EXPLORING PERIPHERAL BLOOD MONONUCLEAR CELLS AS THE SOURCE OF INTERLEUKIN-6 IN POLYMYALGIA RHEUMATICA

HANNAH LOUISE BAZZARD

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Centre for Research in Bioscience (CRIB), Bristol UK
Abstract

Polymyalgia rheumatica (PMR) is a chronic inflammatory condition which affects the elderly, causing aching and stiffness of the neck, shoulders and pelvis, as well as more systemic manifestations such as fever, malaise and fatigue. PMR shares symptoms with rheumatoid arthritis (RA) and both are associated with significantly elevated circulating concentrations of interleukin-6 (IL-6), which is thought to play a key role in the pathogenesis of these diseases. In RA, IL-6 is derived from synovial cells in the joints. In PMR, however, the source of IL-6 is unknown. PMR patients do not exhibit the same joint involvement as in RA but they do have elevated circulating IL-6 concentrations, thus, it was hypothesised that the source of IL-6 in PMR may be one of the circulating peripheral blood mononuclear cell (PBMC) types, which are all capable of producing IL-6.

Blood samples were taken from untreated PMR patients, RA patients with active disease and healthy controls (HC) of similar age and gender. To account for known circadian variations in circulating IL-6, samples were taken at a standard time. IL-6 was quantified in plasma and serum using cytometric bead array (CBA) and enzyme-linked immunosorbent assay. The biological activity of the IL-6 was tested for the first time in PMR using a B cell proliferation assay. Using immunostaining and flow cytometry, constitutive intracellular IL-6 was measured in CD3+, CD14+, CD19+, CD123+ and CD11c+ PBMCs. Intracellular IL-6 was also determined following PBMC stimulation in vitro, to determine potential differences in cell responsiveness. Concentrations of secreted IL-6 in the culture supernatants of resting and stimulated PBMC were determined by CBA in parallel cultures. Other cytokines were also quantified in order to examine PMR and RA pathologies more broadly. Finally, the results of the cytokine assays were compared with patient reported severity of fatigue and the four different components of fatigue (emotional, living, physical and cognitive).

Circulating IL-6 was significantly elevated above HC in both serum and plasma of PMR and RA patients, and this IL-6 was found to be biologically active. PBMC in all subjects constitutively produced low levels of intracellular IL-6 and very low concentrations of secreted IL-6 in parallel cultures. Overall, responses to in vitro stimulation were variable but no significant differences were observed between PMR, RA and HC samples. Secreted IL-6, in contrast, increased dramatically following stimulation of all cultures, suggesting intracellular staining may not reflect the secretory capability of these cells, but also confirming that there were no differences between PBMC responses of PMR, RA and HC groups. A significant correlation was observed between circulating IL-6 concentrations in PMR and RA patients, and physical fatigue, living fatigue and total fatigue. Broader cytokine analysis demonstrated that IL-6 alone was significantly elevated in PMR patients.

Taken together, circulatory IL-6 in active PMR is found to be elevated in the absence of other inflammatory cytokines. It is biologically active and correlates strongly with physical and living aspects of fatigue. Circulating PBMCs are not the source of this elevated IL-6 in PMR patients, suggesting that neutrophils, vascular endothelium or muscle tissue may instead be involved.
Acknowledgements

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Abbreviations and Acronyms

APR - Acute phase response
BRAF-MDQ - Bristol rheumatoid arthritis fatigue multi-dimensional questionnaire
CBA - Cytometric bead array
CRP - C-reactive protein
DC - Dendritic cell
ELISA - Enzyme-linked immunosorbent assay
FL - Fluorescence
g - Gram
GC - Glucocorticoids
GCA - Giant cell arteritis
HC - Healthy control
HPA axis - Hypothalamo-pituitary-adrenal axis
IFN - Interferon
IL - Interleukin
LPS - Lipopolysaccharide
mDC - Myeloid dendritic cell
MFI - Mean fluorescence intensity
mg - Milligram
ml - Millilitre
MTS - (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ng - Nanogram
NK cell - Natural killer cell
NS - Not significant
pDC - Plasmacytoid dendritic cell
PB - Peripheral blood
PBMC - Peripheral blood mononuclear cells
pg - Picogram
PMR - Polymyalgia rheumatica
PV - Plasma viscosity
RA - Rheumatoid arthritis
RF- Rheumatoid factor
RT- Room temperature
SEM- Standard error of the mean
SF- Synovial fluid
SP- Study participant
TNF- Tumour necrosis factor
UWE- University of the West of England
WCC- White cell count
µg- Microgram
µl- Microlitre
Chapter 1: Introduction
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1.1 Demographics and historical perspective of polymyalgia rheumatica and rheumatoid arthritis

Immune-mediated inflammatory diseases are a set of chronic and debilitating conditions that are highly prevalent in the Western world (5%-7% of population) and are grouped according to a characteristic imbalance of cytokines associated with the pathogenesis of inflammation (El-Gabalawy et al., 2010). Included in this group of inflammatory diseases are polymyalgia rheumatica (PMR) and rheumatoid arthritis (RA), both of which are characterised by chronic inflammation, and are strongly associated with significantly elevated levels of the pro-inflammatory cytokine, interleukin-6 (IL-6) (Arvidson et al., 1994, Roche et al., 1993).

The symptoms of PMR were first described by Bruce in 1888, when he identified that the disease shared similarities with rheumatism, gout and rheumatoid arthritis, thus he labelled the disease ‘senile rheumatic gout’ (Bruce, 1888). Years later, studies by Barber described the symptoms of PMR in further detail, including key laboratory findings still used to diagnose PMR today, such as increased erythrocyte sedimentation rate (ESR) (Barber, 1957). The disease was then given its current name ‘polymyalgia rheumatica’, due to characteristic muscular pain, lack of arthritis, but clear association with the rheumatoid diseases. PMR was once considered to be a manifestation of giant cell arteritis (GCA), also known as temporal arteritis, because around half of the patients with GCA also present with symptoms of PMR (Salvarani et al., 2004, Martinez-Taboada et al., 2008). GCA, however, is mainly associated with inflammation of the larger arteries and in particular the branches of the proximal aorta, sometimes causing blindness and stroke (Salvarani et al., 2008). Clinically it is often confused with PMR at initial presentation.

PMR generally affects people over the age of 50, peaking around 60-70 years, with 1 in 1,200 people developing the condition in the UK each year (National Health Service, 2013). It is characterised by morning aching and stiffness of the proximal muscles in the neck, shoulders and pelvis, as well as causing other systemic manifestations such as low-grade fever, weight loss, malaise and depression (Bird et al., 1979, Salvarani et al., 2004, Kaposi and Schmidt, 2006). Women have a fourfold
higher risk of developing the disease compared to men (Zimmermann-Gorska, 2008), which may be influenced by female sex hormones (prolactin and oestrogen) that are known to encourage immune-mediated inflammation (El-Gabalawy et al., 2010).

RA patients, in contrast, have more localised symptoms, including swelling of the proximal interphalangeal (PIP), metacarpophalangeal (MCP) or wrist joints, and also the development of rheumatoid nodules (Arnett et al., 1988) (Figure 1). The symptom of morning stiffness is shared between RA and PMR. Chronic synovitis (inflammation at the synovial sites) in RA results in the severe and permanent destruction of synovium, cartilage and bone, causing great pain and discomfort for sufferers (Kim and Kim, 2010).

