand binding site of FcγRI. Monoclonal antibody, 10.1 also inhibited murine IgG2a anti-CD3 induced T cell proliferation. However, the binding of monomeric human IgG to U937 cells was not inhibited by 10.1.

The mAb 32.2 was a kind gift from Professor PM Lydyard (Department of Immunology, University College London, London). This murine anti-FcγRI mAb was derived from a fusion between the NS-1 myeloma cell line and splenocytes from a mouse previously immunised with partially purified FcR from U937 cells (Anderson et al., 1986). The mAb 32.2 is a murine IgG1 as determined by immunoblot assays using isotype-specific antisera. The specificity of this antibody for FcγRI was confirmed by the ability of intact 32.2 and also its Fab fragments to immunoprecipitate a 72-kDa molecule from radiiodinated U937 cells. Indirect immunofluorescent staining and flow cytometry of human leucocytes and cells lines showed the mAb 32.2 to only bind FcγRI bearing cells (human monocytes, U937s, HL60s and some neutrophil samples). The mAb 32.2 did not react with murine FcRI or FcRII.
Blocking experiments using human IgG demonstrated the mAb 32.2 to bind an epitope distinct from the ligand binding site of FcγRI. Therefore, the mAb 32.2 did not interfere with ligand binding by the receptor, nor did ligand binding interfere with mAb 32.2 binding.

2.3.2. Anti-FcγRII monoclonal antibodies
The murine IgG2b anti-FcγRII mAb named IV.3 was a kind gift from Professor MW Fanger. This murine mAb was generated from a fusion of a BALB/c myeloma cell line (P3. x 63.Ag8.653) with spleen cells from a CAF1 mouse previously immunised with the human erythroblastic cell line K562 (Looney et al., 1986b).

The specificity of this monoclonal antibody for FcγRII was determined by the immunoprecipitation of a 40-kDa surface molecule by IV.3 from both K562 and U937 cells. The complete inhibition of FcR mediated rosette formation between human O⁺ erythrocytes opsonised with human IgG anti-Rh and U937 cells by IV.3 suggested that the mAb was binding to an epitope within the ligand binding site of FcγRII. This was confirmed by the inhibitory effect of IV.3 on FcγR mediated superoxide production by human neutrophils (Looney et al., 1986b).

2.3.3. Anti-FcγRIII monoclonal antibodies
The anti-FcγRIII mAb (3G8) producing cell line was cultured to generate supernatants from which the antibody could be purified for F(ab')₂ and Fab production. Purified 3G8 was purchased from Immunotech (Marseilles, France) to be used for all whole antibody experiments. Purified 3G8 was also purchased from PharMingen (San Diego, CA) for quality control. Endotoxin concentrations were <0.01ng/µg protein, as determined by Limulus amebocyte lysate assay.

The mAb, 3G8 was originally raised following the fusion of the P3U1 myeloma cell line with spleen cells from CD₂F mice previously immunised with human PMN (Fleit et al., 1982). The mAb, 3G8 was typed as a murine IgG1 by Ouchterlony analysis and did not react with mouse FcR.
Using the immunofluorescent staining of human leucocytes and cell lines, 3G8 was found to react with all PMN, all eosinophils and a small population of peroxidase-positive lymphocytes (~16%). All monocytes, U937 cells and HL60 cells and peroxidase-negative lymphocytes were negative for 3G8 binding. The specificity of 3G8 for FcyRIII was confirmed by ability of the mAb to immunoprecipitate a molecule ranging from 53 to 66-kDa from $^{125}$I labelled PMN.

2.3.4. **Anti-TNF monoclonal antibodies**
The murine IgGl anti-human TNFα mAb (CB006) was a kind gift from Celltech Therapeutics Ltd (Slough, Berkshire). This mAb blocks the biological activity of human TNFα (Charpentier *et al.*, 1992) by binding an epitope involved in TNF receptor binding (Dr S Stephens, personal communication).

2.4. **Human monoclonal antibodies used in these studies**

2.4.1. **IgG rheumatoid factor monoclonal antibodies**
The cell lines C1 and C2 were cultured to produce supernatants from which human IgG1 (C2) or IgG2 (C1) rheumatoid factor mAb could be purified. These IgG rheumatoid factor secreting hybridomas were generated following the fusion of synovial fluid mononuclear cells from the knee of a patient with seropositive rheumatoid arthritis (less that 1 year duration) with the heterohybridoma cell line, K6H6/B5 (Lu *et al.*, 1993). The products of the cell lines C1 and C2 were typed as IgG2 and IgG1 respectively by enzyme linked immunosorbant assay (ELISA). Characterisation by ELISA also showed the products of both cell lines to have rheumatoid factor activity and were monospecific since there was no significant binding to human collagen IV, bovine serum albumin, chicken ovalbumin, key-holelimpet hemocyanin, tetanus toxoid or calf thymus single stranded DNA. Fast protein liquid chromatography (FPLC) demonstrated the ability of these human IgG rheumatoid factor mAb to self-associate (Lu *et al.*, 1992). Molecular characterisation of these two mAb showed that both possess λ light chains. The IgG1 mAb (C2) used the heavy chain variable gene family $V_H4.11$ and the IgG2 mAb (C1) used a light chain variable gene from a new $V\lambda$ family designated $V\lambda9$. 

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2.4.2. IgG anti-NIP monoclonal antibodies
Purified chimeric anti-5-iodo-4-hydroxy-3-nitrophenacetyl (NIP) monoclonal antibodies were a generous gift from Professor PJ Lachmann (Medical Research Council Centre, Cambridge University, Cambridge) and Dr J Voice (Department of Immunology, Scripps Research Institute, San Diego, CA). These chimeric mAb (containing mouse variable regions and human constant regions) of each IgG subclass were raised against the hapten, 4-hydroxy-3-nitrophenacetyl (NP) (Brüggemann et al., 1987) and were produced by the following cell lines: THG1-24 (IgG1), JW183/5/1 (IgG2), TH3-MP-2-19-3-8 (IgG3) and JW184/2/1 (IgG4). Each of these monoclonal antibodies were purified by affinity chromatography using a NIP-caproate-o-succinimide Sepharose column. Characterisation of these mAb with respect to their serology, mobility in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and ability to bind Protein A showed their behaviour to be identical to their human counterparts (Brüggemann et al., 1987). Each of the IgG anti-NIP mAb were checked by ELISA against the four human IgG subclasses and mAb purity were confirmed by SDS-PAGE (Voice & Lachmann, 1997).

2.5. Purification of monoclonal antibodies by affinity chromatography

2.5.1. Purification of murine monoclonal antibodies
Cell-free culture supernatants containing the anti-FcγRIII mAb (3G8) were pooled, adjusted to pH8 with 1M sodium hydroxide (NaOH) and filtered (pore size 0.2µm) (Gelman, Portsmouth). The supernatant was then concentrated under pressure (Nitrogen at 15 pounds per square inch/1 kilogram per centimetre squared) with continuous stirring, in an Amicon concentrator over a Diaflow ultrafiltration membrane with a molecular weight (MW) cut-off at 30-kDa (Millipore, Watford, Hertfordshire). Both the concentrated supernatant and the filtrate were collected and tested for protein concentration and total murine IgG content according to the protocols described later in this chapter.

The anti-FcγRIII mAb was then purified from the concentrated supernatant using affinity chromatography. Briefly, the supernatant was loaded onto a 2 ml Protein G-Sepharose 4B column (Sigma) previously equilibrated with phosphate buffered solution (PBS, pH8) at 4°C.
Following washing with PBS, the mAb was eluted with one column volume of 3M magnesium chloride (MgCl) and dialysed into PBS. The purified mAb was then concentrated using a centricon 30 with a MW cut-off at 30-kDa (Millipore). The concentrated monoclonal antibody was then filtered, aliquoted and stored at -70°C. The protein and murine IgG concentrations were then tested.

2.5.2. Preparation of F(ab')\(_2\) fragments of the anti-FcyRIII mAb
Preparation of anti-FcyRIII F(ab')\(_2\) fragments was performed by Cybos Ltd (Southampton). Briefly, the anti-FcyRIII mAb was concentrated to 5mg/ml using a Vivascience Vivapore concentrator and then dialysed into 20mM sodium acetate buffer (pH4.2) overnight at 4°C. Immobilised pepsin (Peirce, Rockford IL) was washed four times in sodium acetate buffer (pH4.2) and 125μl of this was added to the antibody in a glass bottle. The mixture was then incubated at 37°C on an orbital mixer for 12 hours, after which the proteolytic reaction was stopped by microcentrifuging the mixture and removing the supernatant. The digest was purified from the supernatant using a Protein G-Sepharose 4B column. The F(ab')\(_2\) fragments were collected as flow through, dialysed into PBS and concentrated. Purity of the F(ab')\(_2\) digest was confirmed by SDS-PAGE.

2.5.3. Preparation of Fab fragments of the anti-FcyRIII mAb
Preparation of anti-FcyRIII Fab fragments was performed by Cybos Ltd. The anti-FcyRIII mAb was concentrated to 5mg/ml using a Vivascience Vivapore concentrator and then dialysed into 20mM sodium acetate buffer (pH4.2) overnight at 4°C. Immobilised papain (Peirce) was washed four times in sodium acetate buffer (pH4.2) and 125μl of this was added to the antibody in a glass bottle. The mixture was then incubated at 37°C on an orbital mixer for 12 hours, after which the proteolytic reaction was stopped by microcentrifuging the mixture and removing the supernatant. The digest was purified from the supernatant using a Protein G-Sepharose 4B column. The Fab fragments were collected as flow through, dialysed into PBS and concentrated. Purity of the Fab digest was confirmed by SDS-PAGE.
2.5.4. **Purification of human monoclonal antibodies**

Cell-free culture supernatants containing either the IgG1 (C2) or the IgG2 (C1) rheumatoid factor mAb were pooled and adjusted to pH 7.4 by adding one volume of 1M phosphate buffer to nine volumes of supernatant. The supernatant was concentrated as described previously for the murine anti-FcyRIII mAb. Both IgG-RF mAb were purified by affinity chromatography. Briefly, the concentrated supernatant was loaded onto a 2 ml Protein G-Sepharose 4B column previously equilibrated with PBS (pH 7.4) at 4°C. Following washing with PBS, the mAb was eluted with one column volume of 0.1M glycine (pH 2.8) and rapidly concentrated using polyethylene glycol 20,000 at 4°C as a hydroscopic agent. The purified mAb was filtered, aliquoted and then snap frozen after which it was stored at -70°C. Prior to use, the mAb was adjusted to pH 7.4 by adding one volume of 1M phosphate buffer (pH 7.4) to nine volumes of the purified monoclonal antibody. Protein concentration, human IgG concentration and rheumatoid factor activity were tested according to the protocols described below.

2.6. **Measurement of protein concentration**

A modified Bradford assay (Bradford, 1976; Locke, 1994) was used to determine protein concentration. Serial doubling dilutions of bovine serum albumin (BSA; Sigma) dissolved in PBS starting from a concentration of 2mg/ml were used to construct a standard curve. 10μl of the test solution (in PBS) or BSA standards were pipetted into a 96 well Immulon II plate (Dynex Technologies, Chantilly, VA). All samples were assayed in duplicate. 190μl of the modified dye reagent was then added to all wells and the plate left to stand for 5 minutes before absorption was measured at 595nm. A standard curve was constructed by plotting the averaged optical densities of the standards on the y-axis, against concentration on the x-axis. The protein concentration of the test samples was then calculated from the standard curve.
2.7. Measurement of immunoglobulin (IgG) concentration
Murine and human IgG concentrations were determined by ELISA.

2.7.1. Measurement of murine IgG concentration
Immunol II ELISA plates (Dynatech) were coated with F(ab')2 goat anti-mouse IgG antibody (Fab specific; Sigma) at 1μg/ml in bicarbonate buffer (BIC) for 1 hour at 37°C. After washing 3 times with PBS/0.1% twen (PBT) the plates were blocked with 200μl of 2%BSA/PBT at 37°C for 1 hour. Following washing as before, 100μl of test samples at various dilutions or an isotype-matched murine IgG (Southern Biotechnology Associates, Birmingham, AL) in doubling dilutions from 1μg/ml as standards were loaded onto the plates. All dilutions were made in 1% BSA/PBT and all samples were assayed in duplicate. The plates were incubated at 37°C for 1 hour, washed three times with PBT, then 100μl of alkaline phosphatase conjugated goat anti-mouse IgG (whole molecule specific, Sigma) at 1μg/ml was added to each well and the plates incubated as before. Following washing three times with PBT and three times with BIC, 100μl of phosphatase substrate (p-Nitrophenyl phosphate disodium (Sigma) at 1mg/ml in BIC with 10mM MgCl) was added to each well. After a 10 minute incubation at 37°C, absorbance was measured at 405nm with a reference of 490nm. A standard curve was constructed by plotting the averaged optical densities of the standards along the y-axis and ln concentration on the x-axis. Averaged optical densities of the test samples were read off the standard curve and the IgG concentration calculated.

2.7.2. Measurement of human IgG concentration
Immunol II ELISA plates (Dynex) were coated with goat F(ab')2 anti-human IgG (Fc specific; Sigma) at 1μg/ml in BIC for 1 hour at 37°C. After washing 3 times with PBT the plates were blocked with 200μl of 2%BSA/PBT at 37°C for 1 hour. Following washing as before, 100μl of test samples at various dilutions or human IgG (Sigma) in doubling dilutions from 1μg/ml as standards were loaded onto the plates. All dilutions were made in 1% BSA/PBT and all samples were assayed in duplicate. The plates were incubated at 37°C for 1 hour, washed three times with PBT, then 100μl of alkaline phosphatase conjugated goat anti-human IgG (γ chain specific; Sigma) at 1μg/ml was added to each well and the plates incubated as before. Following washing three times with PBT
and three times with BIC, 100μl of phosphatase substrate was added to each well. After a 10 minute incubation at 37°C, absorbance was measured at 405nm with a reference of 490nm. Averaged optical densities of the standards were plotted along the y-axis and ln concentration plotted on the x-axis. Averaged optical densities of the test samples were read off the standard curve and the IgG concentration calculated.

2.8. Measurement of rheumatoid factor activity
To determine rheumatoid factor activity of the purified human IgG rheumatoid factor mAb (C1 and C2), the ability of these lambda (λ) chain containing monoclonal antibodies to bind human IgG Fc was determined by a modification of an ELISA described previously (Lu et al., 1992). Briefly, Immunol II plates were coated with Fc fragments of human IgG (Jackson Immunoresearch Laboratories inc., Westgrove, PA) at 50μg/ml in BIC. The plate was incubated at 37°C for 1 hour and then washed three times with PBT. The plates were then blocked by incubating at 37°C for 1 hour with 200μl of 4% BSA/PBT. Following washing as before, 100μl of the purified IgG-RF mAb samples diluted to known concentrations were loaded onto the plate. A λ chain containing human IgG1 or IgG2 myeloma protein (The Binding Site, Birmingham) was used as a negative control and a λ chain containing rheumatoid factor (Cambridge Life Science, Cambridge) was used as a positive control. All controls were assayed at concentrations equal to the test monoclonal antibody. All dilutions were made in 4%BSA/PBT and all samples were assayed in duplicate. The plates were incubated at 37°C for 1 hour and then washed as before. 100μl of an alkaline phosphatase conjugated goat anti-human λ light chain antibody (bound and free; Sigma) was added to each well at 1μg/ml and the plates incubated as before. Following washing three times with PBT and three times with BIC, 100μl of phosphatase substrate was added to each well. After a 1 hour incubation at 37°C, absorbance was measured at 405nm with a reference of 490nm. Rheumatoid factor activity of the IgG-RF mAb was expressed as a percentage of the positive control.
2.9. **Immune complexes used in these studies**

2.9.1. *Measurement of human IgG rheumatoid factor mAb immune complex size by analytical high-performance liquid chromatography*

High-performance liquid chromatography (HPLC) was used to measure the molecular weights of immune complexes consisting of the IgG1 (C2) or the IgG2 (C1) rheumatoid factor monoclonal antibodies. The purified IgG1 or IgG2 rheumatoid factor mAb was adjusted to a concentration of 1mg/ml in PBS and was then passed through a Biosep size exclusion column S3000 (Phenomenex inc, Torrance, CA) which had been pre-equilibrated with 0.5M Tris/0.25M NaCl (pH 7.2). A λ chain containing human IgG1 or IgG2 myeloma protein (The Binding Site) was also analysed as a control. The column was set to run at 1ml/min and protein was detected at an absorbance of 280nm. Previously the column was calibrated using markers of known molecular weights (Sigma). The data from these calibration markers was used to construct a standard curve from which elution times of the IgG-RF mAb could be read to obtain molecular weights of the IgG-RF containing immune complexes (Appendix 2).

2.9.2. *Separation of human IgG rheumatoid factor mAb immune complexes by preparative high performance liquid chromatography*

To obtain purified IgG-RF mAb containing immune complexes of a known size, HPLC was performed as described above. However, as the IgG-RF mAb complexes passed through the column, fractions were collected every 20 seconds. The time at which each fraction was collected was read off the calibration curve and the size of the sample could be determined. Protein concentration of each fraction was determined by measuring absorbance at 280nm. Approximate immunoglobulin concentrations were determined by dividing this absorbance reading by 1.4 for an immunoglobulin concentration expressed as mg/ml. All fractions were filtered and where necessary, concentrated using a centricon 30 with a MW cut-off at 30-kDa (Millipore). Unless stated otherwise, the fractions were placed on ice and used in further experiments on the same day as preparation. Prior to the incubation of complexes with cell cultures, the fractions were dialysed into PBS.
2.9.3. Preparation of human IgG anti-NIP mAb immune complexes

Immune complexes were formed between human IgG anti-NIP mAb of each IgG subclass and NIP conjugated to BSA. Both were generous gifts from Professor PJ Lachmann and Dr J Voice. The NIP conjugated BSA used in these studies had been previously prepared (Voice & Lachmann, 1997) and stored at -70°C.Briefly, after coupling NIP-caproate-O-succinimide to the BSA at various concentrations, any free NIP was removed and the average molar ratios of NIP:BSA determined. The NIP:BSA molar ratios used in these experiments were 2.4NIP:1BSA, 8.9NIP:1BSA and 21.5NIP:1BSA where the number of NIP molecules per BSA will be referred to as the epitope density of the antigen.

Immune complexes were prepared as described previously (Crockett-Torabi & Fantone, 1990; Voice & Lachmann, 1997). Briefly, the IgG anti-NIP mAb of a selected subclass was diluted to 200μg/ml in Hank's balanced salt solution without phenol red (HBSS; Gibco). The antibody was then mixed with the antigen (NIP/BSA) at a selected epitope density and concentration such that complexes were prepared in either a molar excess of antigen, a molar excess of antibody or in an antigen:antibody molar ratio where the epitope density was equivalent to the number of antibody molecules (referred to as equivalence). The antibody/antigen preparations were incubated at 37°C for 1 hour and then cooled on ice for 20 minutes. The immune complexes were used in further experiments on the same day as preparation.

2.10. Source of human peripheral blood monocytes

Peripheral blood monocytes constitute only 10% of circulating leucocytes (Ziegler-Heitbrock et al., 1988). Therefore, to consistently obtain a highly purified monocyte preparation, large quantities of whole blood were required on a regular basis. Whole blood was generously supplied by both Haematology Day Care (PPW 2) and Haematology Outpatient Departments at University College Hospital (London). Venous blood (300-500ml) was collected from patients with either polycythaemia or haemachromatosis undergoing therapeutic bloodletting, into sterile wet packs (Baxter Healthcare Ltd, Thetford, Norfolk) containing 63ml citrate phosphate dextrose adenine 1 anti-coagulant solution. The majority of blood used in these studies was collected from patients with
haemachromatosis. Blood was stored at 4°C and used within 24 hours of collection.

Although monocyte function has not been examined in patients with haemachromatosis, there have been reports that both neutrophils and monocytes from patients with polycythaemia display abnormally low reactive oxygen species production in response to formyl-methionyl-leucyl-phenylalanine. However, both resting and phorbol-12-myristate 13-acetate (PMA) stimulated neutrophils and monocytes displayed normal free radical production (Samuelsson et al., 1994). Therefore, for all chemiluminescence studies, monocytes were isolated from patients with haemachromatosis.

Whole blood and isolated monocytes from normal donors were also used in a small number of experiments including FACS analysis of whole blood, chemiluminescent studies and some titrations of reagents used to study the production of cytokines in vitro. These experiments were used to corroborate results from similar experiments performed on whole blood and monocytes from patients with polycythaemia and haemachromatosis.

2.10.1. Isolation of human peripheral blood monocytes from whole blood
Nine volumes of whole blood was mixed with one volume of 0.9% sodium chloride/6% dextran to sedimentate erythrocytes. One volume of buffy coat was then layered onto 2 volumes of Histopaque (density 1.077 g/ml; Sigma) and centrifuged at 400g for 30 minutes at room temperature. The mononuclear cell layer lying at the plasma/Histopaque interface was removed and diluted 1:1 with MM. Following mixing by inversion, the cell suspension was centrifuged at 250g for 10 minutes at room temperature. The supernatant was discarded, the pellet resuspended in MM and the suspension centrifuged at 100g for 10 minutes at room temperature. This was repeated another five times to remove contaminating platelets. The mononuclear cell pellet was then resuspended in MM supplemented with 10% heat inactivated FCS to a concentration of approximately 5 x 10⁶ cells/ml. The cell suspension was transferred into 100mm plastic petri dishes (Bibby Sterilin Ltd, Staffordshire) and incubated at 37°C for 2 hours. Nonadherent cells were
removed by vigorously washing six times with warmed HBSS. Adhered cells were gently harvested using a rubber policeman, resuspended in CGM and the concentration adjusted to $5 \times 10^5$ cells/ml (unless stated otherwise).

Monocyte purity was determined by indirect immunofluorescent staining and flow cytometry for CD14 and CD3 expression and was shown to be greater than 95%. CD14 and CD3 expression were determined using the murine mAb TuK4 and UCHT1 respectively (DAKO, Ely, Cambridge). Trypan blue (Sigma) exclusion showed monocyte viability to be greater than 98%. Monocyte purity and viability were checked routinely throughout the study to ensure high levels of both were maintained.

2.11. Immunofluorescent staining and flow cytometry

2.11.1. Indirect immunofluorescent staining of whole blood
Indirect immunofluorescent staining of whole blood was performed as described previously (Maeda et al., 1996) with some modifications. Briefly, 2 ml of whole blood was diluted 1:5 with heparinised (10 units of heparin/ml) staining buffer (1% BSA and 0.1% azide in PBS) and centrifuged at 250g for 5 minutes at 4°C. The supernatant was discarded and the wash repeated. The pellet (plasma-free blood) was then made up to a total volume of 2 ml with staining buffer.

25μl of the test antibody or isotype-matched control (Table 2) at final concentrations as indicated by each experiment, was mixed with 100μl of plasma-free blood and incubated on ice for 45 minutes. 150μl of ice cold staining buffer was added to each sample and mixed by gentle pipetting. The samples were then centrifuged in a bench top microfuge at 1850g for 5 minutes and the supernatants discarded. This wash was repeated and the pellets resuspended with 25μl of fluorescein isothiocyanate (FITC) conjugated goat F(ab')2 anti-mouse Ig (1:20 dilution; DAKO). Following incubation on ice for 45 minutes in the dark and then washing twice as before, the cell pellets were resuspended in 2 ml of lysing buffer (Becton Dickenson, Oxford). After mixing by inversion the samples were incubated at room temperature for 15 minutes in the dark, washed twice with 2 ml of cold staining buffer and the erythrocyte-free pellets then
resuspended in 250μl of 2% paraformaldehyde in PBS to fix the cells. The samples were stored at 4°C in the dark before being analysed by flow cytometry.

2.11.2. Indirect immunofluorescent staining of isolated monocytes
1ml of freshly isolated human monocytes (1x10^6/ml) were seeded into 24 well plastic tissue culture plates (Becton Dickenson) and allowed to adhere at 37°C for 24 hours (unless stated otherwise). Prior to staining, the adhered cells were harvested by cooling on ice and gentle pipetting. The monocyte suspension was washed twice with 1ml of cold staining buffer. The monocyte pellets were then resuspended with 25μl of the test antibody or isotype-matched control (Table 2) at final concentrations as indicated by each experiment, incubated on ice for 45 minutes and then 150μl of ice cold staining buffer added to each sample and mixed by gentle pipetting. The samples were centrifuged in a bench top microfuge at 1850g for 5 minutes and the supernatants discarded. This wash was repeated and the pellets resuspended with 25μl of FITC conjugated goat F(ab')2 anti-mouse Ig (1:20, DAKO). The samples were incubated on ice for 45 minutes in the dark, washed twice as before, and the cells were then fixed with 250μl of 2% paraformaldehyde in PBS. Samples were kept at 4°C in the dark until analysed.

Table 2. Primary monoclonal antibodies used in the indirect immunofluorescent staining of whole blood and/or isolated monocytes.

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Species</th>
<th>Isotype</th>
<th>Specificity for human surface antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCHT1</td>
<td>mouse</td>
<td>IgG1κ</td>
<td>CD3</td>
</tr>
<tr>
<td>TüK4</td>
<td>mouse</td>
<td>IgG2κ</td>
<td>CD14</td>
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<td>FcγRI (CD64)</td>
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<td>IgG2bκ</td>
<td>FcγRII (CD32)</td>
</tr>
<tr>
<td>3G8</td>
<td>mouse</td>
<td>IgG1κ</td>
<td>FcγRIII (CD16)</td>
</tr>
</tbody>
</table>

The mouse monoclonal antibodies IgG1κ (DAK-G01), IgG2κ (DAK-G05) and IgG2bκ (DAK-G09) which are all specific for Aspergillus niger glucose oxidase (all from DAKO) were used as isotype-matched controls.
Aspergillus niger glucose oxidase is an enzyme which is neither present nor induced in mammalian tissues.

2.11.3. Flow Cytometry
Flow cytometry was performed using a Fluorescence Activated Cell Sorter (FACScan; Becton Dickenson). For each sample, 10,000 events were acquired. The instrument settings used for the analysis of whole blood and purified, cultured monocytes are shown in Table 3. The forward light scatter detected cell size while the side 90° light scatter detected cell granularity. The FL-1 channel detected the light emission of the excited fluorochrome, FITC, as fluorescent intensity. For the analysis of whole blood, each cell population was gated on, according to cell size and 90° light scatter. No such gating was necessary for the analysis of isolated monocytes.

Table 3. FACScan instrument settings.

a) FACScan settings for analysis of whole blood.

<table>
<thead>
<tr>
<th></th>
<th>Volts</th>
<th>Gains</th>
<th>Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward scatter</td>
<td>0</td>
<td>1.00 (linear)</td>
<td>50-200</td>
</tr>
<tr>
<td>Side scatter</td>
<td>400</td>
<td>1.00 (linear)</td>
<td>off</td>
</tr>
<tr>
<td>FL1</td>
<td>548</td>
<td>log</td>
<td>off</td>
</tr>
</tbody>
</table>

b) FACScan settings for analysis of isolated monocytes.

<table>
<thead>
<tr>
<th></th>
<th>Volts</th>
<th>Gains</th>
<th>Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward scatter</td>
<td>4</td>
<td>4.76 (linear)</td>
<td>25-200</td>
</tr>
<tr>
<td>Side scatter</td>
<td>400</td>
<td>1.00 (linear)</td>
<td>off</td>
</tr>
<tr>
<td>FL1</td>
<td>548</td>
<td>log</td>
<td>off</td>
</tr>
</tbody>
</table>

The data was analysed using Win MDI software (Microsoft).
2.11.4. Calibration of the FACScan

The FACScan was calibrated using standard fluorescent beads Fluorospheres; DAKO) as described previously (Le-Bouteiller et al., 1983). These beads are a mixture of 5 populations of 3.2μm microspheres, each with a different fluorescent intensity. Each bead population is covered with a known amount of fluorochrome that can be excited at any wavelength between 364 and 650nm. The laser beam of the FACScan excited the fluorochrome at 488nm and the light detection set at 548nm to calibrate the FL-1 channel for FITC. The mean fluorescent intensities (MFI) for each bead population were measured and using the assigned values for molecules of FITC per bead, a calibration curve was constructed (Appendix 3).

2.11.5. Calculation of the number of Fcγ receptor binding sites per cell

The mean fluorescent intensity was directly proportional to the degree of anti-FcγR mAb cellular staining and therefore the levels of Fcγ receptor expression. Using the MFI's previously obtained from the standard fluorescent calibration beads, the equation below for the number of binding sites per cell could then be solved.

\[ \text{Receptor binding sites per cell} = \frac{\text{Test FITC molecules} - \text{Control FITC molecules}}{\frac{f}{p} \text{ ratio}} \]

Where:

FITC molecules = \( \exp \left\{ \frac{(\ln \text{MFI} - c)}{m} \right\} \)

\( \frac{f}{p} \) ratio: \( \frac{E_{495 \text{ nm}}}{E_{278 \text{ nm}}} = 0.61 \pm 0.05 \) corresponding to a molar FITC/protein ratio of 2.3

* Receptor binding sites per cell is equivalent to the number of secondary antibody molecules (F(ab')\(_2\) goat anti-mouse Ig) bound per cell (Fanger et al., 1989), however, by accounting for the \( \frac{f}{p} \) ratio, the actual number of FcγR binding sites were calculated (Maeda et al., 1996).
2.12. Culturing of human monocytes and production of cytokines in vitro

200μl of freshly isolated monocytes were seeded into 96 well plastic tissue culture plates (Becton Dickenson) and incubated at 37°C for 24 hours (unless stated otherwise). The culture supernatants were removed and replaced with fresh CGM.

The adherent human monocytes were then incubated with various monoclonal antibodies (Table 4) at final concentrations as indicated by each experiment, in a total volume of 200μl CGM. As negative controls, cultures were incubated with either isotype-matched controls at concentrations equal to that of the test mAb, or medium alone. As positive controls, cultures were incubated with lipopolysaccharide (LPS) from Escherichia coli 026:B6 (Sigma) in the absence of polymixin B or PMA (Sigma), both at a final concentration of 0.5μg/ml. Possible LPS contamination of reagents was excluded by performing all incubations in the presence of polymixin B (10 μg/ml; Gibco), which consistently abrogated the LPS-induced TNFα response. Since supernatants were to be collected at various time intervals, each culture was prepared in multiples so that each culture corresponded with an individual time point. All cultures were incubated at 37°C and cultures supernatants collected at various time points between 0 and 24 hours (unless stated otherwise). Once collected, the supernatants were centrifuged in a bench top microfuge at 7000g for 5 minutes. Cell-free supernatants were recovered and stored at -70°C.
Table 4. Monoclonal antibodies incubated with adhered human monocytes for measurement of cytokine production in vitro.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Isotype</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.1</td>
<td>Mouse</td>
<td>IgG1</td>
<td>Human FcγRI</td>
</tr>
<tr>
<td>IV.3</td>
<td>Mouse</td>
<td>IgG2bκ</td>
<td>Human FcγRII</td>
</tr>
<tr>
<td>3G8</td>
<td>Mouse</td>
<td>IgG1κ</td>
<td>Human FcγRIII</td>
</tr>
<tr>
<td>F(ab')2 3G8</td>
<td>Mouse</td>
<td>IgG1κ</td>
<td>Human FcγRIII</td>
</tr>
<tr>
<td>Fab 3G8</td>
<td>Mouse</td>
<td>IgG1κ</td>
<td>Human FcγRIII</td>
</tr>
<tr>
<td>C1</td>
<td>Human</td>
<td>IgG2α</td>
<td>Human IgG Fc</td>
</tr>
<tr>
<td>C2</td>
<td>Human</td>
<td>IgG1κ</td>
<td>Human IgG Fc</td>
</tr>
<tr>
<td>THG1-24</td>
<td>Human</td>
<td>IgG1κ</td>
<td>NIP</td>
</tr>
<tr>
<td>JW183/5/1</td>
<td>Human</td>
<td>IgG2α</td>
<td>NIP</td>
</tr>
<tr>
<td>TH3-MP-2-19-2-8</td>
<td>Human</td>
<td>IgG3α</td>
<td>NIP</td>
</tr>
<tr>
<td>JW184/2/1</td>
<td>Human</td>
<td>IgG4α</td>
<td>NIP</td>
</tr>
</tbody>
</table>

The mouse monoclonal antibodies IgG1κ (MOPC-21; Sigma), IgG2ακ (DAK-G05) and IgG2bκ (DAK-G09) and the human IgG1 (κ) and IgG2 (κ) myeloma proteins (The Binding Site) were used as isotype-matched controls. Where cultures were incubated with preformed complexes of human IgG anti-NIP mAb with NIP/BSA, control cultures were incubated with either the IgG anti-NIP mAb alone or NIP/BSA alone. Where necessary, antibodies were predialysed into PBS to remove any traces of azide.

For the inhibitor studies, cultures were incubated with monoclonal antibodies, LPS or medium (all in the absence of polymixin B) in the presence of cycloheximide (50μg/ml), actinomycin D (2μg/ml) or colchicine (1μg/ml) (all from Sigma). All three inhibitors prevented LPS-induced TNFα release in a dose-dependent manner and were used at concentrations according to the manufacturers specification. A gelatinase-specific MMP inhibitor (1746) was a kind gift from Celltech. This MMP inhibitor prevented LPS-induced TNFα release in a dose dependent manner and had an IC50 ~15μM. Maximum inhibition was achieved at 50μM. The presence of the above inhibitors had no effect upon monocyte viability.
2.13. Detection and measurement of cytokines in culture supernatants

The presence and concentrations of TNFα and IL-1α in culture supernatants were determined by ELISA. All assays showed high levels of reproducibility. Additionally, all antibodies and reagents used in determining the production of cytokines were assayed by ELISA in the absence of cells. This was to ensure positive results were not due to any cross-reactivity or interference of reagents with the assay.

2.13.1. ELISA for the detection and measurement of TNFα in culture supernatants

A modification of the ELISA described by Charpentier et al. (1992) was used to detect and measure human TNFα in culture supernatants. This ELISA had an upper limit of 2000pg/ml and detected free, soluble TNFα. 96 well maxisorb plates (Gibco) were coated with 100μl of a murine IgG1 anti-human TNFα monoclonal antibody (CB006, Celltech) at 8μg/ml in 0.02M N, N-bis [2-Hydroxyethyl]-2-aminoethanesulfonic acid (BES at pH7; Sigma). The plates were incubated at room temperature for 16-20 hours, after which the wells were aspirated and replaced with 200μl of blocking buffer (1% BSA in 0.02M BES at pH7). The plates were incubated at room temperature for 1 hour and the wells were aspirated as before. Unless stated otherwise, all samples were diluted in 2% normal mouse serum (Seralab, Loughbrough, Leicestershire)/1%BSA/PBS. Prior to loading the samples, 50μl of this diluent was added to all wells. Recombinant human TNFα (National Institute for Biological Standards and Control (NIBSC), Potters Bar, Hertfordshire) standards in doubling dilutions from 2000pg/ml were used to calibrate the assay. Three concentrations of human TNFα (600, 200 and 60pg/ml) diluted in CGM were also used as controls. 50μl of the standards, controls or the undiluted culture supernatants were then loaded onto the plates in duplicate. The plates were incubated for 1 hour at room temperature with continuous agitation using a microplate shaker (Camlab, Cambridge) set at 800rpm. Following aspiration of the wells, the captured TNFα was detected by incubating with 100μl of a sheep polyclonal anti-human TNF (Celltech) at a 1:10,000 dilution and the plates incubated as before. Following aspiration, 100μl of horseradish peroxidase (HRP) conjugated anti-sheep IgG (H & L) (Jackson Immunoresearch Laboratories) was added to each well at 0.4μg/ml. The plates were incubated for 30 minutes at room
temperature with continuous agitation. After four washes with PBS, 100μl of peroxidase substrate (100μg/ml 3,3',5,5'-tetramethylbenzidine (Sigma) in citrate buffer with 0.05% H₂O₂) was added to all wells. The plates were incubated at room temperature with agitation for 15-30 minutes. The assay was then stopped by adding 50μl of 2M sulphuric acid to all wells and the absorbance was measured at 450nm with a reference of 570nm. A standard curve was constructed by plotting the averaged optical densities of the TNFα standards on the y-axis and the concentration along the x-axis. The equation of the standard curve was then used to calculate the TNFα concentration in the culture supernatants from averaged optical densities.

2.13.2. Alternative ELISA for the detection and measurement of TNFα in culture supernatants.

When TNFα concentrations were tested in supernatants from cultures incubated with either the IgG1 or IgG2 rheumatoid factor mAb, high optical densities were observed. Similarly, when the IgG rheumatoid factor mAb were cultured in the absence of monocytes and the supernatants then tested for TNFα using the above ELISA, comparable optical densities were recorded (Appendix 4). These false positive readings suggested that the IgG rheumatoid factor mAb was interfering or cross-reacting with the TNFα ELISA. Therefore, for experiments where monocytes were incubated with either the IgG1 or IgG2 rheumatoid factor mAb, an alternative ELISA with an upper limit of 2000pg/ml was used to detect TNFα in culture supernatants.

Briefly, 96 well maxisorb plates (Gibco) were coated with murine IgG1 anti-human TNFα mAb (MAb1; PharMingen) at 2μg/ml in BIC and incubated overnight at 4°C. Following aspiration of the wells, the plates were blocked with 200μl of 1%BSA in PBS. The plates were incubated at room temperature for 1 hour and then washed three times with PBT. Unless stated otherwise, all samples were diluted in 1%BSA/PBT. Recombinant human TNFα (NIBSC) standards in doubling dilutions from 2000pg/ml were used to calibrate the assay. Three concentrations of human TNFα (600, 200 and 60pg/ml) diluted in CGM were also used as controls. 95μl of the standards, controls, or the undiluted culture supernatants were then loaded onto the plates in duplicate. The plates
were incubated overnight at 4°C and then washed as before. The captured TNFα was detected by incubating all wells with 100μl of biotin conjugated mouse anti-human TNFα mAb (MAB11; PharMingen) at 1μg/ml for 1 hour at room temperature. The plates were washed 4 times with PBT and 100μl of HRP-conjugated streptavidin (1:500 dilution; DAKO) was then added to all wells. The plates were incubated for 30 minutes at room temperature and then washed 5 times with PBT, after which 100μl of peroxidase substrate was added to each well. Following incubation at room temperature for 1 hour, the reaction was stopped by adding 50μl of 2M sulphuric acid to all wells. Absorbance was measured at 450nm with a reference of 570nm. Averaged optical densities of the TNFα standards were plotted on the y-axis and concentration plotted along the x-axis. The equation of this standard curve was used to calculate the TNFα concentration in the culture supernatants from averaged optical densities.

2.13.3. ELISA for the detection and measurement of IL-1α in culture supernatants
The Quantikine™ IL-1α ELISA (R&D Systems, Oxfordshire) with an upper limit of 250pg/ml and a lower limit of less than 0.5pg/ml was carried out according to the manufacturers protocol. Briefly, 95μl of undiluted culture supernatants were loaded onto 96 well maxisorb plates precoated with a murine anti-human IL-1α mAb. 95μl of recombinant human IL-1α in serial doubling dilutions from 250pg/ml were also assayed to construct a standard curve. All samples were assayed in duplicate. The plates were incubated at room temperature for 2 hours and then washed 3 times with PBT. 200μl of HRP-conjugated anti-IL-1α polyclonal antibody was added to all wells and the plates incubated at room temperature for 1 hour. After washing as before, 200μl of peroxidase substrate was added to each well and the plates incubated at room temperature for 20 minutes. The reaction was stopped by adding 50μl of 2M sulphuric acid to all wells. Absorbance was measured at 450nm with a wavelength correction of 570nm. Averaged optical densities of the IL-1α standards were plotted on the y-axis and concentration plotted on the x-axis. The equation of this standard curve was used to calculate the IL-1α concentration in the culture supernatants from averaged optical densities.
2.14. Detection and measurement of reactive oxygen species production by adhered human monocytes in vitro

Reactive oxygen species production was measured by chemiluminescence as described previously (Assreuy et al., 1994; Epperlein et al., 1998). The chemiluminescent probe lucigenin (bis-N-methylacridinium nitrate) could be used for the detection and measurement of reactive oxygen species. However, lucigenin detects products of the NADPH-oxidase system such as superoxide and also possibly other oxidants such as nitric oxide (Davies & Edwards, 1992). The chemiluminescent probe luminol (3-aminophthalhydrazide), although largely MPO dependent (McNally & Bell, 1996) detects products from both the MPO system and NADPH-oxidase activation (Johansson & Dahlgren, 1989). Therefore, in these experiments reactive oxygen species production was measured by luminol-dependent chemiluminescence (Allen et al., 1972; Allen & Loose, 1976).

Chemiluminescence was measured with a luminometer (Dr A. Nourohu-Dutra, Department of Nephrology, University College London, London) which used a gallium-arsenide photomultiplier tube cooled to -20°C with a linear counting efficiency curve above 10% (most methods have an efficiency of < 0.01%) in the wavelength range of 200 - 900nm. The signal emitted from the tube was fed directly into a frequency counter unit (20 MHz) and the data were presented as average counts per second (cps). 1000 cps represented 0.2 nM of reactive oxygen species per second.

1ml of freshly isolated monocytes were seeded into 35mm plastic petri dishes (Corning, Buckinghamshire) and incubated at 37°C for 24 hours. The cells were then carefully washed with warm HBSS to ensure all traces of serum had been removed. 900µl of HBSS/10mM HEPES (pH 7.2) was added to the adherent monocytes followed by 20µl of 1M luminol (Molecular Probes Europe BV, Leiden, Holland). The petri dishes were then covered with cling film and placed into one of four 37°C light tight mirrored counting chambers. The dishes were left in the chambers for 2 minutes, allowing the temperature of the cells to equilibrate and background light emission to be measured. The dishes were then removed from the chambers and 100µl of the anti-FcyR mAb (Table 5) or
isotype-matched control added at final concentrations as indicated by each experiment. As a positive control, cultures were incubated with 100μl of PMA (0.5μg/ml). All dishes were then quickly returned to the counting chambers and chemiluminescence measured in real time. Polymixin B was not used in these experiments since there were uncertainties of whether this chemical would somehow interfere with the chemiluminescent probe, luminol.

Table 5. Monoclonal antibodies incubated with adhered human monocytes for the measurement of reactive oxygen species production in vitro.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Isotype</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.1</td>
<td>Mouse</td>
<td>IgG1</td>
<td>Human FcγRI</td>
</tr>
<tr>
<td>32.2</td>
<td>Mouse</td>
<td>IgG1</td>
<td>Human FcγRI</td>
</tr>
<tr>
<td>IV.3</td>
<td>Mouse</td>
<td>IgG2bκ</td>
<td>Human FcγRII</td>
</tr>
<tr>
<td>3G8</td>
<td>Mouse</td>
<td>IgG1κ</td>
<td>Human FcγRIII</td>
</tr>
<tr>
<td>3G8 F(ab')2</td>
<td>Mouse</td>
<td>IgG1κ</td>
<td>Human FcγRIII</td>
</tr>
</tbody>
</table>

The mouse monoclonal antibodies, IgG1κ (DAK-G01) and IgG2bκ (DAK-G09) were used as isotype-matched controls.

Where necessary, antibodies were predialysed into PBS to remove any traces of azide which is an inhibitor of MPO activity (Johansson & Dahlgren, 1989).

2.15. **Statistical analysis**
Significance was determined using unpaired T-test with Fisher's correction for small sample size.

2.16. **Data representation**
Results have been presented as either the mean ± standard deviation (S.D.) of three experiments, or as data from one representative experiment.
CHAPTER 3

RESULTS
3.1. **Fcγ receptor expression by human peripheral blood monocytes**

3.1.1. **Murine anti-FcγR mAb: confirmation of antibody specificity and determination of optimal binding levels**

**Experimental design**
Before the murine anti-FcγR monoclonal antibodies could be used for the detection of surface FcγR on human leucocytes, the specificity of each antibody needed to be verified and optimal binding concentrations determined. This was achieved by the indirect immunofluorescent staining and flow cytometry of leucocytes in whole blood, according to the protocol described in materials and methods (2.11.1.). Plasma-free blood was stained with either the anti-FcγRI mAb (10.1), anti-FcγRII mAb (IV.3), anti-FcγRIII mAb (3G8) or murine isotype-matched controls at a final concentration of 50, 20, 10, 5, 2 or 1μg/ml.

**Results**
Figure 6 shows the FACS profile of whole blood following erythrocyte-lysis, with individual gates around the lymphocyte (L), monocyte (M) and granulocyte (G) cell populations. Similar FACS profiles were observed in 5 separate repeat experiments with whole blood from different donors.

The levels of cellular staining were obtained for each of the three anti-FcγR mAb at each concentration. Mean fluorescent intensities for the anti-FcγRI and anti-FcγRII mAb were obtained by gating on the monocyte population. Since monocytes express little if any FcγRIII (Fleit et al., 1982; Ziegler-Heitbrock et al., 1988), levels of cell staining for the anti-FcγRIII mAb were acquired by gating on the granulocyte population. All MFI were corrected for non-specific binding by subtracting the fluorescent intensity from cells stained with the isotype-matched controls. As shown in Table 6, the optimal binding concentration of both the anti-FcγRI (10.1) and the anti-FcγRIII (3G8) mAb was 20μg/ml, while that of the anti-FcγRII mAb (IV.3) was 10μg/ml. Matching optimal concentrations were acquired in 2 repeat experiments with whole blood from different donors.
Figure 6. Dotplot showing the FACS profile of whole blood. Forward scatter (FSC-H) has been plotted along the x axis and side scatter (SSC-H) along the y axis and the three leucocyte populations, lymphocyte (L), monocyte (M) and granulocyte (G), individually gated. Data are from one representative experiment.
Table 6a. Mean fluorescent intensities following the immunostaining of whole blood with each of the three anti-FcγR mAb. Data are from one representative experiment.

<table>
<thead>
<tr>
<th>Monoclonal antibody concentration (µg/ml)</th>
<th>Anti-FcγRI (10.1) binding to monocytes (MFI)</th>
<th>Anti-FcγRII (IV.3) binding to monocytes (MFI)</th>
<th>Anti-FcγRIII (3G8) binding to granulocytes (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>128.73</td>
<td>168.42</td>
<td>1598.88</td>
</tr>
<tr>
<td>20</td>
<td>148.12</td>
<td>173.92</td>
<td>1821.48</td>
</tr>
<tr>
<td>10</td>
<td>80.14</td>
<td>188.87</td>
<td>1482.48</td>
</tr>
<tr>
<td>5</td>
<td>73.42</td>
<td>160.11</td>
<td>1260.41</td>
</tr>
<tr>
<td>2</td>
<td>46.97</td>
<td>146.60</td>
<td>939.28</td>
</tr>
<tr>
<td>1</td>
<td>32.22</td>
<td>122.70</td>
<td>553.22</td>
</tr>
</tbody>
</table>

The samples displaying saturating binding for each of the anti-FcγR mAb were then further analysed for their specificity by comparing the levels of receptor binding sites per cell in each leucocyte population with known FcγR distribution (Fanger et al., 1989). The binding of each of the three anti-FcγR mAb to each of the three cell populations is shown in Figure 7. The percentage of cells stained positive for each anti-FcγR mAb and the average number of receptor sites per cell (rsc) are summarised in Table 7. These data are from one representative experiment and similar results were observed in 2 repeat experiments with whole blood from different donors.

Table 6b. The binding of each anti-FcγR mAb at saturating binding concentrations to human leucocytes in whole blood. The table shows the percentage of cells stained positive (% +) for the anti-FcγR mAb within each gated cell population and the mean number of Fcγ receptor sites per cell (rsc).

<table>
<thead>
<tr>
<th></th>
<th>Anti-FcγRI mAb</th>
<th>Anti-FcγRII mAb</th>
<th>Anti-FcγRIII Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% +</td>
<td>rsc</td>
<td>% +</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Monocyte</td>
<td>95</td>
<td>12904</td>
<td>99</td>
</tr>
<tr>
<td>Granulocyte</td>
<td>50</td>
<td>1458</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 7a shows the levels of anti-FcγRI mAb binding to each of the three gated cell populations. 95% of the gated monocyte population expressed high levels of FcγRI (12904 rsc), while 50% of the granulocyte population beared low FcγRI levels (1458 rsc). However, the histogram for this later result actually appears to show about 95% of granulocytes to stain positive. This discrepancy is due to the placement of the marker which gated from the far right-hand side of the isotype control. The anti-FcγRI mAb did not show significant binding to the gated lymphocyte population, although a slight shift of the anti-FcγRI profile to the right of the isotype control was observed.

Figure 7b shows the levels of anti-FcγRII mAb binding to each cell population. 99% of gated monocyte population and 100% of the granulocyte population expressed high FcγRII levels (19480 and 20617 rsc respectively). The anti-FcγRII mAb did not show significant binding to the gated lymphocyte population although, as for the anti-FcγRI mAb, there was a slight shift of the profile to the right of the isotype control.

Figure 7c shows the levels of anti-FcγRIII mAb binding to each cell population. 30% of the lymphocyte population expressed FcγRIII. Most were expressing low levels, however, approximately 10% of this subpopulation were expressing high FcγRIII levels. 95% of granulocyte population expressed high levels of FcγRIII (154623 rsc). Interestingly, 58% of the gated monocyte population stained positive for the anti-FcγRIII mAb, expressing a mean of 4784 rsc. The anti-FcγRIII mAb binding to monocytes exhibited bimodal staining, suggesting that most of the FcγRIII positive cells expressed low to moderate receptor levels, while a smaller subpopulation (~20%) expressed high receptor levels.

Following enzymatic digestion, both the F(ab')2 and Fab fragments of the anti-FcγRIII mAb bound to the leucocyte populations as observed for the whole antibody by flow cytometry using FITC conjugated F(ab)2 goat anti-mouse Ig as the detecting antibody. This confirmed that following preparation, the receptor binding function of both antibody fragments was maintained. The saturating binding concentrations of the F(ab')2 and Fab fragments were 20μg/ml and 10μg/ml respectively as determined by flow cytometry (Table 7).
Table 7. Mean fluorescent intensities following the immunostaining of whole blood with the whole anti-FcγRIII mAb, anti-FcγRIII F(ab)2 fragments or anti-FcγRIII Fab fragments. Data are from one representative experiment.

<table>
<thead>
<tr>
<th>Monoclonal antibody concentration (µg/ml)</th>
<th>Whole anti-FcγRIII binding to granulocytes (MFI)</th>
<th>F(ab)2 anti-FcγRIII binding to granulocytes (MFI)</th>
<th>Fab anti-FcγRIII binding to granulocytes (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>326.64</td>
<td>450.32</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>861.83</td>
<td>536.73</td>
<td>651.06</td>
</tr>
<tr>
<td>10</td>
<td>757.89</td>
<td>443.53</td>
<td>705.46</td>
</tr>
<tr>
<td>5</td>
<td>651.83</td>
<td>273.78</td>
<td>648.25</td>
</tr>
<tr>
<td>2</td>
<td>502.21</td>
<td>142.50</td>
<td>333.27</td>
</tr>
</tbody>
</table>

Legend for Figure 7. The binding of the three anti-FcγR mAb to leucocytes in whole blood. Histograms show the fluorescent staining of whole blood by the a) anti-FcγRI mAb (red) or murine IgG1 (black), b) anti-FcγRII mAb (red) or murine IgG2b (black) or c) anti-FcγRIII mAb (red) or murine IgG1 (black) at saturating binding concentrations. Anti-FcγR mAb staining to each of the three gated cell populations are represented by a separate histogram. Fluorescent intensity (FL1-H) has been plotted along the x axis and the number of events plotted along the y axis. Data shown are from one representative experiment.
Figure 7a)

Lymphocytes

Monocytes

Granulocytes
Figure 7b)

Lymphocytes

Monocytes

Granulocytes
Figure 7c)

Lymphocytes

Monocytes

Granulocytes
3.1.2. FcγR Expression by isolated and adherent human monocytes in vitro

Experimental design
In the previous experiment, the binding capacities and specificities for each of the three murine anti-FcγR monoclonal antibodies were confirmed. The anti-FcγR mAb were then used to establish a system suitable for the investigation of FcγR-dependent cytokine production by human macrophages in vitro. Such a system required isolated peripheral blood monocytes to express all three Fcγ receptors. Human peripheral blood monocytes constitutively express FcγRI and FcγRII while approximately 10% have been reported to express FcγRIIIa (Ziegler-Heitbrock et al., 1988). However, the results presented in the previous section suggest that this is an oversimplification. Approximately 58% of monocytes expressed FcγRIIIa at low levels, while a subpopulation of approximately 20% of the total, expressed high FcγRIIIa levels. This observation has also been reported by other groups (Anderson CL et al., 1990; Maeda et al., 1996). Since the mean level of FcγRIIIa expression was low in comparison to the other two FcγR, steps were taken to induce more comparable levels by a process of maturation. Klaassen et al. (1990) found the adherence of monocytes to plastic upregulated FcγRIIIa expression. A modification of this system was chosen to be used for the studies of FcγR-mediated macrophage activation. However, it was necessary to confirm the levels of FcγR expression by both isolated and adhered monocytes so that optimum conditions could be established for future experiments.