Figure 1: Hand of an elderly woman suffering from rheumatoid arthritis. The proximal interphalangeal joints (green arrows) and metacarpophalangeal joints (blue arrows) have rheumatoid nodules (red arrows). Inflammation around these joints and the wrist can also be observed (Image courtesy of Science Photo Library 2013 Reprinted with Permission).
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The prevalence of RA in the UK is higher than PMR and again it affects more women (1.16%) than men (0.44%) (Symmons et al., 2002). According to a report published by the National Audit Office (NAO) in 2009, rheumatoid arthritis costs the National Health Service (NHS) around £560 million per year and costs the economy a further £1.8 billion per year through absence from work and work-related disability (NAO, 2009). Facts such as these show the economic importance for research to continue looking into the pathogenesis of these debilitating diseases and new methods to alleviate them.

In addition to suffering from chronic pain, PMR and RA patients suffer from a specific type of fatigue which arguably has the most important impact on the individual’s quality of life (Hewlett et al., 2005). Fatigue has recently been estimated to be experienced by around 98% of RA patients at some point during the disease (Nicklin et al., 2010). In a study by Professor Hewlett and colleagues, patients described RA fatigue as ‘severe’, ‘dramatic’ and ‘overwhelming’, and often regarded the impact of fatigue as being comparable to pain (Hewlett et al., 2005).

1.2 Diagnosis of PMR and RA

Modern diagnosis of PMR and RA is based on criteria developed by researchers and clinicians who have examined the disease in great detail for a number of years. According to Salvarani and co-workers, there was a ‘lack of effort’ to standardise the diagnosis of PMR because for years it was believed to be part of the pathogenesis of GCA (Salvarani et al., 2004). Bird, Chuang, Healey and colleagues have, however, all suggested criteria for the diagnosis of PMR based on different inclusion and exclusion factors, and these have been widely adopted by physicians to help detect and treat PMR globally (Bird et al., 1979, Chuang et al., 1982, Healey et al., 1984).

The Bird diagnostic criteria were developed from a collaboration of results and findings from eleven rheumatology units in the South and West of Great Britain (Bird et al., 1979). Seven key characteristics of PMR were selected, and it was determined
that if the patient was exhibiting three of the seven key symptoms then they would be diagnosed as having ‘probable PMR’ (Bird et al., 1979). As only three features are required to be present out of seven for a PMR diagnosis, the Bird criteria have a more practical application within a clinical setting as they are more broadly inclusive, compared to those which require all the criteria to be satisfied in order to make a diagnosis, e.g. the Healey criteria (Semanta and Kendall, 2002). The Bird criteria were thus selected for use in this study to recruit patients with PMR (Table 1).

Table 1: Bird Criteria for initial diagnosis of PMR (Bird et al., 1979).

<table>
<thead>
<tr>
<th>Bird criterion</th>
<th>Clinical description of symptom*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Bilateral shoulder pain/stiffness</td>
</tr>
<tr>
<td>2.</td>
<td>Duration of symptoms &lt;2 weeks**</td>
</tr>
<tr>
<td>3.</td>
<td>Initial ESR &gt;40 mm/h</td>
</tr>
<tr>
<td>4.</td>
<td>Morning stiffness duration &gt;1 h</td>
</tr>
<tr>
<td>5.</td>
<td>Age &gt;65 years</td>
</tr>
<tr>
<td>6.</td>
<td>Depression and/or weight loss</td>
</tr>
<tr>
<td>7.</td>
<td>Bilateral upper arm tenderness</td>
</tr>
</tbody>
</table>

*Three or more features are required to make a positive diagnosis.

**Time taken for symptoms to become fully apparent.

The American College of Rheumatology (ACR) have described a list of criteria for RA diagnosis which is widely used, and was thus selected for use in the diagnosis and recruitment of RA patients in this study (Arnett et al., 1988) (Table 2). The ACR criteria follow similar principles to the Bird criteria, with four out of seven key criteria needing to be fulfilled in order to diagnose the patient with RA. These criteria are
based on physical symptoms, laboratory findings and also radiographic images (Arnett et al., 1988).

Table 2: ACR Classification criteria for initial diagnosis of RA (Arnett et al., 1988).

<table>
<thead>
<tr>
<th>ACR criterion</th>
<th>Clinical description of symptom†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Morning stiffness duration (≥1 hour)***</td>
</tr>
<tr>
<td>2.</td>
<td>Swelling (soft tissue) of three or more joints***†</td>
</tr>
<tr>
<td>3.</td>
<td>Swelling (soft tissue) of hand joints (PIP, MCP, or wrist)*** †</td>
</tr>
<tr>
<td>4.</td>
<td>Symmetrical swelling (soft tissue)***†</td>
</tr>
<tr>
<td>5.</td>
<td>Subcutaneous nodules†</td>
</tr>
<tr>
<td>6.</td>
<td>Serum rheumatoid factor (RF)</td>
</tr>
<tr>
<td>7.</td>
<td>Erosions and/or periarticular osteopenia in hand or wrist joints seen on radiograph</td>
</tr>
</tbody>
</table>

†A classification as RA requires that four of the seven criteria be fulfilled.

***Must have been continuously present for 6 weeks or longer.

†Must be observed by a physician.

In addition to elevated serum levels of IL-6, PMR is also characterised by an exaggerated acute phase response (APR) (Alvarez-Rodriguez et al., 2010). In order to assess the intensity of an APR, C-reactive protein (CRP) levels and ESR are both measured in patient blood samples. An elevated CRP and an increase in ESR above 40 mm/h are generally regarded as indicative of PMR (Zimmerman-Gorska, 2008). It is not uncommon for PMR patients, however, to exhibit a low ESR and low CRP levels and still have active disease based on the Bird criteria. According to Brooks and McGee, 13% of all patients who have all the typical features of PMR also have a normal ESR (Brooks and McGee, 1997). Therefore it has been suggested that CD8+ T
cell numbers or IL-6 levels could be measured as an alternative method of assessing the acute phase response (Dasgupta et al., 2006), although this has not been routinely adopted. A conclusive diagnosis of PMR is usually made if there is a rapid response to low dose glucocorticoids (GC), despite this not being included in the Bird criteria. Patients with ‘pure’ PMR usually experience a rapid clinical response (>70%) in under a week to 10-20 mg prednisone per day and laboratory inflammatory markers (such as ESR and CRP) return back to normal reference range within four weeks (Dasgupta, 2010, Gonzalez-Gay et al., 2010).

The diagnosis of PMR and RA is not always straightforward, particularly as the Bird criteria are very inclusive and PMR diagnostic criteria are similar to the ACR criteria for the diagnosis of RA. In order to aid the differential diagnosis of PMR and RA and to allow the clinician to get deeper insight into the type and the extent of the symptoms experienced by the patient, questionnaires have been developed to measure other aspects of these diseases. These measurements are based particularly on the level of pain and fatigue the patient experiences. The Health Assessment Questionnaire (HAQ), Disease Activity Score (DAS) and Bristol Rheumatoid Arthritis Fatigue Multi-Dimensional Questionnaire (BRAF-MDQ) are all different forms of such questionnaire and were adopted in this study to aid the diagnosis of PMR and RA and allow further insight into the nature and severity of the patient’s symptoms.

The HAQ (Appendix A) is a method of assessing patient-reported outcomes based on five patient-focused components, namely: pain, disability, the effects of medication, the cost of care, and mortality (Bruce and Fries, 2005). The DAS (Appendix A) was devised in order to measure inflammatory activity in RA, and combines clinical and laboratory-based information to create a score which can be used to measure patients’ response to therapy (Fransen and van Riel, 2005). As the patients in this study are required to have active disease when the sample is taken, use of the DAS ensures that the patient fulfils this criterion.