Methods
The levels of FcγR expression by freshly isolated and cultured monocytes were determined by indirect immunofluorescent staining and flow cytometry. The anti-FcγR monoclonal antibodies were used at the predetermined saturating binding concentrations. Isolated monocytes were adhered to plastic in CGM and cultured at 37°C for 24 and 48 hours. The presence of serum was essential for the survival of monocytes in culture, as well as the induction of FcγRIIIa expression and maintenance of CD14 expression (Andreesen et al., 1990). Following each culture time, the cells were then harvested and stained for each FcγR according to the protocol described in materials and methods (2.11.2.). Monocytes were also stained for FcγR expression immediately following isolation (time 0).
Results
As shown in Figure 8c, when isolated human monocytes were allowed to adhere to plastic, an increase in FcγRIIIa expression was observed with time. In this example, at time 0 (freshly isolated monocytes) 22% of the monocytes were stained with a high fluorescent intensity, suggesting high levels of FcγRIIIa expression by this subpopulation. Following adherence to plastic for 24 hours, 42% of monocytes were expressing FcγRIIIa and after 48 hours this rose to 51%. FcγRI and FcγRII expression levels did not significantly change with time. Following adherence to plastic for 0, 24 and 48 hours, 35%, 37% and 30% of monocytes respectively expressed FcγRI (Figure 8a). Similarly, at time 0, 60% of monocytes were expressing FcγRII. Following adherence to plastic for 24 and 48 hours, 67% and 60% of adhered monocytes respectively expressed FcγRII (Figure 8b). Similar results were obtained in two repeat experiments with monocytes from different donors.

In a separate experiment, monocytes were adhered to plastic and cultured for 3 days and 4 days, after which the cells were immunostained for FcγRIIIa expression as before. Figure 9 shows that following adherence to plastic for 3 days 64% of monocytes expressed FcγRIIIa. Following adherence to plastic for 4 days, 67% of monocytes expressed FcγRIIIa. Similar results were observed when this experiment was repeated. This suggested that by 3 days, FcγRIIIa expression by adhered human monocytes had reached a plateau, confirming observations previously reported by Klaassen et al. (1990). Therefore, at time points from 24 hours to 4 days the levels of expression of all three Fcγ receptors were reasonably comparable, providing a workable system for subsequent studies.
Legend for Figure 8. The effects of culturing adhered human monocytes on FcγR expression. Following adherence to plastic for 0, 24 or 48 hours at 37°C, human monocytes were then immunostained with the a) anti-FcγRI mAb (red) or murine IgG1 (black) b) anti-FcγRII mAb (red) or murine IgG2b (black) or c) anti-FcγRIII mAb (red) or murine IgG1 (black). Fluorescent intensity (FL1-H) has been plotted along the x-axis and the number of events plotted along the y-axis. Data shown are from one representative experiment.
Figure 8a)

**Time 0**

- Events vs. FL1-H
- 35% M1

**24 Hours**

- Events vs. FL1-H
- 37% M1

**48 Hours**

- Events vs. FL1-H
- 30% M1
Figure 8b)

Time 0

24 Hours

48 Hours
Figure 8c)

Time 0

22% M1

24 Hours

42% M1

48 Hours

51% M1
Figure 9. The effects of culturing adhered human monocytes on FcγRIIIa expression. Following adherence to plastic for 3 and 4 days at 37°C, human monocytes were then stained with the anti-FcγRIII mAb (blue) or murine IgG1 (green). Fluorescent intensity (FL1-H) has been plotted along the x-axis and the number of events plotted along the y-axis. Data shown are from one representative experiment.
3.1.3. The effect of TNFα on FcγR expression by adhered monocytes in vitro

Experimental design
Several studies have demonstrated the modulation of monocyte FcγRIIIa expression by cytokines such as TGFβ (Welch et al., 1990), IL-4 (Wong et al., 1991; te Velde et al., 1990) or IL-10 (Calzada-Wack et al., 1996). Two studies have reported the downregulation of macrophage-derived monocyte FcγRIIIa expression by TNFα (Liao & Simon, 1994; Liao et al., 1994), while FcγRI and FcγRII expression remained unaffected. These studies were both performed on monocytes that had been cultured in suspension. Therefore, the effects of TNFα on FcγRIIIa expression by adhered human monocytes was examined.

Methods
Human monocytes were adhered to plastic and cultured in CGM at 37°C for 3 days. After replacing the media with fresh CGM, the cultures were incubated at 37°C with either recombinant human TNFα (2ng/ml) or medium alone for 1, 2 and 24 hours. Following each incubation time, the cells were harvested and FcγRIIIa expression measured by indirect immunofluorescent staining and flow cytometry as described previously. Adhered monocytes cultured for 3 days were also stained for FcγRIIIa expression prior to incubation with TNFα or medium (Time 0).

Results
As shown in Figure 10, the incubation of adhered monocytes with TNFα had no notable effect on FcγRIIIa expression. Following 3 days in culture, 61% of adhered monocytes expressed FcγRIIIa at an average of 1840 rsc (Time 0). Following a 1 hour incubation with TNFα, 62% of adhered monocytes expressed FcγRIIIa (1996 rsc) compared with 69% of untreated monocytes expressing FcγRIIIa (2294 rsc). Following a 2 hour incubation, 40% of TNFα treated monocytes expressed an average of 1238 FcγRIIIa sites per cell, while 39% of monocytes in the absence of TNFα expressed an average of 1543 FcγRIIIa sites per cell. Following an incubation of 24 hours with TNFα, 65% of monocytes expressed an average of 1994 FcγRIIIa rsc, while 53% of untreated cells expressed an average of 1687 rsc. Similar results were observed when this experiment was repeated.
Legend for Figure 10. The effects of TNFα on FcγRIIIa expression by adhered human monocytes. Following adherence to plastic for 3 days (Time 0) monocytes were incubated with and without recombinant human TNFα for 1, 2 or 24 hours at 37°C. Untreated cells were stained with the anti-FcγRIII mAb (blue) or murine IgG1 (green). Similarly, TNFα treated monocytes were stained with the anti-FcγRIII mAb (red) or murine IgG1 (black). Fluorescent intensity (FL1-H) has been plotted along the x-axis and the number of events plotted along the y-axis. Histograms are from a representative experiment.
Figure 10

Time 0

1 Hour

2 Hours

24 Hours
Summary
The three murine anti-FcγR monoclonal antibodies each reacted with human leucocytes in whole blood according to the cellular distribution of each Fcγ receptor (Fanger et al., 1989), thereby confirming the monospecificity of each monoclonal antibody. Saturating binding concentrations for each anti-FcγR mAb were determined for use in subsequent experiments.

The adherence of isolated human monocytes to tissue culture plastic induced the upregulation of FcγRIIIa expression reaching a plateau after 3 days in culture, although a significant increase in FcγRIIIa expression was observed after only 24 hours in culture. Furthermore, FcγRIIIa expression by monocytes adhered to plastic was unaffected by the presence of exogenous TNFα, in agreement with observations by Gessl et al. (1994). FcγRI and FcγRII expression by adhered monocytes remained constant with time.

Adhered monocytes were therefore cultured for 24 hours prior to experimentation. This culture time was selected on the basis of both receptor expression and optimum TNFα production, which will be discussed in the following section (3.2.1. & 3.2.2.). Thus an in vitro system was established where the effects ligating each Fcγ receptor on macrophage activation could be investigated.
3.2. **TNFα production by human peripheral blood monocytes in vitro**

3.2.1. *The effects of peripheral blood monocyte isolation and adherence on TNFα production*

**Experimental design**
The adherence of monocytes to plastic tissue culture plates has been shown to induce cell activity such as the generation of superoxide, the release of β-glucuronidase (Krause *et al.*, 1996-97; Kelley *et al.*, 1987) and TNF gene activation (Darville *et al.*, 1992). Therefore, following the adherence of freshly isolated monocytes to plastic at 37°C, supernatants were collected at various time points over 24 hours and tested for TNFα as described in materials and methods (2.13.1.).

**Results**
As shown in Table 8, TNFα was rapidly released into the culture supernatants following the adherence of isolated monocytes to plastic.

**Table 8.** The effect of adherence to plastic on TNFα release by isolated human monocytes. Values are expressed as the mean ± S.D. from three separate experiments.

<table>
<thead>
<tr>
<th>Time after adherence (hours)</th>
<th>TNFα (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.027 ± 0.05</td>
</tr>
<tr>
<td>1</td>
<td>0.034 ± 0.06</td>
</tr>
<tr>
<td>2</td>
<td>0.244 ± 0.06</td>
</tr>
<tr>
<td>4</td>
<td>0.366 ± 0.06</td>
</tr>
<tr>
<td>6</td>
<td>0.477 ± 0.10</td>
</tr>
<tr>
<td>18</td>
<td>0.437 ± 0.08</td>
</tr>
<tr>
<td>24</td>
<td>0.686 ± 0.28</td>
</tr>
</tbody>
</table>
3.2.2. The effects of culturing adhered monocytes on TNFα production

Experimental design
The adherence of monocytes to plastic, compared with monocytes that have been matured and subsequently stimulated in suspension, may affect the nature of cell activation. For example, compared with monocytes in suspension, adhered monocytes may exhibit enhanced TNFα and IL-1β production, while the ability to release IL-6 is diminished (Krause et al., 1987). There have been reports that the long-term culturing of monocytes in suspension increases phagocytic activity, while peroxidase activity decreases (Fleit et al., 1982). One group reported that following 24 hours in culture, non-adherent monocytes were able to be stimulated to produce maximal levels of TNFα and this ability for cytokine production remained constant with time (Young et al., 1990). Therefore, the effects of culturing adhered monocytes on their capacity for TNFα production was investigated. Adhered human monocytes were incubated with LPS (a known stimulator of TNFα production) or the murine IgG1 anti-FcγRIII mAb (see 3.3. for further details) to determine the optimal conditions for TNFα production.

Methods
Adhered human monocytes were cultured for 1 or 4 days at 37°C. Following each culture time, the medium was removed and the cultures then incubated with LPS (0.5μg/ml; in the absence of polymixin B) or the anti-FcγRIII mAb, 3G8 (50μg/ml). Cell-free supernatants were collected after 4 hours and tested for TNFα as described previously.

Results
When adhered monocytes were cultured for 1 or 4 days and then incubated with LPS, all cultures released TNFα into the supernatants. However, the levels of LPS-induced TNFα released from the 1 day cultures (1.162 ± 0.44 ng/ml) were greater than those released from the 4 day cultures (0.144 ± 0.13 ng/ml). Similarly, levels of TNFα released from the 1 day cultures incubated with the anti-FcγRIII mAb (0.711 ± 0.12 ng/ml) were greater than those from the 4 day cultures (0.269 ± 0.16 ng/ml).
Summary
The adherence of human peripheral blood monocytes to plastic was found to trigger the release of TNFα into culture supernatants. Following 24 hours in culture, adherent human monocytes released TNFα upon stimulation. However, longer culture times resulted in a diminished capacity for TNFα release following stimulation. Therefore, in order achieve a system whereby adherent monocytes were expressing all three Fcγ receptors, were able to produce a strong TNFα response upon stimulation and the baseline TNFα production at time 0 tested negative, the system was standardised as follows: Freshly isolated peripheral blood monocytes were adhered to plastic at 37°C for 24 hours in CGM. Following this incubation, the culture media was removed and replaced with fresh CGM. Appropriate stimulation/incubations could then be performed (see materials and methods for full details).

3.2.3. Reproducibility and donor variability of TNFα production by adhered human monocytes

Experimental design
Monocytes were separated from three different donors on the same day. Following adherence of the monocytes to plastic for 24 hours, the culture media was replaced with fresh and the cells incubated with LPS (0.5 μg/ml; in the absence of polymixin B) in triplicate for 4 hours. Following collection, the supernatants were tested for TNFα as described previously. All samples were assayed on the same ELISA plate.

Results
As shown in Table 9, when monocytes from the three donors were stimulated with LPS, differing levels of TNFα were produced. This diversity in monocyte responsiveness to stimulation reflected the donor variability. However, the levels TNFα released into the supernatants from monocytes isolated from one individual, were highly consistent, indicating reliable reproducibility of both the in vitro system and the cytokine assay.
Table 9. LPS induced TNFα production from adhered monocytes isolated from three different donors.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.487</td>
<td>2.563</td>
<td>2.654</td>
<td>2.668 ± 0.19</td>
</tr>
<tr>
<td>2</td>
<td>0.267</td>
<td>0.287</td>
<td>0.299</td>
<td>0.284 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>1.093</td>
<td>1.272</td>
<td>1.005</td>
<td>1.123 ± 0.14</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>1.282 ± 1.12</td>
<td>1.374 ± 1.14</td>
<td>1.319 ± 1.21</td>
<td></td>
</tr>
</tbody>
</table>

3.3. The effects of Fcγ receptor ligation on TNFα production by adhered human monocytes in vitro

3.3.1. TNFα release from adhered monocytes following incubation with murine anti-FcγR mAb

Experimental design
The ability of each Fcγ receptor to mediate TNFα release from adhered human monocytes was investigated by specifically ligating each FcγR using a murine anti-FcγR monoclonal antibody in the absence of secondary crosslinking, according to the protocol described in materials and methods (2.12.).

Results
When adhered human monocytes were incubated with the anti-FcγRI, anti-FcγRII or anti-FcγRIII mAb (all at 50μg/ml), TNFα was detected only in those cultures incubated with the anti-FcγRIII mAb (Figure 11a). The anti-FcγRIII mAb response was concentration dependent (Table 10) and polymixin B resistant (Figure 11c). Although flow cytometry showed anti-FcγRIII saturating binding levels to granulocytes to be obtained at 20μg/ml, optimum TNFα production from adhered monocytes was achieved with the anti-FcγRIII mAb at a concentration of 50μg/ml. This inconsistency may be due to the ability of the anti-FcγRIII mAb to bind the granulocyte isoform, FcγRIIIb with a greater affinity than the macrophage isoform, FcγRIIIa (Anderson CL et al., 1990). The levels of TNFα produced in response to the anti-FcγRIII mAb rapidly rose with time, peaking at 4 hours, after which the
response declined. TNFα was undetectable in the supernatants from cultures incubated with medium alone or the murine IgG2b (50μg/ml). In this representative experiment minimal levels of TNFα were detected in cultures incubated with the murine IgG1 (50μg/ml) at 2 and 6 hours only. In other experiments TNFα was undetectable. TNFα release from adhered monocytes was induced by both LPS (in the absence of polymixin B) and PMA. The LPS-induced TNFα response rose rapidly with time and peaked after 6 hours, as observed by Matic & Simon (1991), after which the levels of TNFα declined with time. The PMA-induced response displayed a time course which rose continuously over 24 hours. Similar time courses for all the above incubations were observed in 5 repeat experiments with adhered monocytes from different donors. Only the LPS response was polymixin B sensitive (Figure 11b & 11c). Furthermore, the inhibition of LPS-induced TNFα release by polymixin B was consistent in all subsequent experiments.

Table 10. The effects of anti-FcyRIII mAb concentration on TNFα release from adhered monocytes. Culture supernatants were collected for measurement of TNFα 4 hours after incubation with anti-FcyRIII mAb (3G8) at various concentrations. Data from three separate experiments are shown.

<table>
<thead>
<tr>
<th>Anti-FcyRIII mAb (μg/ml)</th>
<th>1 TNFα (ng/ml)</th>
<th>2 TNFα (ng/ml)</th>
<th>3 TNFα (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>0.399</td>
<td>0.876</td>
<td>0.910</td>
</tr>
<tr>
<td>100</td>
<td>0.274</td>
<td>1.641</td>
<td>0.950</td>
</tr>
<tr>
<td>50</td>
<td>0.150</td>
<td>1.776</td>
<td>0.510</td>
</tr>
<tr>
<td>25</td>
<td>0.032</td>
<td>1.036</td>
<td>0.169</td>
</tr>
<tr>
<td>10</td>
<td>0.000</td>
<td>0.431</td>
<td>0.026</td>
</tr>
<tr>
<td>5</td>
<td>0.000</td>
<td>0.079</td>
<td>0.000</td>
</tr>
<tr>
<td>1</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Legend for Figure 11a. TNFα release from adhered monocytes following incubation with anti-FcγR mAb. Figure shows the time courses for TNFα production by adhered monocytes following incubation with the anti-FcγRI mAb (pink open triangle), the anti-FcγRII mAb (light blue open circle), or the anti-FcγRIII mAb (red solid circle). The negative controls included medium alone (black open square), murine IgG1 (pink open diamond) and murine IgG2b (blue solid diamond). The positive controls were LPS in the absence of polymixin B (green solid triangle) and PMA (blue solid square). The data are from one representative experiment.

Legend for Figure 11b. Inhibition of LPS-induced TNFα production from adhered human monocytes by the presence of polymixin B. Adhered human monocytes were cultured with LPS (1μg/ml) in the presence of various concentrations of polymixin B for 4 hours after which supernatants were collected and tested for TNFα.

Legend for Figure 11c. The effect of polymixin B on the anti-FcγRIII mAb-induced TNFα response. Adhered human monocytes were cultured with either LPS (1μg/ml) or the anti-FcγRIII mAb (50μg/ml) in the presence (10μg/ml) or absence of polymixin B. Supernatants were collected after 4 hours and assayed for TNFα.
3.3.2. The effects of blocking FcγRI or FcγRII on the anti-FcγRIII mAb induced TNFα response from adhered human monocytes

Experimental design
The three anti-FcγR mAb can be viewed as surrogates for the interactions between small IgG immune complexes and each individual Fcγ receptor in isolation. Each Fab region of an anti-FcγR mAb can bind one FcγR, thereby allowing the crosslinking of two Fcγ receptors of the same type. A third FcγR of any type may be non-specifically recruited through the Fc fragment of the monoclonal antibody. The three murine anti-FcγR mAb block IgG Fc binding by recognising epitopes in or close to their receptor's binding site (Dougherty et al., 1987; Looney et al., 1986b; Fleit et al., 1982). Therefore, the anti-FcγRI and anti-FcγRII mAb, which were non-stimulatory for TNFα, were used to block binding of the anti-FcγRIII mAb Fc fragment to either FcγRI or FcγRII. This was to determine whether TNFα release induced by the anti-FcγRIII mAb was dependent upon any additional Fcγ receptor recruitment through the mAb Fc region.

Methods
Adhered human monocytes were preincubated at 37°C for 30 minutes with either the anti-FcγRI mAb or the anti-FcγRII mAb (both at the saturating binding concentration of 20μg/ml), or medium alone. The anti-FcγRIII mAb (50μg/ml) was then added to all cultures. Supernatants were collected after a 4 hour incubation with the anti-FcγRIII mAb and tested for TNFα as described previously.

Results
Adhered monocytes incubated with LPS (in the absence of polymixin B) released TNFα into the supernatant (1.308 ± 0.53 ng/ml). As observed previously, no TNFα was detected in cultures incubated with either medium, the anti-FcγRI mAb or the anti-FcγRII mAb alone. TNFα was detected in the supernatants from cultures that had been preincubated with medium alone and then incubated for 4 hours with the anti-FcγRIII mAb (1.279 ± 0.12 ng/ml). Cultures incubated with the anti-FcγRI mAb prior to incubation with the anti-FcγRIII mAb also released similar amounts of TNFα (1.635 ± 0.44 ng/ml), as did those cultures preincubated with the anti-FcγRII mAb (1.424 ± 0.19 ng/ml) (Figure 12). Therefore, the blocking of either FcγRI
or FcγRII with their respective monoclonal antibodies had no statistical significant effect upon the levels of TNFα released in response to the anti-FcγRIII mAb.

**Figure 12.** Barchart showing the effects of blocking FcγRI or FcγRII on the anti-FcγRIII mAb-induced TNFα response. Adhered human monocytes were preincubated with either medium alone, the anti-FcγRI mAb (10.1) or the anti-FcγRII mAb (IV.3). All cultures were then incubated for 4 hours with the anti-FcγRIII mAb (3G8). Values are expressed as the mean ± S.D. from three separate experiments.
3.3.3. TNFα release from adhered monocytes following incubation with F(ab')2 or Fab fragments of the anti-FcyRIII mAb

Experimental design
The previous experiment had not eliminated the possibility of the anti-FcyRIII mAb recruiting another FcyRIIIa receptor through its Fc region. Therefore, to determine whether TNFα release in response to the anti-FcyRIII mAb was dependent upon the Fc region of the monoclonal antibody interacting with an Fcy receptor, adhered monocytes were incubated with either F(ab')2 or Fab fragments of the anti-FcyRIII mAb. To compensate for the differing levels of antigen binding sites in the three antibody preparations, the concentrations of the F(ab')2 and Fab fragments were adjusted. Therefore, the anti-FcyRIII F(ab')2 fragments were adjusted to 2/3 the concentration of the whole antibody and the anti-FcyRIII Fab fragments were adjusted to 1/3 of the whole anti-FcyRIII mAb concentration.

Results
Figure 13 shows that following a 4 hour incubation with the anti-FcyRIII mAb (50μg/ml), adhered monocytes released TNFα into the supernatant (0.499 ± 0.12 ng/ml). Low levels of TNFα were detected in the supernatants from cultures incubated with either the anti-FcyRIII mAb F(ab')2 fragments at 33μg/ml (0.025 ± 0.04 ng/ml) or the anti-FcyRIII Fab fragments at 17μg/ml (0.036 ± 0.06 ng/ml). However, similarly low levels of TNFα were also detected in the supernatants from cultures incubated with medium alone (0.026 ± 0.05 ng/ml). LPS (in the absence of polymixin B) also induced adhered monocytes to release TNFα as observed previously (1.076 ± 0.57 ng/ml). Similar results were observed when cultures were incubated with the anti-FcyRIII mAb F(ab')2 or Fab fragments at a final concentration equal to the whole monoclonal antibody (50μg/ml) (data not shown).

Moreover, when adhered monocytes were preincubated with F(ab')2 fragments of the anti-FcyRIII mAb at binding saturating levels (20μg/ml), the whole anti-FcyRIII induced TNFα response was inhibited by 69.0 ± 6.2%, confirming the functional ability of the anti-FcyRIII F(ab')2 fragment to bind FcyRIII. The lack of total inhibition by the F(ab)2 fragments may be because a slightly higher F(ab)2 concentration was required, again highlighting the differing binding affinity of the anti-FcyRIII mAb for FcyRIIIa and FcyRIIIb.
Figure 13. The effects of anti-FcγRIII F(ab′)2 and Fab fragments on TNFα release. Barchart showing the production of TNFα after adhered monocytes were incubated for 4 hours with either the anti-FcγRIII (3G8) mAb, anti-FcγRIII F(ab′)2 fragments, anti-FcγRIII Fab fragments or medium alone. Values are expressed as the mean ± S.D. from three separate experiments.
3.3.4. **TNFα release from adhered monocytes following incubation with more than one anti-FcγR mAb**

**Experimental design**
Looney *et al.* (1986b) reported that the anti-FcγRII mAb (IV.3) alone had no inhibitory effect upon rosette formation between granulocytes and rabbit IgG-sensitised erythrocytes, whereas rosette formation was partially inhibited by the anti-FcγRIII mAb, 3G8. However, in combination, IV.3 and 3G8 completely inhibited this rosette formation. Although neither the anti-FcγRI nor the anti-FcγRII mAb induced TNFα release from adhered monocytes, an additive effect may be possible if both are used together or in combination with the stimulatory anti-FcγRIII mAb. Therefore, adhered monocytes were incubated for 4 hours with various combinations of the three anti-FcγR mAb (all at a final concentration of 50μg/ml) and the levels of TNFα production were compared.

**Results**
The incubation of adhered human monocytes with more than one anti-FcγR mAb had no statistically significant effect upon the levels of TNFα released into the culture supernatant. Following a 4 hour incubation, cultures incubated with the anti-FcγRIII mAb alone (0.747 ± 0.57ng/ml) released TNFα as observed previously. Similar levels of TNFα were released from cultures incubated with either the anti-FcγRI and the anti-FcγRIII mAb (0.840 ± 0.95ng/ml), the anti-FcγRII and the anti-FcγRIII mAb (0.877 ± 0.60ng/ml) or all three anti-FcγR mAb (0.958 ± 0.82ng/ml). TNFα levels were low if at all detectable in the supernatants from cultures incubated with either the anti-FcγRI mAb alone (0.000 ± 0.00ng/ml) or the anti-FcγRII mAb alone (0.023 ± 0.03ng/ml). TNFα was also undetectable in the supernatants from cultures incubated with both the anti-FcγRI and the anti-FcγRII mAb or medium alone. As observed previously, LPS in the absence of polymixin B induced TNFα release from adhered monocytes (0.779 ± 0.45 ng/ml).

**Summary**
The experiments presented in this section have demonstrated the ability of the murine anti-FcγRIII mAb to induce TNFα release from adhered human monocytes in the absence of secondary crosslinking: a novel finding. The anti-FcγRI and anti-FcγRII mAb were both found to be non-stimulatory for
TNFα release by the adhered human monocytes, supporting previous findings (Debets et al., 1990). Furthermore, neither the F(ab')2 nor the Fab fragments of the anti-FcγRIII mAb were able to induce TNFα release from the adhered monocytes. Using the anti-FcγRI and anti-FcγRII mAb to block receptor binding, it was found that there was no additional recruitment of either FcγRI or FcγRII by the Fc region of the anti-FcγRIII mAb during the induction of a TNFα response.

3.4. The effects of Fcγ receptor ligation on IL-1α production by adhered human monocytes in vitro

3.4.1. IL-1α release from adhered monocytes following incubation with anti-FcγR mAb

Experimental design
The experiments presented in the previous section (3.3.) demonstrated the ability of FcγRIIIa to mediate TNFα release from adhered human monocytes, following receptor ligation with a murine anti-FcγR monoclonal antibody. It therefore seemed appropriate to investigate the ability of each Fcγ receptor to mediate the release of other cytokines following receptor ligation by these anti-FcγR monoclonal antibodies. Work by Chantry et al. (1989) has demonstrated the ability of immune complexes derived from patients with type II mixed cryoglobulin to induce IL-1α and IL-β production from human monocytes. The crosslinking of FcγR by IgG immune complexes had already been shown to induce the release of IL-1β from human monocytes (Polat et al., 1993). Although IL-1α tends to be membrane associated it was decided to see if FcγR ligation could also induce IL-1α release from adhered human monocytes. This could have implications in the local environment of the synovium since this proinflammatory cytokine may play a similar role to TNFα in mediating pathology in rheumatoid arthritis. This is supported by the observation of spontaneous and prolonged IL-1α production from isolated rheumatoid synovial cells (Buchan et al., 1988). Therefore, the ability of each Fcγ receptor to mediate IL-1α release by adhered human monocytes in vitro was investigated by ligating each FcγR using the murine anti-FcγR monoclonal antibodies in the absence of secondary crosslinking as previously described. Culture supernatants were collected at various time points over 72 hours and tested for IL-1α, according to the protocol.
described in materials and methods (2.13.3.). Supernatants were also collected after a 4 hour incubation and assayed for TNFα as described previously.

Results
When adhered human monocytes were incubated with the anti-FcγRI, anti-FcγRII or anti-FcγRIII mAb, only those cultures incubated with the anti-FcγRIII mAb released IL-1α into the supernatant (Figure 14). The time course for IL-1α release in response to the anti-FcγRIII mAb was very different from the anti-FcγRIII-induced TNFα response (see section 3.3.1.). IL-1α was not detectable until after an incubation of 11 hours. The IL-1α levels then gradually rose with time and appeared to plateau after 33 hours. IL-1α was not detected in supernatants from cultures incubated with either medium alone or the murine isotype-matched controls. LPS (in the absence of polymixin B) also induced adhered monocytes to release IL-1α and the time course was again, different from the LPS-induced TNFα response since IL-1α was only detected in supernatants following a 10 hour incubation. Unlike the anti-FcγRIII mAb-induced IL-1α response, the IL-1α response induced by LPS rose steeply and continuously over the 72 hour time course. Interestingly, it was found that although higher IL-1α levels were measured in response to LPS, compared with the IL-1α levels induced by the anti-FcγRIII mAb, the levels of TNFα released into supernatants were similar following a 4 hour incubation with either LPS (1.279ng/ml) or the anti-FcγRIII mAb (1.347ng/ml). Similar results were observed in 2 repeat experiments with monocytes from different donors.
Legend for Figure 14. IL-1α release from adhered monocytes following incubation with anti-FcγR mAb. Figure shows the times course for IL-1α production by adhered monocytes following incubation with the anti-FcγRI mAb (pink solid square), the anti-FcγRII mAb (light blue open square), or the anti-FcγRIII mAb (red solid circle). The negative controls included medium alone (blue open triangle), murine IgG1 (black open circle) and murine IgG2b (black solid diamond). LPS in the absence of polymixin B (green solid triangle) was used as a positive control. The data shown are from one representative experiment.
Figure 14

**IL-1 (pg/ml)**

**Time (Hours)**

- LPS
- Medium
- mIFN-2β
- mIFN-1
- Anti-IFN YRII (3CG)
- Anti-IFN YRII (1V.3)
- Anti-IFN YRI (10.1)
3.4.2. The effect of an anti-TNFα mAb on both the LPS and anti-FcγRIII mAb induced IL-1α release from adhered human monocytes

Experimental design
Dinarello et al. (1986) first described the capacity of human recombinant TNFα to induce the production of IL-1 from human mononuclear cells in vitro. IL-1 production by rheumatoid synovial mononuclear cells in culture has also been shown to be dependent on the presence of TNFα (Brennan et al., 1989). Furthermore, immune complexes may work in synergy with TNFα to enhance IL-1 production from human monocytes (Chantry et al., 1989). The potential dependency upon TNFα of the FcγRIIIa-mediated and LPS-induced IL-1α production by adhered human monocytes shown in the previous experiment (3.4.1.), was investigated using a neutralising anti-TNFα mAb (CB006). The anti-TNF mAb was specific for the active site of TNFα and therefore prevented both its detection by the ELISA used in these studies and its interaction with TNF receptors (Dr S Stephens, personal communication).

Methods
Adhered human monocytes were incubated with either LPS (in the absence of polymixin B) or the anti-FcγRIII mAb (50μg/ml) in the presence of the murine monoclonal anti-TNFα mAb at 100, 10, 1 or 0μg/ml. Following a 4 hour incubation, supernatants were collected and tested for TNFα and after a 48 hour incubation supernatants were collected and tested for IL-1α as described previously.

Results
As shown in Figure 15a, the anti-TNFα mAb efficiently bound soluble TNFα in the culture supernatants in a dose dependent manner, preventing its detection by the TNFα ELISA. This was possible since the neutralising anti-TNFα mAb was the same as the coating antibody in the TNFα ELISA. There was no cross-reactivity between the anti-TNFα mAb and the ELISA which may have given false positive results. However, to have fully ruled out the possibility of the anti-TNFα mAb in solution interfering with the detection of TNFα, recombinant TNFα should have been titrated to show that the ELISA could still detect excess, unbound cytokine. Additionally, to have fully established neutralisation by the anti-TNFα mAb, a TNFα bioassay
should have been performed. Nevertheless as shown in figure 15a, at the anti-TNFα mAb concentration of 1μg/ml, the detection of TNFα release in response to LPS was inhibited by 50% while the detection of the anti-FcγRIII mAb TNFα response was inhibited by 38%. At the anti-TNFα mAb concentration of 10μg/ml, the detection of the TNFα responses following incubation with either LPS or the anti-FcγRIII mAb were both inhibited by 94%. At 100μg/ml, the anti-TNF mAb inhibited TNFα detection in response to LPS and the anti-FcγRIII mAb both by 100%. Similar levels of inhibition were observed when this experiment was repeated.

As shown in Figure 15b, IL-1α release from adhered monocytes following incubation with the anti-FcγRIII mAb was inhibited by the anti-TNFα mAb in a dose dependent manner. At the anti-TNFα mAb concentration of 1μg/ml the anti-FcγRIII mAb-induced IL-1α response was inhibited by 66%. The anti-FcγRIII mAb-induced IL-1α response was inhibited by 82% when cultures were incubated in the presence of the anti-TNF mAb at both 10μg/ml and 100μg/ml. No such inhibition was seen when cultures were incubated with LPS in the presence of the anti-TNFα mAb (Figure 15c). This was not due to the anti-TNFα mAb interfering with the IL-1α ELISA. Additionally, IL-1α was not detected in the supernatants from cultures incubated with recombinant human TNFα alone at concentrations ranging between 0 and 5ng/ml. Similar results were observed when this experiment was repeated.
Figure 15. The effect of a neutralising anti-TNFα mAb on TNFα detection and IL-1α release. Barchart shows the levels of a) TNFα detection in cultures following a 4 hour incubation with LPS (in the absence of polymixin B) or the anti-FcγRIII mAb; b) IL-1α release from cultures following a 48 hour incubation with the anti-FcγRIII mAb; c) IL-1α release from cultures following a 48 hour incubation with LPS (in the absence of polymixin B) all in the presence of an anti-TNFα mAb at various concentrations. Data are from one representative experiment.
Anti-TNF mAb (μg/ml)

b)

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c)

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Summary
The experiments presented in this section have again demonstrated the ability of FcγRIIIa to mediate cytokine production by adhered monocytes following receptor ligation with the anti-FcγRIII mAb in the absence of secondary crosslinking. Of the three anti-FcγR monoclonal antibodies, only the anti-FcγRIII mAb was able to induce IL-1α release from adhered human monocytes in vitro. The time course for IL-1α release from adhered monocytes in response to either the anti-FcγRIII mAb or LPS was different from the profiles observed for TNFα release. Furthermore, the anti-FcγRIII mAb-induced IL-1α production from adhered monocytes appeared to be dependent upon the presence of TNFα, while the LPS induced IL-1α production occurred independently of TNFα.

3.5. The effects of Fcγ receptor ligation on reactive oxygen species production by adhered human monocytes in vitro.

3.5.1. Reactive oxygen species production by adhered monocytes following incubation with anti-FcγR mAb

Experimental design
Phagocytic leucocytes respond to certain stimuli by generating an intense respiratory burst (Babior, 1984b). After establishing the ability of FcγRIIIa to mediate both TNFα and IL-1α production from adhered human monocytes (3.3.1. & 3.4.1.), these studies were further extended to Fcγ receptor-mediated production of reactive oxygen species. Trezinni et al. (1990) had previously demonstrated the production of reactive oxygen species by non-adhered monocytes through an FcγRIIIa pathway, following receptor ligation with the murine anti-FcγRIII mAb (3G8). As with the production of TNFα and IL-1α described previously in this thesis, Trezzini's group found that both the anti-FcγRI (32.2) and the anti-FcγRII mAb (IV.3) failed to induce free radical production by monocytes in suspension. Therefore, the ability of each FcγR to mediate a respiratory burst by adhered human monocytes in vitro was investigated. Each Fcγ receptor was ligated using the murine anti-FcγR monoclonal antibodies in the absence of secondary crosslinking and reactive oxygen species production measured by luminol-dependent chemiluminescence according to the protocol described in materials and methods (2.14.).
Results

When adhered human monocytes were incubated with either the anti-FcγRI (32.2), anti-FcγRII (IV.3), anti-FcγRIII (3G8) mAb or the isotype control murine IgG1 (all at 15μg/ml), only those cultures incubated with the anti-FcγRIII mAb generated a reactive oxygen species response (Figure 16). Cultures incubated with the anti-FcγRI mAb, the anti-FcγRII mAb or the murine IgG1 showed levels of chemiluminescence that fluctuated between 7 and 20 cps over the whole time course. These levels of chemiluminescence were similar to those recorded from the adhered monocytes prior to the addition of the monoclonal antibodies (background light emission ~12 cps). Following the addition of the anti-FcγRIII mAb to the adhered monocytes, the chemiluminescence response rapidly peaked within 2 minutes (126 cps). The light emission remained high for another 8 minutes and then gradually decayed with time, returning to near background levels (21 cps) 60 minutes after the anti-FcγRIII mAb was first added.

In 2 repeat experiments, similar chemiluminescent responses were observed. In these repeated experiments, the anti-FcγRI mAb, 32.2 was substituted by the other anti-FcγRI mAb, 10.1. As with mAb 32.2, the anti-FcγRI mAb 10.1, induced levels of chemiluminescence equal to background light emission. In one experiment the murine IgG1 isotype control was substituted by the murine IgG2b and levels of chemiluminescence were again similar to background light emission.
Legend for Figure 16. Reactive oxygen species production by adhered monocytes following incubation with anti-FcγR mAb. Figure shows the times course for reactive oxygen species production by adhered monocytes following incubation with the anti-FcγRI mAb (green solid square), anti-FcγRII mAb (blue solid triangle), anti-FcγRIII mAb (red solid circle) or murine IgG1 (pink solid diamond). Data are from one representative experiment.
3.5.2. Reactive oxygen species production by adhered monocytes following incubation with PMA or LPS

Experimental design
PMA and LPS are potent stimulators of phagocyte activity including activation of the respiratory burst (Dahlgren, 1987). PMA activates protein kinase C (Niedel et al., 1983). LPS stimulates monocytes through the surface marker, CD14 by first complexing with the LPS binding protein (LBP) which is ubiquitously present in serum (Wright et al., 1990; Ziegler-Heitbrock & Ulevitch, 1993). Serum has been shown to inhibit luminol-dependent PMN chemiluminescence, mainly due to the high levels of albumin in serum acting as a scavenger of oxygen radicals (Hastings et al., 1982; Holt et al., 1984). Since the chemiluminescent experiments outlined in this thesis were performed in the absence of polymixin B, the potential for LPS to stimulate a respiratory burst by adhered monocytes in the absence of serum was determined.

Results
Figure 17a shows the time course for reactive oxygen species production following the incubation of adhered monocytes with the anti-FcyRIII mAb (15μg/ml), murine IgG1 (15μg/ml) or PMA (0.5μg/ml). As observed in the previous experiments (3.5.1.), the anti-FcyRIII mAb induced adhered monocytes to generate a reactive oxygen species response, while chemiluminescence levels induced by the murine IgG1 were similar to those observed for background light emission. Cultures incubated with PMA generated a strong chemiluminescent response that gradually rose with time, peaking after an incubation of 25 minutes (7750cps), after which the light emission gradually decayed with time. At its peak, the PMA induced response was 6 times greater than the anti-FcyRIII mAb induced response at its peak (126cps). Similar time courses were observed in 4 repeat experiments, with the PMA induced response ranging from 9 to 11 times greater than the anti-FcyRIII mAb induced response.

Figure 17b shows the time course for reactive oxygen species production from adhered monocytes following incubation with the anti-FcyRIII mAb, murine IgG1 or LPS (0.5μg/ml). As observed previously, the anti-FcyRIII mAb induced adhered monocytes to generate a reactive oxygen species
response. The levels of chemiluminescence recorded from cultures incubated with LPS were comparable to the levels of chemiluminescence induced by the murine IgG1 and background light emission. This result was reproduced in 2 repeat experiments.
Legend for Figure 17. The effects of PMA and LPS on reactive oxygen species production. Time course for reactive oxygen species production by adhered monocytes following incubation with a) the anti-FcγRIII mAb (red solid circle), murine IgG1 (green solid triangle) or PMA (blue solid square) or b) the anti-FcγRIII mAb (red solid circle), murine IgG1 (green solid triangle) or LPS (blue solid square). Data are from one representative experiment.
Reactive oxygen species production (cps)

Figure 17b

Time (Minutes)

Antigen-FCyRIII (3G8)

Anti-FCyRIII IgG1

LPS

Murine IgG1
3.5.3. Reactive oxygen species production by adhered monocytes following incubation with anti-FcγRIII mAb F(ab')2 fragments

Experimental design
To determine whether the generation of the oxidative respiratory burst in response to the anti-FcγRIII mAb was dependent upon an Fc-FcγR interaction, adhered monocytes were incubated with F(ab')2 fragments of the anti-FcγRIII mAb.

Results
As shown in Figure 18, adhered monocytes incubated with the anti-FcγRIII mAb (15μg/ml) generated a reactive oxygen species response as observed previously. Incubation with the murine IgG1 (15μg/ml) induced levels of chemiluminescence similar to those observed for background light emission. The culture incubated with F(ab')2 fragments of the anti-FcγRIII mAb (15μg/ml) also generated a reactive oxygen species response. Unlike the chemiluminescent time course induced by the whole anti-FcγRIII mAb, incubation of adhered monocytes with the F(ab')2 fragments induced a weaker response. The levels of chemiluminescence rose gradually with time and peaked after an incubation time of 30 minutes (100cps). The response then continued at this level while the whole anti-FcγRIII mAb-induced chemiluminescent response declined with time to a similar level. These observations were confirmed in 2 repeat experiments with monocytes from different donors. In one of the repeat experiments a culture was also incubated with anti-FcγRIII mAb Fab fragments (15μg/ml) and no chemiluminescent response was observed.

Summary
The findings presented in this results section have demonstrated the ability of FcγRIIIa to mediate a respiratory burst from adhered human monocytes following receptor ligation with a monoclonal antibody in the absence of secondary crosslinking. The monoclonal antibodies to FcγRI and FcγRII did not trigger reactive oxygen species production from adhered monocytes. These observations support previous experiments performed on non-adherent human monocytes (Trezinni et al., 1990). F(ab')2 fragments of the anti-FcγRIII mAb also triggered the adhered monocytes to generate a respiratory burst. However, contrary to previous reports (Trezinni et al.,
1990) the anti-FcyRIII F(ab')2 fragments triggered a weaker chemiluminescent response, with a profile distinct from the time course triggered by the whole monoclonal antibody.
Legend for Figure 18. The effects of anti-FcγRIII F(ab')₂ fragments on reactive oxygen species production. Time course for reactive oxygen species production by adhered monocytes following incubation with the anti-FcγRIII mAb (red solid circle), anti-FcγRIII F(ab')₂ fragments (blue solid square) or murine IgG1 (green solid triangle). Data are from one representative experiment.
3.6. The effects of human IgG immune complexes on TNFα production by adhered monocytes in vitro

The work presented in this chapter so far has been focused upon the ability of Fcγ receptor ligation to trigger the release of pro-inflammatory mediators such as cytokines and reactive oxygen species from adhered human monocytes in vitro. This was achieved using murine anti-FcγR monoclonal antibodies which may be viewed as surrogates for small IgG immune complexes. It was necessary to examine the effects of physiological ligands on FcγR-mediated monocyte activation in order to investigate the ability of small IgG immune complexes to induce TNFα production. Complexes of human IgG anti-NIP mAb were used to investigate the effects of IgG immune complex size and subclass on TNFα production by adhered human monocytes in vitro.

3.6.1. TNFα release from adhered monocytes following incubation with immune complexes of human IgG anti-NIP mAb

Experimental design

As discussed previously, the type of immune complex implicated in the pathogenesis of rheumatoid arthritis are small, soluble IgG1 complexes. The ability of small complexes consisting of the human IgG1 anti-NIP mAb to trigger TNFα release from adhered human monocytes was investigated. The NIP/BSA antigen with an average epitope density of 2.4 was selected to create a heteropopulation of small complexes, theoretically comprising IgG monomers, dimers and trimers. Immune complexes were prepared in a molar excess of antigen, a molar excess of antibody (both soluble complexes) and at molar equivalence (insoluble complexes) to establish the effects of IgG1 anti-NIP immune complex preparation on TNFα release.

Methods

Immune complexes of the human IgG1 anti-NIP mAb coupled with 2.4NIP/BSA were prepared in either a molar excess of antigen, with the molar ratio of 1:5, a molar excess of antibody, with the molar ratio of 1:5, or in an antigen:antibody ratio of 1:2 (equivalence) so that the mean number of NIP molecules per BSA was approximately equivalent to the number of antibody molecules. The immune complexes were prepared according to the protocol described in the materials and methods (2.9.3.). Adhered human
monocytes were then incubated with the preformed immune complexes (at a final IgG1 concentration of 50µg/ml) or appropriate controls (see materials and methods 2.12.) and following a 4 hour incubation supernatants were collected and tested for TNFα as described previously.

Results
As shown in Figure 19, when adhered human monocytes were incubated with complexes of human IgG1 anti-NIP mAb coupled to 2.4NIP/BSA, significant levels of TNFα were only detected in supernatants from those cultures incubated with the immune complexes prepared in a molar excess of antigen (1.121 ± 0.46ng/ml). This response was concentration dependent (Figure 20). The incubation of cultures with LPS (in the absence of polymixin B) induced similar levels of TNFα (1.427 ± 0.72ng/ml). Minimal levels of TNFα were detectable in the supernatants from cultures incubated with the immune complexes prepared either in a molar excess of antibody (0.018 ± 0.03ng/ml) or at equivalence (0.014 ± 0.02ng/ml). TNFα was undetectable in the supernatants from cultures incubated with either the 2.4NIP/BSA, the IgG1 anti-NIP mAb or medium alone.

In a separate series of experiments, IgG1 anti-NIP immune complexes were prepared using NIP/BSA with average epitope densities of 8.9 or 21.5. Again, TNFα was only detected in the supernatants from cultures incubated with complexes preformed in a molar excess of antigen.
Figure 19. The effect of IgG1 anti-NIP immune complex preparation on TNFα release. Barchart showing the levels of TNFα released by adhered monocytes following a 4 hour incubation with LPS (in the absence of polymixin B) or small IgG1 anti-NIP immune complexes prepared in either a molar excess of antigen, a molar excess of antibody or at molar equivalence. Values are expressed as the mean ± S.D. from three separate experiments.
Figure 20. The effect of small IgG1 anti-NIP immune complex (prepared in a molar excess of antigen) concentration on TNFα release by adhered monocytes following a 4 hour incubation. Data are from one representative experiment. This result was reproduced when the experiment was repeated.
3.6.2. The effects of IgG immune complex subclass on TNFα production by adhered monocytes

Experimental design
IgG of each subclass bind Fcγ receptors with differing affinities (van de Winkel & Capel, 1993) and therefore complexes of each IgG subclass may differ in their ability to trigger TNFα production from adhered human monocytes. This was investigated by incubating adhered monocytes with small anti-NIP immune complexes of each IgG subclass. The human IgG1, IgG2, IgG3 or IgG4 small anti-NIP immune complexes were preformed in a molar excess of antigen (2.4NIP/BSA) as described in the previous experiment (3.6.1.). Adhered human monocytes were then incubated with the preformed immune complexes (at a final IgG concentration of 50µg/ml) or appropriate controls (see materials and methods 2.12.). Supernatants were collected at various time points over 21 hours and tested for TNFα as described previously.

Results
As shown in Figure 21, all cultures incubated with the small soluble IgG anti-NIP immune complexes released TNFα into the supernatant. The complexes of each IgG subclass induced a TNFα response that rapidly rose with time, peaking after an incubation of 4-6 hours and then gradually declined with time. Cultures incubated with LPS (in the absence of polymixin B) showed a similar time course for TNFα release. The IgG subclass specific complexes induced levels of TNFα production in the order of magnitude: IgG1>IgG3>IgG2>IgG4. TNFα was undetectable in the supernatants from cultures incubated with medium alone (data not shown). TNFα was also undetectable following incubation with either the 2.4NIP/BSA or the IgG anti-NIP mAb of each subclass alone. Similar time courses and subclass specific levels of response were observed in 2 repeat experiments with monocytes from different donors.
Legend for Figure 21. The effects of immune complex subclass on TNFα release. Time course for TNFα production by adhered monocytes following incubation with small IgG anti-NIP complexes of the subclass IgG1 (red solid circle), IgG2 (blue solid square), IgG3 (green solid triangle) or IgG4 (pink solid diamond), all preformed in a molar excess of antigen. Cultures were also incubated with LPS (black open circle) or medium alone (pale blue open square). Data are from one representative experiment.
Figure 21

TNF (ng/ml)

Time (Hours)

IgG1-2ANP
IgG2-2ANP
LPS
IgG+2ANP
Medium

159
3.6.3. The effects of antigen epitope density on human IgG immune complex induced TNFα production by adhered monocytes

**Experimental design**

The effects of immune complex size on the ability of adhered human monocytes to release TNFα was investigated using soluble IgG1 anti-NIP mAb complexes formed with NIP/BSA of different epitope densities.

**Methods**

IgG1 anti-NIP mAb complexes were prepared in a molar excess (1:5) of either 2.4NIP/BSA (small complexes), 8.9NIP/BSA (moderate complexes) or 21.5NIP/BSA (large complexes) as described previously. Adhered human monocytes were then incubated with the preformed immune complexes (at a final IgG1 concentration of 50μg/ml) or appropriate controls (see materials and methods 2.12.). Supernatants were collected after a 4 hour incubation and tested for TNFα.

**Results**

Following a 4 hour incubation, TNFα was detected in supernatants from cultures incubated with the small soluble IgG1 complexes (0.968 ± 0.28ng/ml), the moderate soluble IgG1 complexes (1.475 ± 0.91ng/ml) and also the large soluble IgG1 complexes (1.011 ± 0.52ng/ml). However, there appeared to be no significant relationship between the epitope density (complex size) and the levels of TNFα released.

3.6.4. The effects of blocking FcγR on small human IgG immune complex triggered TNFα production

**Experimental design**

To determine and identify Fcγ receptor involvement in the production of TNFα induced by small soluble IgG1 anti-NIP mAb immune complexes, anti-FcγRI, anti-FcγRII and F(ab')2 fragments of the anti-FcγRIII mAb were used to block FcγRI, FcγRII and FcγRIIIa receptor binding respectively.

**Methods**

Adhered human monocytes were preincubated at 37°C for 30 minutes with either the anti-FcγRI mAb, the anti-FcγRII mAb, F(ab')2 fragments of the anti-
FcγRII mAb (all at 20μg/ml) or medium alone. Small IgG1 anti-NIP complexes were preformed in a molar excess of antigen (1:5) as described previously and were then added to all cultures (at a final IgG1 concentration of 50μg/ml). LPS (in the absence of polymixin B) and the anti-FcγRIII mAb were used as positive controls as described in previous experiments. Culture supernatants were collected 4 hours after the complexes were added and tested for TNFα.

Results
As observed previously, cultures incubated with either LPS (in the absence of polymixin B) or the anti-FcγRIII mAb released TNFα into the supernatant (0.570 ng/ml and 0.478 ng/ml respectively). TNFα was not detected in the supernatants from cultures incubated with either the anti-FcγRI mAb, anti-FcγRII mAb, F(ab')2 fragments of the anti-FcγRIII mAb or medium alone. When either FcγRII or FcγRIIIa were blocked, the TNFα response induced by the small soluble IgG1 complexes was partially inhibited by 21% and 37% respectively. However, the blocking of FcγRI had little effect (1% inhibition) upon the TNFα response following incubation with the small IgG1 immune complexes. Similar levels of inhibition were observed when this experiment was repeated.