The BRAF-MDQ has recently been devised by Nicklin and colleagues as a method of measuring fatigue in RA, but it can also be used to measure fatigue in PMR (Nicklin et al., 2010). Standardised numerical rating scales (NRS) and visual analogue scales
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(VAS) are incorporated into the BRAF-MDQ, which tests fatigue severity, effects of fatigue, and also the patient’s perceived ability to cope with fatigue on a daily basis (Appendix A) (Nicklin et al., 2010). The development of the BRAF-MDQ allows clinicians or researchers to understand the fatigue experience as a whole, as well as focusing on the individual aspects of fatigue, which may be caused by different factors or require different treatment options (Hewlett et al., 2011). As fatigue is such a major feature of RA and PMR, tools such as this are an important aid for the invaluable treatment and support of PMR patients and also for future research.

1.3 Treatment of PMR and RA

GCs such as prednisolone are predominantly used in the treatment of PMR and RA. Despite providing relief from symptoms, however, GCs do not correct the underlying mechanisms causing these diseases (Martinez-Taboada et al., 2008). For the majority of PMR patients, GC treatment is required for around 1-2 years, yet patients who suffer from ‘a more chronic, relapsing course’ often require lower doses of steroid treatment for 5 years or more (Salvarani et al., 2007). The prednisolone dose should be reduced or ‘tapered’ gradually over time to avoid or reduce the detrimental side-effects associated with prolonged steroid use (Dasgupta, 2010). Too rapid a reduction, however, often results in relapse of PMR (Zimmerman-Gorska, 2008).

The adverse side-effects associated with long-term steroid use include chronic conditions such as diabetes and osteoporosis. A study of PMR by Gabriel and colleagues demonstrated that 65% of their 124 patients treated with corticosteroids alone developed at least one secondary condition (Gabriel et al., 1997). Symptoms of potential co-morbidities such as osteoporosis and diabetes are therefore monitored closely and prophylactic treatment for them may be given alongside GC treatment. Alendronic acid, calcium, vitamin D supplementation and bisphosphates, for example, may be given to help prevent the development of osteoporosis (Zimmerman-Gorska, 2008). Furthermore, there are a number of RA patients who do not respond to GC treatment at all, for reasons which are currently unclear (Kirwan, J., personal
A recent study by Clarke and colleagues has suggested, however, that there may be a link between the cytokine profile of the patient and their responsiveness to GC treatment (Clarke et al., 2011).

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen are often the first choice for the treatment of PMR. If there is no improvement in PMR symptoms within 2-4 weeks then steroidal treatment is recommended (Salvarani et al., 2004). It has been suggested that PMR patients with the ‘pure’ form only experience short and mild beneficial effects from NSAIDs (Gonzalez-Gay et al., 2010), making them superfluous. They may, however, have a role in keeping patients in remission after GC therapy has been stopped (Gonzalez-Gay et al., 2010). A study by Gabriel and colleagues demonstrated that even low doses and short duration of combined corticosteroid and NSAID treatment caused a higher incidence of adverse events in PMR patients, such as gastrointestinal bleeding and hypertension (Gabriel et al., 1997), for this reason NSAIDs are not recommended during GC therapy.

It is has been well established that cytokines such as IL-1β, TNFα, IL-6 and IL-8 play a role in the pathogenesis of inflammatory disease, and recently there has been a more targeted therapeutic approach which centres around the blockade of particular cytokines or cytokine receptors in order to reduce the signs and symptoms of these diseases (Houssiau et al., 1988, Luqmani, 2007). The most well-known example is the use of TNFα-antagonists such as the monoclonal antibody Infliximab which is used in the treatment of RA (Blumenauer et al., 2002).

Tocilizumab, a monoclonal antibody which blocks the IL-6 receptor, has also been approved for use in RA patients in the UK (Ohsugi and Kishimoto, 2008). Research by Emery and colleagues was highly significant to this approval, as results from their study showed that the use of Tocilizumab (plus methotrexate) could accomplish swift and continuous improvements in the symptoms of RA in patients who failed to respond to TNFα blockers (Emery et al., 2008). Hagihara and colleagues demonstrated that Tocilizumab can also ameliorate the symptoms of PMR (Hagihara et al., 2010), however, the results presented are from one patient only and serious side-effects have been reported elsewhere (Campbell et al., 2011). Tocilizumab therapy is not currently
approved by the Food and Drug Administration for the treatment of PMR, as it can cause serious infections of the respiratory tract, nasopharynx, skin and soft tissue and gastrointestinal tract (Campbell et al., 2011).

A more recent study by Macchioni and colleagues contradicts this; two newly diagnosed PMR patients were treated with Tocilizumab and it was determined that Tocilizumab is an ‘effective and safe’ way to treat PMR (Macchioni et al., 2013). This is despite having to administer GC therapy to one of the patients alongside the Tocilizumab after the second infusion due to a lack of clinical response (Macchioni et al., 2013). Further trials and larger studies are underway to see if Tocilizumab could be an effective and safe therapy for PMR patients, and to determine whether the benefits outweigh the side effects.

1.4 Pathogenesis of RA

The pathogenesis of RA is complex. The interaction of both environmental factors, such as drinking alcohol and smoking, and genetic factors is thought to increase susceptibility to the disease, by activating the series of immunological events which lead to the onset of RA (Silman et al., 1996, Klareskog et al., 2006). It is suspected that there is a genetic predisposition for both RA and PMR, as the geographical distribution of the diseases are well defined, with Scandinavia and other parts of Northern Europe having the highest incidence of PMR (Cimmino, 1997). Patients with RA and PMR have been found to share an association with a serotype known as HLA-DR4 (i.e. a characteristic set of antigens found on leukocytes and other cells). A similar sequence polymorphism encoded by the second hypervariable region of the allele HLA-DRB1 has been discovered in PMR and GCA patients (Weyand et al., 1994). The exact role of these alleles is yet to be elucidated despite the association of HLA-DR4 with RA being discovered more than 25 years ago (Stastny et al., 1988).

The immunological events that are triggered in RA include the recognition of citrullinated antigens by self-reactive T cells, causing them to ‘attack’ joint tissue
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(Boissier, 2011). This brings about an overwhelming inflammatory response, resulting in severe and permanent destruction of synovial tissue, cartilage and bone (Kim and Kim, 2010). The breakdown of the inflammatory process causes major imbalances in cytokine systems (Boissier, 2011), resulting in an increase in the production of many of the pro-inflammatory cytokines such as IL-6, IL-1β, TNFα and IL-8 (Perry et al., 2009). These are abundant in the affected tissues of RA patients (Feldmann et al., 1996) along with the more recently identified, IL-17 (Chabaud et al., 1999). IL-6 in particular is known to be significantly elevated in the synovial fluid (Hirano et al., 1988, Guerne et al., 1989) and plasma (Perry et al., 2009) of RA patients, whilst a preliminary study suggests circulating IL-1β, TNFα, and IL-4 may also be significantly elevated in a subset of RA patients (Clarke et al., 2011). Clarke and colleagues also observed a potential link between patients with the latter cytokine profile and non-responsiveness to GC therapy in a small scale study (Clarke et al., 2011).

1.5 Cytokines involved in the pathophysiology of RA

It is well established that IL-6 is strongly associated with both RA and PMR, and its exact function and role in the disease pathogenesis is outlined in the following section. Other cytokines which have been strongly implicated in RA disease pathology are discussed here, as well as some cytokines whose involvement in RA disease pathology is still speculative.

1.5.1 TNFα

TNFα is produced by many cell types including macrophages (Clavel et al., 2008), endothelial cells (Ranta et al., 1999), fibroblasts (Fahey et al., 1995) and synovial-derived T cells (Steiner et al., 1999). It has a broad range of biological activities and plays a fundamental role in RA, by bringing about synovitis and inducing the network of other inflammatory cytokines that are seen in RA (Figure 2) (Vasanthi et al., 2007). The blocking or neutralising of TNFα reduces joint inflammation in RA (Perdriger, 2009). TNFα is raised particularly in the synovium of RA patients (Steiner et al., 1999)
and as a result, has been shown to be raised in the serum/plasma of RA patients compared to healthy controls in most but not all studies (Espersen et al., 1991, Cutolo et al., 2006, Perry et al., 2009, Szodoray et al., 2006).

Figure 2: The complex cytokine network involved in RA disease pathogenesis. Th1 and Th17 T cell subsets have a role in promoting inflammatory cytokine production in macrophages and synovial cells. Activated macrophages and fibroblasts in the synovial lining produce inflammatory cytokines that can activate themselves or nearby cells (Firestein, 2003). Diagram adapted from Firestein (2003) and Boissier (2011). Key:- APC: Antigen presenting cell; MHC: Major histocompatibility complex; TCR: T-cell receptor, NK cell; Natural killer cell.
1.5.2 IL-1β

IL-1β is also secreted by a variety of cells including monocytes and macrophages (Figure 2) (Ward et al., 2010). IL-1β is raised in the serum and plasma of RA patients (Szodoray et al., 2006, Perry et al., 2009). Furthermore, concentrations of IL-1β in the plasma and synovial fluid of RA patients strongly correlate with various measures of the disease, such as the length and extent of morning stiffness and the level of pain experienced by the patient (Kay and Calbrese, 2004). Blocking this cytokine also dramatically reduces these symptoms (Kay and Calbrese, 2004), thus strongly implicating IL-1β in the pathogenesis of RA.

1.5.3 IL-8

IL-8 is produced by a range of cell types and plays a key role in recruiting and activating immune cells such as neutrophils at the site of inflammation (Harada et al., 1994). Plasma IL-8 is significantly raised in RA patients compared to normal healthy controls (Perry et al., 2009).

1.5.4 IL-17

IL-17 (or IL17A as it is otherwise known) is a cytokine derived from a relatively newly discovered subset of T cells known as Th17 cells, and is induced by high concentrations of IL-6 in the presence of TGFβ (Harrington et al., 2005, Kimura and Kishimoto, 2010). IL-17 is expressed at high levels in the synovial fluid of RA patients (Chabaud et al., 1999). The principal function of IL-17 is thought to be in the defence against pathogens in the mucosal or epithelial lining (Jin and Dong, 2013) and also in the removal of extracellular (Ishigame et al., 2009) and intracellular (Hamada et al., 2008) pathogens following infection. IL-17, however, has a crucial role in the pathogenesis of RA by strongly inducing many of the pro-inflammatory cytokines, such as IL-8 and IL-6 (Figure 2) (Hwang et al., 2004), whilst also being directly involved in the destruction of joint tissue (Chabaud et al., 2001). Specifically, IL-17 causes degradation of cartilage by increasing the breakdown of proteoglycan and decreasing proteoglycan synthesis, as well as destroying type I collagen (Chabaud et
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It has been suggested that neutrophils are also a critical source of IL-17 in the effector phase of RA, although the trigger for this secretion is still unclear (Katayama et al., 2013). Despite its localised action, high serum concentrations have previously been measured in RA patients compared to healthy controls (Wallis et al., 2011). Studies have shown that treatment with anti-IL17 monoclonal antibodies can reduce RA symptoms, such as joint inflammation and destruction, both in mouse models and in human trials (Lubberts et al., 2004, Genovesse et al., 2010), thus confirming a crucial role for this cytokine in the pathophysiology of RA.

1.5.5 IL-4

IL-4 is produced predominantly by Th2 cells, as well as basophils and mast cells, and has various proinflammatory and anti-inflammatory functions within the immune system (Gessner et al., 2005). IL-4 is a powerful activator of B cells, it mediates the differentiation of naïve helper T cells into Th2 cells (Figure 2), it is a potent suppressor of IFNγ-producing CD4+ Th1 cells, and also induces class II major histocompatibility complex (MHC) expression on macrophages and DCs (Nelms et al., 1999, Ohmura et al., 2005). In terms of having a potentially anti-inflammatory role in RA, the proliferation of synoviocytes has been shown to be suppressed by IL-4, as it inhibits the initial phases of the cell cycle (Dechanet et al., 1993). Moreover, a study by Wallis and co-workers suggests that IL-4 significantly reduces the production of IL-17 in healthy subjects (Wallis et al., 2011) and therefore may down-regulate RA.

Ohmura and colleagues, in contrast, suggest that IL-4 may actually promote RA, perhaps because of its ability to activate B cells (Ohmura et al., 2005), which produce RF, anti-citrullinated antibodies and a range of pro-inflammatory cytokines involved in RA pathogenesis (Silverman and Carson, 2003). Ohmura and colleagues demonstrated that IL-4 is vital for the full development of RA disease in mouse models; however it was not directly involved in the effector phase of the disease (Ohmura et al., 2005).

According to Wallis and colleagues, some individuals possess an inherited gene polymorphism in the coding region of the IL-4 receptor that would normally function to suppress or control the immune responses of Th17 cells, and hence the amount of
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IL-17 produced (Wallis et al., 2011). With this genetic polymorphism in its receptor, IL-4 cannot function to suppress Th17 cells as it would normally (Wallis et al., 2011). A recent study has confirmed the association between genetic variants of the IL-4 receptor and the progression rate of joint damage in RA patients (Krabben et al., 2013). Furthermore, despite IL-4 being capable of down-regulating IL-17 secretion by Th17 cells, Cooney and colleagues have shown that Th17 cells which have been subjected to ‘multiple rounds of stimulation’ (as would be happening in RA), are no longer affected by IL-4 and cannot signal through the IL-4 receptor (Cooney et al., 2011).

Overall then, IL-4 may have both anti-inflammatory and pro-inflammatory roles in RA. IL-4 suppresses synoviocyte proliferation and IL-17 production by Th17 cells. Yet, a genetic polymorphism in the IL-4 receptor in some individuals and the desensitisation of Th17 cells to IL-4 signalling may inhibit the anti-inflammatory activities of IL-4 and promote a more sinister role for IL-4 in RA disease pathogenesis.

1.5.6 IL-2

IL-2, produced mainly by CD4+ T cells, predominantly exerts its effects on T cells, inducing rapid expansion, proliferation and differentiation (Gaffen and Liu, 2004). Early research suggests that IL-2 production from synovial and peripheral blood lymphocytes is diminished in RA patients compared to healthy controls (Combe et al., 1985). Other studies have also demonstrated that plasma IL-2 concentrations are decreased in RA patients compared to healthy controls (Kitas et al., 1988, Espersen et al., 1991). Espersen and colleagues hypothesise that this may be due to RA patients having greater concentrations of the IL-2 receptor in serum and synovial fluid (Symons et al., 1988, Espersen et al., 1991). More recently, normal lymphocytes have been shown to produce decreased amounts of IL-2 in the presence of RA synovial fluid as well as having a decreased response to IL-2; again attributed to the presence of an inhibitor of IL-2 in RA patient synovial fluid (Miossec et al., 2005). In contrast, it has been suggested that IL-2 is significantly elevated in the synovium particularly in the early stages of RA, compared to patients with ‘established RA’ (Raza et al., 2005). Thus, the role of IL-2 in RA disease pathogenesis is unclear.
1.5.7 IL-12p70

IL-12p70 is the biologically active form of IL-12 (Feinberg et al., 2004). Phagocytic cells (especially DCs), are the main producers of IL-12, with B cells also capable of IL-12 secretion (Trinchieri, 1995). The major function of IL-12 is to induce IFNγ production from CD4+ T cells and to favour the Th1 response in the early stages of selection, promoting the production and proliferation of Th1 cells (Trinchieri, 1995). Th1 cells secrete IFNγ and TNFα in order to activate macrophages and cytotoxic T cells to destroy intracellular pathogens, as well as stimulating B cells to produce appropriate antibodies (Alberts et al., 2002).