Summary
The results presented in this section have demonstrated the ability of small, soluble human IgG anti-NIP immune complexes to induce the release of TNFα from adhered human monocytes in vitro. Those complexes formed at equivalence (insoluble) or in a molar excess of antibody failed to trigger a significant TNFα response. The levels of TNFα released following the incubation of adhered monocytes with small immune complexes appeared to be subclass dependent, however, complex size did not influence the magnitude of the TNFα response. The blocking of FcγRII or FcγRIIIa resulted in a partial inhibition of the release of TNFα from adhered monocytes in response to the small IgG1 anti-NIP complexes. Furthermore, FcγRIIIa appeared to play a more dominant role than FcγRII in the induction of TNFα release from cultures in response to these small IgG1 immune complexes.
3.7. Human IgG rheumatoid factor mAb

This study has therefore established that small soluble human IgG immune complexes could induce the release of TNFα from adhered human monocytes under the conditions of the in vitro system used in this project. However, the actual complex relevant to the hypothesis of this thesis, was the self-associating IgG rheumatoid factor. Two IgG rheumatoid factor monoclonal antibodies (an IgG1 and an IgG2) produced by Professor PP Chen and colleagues (Lu et al., 1993) were used to investigate the ability of small IgG rheumatoid factor immune complexes to induce TNFα production by adhered human monocytes in vitro.

3.7.1. Determining the potential for the IgG1 rheumatoid factor mAb to self-associate

Experimental design

In previous studies using these monoclonal antibodies, Lu et al. (1992; 1993) analysed the ability of both the IgG1 and the IgG2 rheumatoid factor mAb to self-associate by FPLC. Briefly, following a 30 hour incubation at 37°C, a shift towards the higher molecular weight of 300-kDa was observed which corresponded to IgG rheumatoid factor homodimers. Prior to any functional experiments, it was essential to confirm the ability of the IgG1 and the IgG2 rheumatoid factor monoclonal antibodies to self-associate. HPLC analysis of the IgG1 rheumatoid factor monoclonal antibody and, as a control the IgG1 myeloma protein, was performed according to the protocol described in materials and methods (2.9.1.).

Results

At time 0 the elution profile of the IgG1-RF mAb showed a large peak with an estimated molecular mass of 150-kDa, corresponding to IgG1-RF monomers. There was also a smaller peak with a MW of 470-kDa, corresponding to IgG1-RF trimers and a shoulder ranging from 580-kDa to a MW of greater than 660-kDa corresponding to IgG1-RF aggregates (Figure 22a).

As shown in Figure 22b, following a 15 hour incubation at 37°C, the elution profile of the IgG1-RF mAb exhibited a large peak of IgG1-RF monomers, a
smaller peak of IgG1-RF trimers and a slightly larger peak occurring at a MW of more than 660-kDa (IgG1-RF aggregates).

As shown in Figure 22c, after a 40 hour incubation at 37°C, the IgG1-RF mAb exhibited a similar profile to the 15 hour incubation, however, the peak at the MW of greater than 660-kDa was equal to the peak of IgG1-RF trimers.

The elution profiles of the IgG1 myeloma protein following an incubation at 37°C for 0, 15 and 40 hours all exhibited a peak at the molecular weight of 150-kDa corresponding to IgG monomers (Figure 22d-f).
Legend for Figure 22. HPLC analysis of the IgG1 rheumatoid factor mAb. HPLC elution profiles for the IgG1-RF mAb following incubation at 37°C for a) 0 hours, b) 15 hours or c) 40 hours and the human myeloma IgG1 protein after an incubation at 37°C for d) 0 hours, e) 15 hours or f) 40 hours. Data are from one representative experiment.
3.7.2. Determining the potential for the IgG2 rheumatoid factor mAb to self-associate

Experimental design
In the previous experiment (3.7.1.), the IgG1 rheumatoid factor monoclonal antibody appeared to self-associate spontaneously. The potential for the IgG2 rheumatoid factor monoclonal antibody was therefore analysed by HPLC without preincubation at 37°C and the elution profile was compared with that of the IgG1 rheumatoid factor monoclonal antibody as described previously.

Results
Figure 23a shows the elution profile of the IgG1-RF mAb. As observed previously, there was a large peak of IgG1-RF monomers, a smaller peak of trimers and a shoulder ranging from 580-kDa to a MW greater than 660-kDa (IgG1-RF aggregates).

Figure 23b shows the elution profile for the IgG2-RF mAb. Interestingly, the profile was very different when compared with the elution profile for the IgG1-RF mAb. The IgG2-RF mAb exhibited a large peak at a MW of 270-kDa, corresponding with IgG2-RF dimers. Two larger peaks were observed at the MW of 610-kDa and greater than 660-kDa, both corresponding to IgG2-RF aggregates.

The IgG2 myeloma protein exhibited an elution profile identical to that obtained for the IgG1 myeloma protein in the previous experiment (data not shown).
Figure 23. HPLC elution profiles for the a) IgG1-RF mAb and b) IgG2-RF mAb. Data are from one representative experiment.
3.7.3. Determining the effects of concentration on the elution profile of the IgG1 rheumatoid factor mAb

When self-associating IgG rheumatoid factors from patients with rheumatoid arthritis were first characterised, it was found that their aggregation appeared to be concentration dependent (Pope et al., 1975). Therefore, the effect of immunoglobulin concentration on the elution profile of the IgG1 rheumatoid factor mAb was analysed.

Methods

The IgG1 rheumatoid factor monoclonal antibody was diluted in PBS to a concentration of 1mg/ml and 100µg/ml and the two samples were then analysed by HPLC according to the protocol described in materials and methods (2.9.1.).

Results

As shown by Figure 24, the elution profiles for the IgG1 rheumatoid factor monoclonal antibody at 1mg/ml and 100µg/ml both displayed a large peak of IgG1-RF monomers. The elution profile of the IgG1-RF at the higher concentration exhibited a peak of IgG1-RF trimers and a shoulder corresponding to IgG1-RF aggregates. However, the IgG1-RF at the lower concentration exhibited a shift to less trimers and more aggregates.
Figure 24. The effect of concentration on the HPLC elution profile of the IgG1 rheumatoid factor mAb. HPLC elution profiles for the IgG1-RF mAb at 1mg/ml and 100μg/ml. Data are from one representative experiment.
3.7.4. Determining the effects of fractionation on the stability of the IgG1 rheumatoid factor mAb

Experimental design
Preparative HPLC was performed on the IgG1 rheumatoid factor monoclonal antibody as described in materials and methods (2.9.2.), to separate IgG1-RF complexes of various sizes. Once separated, the stability of these complexes was determined by re-analysing each fraction by analytical HPLC, as described previously. Following collection, each fraction was analysed immediately. Additionally, each fraction was stored at 4°C in 0.5% azide for 4 days and then re-analysed by analytical HPLC.

Results
Fractions of IgG1-RF complexes were collected and HPLC analysis demonstrated certain fractions to contain only monomers, trimers or aggregates. Following a 4 day incubation at 4°C, HPLC analysis of these fractions demonstrated no significant shifts in the elution profiles, indicating the stability of the complexes once isolated from the heteropopulation.

Summary
The HPLC analyses described in these experiments have shown that the IgG1 rheumatoid factor monoclonal antibody spontaneously self-associated forming a mixed population of monomers, trimers and large aggregates. The IgG2 rheumatoid factor monoclonal antibody spontaneously self-associated forming a mixture of dimers and larger aggregates. When the heteropopulation of the IgG1 rheumatoid factor monoclonal antibody was separated into fractions of known size and stored at 4°C for 4 days, there was no significant difference in the complex size within each fraction compared with those analysed on the day of preparation. This suggested the IgG1-RF mAb at each complex size to be stable when separated from the heteropopulation. The only dependency on concentration found was a slight shift of the large aggregates to form larger complexes as the IgG1-RF concentration decreased.
3.7.5. TNFα release from adhered monocytes following incubation with human IgG rheumatoid factor mAb

The HPLC analyses have shown that both the IgG1 and IgG2 rheumatoid factor monoclonal antibodies spontaneously self-associated. The IgG1 rheumatoid factor mAb formed a heteropopulation of monomers, trimers and aggregates, while the IgG2 rheumatoid factor mAb formed a mixed population of dimers and aggregates. The ability of these rheumatoid factor preparations to induce TNFα release from adhered human monocytes was then examined.

Methods

Adhered human monocytes were incubated with either the IgG1-RF mAb, the IgG2-RF mAb (both at 250 µg/ml) or the appropriate controls as described in materials and methods (2.12.). Culture supernatants were collected at various time points over 24 hours and tested for TNFα according to the protocol described in materials and methods (2.13.2.).

Results

Following the incubation of adhered human monocytes with either the IgG1-RF mAb or the IgG2-RF mAb, significant levels of TNFα were only detected in the supernatants from cultures incubated with the IgG1-RF mAb (Figure 25). The levels of TNFα released from cultures in response to the IgG1-RF mAb rapidly rose with time and peaked at 4 hours. The TNFα response then decreased with time. The LPS (in the absence of polymixin B) induced TNFα response rose rapidly with time, peaking after 6 hours, as observed previously. Low levels of TNFα were detectable in the supernatants from cultures incubated with the IgG2-RF mAb. However, these were comparable to TNFα levels detected in the supernatant from cultures incubated with medium alone.

This experiment was repeated six times by incubating adhered monocytes with the IgG1-RF or the IgG2-RF mAb at a range of concentrations (from 1mg/ml to 1µg/ml). In all subsequent experiments, TNFα was not detected in any of the supernatants. Cells did however, secrete TNFα following incubation with either LPS (in the absence of polymixin B) or the anti-FcyRIII mAb (3G8) as observed previously.
Further investigation was carried out by incubating adhered monocytes with the separated IgG1-RF complexes of various sizes, prepared by HPLC as described previously. Adhered human monocytes were incubated with either IgG1-RF monomers, trimers or aggregates, at a final concentration of 50μg/ml. Again, TNFα was undetectable in the supernatants from cultures that had been incubated with IgG1-RF complexes of any size. Cells were however viable and able to release TNFα following incubation with either LPS (in the absence of polymixin B) or the anti-FcγRIII mAb. Similar results were obtained when this experiment was repeated.

Summary
Although the initial result showing the IgG1 rheumatoid factor complexes to trigger TNFα release from adhered human monocytes was promising, the subsequent experiments probably reflected the inherent problems of trying to study stable IgG rheumatoid factor oligomers. This will be referred to in more detail in the discussion (4.3.2.).
Legend for Figure 25. The effects of IgG1 and IgG2 rheumatoid factor mAb on TNFα release. The time course for TNFα production by adhered monocytes following incubation with the IgG1-RF mAb (red solid circle), the IgG2-RF mAb (pink closed square), LPS in the absence of polymixin B (blue closed triangle) or medium alone (green closed diamond). Data are from one representative experiment.
3.8. Inhibitor studies

As shown previously in this chapter, the anti-FcγRIII mAb (3G8), LPS (in the absence of polymixin B) and the small IgG1 anti-NIP mAb complexes were all able to elicit a TNFα response from adhered human monocytes, similar in both kinetics and magnitude. Therefore, the nature of the TNFα response from adhered human monocytes in vitro was further analysed using various inhibitors.

All of the following inhibitor studies were performed in the absence of polymixin B, as the effects of this chemical upon inhibitor function were unknown. Therefore, prior to these inhibitor studies, adhered monocytes were incubated with the anti-FcγRIII mAb or the small IgG1 anti-NIP immune complexes in both the presence and absence of polymixin B to ensure there would be no LPS induced TNFα response occurring as a result of reagent contamination. Cultures incubated with the anti-FcγRIII mAb or the small IgG1 anti-NIP complexes in the absence of polymixin B induced levels of TNFα equivalent to those released from similar cultures incubated in the presence of polymixin B.

3.8.1. The effects of inhibitors on TNFα release from adhered monocytes

Experimental design

The inhibitors cycloheximide, actinomycin D and colchicine were used to determine whether the TNFα response from adhered monocytes following incubation with the anti-FcγRIII mAb, LPS or the small IgG1 anti-NIP complexes was due to the release of preformed or newly synthesised TNFα, and also to assess the role of microtubules and secretory vesicles during the release of this cytokine into culture supernatants. Matrix metalloproteinases such as the gelatinase, in addition to matrix degradation, also process surface bound pro-TNFα into the mature, soluble cytokine (Gearing et al., 1994). Therefore the effects of a gelatinase-specific MMP inhibitor on the release of soluble TNFα was also investigated.

Methods

Adhered human monocytes were incubated with the anti-FcγRIII mAb (50μg/ml), LPS (0.5μg/ml) or the small IgG1 anti-NIP complexes (50μg/ml) as described previously. All cultures were incubated in the presence or
absence of either cycloheximide (50µg/ml), actinomycin D (1µg/ml), colchicine (2µg/ml) or the MMP inhibitor (1746; 50µM). Supernatants were collected following an incubation of 4 hours and tested for TNFα as described previously.

Results
As seen in Figure 26a, following a 4 hour incubation, cycloheximide significantly inhibited TNFα release in response to the anti-FcyRIII mAb (100.0 ± 0.1%; p<0.001) and LPS (99.9 ± 0.3%; p<0.005). Treatment of adhered monocytes with actinomycin D also significantly inhibited both the anti-FcγRIII mAb-induced TNFα response (93.8 ± 5.2%; p<0.001) and the LPS-induced TNFα response (74.2 ± 10.4%; p<0.01). The presence of colchicine did not significantly inhibit the anti-FcγRIII mAb response (30.1 ± 22.3%) or the LPS response (32.2 ± 11.1%). Presence of the MMP inhibitor significantly inhibited TNFα release in response to both the anti-FcγRIII mAb (84.2 ± 15.2%; p<0.005) and LPS (76.8 ± 13.3%; p<0.005).

In a separate experiment, following a 4 hour incubation, cycloheximide inhibited TNFα release in response to both the small IgG1 anti-NIP complexes and LPS by 100% (Figure 26b). Treatment of adhered monocytes with actinomycin D inhibited the small IgG1 anti-NIP complex-induced response by 90% and the LPS-induced response by 68%. The presence of colchicine did not inhibit the small IgG1 anti-NIP complex-induced response, but instead enhanced TNFα release by 28%. The LPS-induced response in the presence of colchicine was partially inhibited by 22%. Presence of the MMP inhibitor significantly blocked the TNFα response to the small IgG1 anti-NIP mAb complexes by 87% and the TNFα response to LPS by 77%. These results were confirmed when this experiment was repeated.

Summary
The presence of cycloheximide abrogated TNFα release in response to LPS, the anti-FcγRIII mAb and the small IgG1 anti-NIP complexes. However, actinomycin D and the MMP inhibitor had a stronger inhibitory effect upon both the anti-FcγRIII mAb and the small IgG1 anti-NIP complex induced TNFα response compared with the LPS-induced response. Colchicine had a greater inhibitory effect upon LPS-induced TNFα release compared with the
anti-FcγRIII mAb response. Interestingly, colchicine enhanced the TNFα response following incubation with the small IgG1 anti-NIP complexes. The inhibitors did not interfere with the TNFα ELISA.

Legend for Figure 26. The effect of inhibitors on TNFα release.
a) Barchart showing the levels of TNFα released by cultures following a 4 hour incubation with the anti-FcγRIII mAb or LPS in the presence of medium, cycloheximide, actinomycin D, colchicine or the MMP inhibitor. Values are expressed as the mean ± S.D. from three separate experiments.
b) Barchart showing the levels of TNFα released by cultures following a 4 hour incubation with the small IgG1 anti-NIP complex or LPS in the presence of medium, cyclohexamide, actinomycin D, colchicine or the MMP inhibitor. Data are from one representative experiment.
TNF (ng/ml) at 4 hours

Figure 26a

- Medium: 1.5
- Cycloheximide: 0.001 +/− 0.002
- Actinomycin D: 0.002 +/− 0.004
- Colchicine: 0.001 +/− 0.002
- MMP inhibitor: 0.002 +/− 0.004

Anti-FcγRIII

LPS
CHAPTER 4

DISCUSSION
The aim of this thesis was to test a hypothetical model proposing an IgG rheumatoid factor-triggered effector mechanism for inflammation in rheumatoid arthritis (Edwards & Cambridge, 1998). This model predicts that small immune complexes comprising self-associating IgG rheumatoid factors, owing to their small size, may evade complement-mediated clearance from the circulation and access tissue macrophages. The model further predicts that these immune complexes initiate inflammation within the synovium by preferentially binding to the surface immunoglobulin receptor, FcγRIIIa. FcγRIIIa crosslinking may trigger macrophage activation and the subsequent production of proinflammatory mediators such as TNFα and reactive oxygen species.

4.1. Fcγ receptor ligation by murine anti-FcγR mAb

4.1.1 Production of cytokines and free radicals following incubation with anti-FcγR mAb

The initial objective of this study was to determine the ability of the macrophage receptor, FcγRIIIa to mediate TNFα release following receptor ligation. Previous work had shown the release of TNFα from human peripheral blood monocytes following non-specific Fcγ receptor ligation with IgG immune complexes (Debets et al., 1988; Polat et al., 1993). Additionally, the production of TNFα by monocytes was confirmed to be a result of Fcγ receptor crosslinking rather than the process of immune complex ingestion (Debets et al., 1988). Further work by Debets et al. (1990) investigated the ability of FcγRI and FcγRII to mediate TNFα release from monocytes. Their observations showed that TNFα release from monocytes was only achieved through FcγRI following receptor crosslinking with murine IgG2a in solid phase. Similarly, FcγRII only mediated TNFα release following receptor crosslinking with murine IgG1 in solid phase after the monocytes were pretreated with either proteases or neurominidase. FcγRIIIa crosslinking had also been shown to mediate TNFα production, however, this had only been demonstrated in natural killer cells (Anégón et al., 1988; Hendrich et al., 1991).
Originally it was decided to repeat the protocol described by Debets et al. (1990) for FcγRIIIa. However, this raised complications. Monocytes required adherence to plastic for at least 24 hours for all three FcγR to be expressed. This is possibly why Debets et al. (1990) only studied TNFα production mediated through FcγRI and FcγRII. As shown in this dissertation and by others (Kelley et al., 1987; Krause et al., 1996-97), the adherence of monocytes to tissue culture plastic over 24 hours was itself a stimulus for TNFα production, as was the procedure of removing and reseeding monocytes following initial maturation. Therefore, TNFα production by pre-matured monocytes seeded into plates coated with either the anti-FcγRIII mAb or murine IgG1 would be uninterpretable and it is unclear how Debets et al. (1990) avoided this. Upon further examination of the literature, work by Trezzini et al. (1990) suggested that for FcγRIIIa-mediated monocyte activation, multiple crosslinking might not be necessary. It was found that of the three anti-FcγR mAb, only the anti-FcγRIII mAb in fluid phase was able to trigger reactive oxygen species production from non-adhered monocytes (Trezzini et al., 1990).

Indeed, when adhered human monocytes were incubated with anti-FcγR mAb in fluid phase, in the absence of secondary crosslinking, only the anti-FcγRIII mAb triggered TNFα release. Similarly, of the three anti-FcγR mAb, only that directed against FcγRIII triggered adhered monocytes to release IL-1α and reactive oxygen species. These observations, in addition to those reported by Debets et al. (1990) and Trezzini et al. (1990), implied that FcγRI and FcγRII both required the crosslinking of multiple receptors, whilst only two or three FcγRIIIa receptors needed to be co-ligated for the signalling of TNFα, IL-1α and reactive oxygen species production.

It had also been decided to attempt to modulate FcγRIIIa expression and see how this may have affected levels of TNFα production following FcγRIIIa ligation. TGFβ has been reported to upregulate FcγRIIIa expression (Welch et al., 1990; Wong et al., 1991), however, preliminary results suggested that TGFβ acts to inhibit FcγRIIIa expression in vitro. This conflict was further supported by the results presented in this thesis showing the presence of TNFα to have no effect upon FcγRIIIa
expression, while others have shown this cytokine to downregulate receptor levels (Liao & Simone, 1994; Liao et al., 1994). In light of these varying reports, such studies were not pursued.

The anti-FcγRIII mAb would have the potential to ligate up to three FcγR. Two FcγRIIIa receptors could be ligated specifically through the mAb Fab regions, while a third FcγR could be non-specifically ligated through the antibody's Fc fragment (Figure 27). It therefore became clear that the anti-FcγRIII mAb might be a good surrogate for the specific type of complex that may be involved in rheumatoid arthritis i.e. a small IgG complex with two or three available FcγR binding sites.

**Figure 27.** A schematic diagram showing that an anti-FcγR mAb has the potential to ligate up to three Fcγ receptors.

4.1.2. **FcyRIIIa crosslinking by anti-FcyRIII mAb and TNFα release**
As suggested by others (Debets et al., 1990), a true IgG Fc-FcγR interaction may be required for intracellular signalling to result in TNFα release. Alternatively, it has been suggested that signalling is dependent upon receptor aggregation and crosslinking rather than ligand binding (van de
Winkel & Capel, 1993). Using blocking antibodies to FcγRI or FcγRII it was found that anti-FcγRIII mAb-mediated TNFα release occurred independently of these two receptors, suggesting there was no additional recruitment of either FcγRI or FcγRII by the Fc region of the anti-FcγRIII mAb.

When the anti-FcγRI mAb used in these studies was originally generated it was found to completely inhibit the binding of opsonised erythrocytes to U937 cells and also murine IgG2a anti-T3 mAb induced T cell proliferation. However, it was also found that this anti-FcγRI mAb did not block the binding of monomeric IgG to U937 cells (Dougherty et al., 1987). This later observation casts some doubt over the ability of this monoclonal antibody to efficiently block FcγRI binding of soluble antibody, such as the Fc region of the anti-FcγRIII mAb. Although FcγRI binds murine IgG2a with high affinity, the anti-FcγRIII mAb was of subclass IgG1 for which FcγRI has a relatively low affinity (Jones et al., 1985). Additionally, in vivo FcγRI is normally saturated with monomeric IgG and to displace this requires a large immune complex (Tax & van de Winkel, 1990). It is possible that the FcγRI expressed by adhered human monocytes in these studies may have been saturated with human IgG. This rules out the ability of FcγRI to be recruited by the Fc region of the anti-FcγRIII mAb for the intracellular signalling of TNFα. However, this then raises the argument that the lack of an anti-FcγRI mAb-induced TNFα response may be due to the receptor being already saturated with human IgG. FcγRI expressed by adhered monocytes that have been cultured for at least 48 hours should be free of monomeric IgG owing to receptor recycling (Dr PK Wallace, personal communication). When adhered monocytes were cultured for 48 or 72 hours and subsequently incubated with the anti-FcγRI mAb, no TNFα response was observed (data not shown). Based on these arguments one may infer that FcγRI could neither signal following ligation by the anti-FcγRI mAb, nor be recruited by the anti-FcγRIII mAb for TNFα release in these studies.

Although FcγRI and FcγRII appeared to not be involved in the anti-FcγRIII mAb induced TNFα response, the Fc fragment of this monoclonal antibody may have ligated another FcγRIIIa receptor. It was
found that neither F(ab')\textsuperscript{2} nor Fab fragments of the anti-FcγRIII mAb were able to induce TNFα production. This could imply that for signalling and subsequent TNFα release, stimulation through FcγRIIIa requires recruitment of three receptors. Alternatively, ligation of only two FcγRIIIa receptors may be mandatory, but, if so, one interaction must involve binding through an Fc domain (Figure 28).

The crosslinking of either two or three FcγRIIIa receptors by the anti-FcγRIII mAb relies in both cases upon an interaction between the Fc region of the anti-FcγRIIIa mAb and a FcγRIIIa receptor. Kipps et al. (1985) showed that FcγRIIIa expressed by Killer cells was unable to mediate ADCC in response to monomeric murine IgG1, suggesting a low affinity of FcγRIIIa for this mouse subclass. However, recent data has shown that the Fc fragment of murine IgG1 can bind FcγRIII. Kocher et al. (1998) showed that the Fc fragment of murine IgG1 monoclonal antibodies directed against surface bound neutrophil granule proteins preferentially ligated the low affinity FcγRIIIb following initial Fab binding. The interactions of these monoclonal antibodies are closely analogous with what appears to be happening with the anti-FcγRIII mAb, where one or two Fab interactions may be stabilising a further interaction through Fc, particularly since FcγRIIIa has a higher affinity for monomeric IgG than FcγRIIIb (Vance et al., 1993). Moreover, since the anti-FcγRIII mAb would initially bind FcγRIIIa through its Fab region(s), the affinity of the anti-FcγRIII Fc region for subsequent FcγRIIIa ligation may be greater than that of the unbound antibody.
**Figure 28.** The signalling for TNFα release through FcγRIIIa requires recruitment of either a) three FcγRIIIa receptors or b) two FcγRIIIa receptors where one interaction must involve binding through an Fc domain.

4.1.3. **FcyRIIIa crosslinking by anti-FcyRIII mAb and reactive oxygen species production**

Trezzini *et al.* (1990) found that anti-FcγRIII F(ab')₂ fragments were able to trigger monocytes to release reactive oxygen species of a magnitude similar to the response induced by the whole antibody. In this thesis it was found that the anti-FcγRIII F(ab')₂ fragments also triggered adhered human monocytes to generate reactive oxygen species, however, the response was markedly weaker than that induced by the whole antibody. Both the findings of Trezzini *et al.* (1990) and those reported in this thesis suggest that the FcγRIIIa mediated respiratory burst may be dependent upon the number of FcγRIIIa receptors crosslinked rather than an IgG Fc-FcγRIIIa interaction. These results suggest that the aggregation of two FcγRIIIa receptors may be sufficient for activation of the membrane-
bound NADPH-oxidase, whereas intracellular signalling and subsequent cytokine production is less readily attained.