Kim and co-workers measured serum and synovial fluid levels of IL-12p70 in 152 RA patients (as well as 69 OA patients) and 50 healthy controls (Kim et al., 2000). A subgroup of 64 RA patients had detectable serum concentrations of IL-12p70, whereas only 5 healthy controls had detectable serum concentrations of the cytokine (P<0.001). When measuring synovial fluid IL-12p70, the number of RA patients with detectable IL-12p70 was again significantly higher than healthy controls (Kim et al., 2000). In the study by Kim and colleagues, patients with elevated concentrations of IL-12p70 also had raised levels of IL-2, IL-6 and TNF (Kim et al., 2000). It has also been reported that serum concentrations of IL-12p40 (one of the subunits of IL-12p70) are significantly elevated in RA compared to healthy controls (Szodoray et al., 2006). This combined evidence indicates a role for IL-12 in RA disease pathogenesis.

1.5.8 IL-10

IL-10 is generally considered as having an anti-inflammatory function in the immune system, by inhibiting the production of pro-inflammatory cytokines IL-1β, TNFα and IL-8 by macrophages and leukocytes (Fiorentino et al., 1991, Cassatella et al., 1993). In RA it has been shown that IL-10 also has an anti-inflammatory role by down-regulating production of IL-1β and TNF by synovial fluid mononuclear cells (Isomaki et al., 1996). Studies have shown that concentrations of IL-10 are raised in RA patients compared to healthy controls (Perry et al., 2009, Szodoray et al., 2006), possibly to suppress rising concentrations of potentially damaging pro-inflammatory cytokines.
1.5.9 Other cytokines involved in RA

IL-23 is produced by macrophages, dendritic cells (DCs) and natural killer (NK) cells and was once thought to promote Th17 cell expansion and IL-17 secretion (Aggarwal et al., 2003). Studies have since shown that IL-23 alone does not promote these activities (Betteli et al., 2006). The generation of IL-17-secreting pathogenic Th17 cells does, however, require stimulation from IL-23 along with IL-6 and TGF-β1 in order to produce TGF-β3, which then (along with IL-6) further drives the development of pathogenic Th17 cells (Jin and Dong, 2013). IL-23 is also thought to stabilise the Th17 phenotype, stopping the conversion of Th17 cells to a Th1 phenotype (Taherian et al., 2014). An increased expression of IL-23p19 mRNA has been observed in RA synovial fibroblasts, as well as an increase in serum IL-23 (Kim et al., 2007) indicating a role for this cytokine in RA disease development.

The cytokine IL-18, which is closely related to IL-1α and IL-1β, is raised in the serum of RA patients and is thought to play a pathophysiological role in RA by inducing various pro-inflammatory cytokines (Figure 2) (Bresnihan et al., 2002). Recently, a gene polymorphism in the IL-18 gene was confirmed as a risk factor for RA (Wen et al., 2014). The recently discovered cytokine IL-32 has also been found to be highly-expressed in the synovium in RA (but not osteoarthritis or healthy controls) after synovial tissue biopsies were performed on these patients (Joosten et al., 2006). IL-32 is also thought to promote secretion of IL-6, IL-1, TNFα and IL-17, acting upon the monocytes, dendritic cells, macrophages and T cells (Figure 2) (Xu et al., 2013). IL-26 has also been identified as a crucial mediator of rheumatoid arthritis as another initiator of this typical inflammatory cytokine cascade (Corvaisier et al., 2012). IL-26 is constitutively produced by synoviocytes and stimulates monocytes to produce inflammatory cytokines, however it also favours the generation of pathogenic Th17 cells (Corvaisier et al., 2012), making it yet another cytokine involved in this complex dysfunction of the immune system.
1.6 Cytokines involved in PMR

Whilst cytokine profiles for RA have been well studied, those for PMR have not. Only a handful of studies have looked at elements of the circulating PMR cytokine profile, the results of which are discussed here.

Studies have shown that IL-10 concentrations in the serum of PMR patients are raised and correlate negatively with the severity of symptoms, indicating an ameliorative role for IL-10 much like in RA (Straub et al., 1999, Alvarez-Rodriguez et al., 2010). In two studies, serum concentrations of IL-12p70 were suppressed in PMR compared to healthy controls (Szodoray et al., 2006, Alvarez-Rodriguez et al., 2010). Alvarez-Rodriguez and co-workers also showed that levels of the inflammatory cytokines IL-1β and TNFα in the serum of PMR patients were not elevated above healthy controls (Alvarez-Rodriguez et al., 2010), indicating that RA and PMR pathogenesis are very different, despite sharing clinical symptoms and raised circulating IL-6. Similarly, cytokines such as IL-17, IL-8, IL-1β, IL-4 and TNFα, which are all elevated in RA and most likely to be produced in the synovium, may not be elevated in PMR, which does not appear to have similar levels of joint involvement. Recently, however, Samson and colleagues showed that Th17 cell numbers are increased in PMR and GCA patients, indicating a potential role for IL-17 in the development of these diseases (Samson et al., 2012). A conflicting report by van der Geest and colleagues proposes that there are no clear alterations in Th subsets or regulatory T cells in PMR patients and thus IL-17 may not be elevated at all (van der Geest et al., 2012).

Evidently, the circulating cytokine profile of PMR patients is not well studied, and requires further research in order to address this lack of knowledge of the cytokines involved in PMR pathogenesis. Hence, one of the aims of this study is to characterise the serum/plasma cytokine profile of PMR patients and directly compare this with RA patients and healthy controls.
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1.7 The crucial role of interleukin-6 in RA and PMR

The cytokine interleukin-6 (IL-6) has long been highlighted as a major contributor to the pathophysiology of both PMR and RA, by promoting synovitis and joint destruction in RA as well as controlling many of the systemic symptoms of both PMR and RA (Srirangan and Choy, 2010). Originally, IL-6 was known as B cell stimulatory factor. It was identified as a T cell line-derived interleukin which stimulated B cell differentiation and maturation (Andersson and Matsuda, 1989). Since then, however, its pleiotropic capabilities have been identified and its name changed to interleukin-6 (IL-6). IL-6 can be synthesised by a range of different cell types, such as neutrophils and eosinophils (Melani et al., 1993), fibroblasts (Cichy et al., 1996), synoviocytes (Guerne et al., 1989), endothelial cells (Dasgupta and Panayi, 1990), monocytes (Roche et al., 1993), macrophages (Martin and Dorf, 1991), T cells (Andersson and Matsuda, 1989), B cells (Barr et al., 2012), DCs (Jego et al., 2003, Leech et al. 2013) and myoblast cells (De Rossi et al., 2000), amongst others.