FcγRI and FcγRII may still require multiple receptor crosslinking for a similar reactive oxygen species response. Studies by Anderson et al. (1986) have shown that reactive oxygen species production by U937 cells through FcγRI could be achieved using the anti-FcγRI mAb (32.2) which binds an epitope outside of the receptor ligand-binding site. However, a response was only achieved when this anti-FcγRI mAb was further crosslinked with a F(ab')2 anti-murine immunoglobulin. This implies that the FcγRI-mediated reactive oxygen species production may have been dependent upon multiple FcγRI aggregation rather than an IgG Fc-FcγR interaction, further supporting the findings of this thesis.

4.1.4. Fcγ receptor crosslinking by anti-FcγR mAb and subsequent signalling

It may be difficult to understand why FcγRIIIa should function differently from FcγRI and FcγRII in response to a binding site-specific ligand such as the murine anti-FcγR mAb, particularly since FcγRIIIa signal transduction is dependent upon the identical gamma chain homodimer that is utilised by FcγRI (Ra et al., 1989; Ernst et al., 1993). All three Fcγ receptors can mediate similar functions. However, there are differences in both their binding capacities for human and murine IgG and their signalling pathways.

FcγRIIa is the only Fcγ receptor to possess its own cytoplasmic signalling motif (van den Herik Oudijk et al., 1995b) but it can also utilise the gamma chain for signalling (Masuda et al., 1993). Owing to its low affinity for IgG, which can be increased following proteolytic activity, FcγRII has been suggested to act as a "bystander" receptor (Tax & van de Winkel, 1990). Additionally, recent crystal structure data suggests that the Fc region of a single IgG molecule can bind either one or two FcγRII receptors (Sondermann et al., 1999a; Sondermann et al., 1999b). FcγRIIIa is only known to signal through the gamma chain homodimer (Wirthmueller et al., 1992), while FcγRI has been shown to signal through either the gamma chain or by recruiting FcγRIIa, depending on the differentiation state of the cell (Melendez et al., 1998). These differentials between the three Fcγ receptors may have a significant
impact on their functional properties. Fcγ receptor clustering is known to result in the phosphorylation of the ITAM signalling motif within the cytoplasmic domains of the gamma chain and FcγRIIa. Therefore, the number of receptors crosslinked may determine the number of tyrosines that are phosphorylated and this in turn may influence the signalling potential of each Fcγ receptor. Fcγ receptor aggregation may therefore only result in the signalling of, for example TNFα release, through a particular FcγR if sufficient receptor numbers have been crosslinked.

Overall, these findings indicate that for the signalling of TNFα and IL-1α release from adhered human monocytes, FcγRIIIa requires a maximum of three receptors to be crosslinked, while the crosslinking of only two FcγRIIIa receptors is required for reactive oxygen species production. FcγRI and FcγRII may require multiple receptor crosslinking for TNFα, IL-1α and reactive oxygen species production. This would support the concept that a small IgG immune complex may have the potential to trigger the production of TNFα and other proinflammatory mediators by macrophages through FcγRIIIa but not through FcγRI or FcγRII.

4.1.5. Effect of inhibitors on anti-FcγRIII mAb and LPS triggered TNFα release

Both the LPS and anti-FcγRIII mAb induced TNFα responses were abrogated by cycloheximide and significantly inhibited by the presence of actinomycin D. This confirmed that the TNFα detected in supernatants required both mRNA transcription and protein translation, and was not due to mobilisation of intracellular stores of TNFα. The two major matrix metalloproteinases secreted by activated monocytes are gelatinase B (MMP-9) and interstitial collagenase (MMP-1) and gelatinase B is spontaneously released from macrophage-derived monocytes in vitro (Saren et al., 1996). Although matrix metalloproteinases have been shown to process pro-TNFα into its mature form (Gearing et al., 1994), there have been reports suggesting otherwise (Black et al., 1996). The gelatinase-specific matrix metalloproteinase inhibitor significantly reduced both LPS and anti-FcγRIII mAb induced TNFα release from adhered monocytes. This indicated that the processing of membrane bound pro-TNFα was dependent upon this particular class of matrix
metalloproteinase. However, evidence from TACE knockout mice suggests that the processing of pro-TNFα is dependent upon this membrane-bound disintegrin-metalloproteinase in vivo (Black et al., 1997; Killar et al., 1999). Furthermore, TACE is known to be sensitive to MMP inhibitors (Black et al., 1996). Therefore, the results presented in this thesis suggest that the gelatinase-specific inhibitor used in these studies also inhibited TACE thereby blocking TNFα release from adhered monocytes. Although not statistically significant, colchicine was found to reduce both LPS and the anti-FcγRIII mAb induced TNFα production by approximately 30%, suggesting some dependence upon microtubule polymerisation.

4.2. **Fcγ receptor ligation by human IgG immune complexes**

IgG containing immune complexes have been shown to trigger human peripheral blood monocytes to produce TNFα (Polat et al., 1993) and IL-1 (Chantry et al., 1989). There have also been a number of studies comparing IgG immune complex characteristics with their binding to Fcγ receptors on human monocytes and neutrophils (Kurlander & Batker, 1982; Klaassen et al., 1988; Huizinga et al., 1989b; Voice & Lachmann, 1997). The effects of different IgG immune complexes on the activation of neutrophils, primarily the respiratory burst, through Fcγ receptors have been examined (Huizinga et al., 1989a; Crockett-Torabi & Fantone, 1990; Walker et al., 1991; Brunkhorst et al., 1992; Hundt & Schmidt, 1992). However, similar studies have not been performed on monocytes. It is difficult to extend the information regarding the Fcγ receptor binding of differing IgG immune complexes and subsequent neutrophil activation to monocytes since untreated neutrophils express only FcγRII and FcγRIIIb. Furthermore, although the neutrophil GPI-linked FcγRIIIb may have the potential to signal, the pathways involved are distinct from the signalling mechanism of the transmembrane isoform, FcγRIIIa (Brunkhorst et al., 1992; Horejsí et al., 1999).

**4.2.1. Human IgG anti-NIP immune complexes and TNFα release from adhered monocytes**

The formation of immune complexes using a multivalent antigen, such as the NIP coupled to BSA used in these studies, can be manipulated to alter lattice formation (Mannik, 1980). Small, soluble human IgG
immune complexes were prepared from human IgG1 anti-NIP monoclonal antibodies in an excess of antigen (2.4NIP/BSA) and also in an excess of antibody. Insoluble complexes of human IgG1 anti-NIP were prepared at molar equivalence. Only the soluble IgG anti-NIP mAb complexes prepared in an excess of antigen were able to trigger adhered human monocytes to release TNFα. Soluble and insoluble IgG immune complexes have been shown to activate the respiratory burst in neutrophils through different pathways (Crockett-Torabi & Fantone, 1990), however, the lack of significant TNFα release in response to the insoluble IgG anti-NIP immune complexes and the soluble complexes prepared in an excess of antibody, was surprising. One possible explanation for this observation is that the insoluble complexes may have formed a lattice which had precipitated during preparation, and therefore may not have been able to activate sufficient number of cells needed to generate a detectable TNFα response. However, in relation to the proposed mechanism of pathogenesis of rheumatoid arthritis, it was the soluble IgG immune complexes that were of interest. The lack of response following incubation with the soluble complexes prepared in antibody excess may have been a result of the complex preparation. In light of previously described protocols (Crockett-Torabi & Fantone, 1990; Voice & Lachmann, 1997) it was decided to keep the IgG anti-NIP mAb concentration constant and alter the antigen's concentration to generate antibody and antigen excess. In retrospect, this may not have been the ideal methodology. In the case of the antibody excess preparation, there were possibly not enough complexes formed to have been able to trigger a TNFα response. Alternatively, the anti-NIP mAb prepared in antibody excess, unlike in antigen excess, may not have been sufficiently crosslinked to have been able to elicit an FcγR-mediated response.

No obvious relationship was found between soluble IgG1 anti-NIP complex size and the levels of TNFα released from adhered human monocytes. However, the subclass specificity of the small soluble immune complexes did influence the magnitude of the TNFα response. Fcγ receptors have differing affinities for each human IgG subclass. Small soluble IgG1 and IgG3 anti-NIP immune complexes both evoked a strong TNFα response from adhered human monocytes. This is consistent with previous studies showing IgG1 and IgG3 complexes to bind human
phagocytes (Kurlander & Batker, 1982; Klaassen et al., 1988; Huizinga et al., 1989b). It has been shown by others that complexes of subclass IgG2 or IgG4 cannot efficiently bind human monocytes or neutrophils (Kurlander & Batker, 1982; Huizinga et al., 1989b; Voice & Lachmann, 1997). Therefore, the observation that the small soluble IgG2 and IgG4 anti-NIP complexes were able to trigger TNFα release from adhered human monocytes was unexpected. The relative levels of TNFα released in response to each subclass did, however, correlate with the Fcγ receptor binding affinities of both FcγRII (Salmon et al., 1992) and FcγRIII (van de Winkel & Capel, 1993) for human IgG.

4.2.2. Human IgG immune complex-induced TNFα release and the involvement of Fcγ receptors

Blocking antibodies to each FcγR were used to determine which receptors were involved in small IgG1 anti-NIP complex-induced TNFα release from adhered monocytes. Using blocking antibodies to FcγRI, FcγRII and FcγRIII, the TNFα response induced by these small, soluble IgG1 immune complexes was found to be dependent upon both FcγRII and FcγRIIIa, where FcγRIIIa appeared to play a dominant role. These immune complex studies therefore support the hypothesis that small IgG immune complexes may trigger the production of TNFα from macrophages in rheumatoid arthritis, and implicate an important role for FcγRIIIa in this inflammatory process.

4.2.3. Effect of inhibitors on human IgG immune complex and LPS induced TNFα release

As observed for both the LPS and anti-FcγRIII mAb-induced TNFα responses, the TNFα response induced by soluble small IgG1 anti-NIP immune complexes was abrogated by the presence of cycloheximide and significantly inhibited by actinomycin D. This confirmed the soluble IgG1 immune complex-induced TNFα response to be dependent upon both mRNA transcription and protein translation. Furthermore, the inhibitory effect of the MMP inhibitor, as for LPS and anti-FcγRIII mAb induced TNFα responses, again suggested the dependency upon gelatinase activity for TNFα processing. Interestingly, the presence of colchicine enhanced the small IgG1 immune complex-induced TNFα release from adhered monocytes. This effect was unlikely to be due to the
prevention of Fcγ receptor internalisation, which may have possibly resulted in prolonged signal transduction. Work by others have shown that Fcγ receptor internalisation occurs independently of microtubule function and instead occurs by receptor diffusion into the plasma membrane (Michl et al., 1983). Furthermore, colchicine was found to inhibit, rather than enhance, the anti-FcγRIII mAb induced TNFα response. However, colchicine has been shown by others to increase IL-1β production from LPS-stimulated monocytes (Allen et al., 1991) and the production of platelet derived growth factor B mRNA by macrophages (Wangoo et al., 1992).

4.2.4. Human IgG anti-NIP immune complexes

Although the above human IgG immune complex studies generated some encouraging data, there are a number of drawbacks in using these anti-NIP complexes. Owing to the nature of the antigen, there were uncertainties regarding complex size. The NIP/BSA antigen used to generate small complexes had an average NIP density of 2.4 molecules per BSA, forming a heteropopulation of monomers, dimers and trimers. Steric hindrance would possibly prevent the formation of tetramers. It was not possible to predict the precise proportions of monomers, dimers and trimers in each preparation. Routine HPLC analysis of the immune complexes prior to incubation with adhered monocytes was unfortunately not possible since the HPLC equipment used in these studies was based in a collaborative laboratory which could only supply restricted use. Furthermore, reagents were limited.

Another consideration to be made when interpreting the data is the stereochemistry of these anti-NIP complexes in relation to IgG rheumatoid factor complexes. The formation of an IgG anti-NIP dimer would be of a similar size to an IgG rheumatoid factor dimer. In addition, both types of dimers would have equal numbers of available FcγR binding sites. However, steric differences may affect the accessibility of these sites (Figure 29). Furthermore, it was impossible to control the position of the NIP molecules on the surface of the BSA. Therefore, the position of the two IgG anti-NIP antibodies in relation to each other could not been controlled (Figure 30).
Figure 29. Schematic diagram showing formation of a) human IgG anti-NIP dimer and b) human IgG rheumatoid factor dimer.
4.3. Human IgG rheumatoid factors

4.3.1. IgG rheumatoid factor mAb self-association

Lu et al. (1993) generated the two human IgG rheumatoid factor mAbs used in these studies and showed that both the IgG1 and the IgG2 rheumatoid factor mAb self-associated to form dimers following incubation at 37°C for 30 hours. The dissociation constant of the IgG1 rheumatoid factor mAb was found to be $5.7 \times 10^{-7} M$ and that of the IgG2 rheumatoid factor mAb was $1.6 \times 10^{-7} M$. When these experiments were repeated in this thesis, it was found that the self-association of both rheumatoid factor subclasses occurred spontaneously. The human IgG1 rheumatoid factor mAb formed a heteropopulation of monomers, trimers and aggregates, while the IgG2 rheumatoid factor mAb self-associated to form dimers and larger complexes. These immune complexes appeared stable both within the heteropopulations and following separation.
4.3.2. Human IgG rheumatoid factor-induced TNFα release

TNFα production from adhered human monocytes was only detected in response to the IgG1 rheumatoid factor mAb complexes, as expected. However, this result was only observed once, implying this response might be an artefact. Alternatively, the particular conditions during this experiment may have been optimal for immune complex formation and subsequent TNFα production. Although the HPLC data indicated that once formed these complexes appeared stable, as for the IgG anti-NIP complexes HPLC analysis prior to incubation with adhered monocytes was not feasible. Therefore, it was not possible to accurately determine the proportion of IgG rheumatoid factor dimers in each preparation. Moreover, the presence of fetal calf serum in cultures necessary for TNFα release from adhered human monocytes, may have influenced the stability of the IgG rheumatoid factor complexes.

These experiments were not pursued at this stage since there is a need for a better control over complex stability and size. Furthermore, there is the question of whether these rheumatoid factors are truly representative. Most rheumatoid factor mAb are of the class IgM since IgG rheumatoid factor mAb are difficult to generate and analyse in detail. This may be due to low levels of immunoglobulin secretion or the instability of EBV-transformed B cells (Lu et al., 1992). It is possible that the IgG rheumatoid factor mAb used in these studies, although generated from rheumatoid synovial cells, are not representative of "arthritogenic" rheumatoid factors.

4.4. TNFα and IL-1α production by adhered human monocytes

As already discussed, of the three anti-FcyR mAb only the anti-FcyRIIIa mAb induced TNFα and IL-1α production from adhered human monocytes. It was interesting to note that IL-1α time course differed from that for TNFα. The TNFα response peaked between 4 and 6 hours after stimulation, and subsequently declined with time. The IL-1α response was only detectable from 10 hours following stimulation, as observed by others (Hazuda et al., 1988), and then continued to rise with time. This lag observed for the IL-1α response suggests a possible dependency upon the TNFα response.
Dinarello et al. (1986) demonstrated the induction of IL-1 production by human mononuclear cells in response to human recombinant TNFα, as did Chantry et al. (1989) from human monocytes. However, both studies tested for IL-1, rather than the individual isoforms. Nevertheless, this was further supported by the observation that the spontaneous production of IL-1 by isolated rheumatoid synovial mononuclear cells in vitro was TNFα dependent (Brennan et al., 1989). In agreement with these findings, the anti-FcyRIII mAb-induced IL-1α production by adhered human monocytes was inhibited by the presence of a neutralising anti-TNФα mAb. However, recombinant human TNFα alone failed to induce IL-1α production from the adhered human monocytes. This may imply that TNFα was involved in the release of intracellular IL-1α that had already been synthesised following the ligation of FcyRIIIa, rather than the TNFα itself being the stimulus for IL-1α production.

In contrast to the anti-FcyRIII mAb, LPS-induced IL-1α production was not inhibited by the neutralising antibody to TNFα. LPS may itself trigger both IL-1α synthesis and release therefore avoiding the dependency upon TNFα. Alternatively, this may be explained by the downregulation of both TNF receptors, TNF-RI and TNF-RII, together with the release of soluble TNF-RII, previously observed with LPS-mediated activation of monocytes (Leeuwenberg et al., 1994). TNF receptor expression by human monocytes and macrophages following FcγR-mediated activation has yet to be investigated. However, the expression of TNF receptors in rheumatoid synovium have been studied using immunostaining techniques. It was found that both TNF-RI and TNF-RII receptor expression levels were increased by rheumatoid synovial cells in comparison with controls and rheumatoid peripheral blood mononuclear cells (Deleuran et al., 1992; Brennan et al., 1992b). Furthermore, some of these cells were secreting TNFα (Deleuran et al., 1992).

The findings presented in this dissertation suggest that unlike LPS stimulation, ligation of FcyRIIIa may favour the establishment of a pro-inflammatory cytokine loop initiated by TNFα, resulting in the release of other cytokines such as IL-1α.
4.5. Rheumatoid factor, Fc receptors and rheumatoid arthritis

The targeting of inflammation to synovium and other tissues in rheumatoid arthritis has previously been difficult to explain. Although circulating IgG rheumatoid factor-based immune complexes have long been suspected to be involved in the disease process, the effector mechanism has been unclear. The findings presented in this dissertation indicate that macrophage FcγRIIIa is the most likely Fcγ receptor to trigger TNFα, IL-1α, and reactive oxygen species production following ligation by a small IgG-based immune complex. Unlike FcγRI and FcγRII, FcγRIIIa expression by macrophages is tissue and site specific. A close correlation between macrophage FcγRIIIa expression and the location of both synovitis and extra-articular features has been demonstrated (Bhatia et al., 1998). The small (intermediate) IgG rheumatoid factor-containing immune complexes first described by Kunkel et al. (1961) may therefore be the pro-inflammatory stimulus for macrophage activation preceding T cell infiltration in the rheumatoid synovium. Once established, this inflammatory process may be subsequently amplified by the local generation of large complement-fixing complexes based on rheumatoid factors of all isotypes (Winchester et al., 1970; Mannik & Nardella, 1995). Furthermore, the formation of large IgG rheumatoid factor complexes within the synovium may activate synovial macrophages through any of the three Fcγ receptors.

It may also be possible for IgA rheumatoid factor complexes to activate macrophages in rheumatoid arthritis through FcαR. This is supported by in vitro studies showing FcαR aggregation by IgA to induce TNFα, IL-1β and IL-6 release from human monocytes (Polat et al., 1993; Patry et al., 1995). Furthermore, FcαR expression by human monocytes can be upregulated by TNFα and IL-1β (Shen et al., 1994). However, although little is known about the tissue distribution of FcαR, an immunohistochemical study of tissues from both normal individuals and patients with rheumatoid arthritis has shown that FcαR is only expressed by gut macrophages (S Blades, personal communication). This suggests that IgA rheumatoid factor-based complexes may not activate macrophages through FcαR in rheumatoid arthritis.
In conclusion, the findings presented in this thesis, together with previous evidence (Edwards & Cambridge, 1998; Edwards et al., 1999) support a specific role for macrophage FcγRIIIa in the induction of inflammation induced by small circulating IgG rheumatoid factor immune complexes in rheumatoid arthritis.

4.6. B cell survival and IgG rheumatoid factor production in rheumatoid arthritis

To complete the above model for the initiation and persistence of inflammation in rheumatoid arthritis, the continuous production of IgG rheumatoid factor requires explanation. The hypothesis has recently been extended to predict that IgG rheumatoid factors have the ability to perpetuate their own existence (Edwards et al., 1999). Moreover, the hypothesis suggests that pathogenic IgG rheumatoid factors do not arise from natural IgM rheumatoid factors as a result of a failure in the constraints that normally prevent these physiological autoantibodies from undergoing affinity maturation and class switching (Børretzen et al., 1994). Instead, the suggestion is that IgG rheumatoid factors with the potential for pathogenicity and natural IgM rheumatoid factors are derived from distinct origins.

B cell survival within the follicle centre requires positive survival signals in the form of T cell help for a source of cytokines and antigen complexed with the complement component, C3d (Lindhout et al., 1997; Fearon et al., 1995). A B lymphocyte recognising self should be deleted owing to a lack of T cell help (Bikoff, 1983). Autoreactive B cells might survive owing to the failure of T cell tolerance to self antigen. However, in the case of rheumatoid factor producing B cells, T cell help may be obtained without a failure in tolerance. As shown by Roosnek & Lanzavecchia (1991), antigen-antibody complexes containing foreign antigen can be efficiently taken up by rheumatoid factor B cells. The foreign antigen is then processed and presented, complexed with class II MHC, to T cells.

Normal individuals make physiological rheumatoid factors as part of a normal immune response. These autoantibodies are mostly low affinity IgM and are likely to be produced by B-1a cells (Casali & Notkins, 1989b).
B-1a cells produce "natural" autoantibodies which are often prevented from affinity maturation and class switching by restrictive mechanisms, possibly a result of the constitutive expression of the nuclear protein STAT-3 (Karras et al., 1997). In contrast, rheumatoid factors from patients with rheumatoid arthritis display high levels of somatic hypermutation involving amino acid substitutions (Thompson et al., 1995a). There is also evidence suggesting that rheumatoid factors from normal individuals and patients with rheumatoid arthritis are derived from distinct germ line gene families (Thompson et al., 1994). Potentially pathogenic IgG rheumatoid factors may therefore be derived from conventional B-2 cells of bone marrow origin and may acquire rheumatoid factor activity by a chance mutation event during an immune response to an unrelated foreign antigen (Pulendran et al., 1997). IgM antibodies against an irrelevant foreign antigen may therefore enter affinity maturation and class switching, during which a chance mutation may give rise to a high affinity IgG rheumatoid factor.

These IgG rheumatoid factors may have the ability to perpetuate their own production owing to their self-association properties (Pope et al., 1974). For a B cell to survive, it must receive two positive signals. The first positive signal is from helper T cell derived cytokines (Lindhout et al., 1997). The second is in the form of complexed antigen in association with C3d which provides a B cell with a survival signal by interacting with surface immunoglobulin and CR2 (Cooper et al., 1990). Soluble antigen provides a negative survival signal to B cells within the germinal centre (Pulendran et al., 1995) and therefore an autoreactive B cell should be deleted.

B cell survival is also regulated by the inhibitory receptor, FcγRIIb1. Co-ligation of FcγRIIb1 and B cell receptor (BCR) by complexed IgG provides a negative signal to the B cell, resulting in a downregulation of antibody production (Gergely & Sarmay, 1996). In contrast, the crosslinking of FcγRIIb2 results in receptor internalisation, delivering complexed IgG to lysosomes for antigen processing and presentation (Sandilands et al., 1997). Since FcγRIIb1 can only move within the plasma membrane and not mediate internalisation, FcγRIIb1 crosslinking may also inhibit antigen processing and presentation to T cells (Minskoff et al., 1998).
Normally a self reactive B cell may not recognise complexed antigen since the epitope that surface immunoglobulin (BCR) recognises is shared by free antibody. However, in the case of IgG rheumatoid factors, the antigen is the antibody. Therefore, while one epitope may be bound, the "antigen" can still present another to surface immunoglobulin. Furthermore, surface IgG rheumatoid factor may compete with FcyRIIb for IgG rheumatoid factor complexes. Thus positive signals may dominate and IgG rheumatoid factor producing B cells will survive and proliferate. Involvement of specific T cell reactivities is still possible, however, IgG rheumatoid factor production may be a self perpetuating process arising against the background of a normal T cell repertoire.

4.7. A new treatment for rheumatoid arthritis

The hypothesis presented in this thesis provides the possibility of a new therapeutic strategy for rheumatoid arthritis. The pathogenic mechanism implicates IgG rheumatoid factors as the primary inflammatory trigger. Therefore, removal of these antibodies would prevent further disease progression. The obvious way to achieve this would be by destroying the rheumatoid factor-producing B cells. To specifically kill rheumatoid factor-producing B cells would be highly complicated. However, total B cell depletion would also result in the desired effect. Recently, a chimeric mouse human IgG1 anti-CD20 monoclonal antibody has been generated (Reff et al., 1994). CD20 is a surface antigen expressed by all B cells from the stage of cytoplasmic μ heavy chain expression until plasma cell formation. It is not expressed by stem cells or early B cell precursors. CD20 is involved in the activation of cell cycle initiation and differentiation and ligation with the anti-CD20 monoclonal antibody results in B cell death. In recent trials, following a single infusion of the anti-CD20 monoclonal antibody, B cells were depleted from the peripheral blood, the bone marrow and the lymph nodes with no significant side effects. Over a period of time the B cell population then recovered to baseline levels, while immunoglobulin levels were maintained (Reff et al., 1994; Maloney et al., 1994).

Such a therapy would allow the repopulation of B lymphocytes, without the reappearance of the pathogenic rheumatoid factor producing B cells. Indeed such a trial is currently ongoing with positive results. Patients
with rheumatoid arthritis, following anti-CD20 treatment, have shown a
dramatic and prolonged clinical improvement (Edwards, in preparation).
This supports the hypothesis that rheumatoid arthritis is an antibody-
driven disease.

4.8. Future work
A number of questions regarding the structure and signalling properties
of Fcγ receptors have been generated from the work presented in this
thesis. Following the crystal structure studies of FcγRII, it would be of
interest to generate similar data for FcγRIII. This may help to elucidate
the relevance of IgG Fc valency for FcγRIIIa signalling. Alternatively, the
generation of an anti-FcγRIII mAb consisting of an Fc fragment and a
single Fab region may clarify whether the crosslinking of two or three
FcγRIIIa receptors are required for the signalling of TNFα production.

Recent data has shown that following crosslinking, FcεRI (Stauffer &
Meyer, 1997) and FcαR (Lang et al., in press) re-distribute to membrane
glycosphingolipid-cholesterol rafts, which are rich in signalling
molecules such as PTK. A collaboration is currently being organised with
Dr W. Wade (Department of Immunology & Microbiology, Dartmouth
College, Lebanon, NH) to determine whether Fcγ receptors, in particular
FcγRIIIa, also enter rafts following crosslinking with murine anti-FcγR
mAb.

LPS and FcγRIIIa appear to mediate IL-1α release from adhered
monocytes through distinct pathways. FcγRIIIa-mediated IL-1α release
was found to be TNFα-dependent, while LPS-triggered IL-1α release
occurred independently of TNFα. It would therefore seem appropriate to
stimulate adhered monocytes with both the anti-FcγRIII mAb and LPS in
the presence of the anti-TNFα mAb to see if IL-1α release could be
restored. Furthermore, it may be possible to establish whether LPS
activates intracellular signalling molecules distinct from those activated
following FcγRIIIa ligation, such as specific mitogen-activated protein
kinases or phospholipases. If such a molecule could be identified it may
then be possible to inhibit its activation and observe the effects of this on
LPS-mediated IL-1α release in the presence of the anti-TNFα mAb.
Using an *in vitro* system, the work presented in this thesis supports a role for IgG rheumatoid factor complexes as a trigger of macrophage activation in rheumatoid arthritis. Human *in vivo* trials have demonstrated B cell depletion as a positive treatment for rheumatoid arthritis. The ultimate test would therefore be to demonstrate the presence and absence of circulating IgG rheumatoid factor complexes in patients with rheumatoid arthritis before and after anti-CD20 therapy. For this a highly sensitive and accurate method for the detection of IgG rheumatoid factor-based complexes is required. A novel approach to this problem is currently under investigation.
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APPENDICES
Appendix 1.

Relevant publications


Do self-perpetuating B lymphocytes drive human autoimmune disease?

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SUMMARY
Normal immunological memory is thought to be underpinned by T lymphocytes. However, in rheumatoid arthritis there are indications that T-lymphocyte control has been subverted by self-perpetuating B lymphocytes. Potential mechanisms in other autoimmune states are less clear, but a number of observations suggest that misappropriation of immunological memory by B lymphocytes may be a common feature of human autoantibody-associated disease. Put simply, autoantibodies drive their own production. If so, the availability of safe B-lymphocyte-depleting agents provides a potential means for reversal of autoimmunity.

HOW DO AUTOACTIVE B LYMPHOCYTES SURVIVE?
Whatever its significance, the presence of autoantibodies remains the one piece of hard evidence about human autoimmunity which has to be built into a pathogenic mechanism. Current dogma states that a B lymphocyte can only avoid early death if it obtains two positive survival signals; cytokines from helper T lymphocytes and, in the follicle centre, antigen, complexed to the complement fragment C3d.1,2 In theory, autoreactive B lymphocytes cannot survive because of T-lymphocyte anergy to self and because any positive survival signal obtained from complexed self antigen is outweighed by a negative signal from uncomplexed antigen. Yet B lymphocytes recognizing one or more of about 50 self proteins do survive in some individuals, and may be associated with disease.3

The prevailing view is that autoreactive B-cell survival is secondary to a failure in the maintenance of T-lymphocyte self-tolerance, induced by an external 'trigger' immunogen. This view derives from early ideas about cross-reactivity between bacterial and tissue-specific antigens in rheumatic fever.4 However, this may be a very misleading model for spontaneous autoimmunity.

Enthusiasm for loss of T-lymphocyte tolerance in autoimmunity was bolstered by animal models transferrable by T lymphocytes, such as collagen II arthritis.5 However, there are two problems with these models. The pattern of disease reflects the antigen used and, in virtually every case, fits poorly with the putative human equivalent. Collagen II arthritis affects cartilage structures, including growth plate. Rheumatoid arthritis targets synovium, and also structures such as pericardium and alveoli.6 Cartilage is only affected if adjacent to synovium.

The second problem is that most T-lymphocyte transferrable animal models require immunization with a massive antigen and/or adjuvant load. In human autoantibody-associated diseases, unlike rheumatic fever or post-dysenteric arthritis,7 there is rarely any evidence for recent exposure to a particular foreign antigen. Rheumatoid arthritis occurs at random during adult life, with little or no geographic or temporal clustering. In the absence of evidence of recent antigen exposure, it is difficult to see why a failure of T-lymphocyte tolerance should occur in mid life. What seems more likely is that the random onset of disease reflects a random process in the immune system; we suggest that immunoglobulin gene mutation is the obvious candidate.

AUTOANTIBOIDS MAY DRIVE THEIR OWN PRODUCTION
As noted by Pulendran et al.,8 autoantibodies may arise by random mutation during immune responses to any antigen. The autoantigens subsequently recognized need not resemble the original antigen. A single amino acid substitution may cause dramatic changes in the conformation of the antigen-binding site. In most cases dramatic changes in antibody specificity will lead to loss of affinity for foreign antigen and death of the B lymphocyte. However, if the new antigen-binding site interacts with a self antigen in such a way as to generate positive survival signals, the B lymphocyte may survive and proliferate (Fig. 1).

Many of the autoantigens associated with rheumatic disease are proteins implicated in survival, differentiation and function of immune cells; notably immunoglobulin G (IgG) Fc, C1q, and nucleoproteins such as topoisoamerase-I and the leucocyte
Autoimmunity and self-perpetuating B lymphocytes

A generalized mechanism for autoantibody-associated disease.

Figure 1. A generalized mechanism for autoantibody-associated disease.

differentiation-related oncogene product deck. Antibodies to these antigens may have a particular opportunity to upset regulatory mechanisms. If an antibody, by modifying the normal function of one of these proteins, stimulated or mimicked T-lymphocyte cytokine activity, whether directly or indirectly, and shifted the balance of unbound and complexed antigen, then its parent clone could become self-perpetuating (Fig. 1). The best characterized example of such a mechanism is in rheumatoid arthritis. Subsets of rheumatoid factors may keep their parent B-lymphocyte clones alive by disturbing the control of both T-lymphocyte help and survival signals given by immune complexes in the follicle centre. If rheumatoid factors can, perhaps other autoantibodies can.

RHEUMATOID FACTORS AND THEIR RELATIONSHIP TO DISEASE

Approximately 80% of subjects with rheumatoid arthritis (RA) develop circulating antibodies to IgG Fc, or rheumatoid factors (RF). Seronegative cases occur, but in many, RF are present in joints. Seronegative cases probably also comprise conditions which can be clinically indistinguishable from RA, such as spondarthropathies. RF are present in the circulation of a few normal individuals and can also be induced by immunization to foreign antigen or by chronic infection. This dictates that if RF are pathogenic in RA, pathogenic potential must be restricted to a subset of RF.

There are recognized differences between RF from normal and RA subjects. ‘Physiological’ RF from normal subjects are chiefly of IgM class and low affinity. RF in RA are of all classes, and are structurally and genetically distinct, as shown by analysis of immunoglobulin V_H regions. RF from normal individuals tend to come from a common germ line. They show evidence of immunoglobulin gene mutation, but mutations tend to be silent. In contrast, RF from RA subjects derive from a wide range of immunoglobulin germ line genes and show a high frequency of substitution mutations, indicating affinity maturation.

It is possible that physiological RF come from the B-1 subset of B lymphocytes, associated with ‘natural’ autoantibodies and that pathogenic RF arise from the B-2 subset. B-1 cells show constitutive expression of the nuclear protein STAT3 and distinct regulatory mechanisms in B-1 cells may relate to the blocks to affinity maturation and class switching of physiological RF. However, B-1-derived antibodies can show both these features and the means by which subtypes of RF may persist, discussed later, do not necessarily require either a B-1 or B-2 origin.

Class switching, high affinity and fine specificity may all be important in RF pathogenicity. A critical test of this concept comes from hypergammaglobulinaemic purpura of Waldenström (HPW) in which high levels of polyclonal RF occur in the absence of a recognizable stimulus, as in RA, but without arthritis. This raises the issue of RF as inflammatory mediators.

RF AND INFLAMMATION

The pathogenic significance of RF has been doubted because of the difficulty in finding a RF-based effector mechanism which explains the pathology of RA. However, new information on the immunological microenvironment in synovium has led to the identification of a plausible effector pathway. IgG RF exist in the circulation of rheumatoid subjects as
oligomers and predominantly dimers.¹⁹ These dimers fix complement poorly and, consequently, can escape clearance by red cell complement receptors (CR1). Unlike IgM-based complexes, IgG dimers are small enough to pass out of the circulation and access tissue macrophages. Histological studies indicate that macrophage activation is the initial event in RA synovium, preceding T-lymphocyte accumulation.²⁰

Recent studies indicate that high-level expression of the IgG Fc receptor FcγRIIa is restricted to macrophages in tissues affected by RA.²¹ Of the three classes of FcγR, FcγRII is believed to be particularly important for the binding of small, and specifically dimeric, complexes and the consequent generation of mediators such as tumour necrosis factor-α (TNF-α).²² FcγRI expression is up-regulated by interferon-γ and may be chiefly involved in a mature inflammatory response. Its high affinity allows it to bind free, locally synthesized antibody to a specific pathogen and interact with the pathogen subsequently, via multiple receptors. FcγRII is of low affinity and is implicated in binding large complexes, again via multiple receptors.²³

A specific role for FcγRIIa in response to small complexes is supported by the fact that a monoclonal antibody to FcγRIIa in free soluble form is capable of inducing release of both TNF-α and reactive oxygen species, whereas monoclonal antibodies to FcγRI and FcγRII do not induce TNF-α release under the same conditions.²⁴ TNF-α release has only been achieved via FcγRI and FcγRII by secondary cross-linking following ligation of the receptors by IgG Fc.²⁵ This suggests that FcγRI and FcγRII may require multiple cross-linking in order to induce signalling but that FcγRIIa may induce signalling in response to a soluble ligand capable of engaging two, or at most three (two Fab- and one Fc-based interactions), receptors. This would be closely analogous to the situation for an IgG RF dimer with which additional non-RF IgG molecules may be in dynamic association.

On this basis it can be argued that RA is precisely the inflammatory state that IgG RF dimers should be expected to generate. There are, however, further issues about IgG subclass. Voice and Lachmann²⁶ recently highlighted the importance of subclass and complex size in interactions between complexed IgG and Fc and complement receptors. IgG1 and IgG3 RF dimers may interact differently with FcγRIIa. Small IgG3-based complexes induce FcγRIIa-dependent TNF-α production only slightly less well than equivalent IgG1-based complexes in our hands (Abrahams, in preparation). However, self-association may raise specific steric considerations. One possibility relates to the long hinge of IgG3 (Fig. 2). Modelling studies predict that the Fc receptor binding sites on an IgG1 dimer would be approximately 75 Å apart, whereas for IgG3 dimers the distance would be approximately 200 Å with the hinge extended.²⁷,²⁸ There is debate about whether the cysteine-rich segment of the IgG3 hinge is normally extended or compressed. However, steric interference in a self-associated dimer may reduce the likelihood of a compressed configuration. A distance of 75 Å would allow direct apposition of the FcγRIIa y-chains responsible for signalling, but 200 Å would not (Fig. 3). This provides one possible explanation for the absence of synovitis in subjects with HPW. 'Intermediate' complexes, consisting of self-associated RF, exist in HPW sera, but in patients with no arthritis they were found to be restricted to IgG3.²⁹ This would be consistent with an inability of IgG3 RF dimers to induce signalling via FcγRIIa.

The above discussion only relates to the initiation of inflammation in synovium by circulating complexes. The dominant involvement of synovium in RA is likely to involve a series of secondary events. Synovial fibroblasts are unusually responsive to cytokines such as TNF-α in terms of the induction of expression of molecules involved in B-lymphocyte survival: vascular cell adhesion molecule-1 (VCAM-1), decay accelerating factor and complement receptor 2.³⁰ Induction of the expression of these molecules on synovial subintimal fibroblasts by cytokines released from FcγRIIa macrophages would explain the survival of B lymphocytes in RA synovium, with follicle formation in some cases. A proportion of these B lymphocytes are known to generate RF.³¹ High concentrations of IgG RFs within the confines of the joint are associated with the formation of larger, complement-fixing complexes, which, together with IgM RF-based complexes, are likely to amplify the inflammatory process.²⁹

**RF AND T-LYMPHOCYTE HELP**

In order to obtain T-lymphocyte help, B lymphocytes normally take up antigen bound to surface immunoglobulin and present it to a T lymphocyte. It is recognized that B lymphocytes that carry surface antibody to IgG Fc, i.e. RF, can obtain help without requiring T lymphocytes responsive to IgG.³² By taking up IgG attached to a non-self antigen they can present that antigen to a responsive T lymphocyte (Fig. 4).³³ RF-producing B cells survive for short periods in everyone, if foreign antigen is available, as after immunization (Fig. 5a). However, the RF produced is mostly IgM. Moreover, there is little evidence of immunoglobulin gene mutations leading to amino acid substitution.³⁴ There seems to be a block to class switching and affinity maturation for RF in normal individuals, suggesting that the acquisition of T-cell help is not enough to allow RF-secreting B lymphocytes to survive long-term. This suggests that there is a protective mechanism operating in the follicle centre.

**SURVIVAL IN THE FOLLICLE CENTRE**

The survival of B lymphocytes in follicle centres depends on the balance between positive and negative signals provided by antigen in different forms (Fig. 6). B-lymphocyte clones recognizing soluble autoantigens should not survive because they should receive a negative survival signal from uncomplexed antigen. However, antigen available in the form of an immune complex can give a positive survival signal. This signal is modulated by the attachment of C3d molecules to the antigen following complement fixation.² The combined interactions between C3d and its receptor, CR2, and antigen with surface immunoglobulin lead to a positive signal which is amplified tenfold for each C3d molecule attached to the complex. The situation is further modulated by FcγRIIb which binds large immune complexes and provides a negative signal.³⁰

Although IgG RF exist in the circulation in rheumatoid subjects largely as non-complement-fixing dimers, this probably reflects both their relatively low concentration in serum and the preferential survival of non-complement-fixing complexes. At sites of IgG RF synthesis, as in rheumatoid syn-
Figure 2. Comparison of IgG1 and IgG3 RF dimers showing the difference in hinge length.

Figure 3. A suggested basis for how the long hinge of IgG3 may inhibit IgG3 RF dimers from bringing the signalling γ-chains of FcγRIIa into apposition.

Ovarium, higher concentrations of IgG RF lead to the formation of complement-fixing multimers, with the potential to provide RF-specific B lymphocytes with a positive survival signal (Fig. 5b).

In theory, any B-cell clone recognizing a soluble autotigen could keep itself alive by generating sufficient antibody to provide a supply of complexed antigen. However, it appears that the regulation of positive and negative signals is such that the negative signal from soluble antigen normally dominates. Two aspects of this regulation may fail in the case of
RF-specific B cell

Figure 4. The basis of ‘bystander’ T-cell help to RF-specific B lymphocytes.

RF-secreting clones. An isolated autoreactive B-lymphocyte clone will normally have to compete for the same epitope on an autoantigen with its own secreted immunoglobulin. Thus it may be unable to bind complexed antigen through surface immunoglobulin except if other clones reactive with other epitopes on the same autoantigen are present. This does apply to RF-secreting clones (Fig. 5b) because an IgG RF molecule that masks an antigenic epitope on IgG provides the same epitope on itself. Secondly, surface RF may compete with FcγRIIb for large complexes, reducing the negative signal from this receptor.32 It would appear that, when it comes to preventing survival of IgG RF-secreting B lymphocytes, there are weaknesses in regulatory mechanisms in the follicle centre as well as in the context of T-lymphocyte help.

Multimeric IgG1- and IgG3-based complexes are both capable of fixing complement and RF of both subclasses are likely to enhance RF-specific B-lymphocyte survival. This may contrast with the relative capacity of their dimers to cross-link FcγRIIa and initiate synovitis and would explain why in HPW RF production is perpetual (multimer-driven), but arthritis (dimer-initiated) does not occur (Fig. 5c).

IgG3 also differs from other IgG subclasses in lacking the Gα epitope on Fc, a common recognition site for RF.33 Even within subclasses, pathogenic potential may depend on the detailed stereochemistry of the CDR antigen interaction. These considerations may help explain the variable anatomical and temporal patterns seen in different cases of the disease.

The prediction is that IgG RF capable of supporting survival of their parent B-lymphocyte clone would also assist survival of RF-specific clones secreting other isotypes. This would explain why in both RA and HPW large amounts of IgM RF are also produced, to the extent that they have tended to obscure the significance of IgG RF (Fig. 5).

B LYMPHOCYTES AND MEMORY IN OTHER AUTOIMMUNE DISEASES

The ways in which B-lymphocyte clones might become self-perpetuating in other autoimmune states cannot be traced as fully as in rheumatoid arthritis. However, several observations and proposed mechanisms which appear to fit such a framework will be briefly reviewed.

Davies and colleagues have suggested that the formation of antibodies to C1q is a crucial early step in the common sporadic form of systemic lupus.10 Anti-C1q antibodies can trigger complement consumption. In rare genetically determined cases complement components are primarily deficient. In either case, limited availability of complement components may both impair clearance of immune complexes and nuclear material normally cleared via complement and, by limiting generation of C3d, reduce the efficiency of B-lymphocyte clonal selection, allowing the genesis of autoantibodies. Anti-C1q antibodies have the potential to interfere with the rules of antibody-antigen interactions in a way similar to RF. An anti-C1q-specific B lymphocyte can endocytose any antigen complexed with C1q and present it to T cells. C1q-anti-C1q complexes could disturb regulation in the follicle centre in a similar way to RF polymers. The kinetics of self-perpetuation of anti-C1q-specific B lymphocytes would differ from RF-specific B lymphocytes because the clone would not generate both antigen and antibody locally, e.g. in a joint. C1q-anti-C1q complexes would probably also be too big to
cross vessel walls, suggesting that the synovitis of lupus may be due to immune complexes present as a result of mechanisms downstream of complement depletion.

In myasthenia gravis B lymphocytes generating antibodies to nicotinic acetylcholine receptors (AChR) survive in the thymus. The interesting question may be not why B lymphocytes of this specificity arise, but why, once generated, they persist. In what way does their surface or secreted immunoglobulin make them able to survive within the thymic environment? AChR are present on myoid cells which are found in close contact with professional antigen-presenting cells in myasthenic, but not normal, thymus. Could anti-AChR modulate myoid cell function in such a way that the thymic environment becomes favourable to B-lymphocyte survival?
B cell committed to production of IgG1 to foreign antigen

Further random Ig gene mutation

T cell help via surface RF binding complexed foreign antigens

Any other B cell committed to lower affinity

Affinity maturation and persistence of any RF-specific B cell supported by positive signal from complexes (class switching to IgG1 still ineffective)

Formation of large IgG3 RF complexes at sites of local synthesis

No small immune complex mediated inflammation

IgG3 RF plus IgM RF of heterogeneous origins

If extreme: hyperglobulinaemic purpura/vasculopathy

Figure 5c. RF in HPW

Figure 6. Relative potency of survival signals for autoantigen-specific and RF-specific B cells. Cross-hatching of elements indicates reduced availability because of competition by other ligands.

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There have to be answers to these questions; answers likely to make additional reasons for loss of T-lymphocyte tolerance to AChR redundant.

In multiple sclerosis T-cell responses to myelin antigens are often invoked. However, in contrast to experimental allergic encephalomyelitis, the pathological domain is not that of myelin, but of the blood-brain barrier. The only established immunological abnormality is the persistent survival of a few B-lymphocyte clones in the central nervous system (CNS). The key question is how these clones live within the CNS, generating the same oligoclonal immunoglobulin bands over many years. CNS damage may simply reflect intolerance of high levels of immunoglobulin, which induce macrophage phagocytosis of myelin in vitro. Recent immunohistochemical studies indicate that initial activation of microglia occurs in the absence of T lymphocytes. B-lymphocyte survival in the CNS should be precluded by the absence of stromal cell cation is that autoimmunity would collapse and lymphoid perpetuating autoreactive cells could be destroyed the implications are enormous. It is now possible to ablate B self-perpetuating B lymphocytes with an anti-CD20 antibody. If enough self-perpetuating autoreactive cells could be destroyed the implication is that autoimmunity would collapse and lymphoid tissue be repopulated by B lymphocytes innocent of any autoimmune tendency. Anti-CD20 therapy is licensed for lymphoma and, apart from transient symptoms due to cytolyis, is well tolerated. Up to 95% of B lymphocytes can be cleared with repopulation in 100 days. This is in stark contrast to anti-T-lymphocyte therapy, for which the justification is unclear and which, even if successful, carries a risk of long-term immunodeficiency. Anti-T-lymphocyte strategies have been disappointing. Interestingly, pan-lymphocyte depletion in RA with anti-CD32 produced benefit limited approximately to the period of B lymphopenia suggesting that autoreactive B-lymphocyte depletion did not reach the putative threshold. Anecdotal reports suggest that RA is about as amenable to long-term remission as lymphoma. Significant long-term remission rates in lymphoma probably require maximal doses of depleting antibody (2–3 g is recommended for anti-CD20) combined with conventional cytotoxic agents. Specific anti-B-cell therapy of this type has not been explored. It might just produce long-term cure.

A DIGITAL ANALOGY

The case has been made for autoimmune disease being driven, not by a primary failure of T-lymphocyte tolerance but by antibody. As for the genome or computer software, the true danger to the immune system may be a chance mutation in an information string which converts 'data' to 'command'. As a transcribed sequence of DNA may become a lethal stop codon and a misread floppy disk may lead to a system crash, if an antibody becomes a B-lymphocyte growth promoter it may be very difficult for the immune system to re-establish control.

THERAPEUTIC IMPLICATIONS

If self-perpetuating B lymphocytes exist, their therapeutic implications are enormous. It is now possible to ablate B lymphocytes with an anti-CD20 antibody. If enough self-perpetuating autoreactive cells could be destroyed the implication is that autoimmunity would collapse and lymphoid tissue be repopulated by B lymphocytes innocent of any autoimmune tendency. Anti-CD20 therapy is licensed for lymphoma and, apart from transient symptoms due to cytolyis, is well tolerated. Up to 95% of B lymphocytes can be cleared with repopulation in 100 days. This is in stark contrast to anti-T-lymphocyte therapy, for which the justification is unclear and which, even if successful, carries a risk of long-term immunodeficiency. Anti-T-lymphocyte strategies have been disappointing. Interestingly, pan-lymphocyte depletion in RA with anti-CD32 produced benefit limited approximately to the period of B lymphopenia suggesting that autoreactive B-lymphocyte depletion did not reach the putative threshold. Anecdotal reports suggest that RA is about as amenable to long-term remission as lymphoma. Significant long-term remission rates in lymphoma probably require maximal doses of depleting antibody (2–3 g is recommended for anti-CD20) combined with conventional cytotoxic agents. Specific anti-B-cell therapy of this type has not been explored. It might just produce long-term cure.

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INDUCTION OF TNF-α PRODUCTION BY ADHERED HUMAN MONOCYTES: A KEY ROLE FOR FcγRIIIa IN RHEUMATOID ARTHRITIS

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Relevance to rheumatologists

Monoclonal antibodies to immunoglobulin receptors (FcγR) on human macrophages were used to probe the mechanisms by which IgG rheumatoid factor-based immune complexes might induce cytokine production. Evidence was obtained for specific involvement of the receptor FcγRIIIa in the production of the cytokines tumour necrosis factor alpha and interleukin 1 alpha, supporting previous evidence for a key role for this receptor in the pathogenesis of rheumatoid arthritis.
control. After washing twice with buffer the cells were incubated with fluorescein isothiocyanate-(FITC) conjugated goat F(ab)\(_2\) anti-mouse Ig (DAKO, Cambridge, UK) at 4°C for 45 minutes. Following two washes with buffer the cells were fixed with 2% paraformaldehyde in PBS and analysed by flow cytometry using a FACScan (Becton Dickenson) with Win MDI software (Microsoft).

**Culture and stimulation of monocytes.** Freshly isolated monocytes (5 x 10\(^5\)/ml) were seeded into 96 well plastic tissue culture plates (Becton Dickenson) and incubated at 37°C for 24 hours. The culture supernatants were removed and replaced with fresh growth medium to ensure that cytokine levels were at baseline. The adherent human monocytes were then incubated with the anti-FcγR mAb. Medium alone and isotype-matched controls were used as negative controls. Lipopolysaccharide (LPS) from *Escherichia coli* 026:B6 (Sigma; in the absence of polymixin B), and phorbol myristate acetate (PMA; Sigma) were both used as positive controls. Possible LPS contamination of reagents was excluded by performing all incubations in the presence of polymixin B (10 µg/ml; Gibco BRL), which consistently abrogated the LPS-induced TNFα response. Culture supernatants were then collected at various time points and the cell-free samples stored at -70°C.

**TNFα ELISA.** Cell-free culture supernatants were loaded onto 96 well maxisorb plates (Gibco BRL) previously coated with murine IgG\(_1\) anti-human TNFα mAb and blocked with 0.02M Bes (pH 7; Sigma)/1% BSA. Human recombinant TNFα (National Institute for Biological Standards Control, Hertfordshire, UK) standards were used to calibrate the assay. The plate was incubated for 1 hour at room temperature with continuous agitation. Following aspiration of the wells, the captured TNFα was detected by incubating with a sheep anti-human TNFα polyclonal antibody. Following aspiration the captured TNFα was then detected with horseradish peroxidase (HRP) labelled anti-sheep IgG (H & L). The plate was incubated for 30 minutes at room temperature with
F(ab)2 and Fab fragment binding to FcγRIIIa was tested by flow cytometry and saturating binding concentrations were 20 µg/ml and 10 µg/ml respectively.

**Isolation of monocytes from whole blood.** Citrated venous blood was collected and buffy coat prepared following sedimentation with 6% Dextran. This was layered onto Histopaque (density 1.077 g/ml; Sigma) and centrifuged at 400g for 30 minutes. The mononuclear cell-containing layer was removed, washed six times with RPMI-1640 medium (Gibco BRL, Paisley, UK) and the mononuclear cells resuspended in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum (Sigma). The cell suspension was transferred into plastic petri dishes (Bibby Sterilin Ltd, Staffordshire, UK) and incubated at 37°C/5% CO2 for 2 hours. Nonadherent cells were removed by washing six times with Hank's balanced salt solution (Gibco BRL). Adhered monocytes were gently harvested using a rubber policeman and resuspended in growth medium (RPMI-1640 supplemented with 25 mM Hepes, 20 µg/ml gentamycin, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 2% non-essential amino acids, 1 mM sodium pyruvate (all from Gibco BRL) and 10% fetal calf serum) and the cell concentration adjusted to 5 x 10^5/ml.