One of the key roles of IL-6 is to stimulate the acute phase response during the early stages of inflammation by inducing the expression of the acute phase genes (Kishimoto et al., 1995). This systemic response involves the onset of fever, leukocytosis, and the synthesis of acute-phase proteins by hepatocytes in the liver (Heinrich et al., 1990). IL-6 is also thought to play a possible role in the shift from acute to chronic inflammation, by changing the nature of the leukocyte infiltrate at the site of inflammation from neutrophils to monocytes (i.e. the mononuclear shift) (Gabay, 2006). Under normal circumstances, the action of IL-6 is thought to resolve inflammation by removing neutrophils from the inflammatory site and encouraging the recruitment of monocytes and macrophages in order to accomplish an effective and complete immune response (Gabay, 2006). IL-6 also has the potential to exert an anti-inflammatory effect. This is demonstrated by studies looking at the increased production of IL-6 in interstitial muscle during exercise, where IL-6 is considered to suppress TNFα production by negative feedback and thus dampen the inflammatory response (Starkie et al., 2003, Rosendal et al., 2005). Furthermore, IL-6 has recently been suggested to have a crucial role in anti-viral immunity by limiting pathological inflammation in the lung following exposure to influenza (Lauder et al., 2013).
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IL-6 signals principally through the membrane-bound IL-6 receptor which is present on hepatocytes, neutrophils, monocytes, macrophages and some lymphocytes (Rose-John et al., 2006). IL-6 signalling, however, also requires two sub-units of a signal transducing receptor component called glycoprotein130 (gp130), which is ubiquitously expressed (Taga and Kishimoto, 1997). This method of signalling is known as the classical signalling pathway (Dayer and Choy, 2010) (Figure 3a).

IL-6, unlike most other cytokines, is also capable of signalling through a soluble form of the IL-6 receptor, which is thought to be released by naïve and memory CD4 T cells (Briso et al., 2008) through proteolytic cleavage of the membrane bound IL-6 receptor protein (Jones et al., 2001) or by alternative mRNA splicing (Horiuchi et al., 1994). Consequently, IL-6 and the soluble IL-6 receptor form a complex which is then able to bind to gp130 (present on most cells) in order to stimulate several types of cells which would not ordinarily be responsive to IL-6 alone (Rose-John et al., 2006). For example, endothelial cells (Romano et al., 1997), fibroblasts (Sporri et al., 1999) and synoviocytes (Dayer and Choy, 2010) do not express the IL-6 receptor, but do express gp130 and are thus able to respond to IL-6 when it is bound to the soluble IL-6 receptor. This alternative method of signalling is known as ‘trans-signalling’ (Rose-John et al., 2006) (Figure 3b).

A soluble form of gp130 also exists and acts as a natural inhibitor to the IL-6/sIL-6R complex thus blocking trans-signalling (Jostock et al., 2001) (Figure 3c). Concentrations of circulating sgp130 are relatively high in human blood (Jones et al., 2001). In a study by Jostock and colleagues it was found that sgp130 only inhibited IL-6 when it was associated with its soluble receptor, and did not inhibit IL-6 alone, nor did it interfere with bound IL-6 on the surface of cells (Jostock et al., 2001) and thus sgp130 provides a method of removing excess circulating IL-6 in order to stop inflammation.
Figure 3: IL-6 signalling: classical and alternative pathways. (a) Classical signalling. IL-6 binds to the membrane-bound IL-6 receptor, in conjunction with two gp130 molecules. Once bound, transcription factors are activated and gene expression changes. (b) Trans-signalling. CD4+ T cells synthesise the soluble IL-6 receptor (sIL-6R). IL-6 binds the sIL-6R to form a complex, which then binds to ubiquitously expressed membrane bound gp130. (c) Soluble gp130 is a natural antagonist to IL-6 which binds the IL-6/sIL-6R complex and selectively inhibits the trans-signalling process. (Diagram adapted from Rose-John et al., 2006. Reprinted with permission).
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When these tightly-controlled immunological responses break down, the function of IL-6 becomes ‘more sinister’ (Jones, 2005). There is growing evidence to suggest that this pleiotropic cytokine plays a crucial role in the development of inflammatory diseases such as PMR and RA, as well as being linked to certain cancers associated with chronic inflammation, such as colon cancer (Grivennikov and Karin, 2011). IL-6 is raised significantly in the serum/plasma of RA and PMR patients, and also in the synovial fluid of RA patients (but not PMR) (Houssiau et al., 1988, Hirano et al., 1988, Madhok et al., 1993 and Perry et al., 2009). Serum levels of IL-6 have been shown to reach up to 234 pg/ml in RA patients (Arvidson et al., 1994, Robak et al., 1998), and up to 400 pg/ml in PMR patients (Kirwan, J., personal communication, 2011), although published values of up to approximately 54 pg/ml in PMR are quoted (Cutolo et al., 2006). This compares to mean IL-6 concentrations of <5 pg/ml in the serum and plasma of healthy individuals (Arvidson et al., 1994, Robak et al., 1998, Straub et al., 2000).

The concentrations of IL-6 measured in the serum and plasma of PMR and RA patients thus varies dramatically within the literature but similar trends are apparent, in that IL-6 concentrations are consistently significantly elevated in PMR and RA patients above that of healthy controls. One issue is that there is no standardised way of measuring cytokines, making meaningful comparisons between laboratories very difficult. Furthermore, some studies measure serum IL-6 concentrations (Arvidson et al., 1994, Robak et al., 1998, Straub et al., 2000, Alvarez-Rodriguez et al., 2010), whilst others measure plasma concentrations (Roche et al., 1993, Perry et al., 2009). As plasma and serum have different constituents this may affect cytokine measurement. Thus one of the aims of this study is to determine whether a correlation exists between plasma and serum concentrations of IL-6 in PMR patients, RA patients and healthy controls.

In both RA and PMR, IL-6 levels fall rapidly in response to treatment with glucocorticoids (GC) (Cutolo et al., 2006, Martinez-Taboada et al., 2008) and the amelioration of patient symptoms strongly correlates with this decline in serum IL-6, confirming a special role for IL-6 in the pathogenesis of PMR and RA (Arvidson et al., 1994). Similarly, when IL-6 signalling mechanisms in RA (and PMR in some cases) are blocked by drugs such as Tocilizumab, the patient goes into remission, again
demonstrating IL-6 is a major contributor to disease pathogenesis of RA. Whilst the biological activity of synovium-derived IL-6 in RA has been confirmed using an IL-6-dependent cell line, known as B9 cells (Guerne et al., 1989), the biological activity of raised circulating IL-6 in PMR has not yet been confirmed. Thus a further aim for this study is to confirm the biological activity of circulating IL-6 in PMR patients in order to establish whether it is capable of causing the symptoms associated with the disease.

1.8 Circadian variation in IL-6 and RA and PMR symptoms

Early morning joint stiffness in RA patients was described over 50 years ago by Scott who noticed a diurnal variation of grip strength, as well as the usual stiffening of the joints and general immobility experienced by RA patients (Scott, 1960). This was later attributed to circadian alterations in immune and inflammatory responses (Harkness et al., 1982). A distinct circadian variation in serum IL-6 levels in RA patients was initially identified by Arvidson and colleagues in 1994, and later confirmed by Crofford and colleagues in 1997 who explored the relationship between these fluctuating levels of IL-6 and changing concentrations of adrenocorticotrophic hormone (ACTH) and cortisol in the plasma of RA patients (Arvidson et al., 1994, Crofford et al., 1997). In more recent years, circadian variation in specific RA symptoms such as pain and fatigue has been directly linked to the fluctuation in levels of IL-6 in the blood during different times of the day, with a peak early in the morning (07:15am) (Perry et al., 2009). No other cytokines that were tested in this study (TNFα, IL-1β, IL-8, IL-4, IL-5, IL-13, IL-10 or IFNγ) were found to fluctuate in the plasma throughout the day in this manner (Perry et al., 2009).

Knowledge of circadian fluctuations in IL-6 levels has some benefit for the patient. It has since been shown that the timing of therapeutic drug application can be optimised to better alleviate early morning stiffness, specifically 02:00am delivery has been found to not only improve symptoms, but it also improves responsiveness to lower GC doses (Spies et al., 2010, Clarke et al., 2011). Additionally, lack of controlling for this circadian variation in IL-6 may also explain differences in cytokine results from
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different studies, and a move towards standardisation of cytokine measurement should include standardising the timing of sampling. Hence, in the present study, all participant blood samples were taken at a fixed specific time in the day (between 9-11am), soon after the peak in circulating IL-6 concentration, but still at a convenient time for participants.