Monocyte purity was determined by flow cytometric analysis of CD14 and CD3 expression and shown to be greater than 95%. Trypan blue (Sigma) exclusion showed monocyte viability to be greater than 98%.

**Expression of FcγR by cultured adhered human monocytes.** Freshly isolated monocytes (1 x 10^6/ml) were seeded into 24 well plastic tissue culture plates (Becton Dickenson, Oxford, UK) and incubated at 37°C for 0, 24 or 48 hours. The cultured monocytes were then harvested by cooling on ice and gentle pipetting. FcγR expression was measured by indirect immunofluorescence flow cytometry. Briefly, the harvested cell suspension was washed twice with ice cold buffer (PBS, 1% bovine serum albumin (BSA), 0.1% sodium azide) and then incubated at 4°C for 45 minutes with an anti-FcγR mAb or an isotype-matched.
MATERIALS AND METHODS

Antibodies. The anti-FcγRIII murine mAb-producing cell line, 3G8 (IgG1) and purified murine anti-FcγRII mAb, IV.3 (IgG2b) were generous gifts from Dr M. Fanger (Dartmouth, Hanover, NH). The 3G8-producing cell line was used to generate purified anti-FcγRIII mAb from which F(ab)2 and Fab fragments were prepared. For all experiments, purified anti-FcγRIII mAb (3G8), purchased from Immunotech (Marseille, France) was used. The anti-FcγRI mAb, 10.1 (IgG1) was kindly donated by Dr N. Hogg (ICRF, London, UK). Saturating binding concentrations of the anti-FcγRI, anti-FcγRII mAb (both 10 µg/ml) and anti-FcγRIII mAb (20 µg/ml) were pre-determined by flow cytometry. Murine IgG1 and IgG2b were purchased from Southern Biotechnology Associates, Inc (Birmingham, Al). These antibodies were dialysed before use to remove azide. Horseradish peroxidase conjugated donkey anti-sheep Ig (H & L) was purchased from Jackson Immunoresearch Laboratories, Inc (West Grove, PA). A murine anti-human TNFα mAb (CB006, IgG1) and a sheep anti-human TNFα polyclonal antibody were kindly provided by Celltech Therapeutics (Berkshire, UK).

Preparation of 3G8 F(ab)_2 and Fab fragments. Anti-FcγRIII mAb (3G8) was purified from culture supernatant by affinity chromatography. Briefly, the supernatant was adjusted to pH 8 and loaded onto a 2 ml Protein G-Sepharose 4B column (Sigma, Poole, UK) previously equilibrated with phosphate buffered saline (PBS, pH 8) at 4°C. Following washing with PBS, the mAb was eluted with 3M magnesium chloride. The purified mAb was then dialysed into PBS and concentrated.

F(ab)_2 fragments of the purified anti-FcγRIII mAb were prepared by pepsin cleavage and Fab fragments were prepared by papain digestion (Pierce, Rockford, IL). The digests were purified using protein G-Sepharose 4B. The F(ab)_2/Fab fragments were collected as flow through, dialysed into PBS and concentrated. Purity of the F(ab)_2 and Fab digests was confirmed by SDS-page. Anti-FcγRIII
There are three classes of receptor for IgG (FcγR) (13), all of which can mediate a number of effector functions including phagocytosis, antibody dependent cytotoxicity and the release of inflammatory mediators (14,15). Monocytes and macrophages constitutively express FcγRII (CD32), which is functionally associated with large immune complexes. FcγRI (CD64), also functionally associated with large complexes, is only expressed at low levels, being inducible by interferon-γ (15). Macrophage FcγRIIIa (CD16) has a restricted tissue distribution (16), being expressed at high level only in synovial intimal and other tissues, such as pericardium, involved in rheumatoid arthritis. Furthermore, FcγRIII has been shown to preferentially bind small immune complexes (17).

These considerations led to the hypothesis that FcγRIIIa has a distinct functional role in the generation of inflammation by small immune complexes, in the context of rheumatoid arthritis (12,18). It has been demonstrated that ligation of FcγRI and FcγRII can result in TNFα production by human monocytes (19). However, TNFα production through FcγRI was only observed in response to murine IgG2a in solid phase, while FcγRII only evoked a response to murine IgG1 in solid phase following treatment with proteolytic enzymes. Human FcγRIIIa has also been shown to mediate TNFα production but data have only been available for natural killer cells (20,21). In this study we have investigated the ability of monocyte-derived macrophages to produce TNFα in vitro following ligation of each of the three human FcγR, using murine monoclonal antibodies (mAb) to each receptor as surrogates for small immune complexes.
Macrophage-derived tumour necrosis factor alpha (TNFα) is thought to be a dominant mediator of synovitis in rheumatoid arthritis (1). The stimulus for the production of TNFα by macrophages is not known. Recent debate has focussed on signals generated as part of putative T cell responses to articular or microbial antigens (2). However, synovial intimal macrophage activation precedes, and is subsequently spatially dissociated from, T cell infiltration (3,4). In addition, it is difficult to integrate mechanisms based on T cell responses with the high levels of circulating rheumatoid factors and frequent presence of extra-articular features in rheumatoid arthritis (5).

For many years, rheumatoid factor-based immune complexes were considered the likely inflammatory stimulus in rheumatoid synovium (6,7). However, two factors made this view unpopular. The large complement-fixing circulating complexes thought to be most phlogistic, do not deposit in vessel walls in rheumatoid arthritis in the way seen in lupus nephritis. Nor are they likely to be able to cross endothelium from the circulation to access and activate synovial macrophages. Consequently, immune complexes have been viewed as having only a secondary role in rheumatoid arthritis, when generated locally within synovium already infiltrated with lymphoid cells.

Recently, it has become clear that complement fixation within the circulation is anti-phlogistic, since it results in immune complex clearance rather than inflammation (8). The pathogenic potential of circulating complexes may actually be enhanced by a failure to fix complement. IgG rheumatoid factor have the ability to self-associate and rheumatoid arthritic sera contain, amongst others, “intermediate” IgG rheumatoid factor based complexes (9,10) which, owing to their small size, fail to fix complement and can, therefore, escape clearance (11). They are also small enough to cross endothelium, with the resulting potential to bind to immunoglobulin receptors on tissue macrophages (12).
Objective. Small IgG rheumatoid factor immune complexes may provide the trigger for macrophage-derived TNFα production in rheumatoid arthritis. Immune complexes may bind to any of three IgG Fc receptors (FcγR). Therefore, the ability of monocyte-derived macrophages to produce TNFα was examined following ligation of each of the three human FcγR, using murine monoclonal antibodies (mAb) to each receptor as a model for small immune complexes.

Methods. Adhered human monocytes expressing all three FcγR were incubated with murine anti-FcγR mAb directed against FcγRI, FcγRII or FcγRIII. Supernatants were collected at various time points and tested for the presence of TNFα and IL-1α by ELISA.

Results. The anti-FcγRIII mAb induced adhered human monocytes to release TNFα. However, F(ab)2 and Fab fragments of the anti-FcγRIII mAb failed to induce TNFα production. TNFα was undetectable following incubation with the anti-FcγRI or anti-FcγRII mAb. Furthermore, blocking FcγRI or FcγRII had no effect on the levels of TNFα released in response to the anti-FcγRIII mAb. Of the three anti-FcγR mAb, only the anti-FcγRIII induced IL-1α production from adhered human monocytes and this was inhibited by the presence of a neutralising anti-TNFα mAb.

Conclusions. This study suggests a dominant role for FcγRIIIa in the induction of both TNFα and IL-1α production by human macrophages in rheumatoid arthritis following receptor ligation by small immune complexes. The signalling of TNFα production, may require either the ligation of three FcγRIIIa receptors or only two FcγRIIIa receptors, where one interaction must involve binding via an Fc domain. Additionally, IL-1α production following FcγRIIIa ligation appears to be dependent upon the presence of TNFα.
continuous agitation. The plate was then washed four times with PBS and tetramethylbenzidine (TMB) peroxidase substrate (Sigma) added. The reaction was stopped with 2M sulphuric acid and optical densities read at 450 nm.

**Interleukin-1 alpha (IL-1α) ELISA.** Cell free supernatants were loaded onto 96 well maxisorb plates precoated with a mouse anti-human IL-1α mAb. Recombinant human IL-1α was used as a standard. The assay was then performed as instructed by the manufacturer (R & D Systems, Oxfordshire, UK).

**Statistical analysis.** Significance was determined using unpaired T-test with Fisher’s correction for small sample size.

**RESULTS**

**Expression of FcyR by adhered human monocytes.** When isolated human monocytes were allowed to adhere to plastic for 48 hours, an increase in FcyRIIIa expression was seen (from 10% to 50% cells positive for FcyRIIIa). FcyRI and FcyRII expression levels did not change over the period of incubation (30% and 90% cells positive for FcyRI and FcyRII respectively) (Figure 1). In other experiments, CD14 levels declined after 24 hours (data not shown) therefore adherence for 24 hours was chosen for subsequent experiments.

**TNFα release from adhered monocytes following incubation with anti-FcyR mAb.** When adhered human monocytes were incubated with the anti-FcyRI, anti-FcyRII or anti-FcyRIII mAb (Figure 2) TNFα was detected only in those cultures incubated with the anti-FcyRIII mAb. The response was concentration dependent (Table 1) and polymixin B resistant. Levels of TNFα produced in response to the anti-FcyRIII mAb peaked at 4 hours and then declined with time. TNFα was undetectable in the supernatants of cultures incubated with medium alone, mouse IgG1 or mouse IgG2b. TNFα release was induced by both LPS (in the
absence of polymixin B) and PMA. The LPS induced TNFα response peaked at 6 hours and then declined with time, while the PMA response rose continuously.

All three anti-FcγR mAb block ligand binding and recognise epitopes in or close to their receptor's binding site (22-24). Therefore, in order to determine whether any additional FcγR recruitment was occurring during the anti-FcγRIII response, anti-FcγRI and anti-FcγRII mAb were used to block receptor binding. Adhered human monocytes were preincubated at 37°C for 30 minutes with either medium alone or the anti-FcγRI mAb or the anti-FcγRII mAb at saturating concentrations (10 μg/ml), as determined by indirect immunofluorescence and flow cytometry. The anti-FcγRIII mAb was then added to all cultures. Blocking either FcγRI or FcγRII receptors had no significant effect on the levels of TNFα released in response to anti-FcγRIII mAb stimulation (Figure 3).

Effect of anti-FcγRIIIa F(ab)2 and Fab fragments on TNFα secretion by monocytes. To determine whether TNFα release in response to the anti-FcγRIII mAb was dependent upon an Fc-FcγRIIIa interaction, adhered monocytes were incubated with F(ab)2 or Fab fragments of the anti-FcγRIII mAb. Neither the anti-FcγRIII F(ab)2 or Fab fragments induced adhered monocytes to secrete TNFα (Figure 4). LPS (in the absence of polymixin B) also induced adhered monocytes to release TNFα as observed previously (1.076 ± 0.57 ng/ml).

In a separate series of experiments, when adhered monocytes were preincubated with F(ab)2 fragments of the anti-FcγRIII mAb at binding saturating levels (20 μg/ml), the whole anti-FcγRIII induced TNFα response was inhibited by 69.0 ± 6.2%.

Effect of various inhibitors on TNFα release. To determine whether the presence of TNFα in supernatants was due to the release of preformed or newly synthesised cytokine, cultures were pre-treated with cycloheximide, actinomycin
D or colchicine. As seen in Figure 5, cyclohexamide, an inhibitor of protein translation, significantly inhibited TNFα secretion in response to the anti-FcγRIII mAb (99.9 ± 0.1%; p < 0.01) and LPS (99.9 ± 0.3%; p < 0.01). Treatment of adhered monocytes with actinomycin D, which blocks transcription, also inhibited the LPS response (70.2 ± 7.8%) and significantly inhibited the anti-FcγRIII mAb response (93.3 ± 6.2%; p < 0.01). The presence of colchicine, an inhibitor of microtubule integrity and secretory vesicle function, did not significantly inhibit the anti-FcγRIII mAb response (24.1 ± 23.0%) or the LPS response (37.3 ± 5.6%).

**IL-1α production by monocytes following incubation with anti-FcγR mAb.** When adhered human monocytes were incubated with the anti-FcγRI, anti-FcγRII or anti-FcγRIII mAb, only the anti-FcγRIII mAb induced secretion of IL-1α (Figure 6). IL-1α production in response to the anti-FcγRIII mAb was not detectable for at least 11 hours incubation and rose gradually over the next 61 hours. IL-1α was not detected in supernatants from cultures incubated with medium alone or mouse isotype. LPS (in the absence of polymixin B) also induced adhered monocytes to release IL-1α (Figure 6), detectable from 10 hours after stimulation and again, levels rose with time. In addition, it was found that although the TNFα levels in supernatants were similar in the presence of LPS or the anti-FcγRIII mAb (data not shown), higher IL-1α levels were found in response to LPS compared with anti-FcγRIII mAb.

**Inhibition of IL-1α production by anti-TNFα mAb.** It has previously been shown that IL-1α production by rheumatoid synovial mononuclear cells may be dependent upon the presence of TNFα (25). Therefore, adhered human monocytes were incubated for 48 hours with either LPS or the anti-FcγRIII mAb in the presence of increasing concentrations of a neutralising murine monoclonal anti-TNFα antibody (CB006). In the representative experiment shown in Figure 7a and b, IL-1α secretion was inhibited by the anti-TNFα antibody when cultures were stimulated with the anti-FcγRIII mAb (Figure 7a).
No such inhibition was seen in the presence of LPS (Figure 7b).

**DISCUSSION**

This study examined the ability of ligand binding-site specific murine anti-FcγR monoclonal antibodies to induce the production of TNFα from human monocyte-derived macrophages, in the absence of secondary crosslinking. Of the three anti-FcγR mAb, only that directed against FcγRIIIa triggered adhered human monocytes to release TNFα. This suggests that FcγRI and FcγRII both require the crosslinking of multiple receptors, whilst only two or three FcγRIIIa receptors need to be co-ligated for signalling of TNFα production. This is consistent with previous observations that FcγRI and FcγRII ligation only results in TNFα release by human peripheral monocytes when multiple receptors were crosslinked by immobilised murine IgG (19).

Both LPS and anti-FcγRIII mAb-induced TNFα responses were inhibited by cyclohexamide and actinomycin D. This confirmed that TNFα detected in supernatants required both transcription and protein translation, and was not due to mobilisation of intracellular stores of TNFα. Colchicine reduced both LPS and anti-FcγRIII mAb-induced TNFα production by approximately 30%, suggesting some dependence upon microtubule polymerisation.

As suggested by others (19), a true IgG Fc-FcγR interaction may be required for intracellular signalling to result in TNFα production. Using blocking antibodies to FcγRI or FcγRII we found that anti-FcγRIIIa mAb-mediated TNFα production was independent of these two receptors. Furthermore, neither F(ab)_2 nor Fab fragments of the anti-FcγRIII mAb were able to induce TNFα production. This could imply that for signalling and subsequent TNFα release, stimulation through FcγRIIIa requires recruitment of three receptors. Alternatively, ligation of only two receptors may be mandatory, but, if so, one interaction must involve binding via an Fc domain. This may be due to the additional requirement for
certain sequences or associated carbohydrate structures present within the Fc region to stabilise the interaction.

IL-1α is present at high levels in rheumatoid synovial fluids (26), and was the first pro-inflammatory cytokine to be implicated in rheumatoid synovitis, and particularly joint destruction (27). Therapeutic blockade of IL-1 is effective, but perhaps less strikingly so than for TNFα (28). As for TNFα release, only the anti-FcγRIIIa mAb induced IL-1α production from adhered human monocytes. Furthermore, its inhibition by a neutralising anti-TNFα mAb, suggests that FcγRIIIa mediated IL-1α production is dependent upon the presence of TNFα. In contrast, LPS-induced IL-1α production was not inhibited by the neutralising antibody to TNFα. This may be explained by the downregulation of both TNF receptors TNF-R55 and TNF-R75, together with the release of soluble TNF-R75, previously observed with LPS-mediated activation of monocytes (29). Our novel findings suggest that ligation of FcγRIIIa may favour the establishment of a pro-inflammatory cytokine loop initiated by TNFα. This is very much in keeping with previous observations on rheumatoid synovial cell cultures suggesting a dominant role for TNFα (25).

The targeting of inflammation to synovium and other tissues in rheumatoid arthritis has previously been difficult to explain. Although circulating IgG rheumatoid factor-based immune complexes have long been suspected to be involved in the disease process, the effector mechanism has been unclear. Our findings indicate that macrophage FcγRIIIa is the most likely FcγR to be triggered to produce TNFα following ligation by a small IgG-based immune complex. Ligation of FcγRIIIa, but not FcγRI or FcγRII, on monocytes, has also been shown to induce the generation of reactive oxygen species (30), which may contribute further to tissue injury in areas of FcγRIIIa expression.

This conclusion is consistent with previous observations highlighting a possible role for FcγRIIIa in the pathogenesis of rheumatoid arthritis (12,18).
Unlike Fc\(\gamma\)RI and Fc\(\gamma\)RII, Fc\(\gamma\)RIIIa expression by macrophages is tissue and site specific. A close correlation between Fc\(\gamma\)RIIIa expression and the location of both synovitis and extra-articular features has been demonstrated (16). The small IgG rheumatoid factor-containing ("intermediate") immune complexes first described by Kunkel et al. (31) may therefore be the pro-inflammatory stimulus to macrophage activation preceding T cell infiltration in rheumatoid synovium. This inflammatory stimulus may subsequently be amplified by T-B lymphocyte interaction and the local generation of large complement-fixing complexes based on rheumatoid factor of all isotypes (6,7).

In conclusion, the pattern of induction of TNF\(\alpha\) production by human monocyte/macrophages supports previous evidence for a specific role for Fc\(\gamma\)RIIIa in inflammation induced by small circulating immune complexes (12,18). Taken as a whole these findings suggest that the clinical syndrome of rheumatoid arthritis may reflect a pro-inflammatory mechanism based on such small immune complexes quite distinct from that seen in lupus nephritis and other forms of basement membrane-associated complex deposition.

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LEGENDS FOR FIGURES

Figure 1. Effects of culturing adhered human monocytes on FcγR expression. Following adherence to plastic for i) 0, ii) 24 or iii) 48 hours at 37°C, human monocytes were then incubated with predetermined optimal binding concentrations of a) anti-FcγRI mAb (10.1) or murine IgG1 b) anti-FcγRII mAb (IV.3) or murine IgG2b or c) anti-FcγRIII mAb (3G8) or murine IgG1. Surface bound antibody was detected using FITC conjugated goat F(ab')2 anti-mouse Ig and fluorescence intensity measured by flow cytometry. Data shown are from a representative experiment with fluorescent intensity along the X axis and number of events along the Y axis. Similar results were observed in two repeat experiments.

Figure 2. The time course of TNFα production by adhered human monocytes. Cultures were incubated at 37°C with anti-FcγRI (10.1), anti-FcγRII (IV.3), anti-FcγRIII (3G8), mouse IgG1, mouse IgG2b, all at 50 µg/ml, LPS (0.5 µg/ml in the absence of polymixin B), PMA (0.5 µg/ml) or medium alone. Cell free culture supernatants were collected at various time points over 24 hours and assayed for TNFα. Data shown are from one representative experiment and similar time courses for TNFα were observed in five repeat experiments.

Figure 3. Effects of blocking FcγRI or FcγRII on anti-FcγRIII mAb induced TNFα production. Adhered human monocytes were preincubated with anti-FcγRI (10.1) or anti-FcγRII (IV.3) at saturating binding levels or medium alone for 30 minutes at 37°C. After 4 hours subsequent incubation with anti-FcγRIII (3G8) at 50 µg/ml, supernatants were collected and assayed for the presence of TNFα. Values are expressed as the mean ± S.D. from three experiments.

Figure 4. Effects of anti-FcγRIII mAb F(ab')2 or Fab fragments on TNFα production.
production. Adhered human monocytes were incubated at 37°C with anti-FcγRIII mAb (3G8) (50 µg/ml), F(ab)_2 fragments of anti-FcγRIII (33 µg/ml) or Fab fragments of anti-FcγRIII (17 µg/ml). After 4 hours, cell-free supernatants were collected for measurement of TNFα concentration. Values are expressed as the mean ± S.D. from three experiments.

Figure 5. Effects of various inhibitors on TNFα production. Adhered human monocytes were incubated at 37°C with anti-FcγRIII (3G8) (50 µg/ml) or LPS (0.5 µg/ml) in the presence of medium alone, cyclohexamide (150 µg/ml), actinomycin D (2 µg/ml) or colchicine (1 µg/ml) (all from Sigma). After 4 hours, cell-free supernatants were collected and assayed for TNFα. Values are expressed as the mean ± S.D. from three experiments.

Figure 6. Time course of IL-1α production by adhered human monocytes. Cultures were incubated with anti-FcγRI (10.1), anti-FcγRII (IV.3), anti-FcγRIII (3G8), mouse IgG1, mouse IgG2b (all at 50 µg/ml), LPS (0.5 µg/ml, in the absence of polymixin B) or medium alone. Culture supernatants were collected at various time points over 72 hours and the cell free samples were then assayed for IL-1α. Data shown are from one representative experiment and similar time courses for IL-1α were observed in two repeat experiments.

Figure 7 a) & b). Effects of an anti-TNFα mAb on IL-1α production. Adhered human monocytes were incubated at 37°C with a) anti-FcγRIII mAb (3G8; 50 µg/ml) or b) LPS (0.5 µg/ml in the absence of polymixin B) in the presence of a mouse anti-human TNFα mAb (CB006) at 0, 1, 10 or 100 µg/ml. Supernatants were collected following 48 hours incubation and tested for IL-1α. Data shown are from one representative experiment and similar results were observed when this experiment was repeated.
Medium  Anti-FcyRI (10.1)  Anti-FcyRII (IV.3)

TNF (ng/ml) at 4 hours

Figure 3

Medium
Anti-FcyRI (10.1)
Anti-FcyRII (IV.3)
Figure 5

TNF (ng/ml)

Medium

0.001 +/- 0.002

Cyclohexamide

0.002 +/- 0.004

Actinomycin D

Colchicine

Anti-FcγRIII (3G8)

LPS
Anti-TNF mAb (μg/ml)

IL-1 (pg/ml)

Figure 7(a)

0 0.2 0.4 0.6 0.8 1.0

0

0 10 100

Anti-TNF mAb (μg/ml)
Figure 7 b)

<table>
<thead>
<tr>
<th>IL-1 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Anti-TNF mAb (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>100</td>
</tr>
</tbody>
</table>
Appendix 2.

Calibration of the HPLC column

HPLC elution times of calibration markers of known molecular weights.

<table>
<thead>
<tr>
<th>Calibration marker</th>
<th>Molecular weight (kDa)</th>
<th>Elution time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>66</td>
<td>9.63</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>150</td>
<td>9.20</td>
</tr>
<tr>
<td>Bamalyase</td>
<td>200</td>
<td>8.76</td>
</tr>
<tr>
<td>Apo ferritin</td>
<td>443</td>
<td>8.16</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>669</td>
<td>7.26</td>
</tr>
</tbody>
</table>
Appendix 3.

Calibration of the FACS\textsuperscript{can}

MFIs for each Fluorosphere bead population. Values are expressed as the mean ± S.D. from three separate readings.

<table>
<thead>
<tr>
<th>Molecules of FITC per bead</th>
<th>Mean fluorescent intensity (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2500</td>
<td>14.647 ± 10.824</td>
</tr>
<tr>
<td>6500</td>
<td>36.767 ± 30.951</td>
</tr>
<tr>
<td>19000</td>
<td>104.96 ± 86.684</td>
</tr>
<tr>
<td>55000</td>
<td>283.87 ± 233.15</td>
</tr>
<tr>
<td>150000</td>
<td>619.75 ± 350.75</td>
</tr>
</tbody>
</table>

The equation of above standard curve was $y = -4.4999 + 0.92352x$, where:

$y =$ MFI
$m = -4.4999$
$c = 0.92352.$
Appendix 4.

TNFα ELISA

Optical densities for the IgG1 rheumatoid factor mAb incubated at 37°C in the presence (+) or absence (−) of adhered monocytes.

<table>
<thead>
<tr>
<th>IgG1-RF mAb</th>
<th>2 Hrs</th>
<th>6 Hrs</th>
<th>24 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ monocytes</td>
<td>1.085</td>
<td>0.964</td>
<td>1.034</td>
</tr>
<tr>
<td>- monocytes</td>
<td>1.063</td>
<td>1.196</td>
<td>1.841</td>
</tr>
</tbody>
</table>
Appendix 5.

Buffers and media

**Bicarbonate buffer (BIC)**
8g Na₂CO₃, 15.5g NaHCO₃ and make up to 10 litres with distilled water. Adjust to pH9.6.

**Bradford assay dye**
600mg of Coomassie blue G-250 in 1L of 2% perchloric acid.

**Citrate buffer (0.05M)**
25.7ml 0.2M Na₂HPO₄, 24.3ml 0.1M citric acid and make up to 100ml with distilled water. Adjust to pH5.

**Hank’s Balanced salt solution (HBSS)**
Dilute 10X HBSS without phenol red (Gibco) 1:10 with distilled water.

**Phosphate buffered solution (PBS)**
PBS 10X: 20g KCl, 20g KH₂PO₄, 114.8g Na₂HPO₄, 800g NaCl. Make up to 10 litres with distilled water. Dilute PBS 10X 1:10 and adjust to pH7.4.

**PBS/Tween**
Dilute PBS 10X 1:10 and adjust to pH7.4.
Add 0.1% Tween 20 (Sigma)