1.9 IL-6 and fatigue

In the last ten years the clinical importance of fatigue in RA and PMR has become better recognised. The recent development of the BRAF-MDQ has allowed clinicians to gain a more comprehensive understanding of the individual aspects of fatigue particularly in patients with RA, but also other inflammatory diseases such as PMR (Nicklin et al., 2010). The BRAF-MDQ assesses the different dimensions of fatigue, such as physical fatigue, the experience of day-to-day living with fatigue, cognitive fatigue (i.e. the ability to concentrate with fatigue), emotional fatigue, as well as the overall impact of fatigue (Appendix A) (Hewlett et al., 2011).

IL-6 has been implicated as a major cause of fatigue, mainly because of the link between fluctuating IL-6 concentrations in the circulation and the changing severity of fatigue symptoms in RA. Two studies in particular have confirmed a link between IL-6 and fatigue in RA (Davis et al., 2008 and Helal et al., 2012), the latter using the BRAF-MDQ in order to assess fatigue. The author of the latter paper, however, looked only at the correlation between IL-6 and overall fatigue, and did not consider that there may be correlation between IL-6 and the individual aspects of fatigue. A study by Späth-Schwalbe and colleagues strongly implicates IL-6 in causing the key symptoms associated with fatigue, as low-dose IL-6 was administered to a group of healthy male subjects who reported fatigue, inactivity and an inability to concentrate for long periods of time (compared to those who were given a placebo) (Späth-Schwalbe et al., 1998). Other studies have highlighted pro-inflammatory cytokines (such as TNFα and IL-1) as a cause of the fatigue experienced by cancer patients (Bower et al., 2002, Bower et al., 2013 and Fung et al., 2013).
IL-6 and other pro-inflammatory cytokines are capable of stimulating the hypothalamo-pituitary-adrenal (HPA) axis, which is thought to be linked to the development of fatigue (Tsigos and Chrousos, 2002 and Sriringan and Choy, 2010). The exact mechanisms involved, however, are yet to be elucidated. Further research into the association between IL-6 and fatigue in RA and PMR is essential to work out exactly how IL-6 is causing fatigue in these diseases and how to reduce this chronic and debilitating symptom. Hence, one of the aims of this study is to determine whether there is correlation between circulating IL-6 concentrations in PMR and RA patients and overall fatigue, as well as the individual aspects of fatigue, as categorised by the BRAF-MDQ, which has not previously been examined.

1.10 Potential sources of IL-6 in RA and PMR

In RA, the synovium is the likely source of IL-6, after cells from the synovial fluid of RA patients were found to express IL-6 mRNA (Hirano et al., 1988). Guerne and co-workers substantiated this theory by detecting higher concentrations of IL-6 in the synovial fluids of RA patients than osteoarthritis (OA) patients, and confirming the biological activity of this synovial-derived IL-6, demonstrating that synoviocytes were in fact a potent source of IL-6 in RA (Guerne et al., 1989). Since then, IL-6 levels of around 5000 pg/ml have been measured in the synovial fluid of the knee in patients with active RA (Perry et al., 2009). By measuring levels of IL-6 in the synovium and in the plasma in parallel, it was hypothesised by Perry and colleagues that in RA, IL-6 is produced initially in the joints, but subsequently diffuses across the synovial membrane into the plasma with a constant rate of transport across the membrane (Perry et al., 2009). This explains why IL-6 concentrations in the serum are much higher in RA patients (up to 234 pg/ml) than in healthy controls (<5 pg/ml) (Arvidson et al., 1994, Robak et al., 1998, Straub et al., 2000).

In PMR patients, serum IL-6 levels have been measured at up to 400 pg/ml (Kirwan, J., personal communication 2011), despite limited joint inflammation. PMR is largely a muscular and systemic inflammatory disease, and there is little evidence of joint or
vascular involvement. This suggests that the source of IL-6 in PMR is unlikely to be the synovium as in RA, but may be derived from peripheral blood mononuclear cells (PBMC) (which includes T cells, B cells, monocytes and DCs). The principal aim of this study is to individually explore the PBMCs as potential sources of elevated circulating IL-6 in PMR.

1.11 Peripheral sources of IL-6

Only two studies have explored IL-6 production in the PBMC of PMR patients, both focusing on the monocytes (Roche et al., 1993, Alvarez-Rodriguez et al., 2010) and/or the T cells (Roche et al., 1993). This section examines the strength of the evidence that suggests that monocytes and T cells are (or are not) the source of IL-6 in PMR, as well as providing evidence that one of the other PBMCs may be a potential source.

1.11.1 Monocytes

Two studies have looked specifically at circulating monocytes as a potential source of elevated circulating concentrations of IL-6 in PMR. Firstly, Roche and colleagues used the polymerase chain reaction (PCR) to demonstrate that peripheral blood derived CD14+ monocytes express IL-6 specific mRNA (Roche et al., 1993). Based on this, the authors conclude that monocytes are the source of IL-6 in PMR. Yet, no difference was observed between PMR patients and controls, and although the monocytes expressed IL-6 mRNA, the authors did not prove that the monocytes can actually secrete IL-6, and thus have no conclusive evidence that the monocytes are the major source of IL-6 in PMR.

In contrast, a more recent study by Alvarez-Rodriguez and co-workers concluded that monocytes are not the source of raised circulating IL-6 in PMR. The authors used intracellular antibody staining and flow cytometry to demonstrate that circulating CD14+ monocytes in PMR patients did not produce greater amounts of pro-
inflammatory cytokines (including IL-6) than circulating monocytes in healthy controls (Alvarez-Rodriguez et al., 2010). They hypothesise that the increased concentrations of IL-6 in PMR are instead produced by another subset of blood cells or at a specific site of inflammation, possibly in the tissue (Alvarez-Rodriguez et al., 2010). Alvarez-Rodriguez and co-workers used intracellular staining and flow cytometry techniques in order to look at monocytes at a single cell level, however, they only presented half of the data which was available to them. The authors only took into account the percentage of monocytes which were stained positively for IL-6, and did not take into consideration the mean fluorescence intensity (MFI) of IL-6 antibody staining. This would have indicated the quantity of IL-6 within the monocytes, and not just the percentage of cells containing it. Furthermore almost 100% of control cells were stimulated making observation of increases during disease unlikely. Thus these assays need repeating, measuring both the percentage of IL-6 positive cells and the MFI of IL-6 antibody staining.

1.11.2 T cells

A clear role for T cells has already been highlighted in the pathogenesis of RA in the synovium, however, there is limited research on the role of T cells in the pathogenesis of PMR. It has been suggested that T cells could be a potential source of this elevated IL-6, as memory CD3+ T cell numbers are increased during active PMR (Dejaco et al, 2010) and a subset of T cells are able to produce IL-6 (Andersson and Matsuda, 1989). Various studies have looked at CD8+ T cells in PMR, with conflicting outcomes. Some say there is a significant reduction in the number of CD8+ T cells in active PMR (Dasgupta et al., 1989, Boiardi et al., 1996) whereas others suggest that CD8+ T cells are not reduced in active PMR (Pountain et al., 1993, Martinez-Toboada et al., 2001).

Roche and colleagues claim that CD4+ T cells do not contribute to IL-6 production in PMR as they could not detect IL-6 mRNA in unstimulated or stimulated cells, instead they concluded that CD14+ monocytes are the ‘exclusive source’ of IL-6 in PMR (Roche et al., 1993). Again, the presence or absence of IL-6 mRNA is not conclusive evidence as to whether cells are capable of producing IL-6, and the stimulant used here
(immobilised anti-CD3 antibody) may not provide an accurate reflection of the situation *in vivo*.

In a recent study, Samson and colleagues (2012) provide evidence that patients with PMR have reduced numbers of Treg and Th1 cells and significantly increased numbers of Th17 cells compared to controls (Samson *et al*., 2012). This suggests that chronic inflammation may be induced or initiated through the release of IL-17 from Th17 cells, triggering the production of IL-6 from other cell types. This study does not, however, provide evidence as to whether T cells contribute to the production of IL-6 and this finding is strongly contested by others (van der Geest *et al*., 2012). Further research is required to confirm if T cells are a potential source of IL-6 in PMR.

### 1.11.3 B cells

B cells are capable of IL-6 secretion. Using experimental autoimmune encephalomyelitis (EAE) mouse models, Barr and colleagues showed that when IL-6-secreting B cells are depleted, autoimmune disease ameliorates- indicating that IL-6 production may be a major pathogenic function of B cells in diseases such as EAE and multiple sclerosis (MS) (Barr *et al*., 2012). Whether this extends to diseases such as PMR (which has a very different pathogenesis to MS) is unknown, however this evidence suggests that B cells are capable of producing IL-6 in large enough quantities to drive autoimmune disease pathogenesis.

It has been shown that Human Immunodefiency Virus (HIV) can induce spontaneous secretion of IL-6 from peripheral B cells, in the presence of IL-4 (Boue *et al*., 1992). The direct involvement of HIV remains unclear but this increased production of IL-6 leads to the large-scale B cell malfunction which HIV patients’ exhibit. This suggests that an infectious agent, such as a virus, could be involved in the pathogenesis of autoimmune diseases such as PMR, perhaps suggesting that a malfunctioning B-cell subset could be the source of spontaneous, elevated concentrations of IL-6 in the serum. Others have also proposed that a link may exist between infection and the onset of PMR (Perfetto *et al*., 2005). With evidence to suggest that B cells are capable of, and are known to produce excessive levels of IL-6 in patients with known infectious
diseases, and with such a crucial role in the onset of RA, it is plausible that B lymphocytes could be the source of elevated concentrations of IL-6 in PMR.

1.11.4 Dendritic cells

DCs may also be a possible source of IL-6 in PMR. There are two principal subsets of DC in humans; plasmacytoid DCs (pDCs) and myeloid DCs (mDCs). pDC are a ‘rare’ type of immature DC, with a capacity to produce large amounts of TNF-α and IFN-α (Lande et al., 2004, Cavanagh et al., 2005). Myeloid DCs are considered important antigen-presenting cells needed for triggering primary T cell responses (Morelli et al., 2001). mDCs are present in normal peripheral blood and RA patients but are found to be significantly elevated in the synovium of RA patients (Thomas et al., 1999). It is thought that the number of DCs correlates directly (pDCs) or inversely (mDCs) with the severity of RA disease depending on the subtype. pDCs (subtype CD123+) have an ameliorative effect on RA symptoms (Jongbloed et al., 2009) whereas circulating mDCs (subtype CD11c+) contribute to RA activity (Richez et al., 2009). A recent study by Leech and co-workers has shown that in models of EAE, early T cell activation was driven by IL-6 which was exclusively derived from DCs (Leech et al., 2013). Nonetheless, overall no studies to date have examined DCs as the source of IL-6 in PMR. It is also possible that viral infection could be the DC activating factor as viral infection can induce release of IL-6 from DCs (Jego et al., 2003).

1.11.5 Endothelium

Activated endothelial cells are capable of producing significant amounts of IL-6 (Dasgupta and Panayi, 1990). Furthermore, IL-6 mRNA has been detected in human dermal microvascular endothelial cells after 24 hours stimulation with LPS and TNFα (Hettmansperger et al., 1992). In a study by Loppnow and colleagues, following incubation with LPS or IL-1β, in vitro endothelial cells produced IL-6 for an extended period of at least 144 hours- which may have had an effect on local immune and inflammatory responses (Loppnow et al., 1989). Thus, endothelial cells are a potential source of elevated concentrations of IL-6 in PMR, but will not be tested in this study due to the invasive and difficult process of taking a blood vessel biopsy.
1.12 Potential sources of IL-6 in the tissue

Generally PMR patients are not thought to suffer severely from synovitis (Kyle et al., 1990). There is limited evidence, however, to suggest that a tissue component such as the synovium, muscle or ligaments could be the source of IL-6 in PMR. Two studies have suggested that a synovitis component is not only present in PMR but is a major feature; causing many of the musculoskeletal symptoms that PMR patients present (Meliconi et al., 1996, Frediani et al., 2002). Meliconi and colleagues state that in their study, ‘synovitis was present in 10 of 12 (83%) untreated PMR patients’, whilst Frediani and colleagues claim that synovitis was present in at least one site in 100% of PMR patients following their sonographic study (Meliconi et al., 1996, Frediani et al., 2002). Bursitis (inflammation of the bursae) is also considered by some to contribute to the pain and discomfort felt in the shoulders and other proximal extremities of PMR patients (Salvarani et al., 1997). In two PMR case studies, radio-labelled glucose (18FDG) was found to accumulate in specific locations within the joints, particularly the ligament attachment sites, leading the authors to believe that inflammation of the ligament attachment sites may be an important element of PMR (Kotani et al., 2011). PMR, however, has significantly less evidence of joint inflammation than RA, despite having equivalent circulating IL-6 levels, making this an unlikely source of IL-6 in PMR.

Kreiner and co-workers suggest that there may also be a muscle component involved in PMR (Kreiner et al., 2010). Increased interstitial levels of inflammatory cytokines IL-6, IL-8, IL-1β were measured in symptomatic muscles of 20 glucocorticoid-naïve PMR patients compared to 20 healthy controls, by microdialysis (Kreiner et al., 2010). The authors suggest that cytokines may be released locally, causing muscle pain in very specific areas where inflammatory cytokine production is high (Kreiner et al., 2010). The study did not, however, perform internal controls for affected muscle and unaffected muscle within individuals. In addition, others have found that human myoblast cells constitutively express IL-6 mRNA and secrete IL-6 (De Rossi et al.,
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2000). Overall, the evidence does not point towards muscular or joint involvement in PMR.

1.13 Methodologies

1.13.1 Measuring circulating IL-6

Previous studies have shown that circulating IL-6 is raised in PMR patients compared to healthy controls using different methods, including enzyme-linked immunosorbent assays (ELISAs) (Roche et al., 1993, Straub et al., 2000, Cutolo et al., 2006) or cytometric bead array (CBA) (Alvarez- Rodriguez et al., 2010). Circulating concentrations of IL-6 have also been measured in either plasma or serum, depending on the study. In order to determine how comparable the results are from these different methods of cytokine measurement and in these different blood components, in the present study both plasma and serum IL-6 were measured by ELISA and CBA. To my knowledge, no previous study has confirmed the biological activity of the raised circulating IL-6 in PMR. Hence, this was also tested using a cell-line based bioassay, with a cell-line known as B9 cells. This cell line is dependent on biologically active IL-6 for metabolism and growth (Helle et al., 1988), and was thus able to establish the biological activity of the circulating IL-6 present in participant samples.

1.13.2 Measuring fatigue vs IL-6

As previous studies have shown evidence for a relationship between IL-6 and fatigue in RA (Davis et al., 2008, Helal et al., 2012), this study set out to determine if a correlation exists between circulating IL-6 concentrations (measured by CBA) and fatigue (measured using the recently-devised BRAF-MDQ) in PMR. For the first time, the correlation coefficients between circulating IL-6 concentrations and individual aspects of fatigue (as determined by the BRAF-MDQ) were determined in order to establish if IL-6 is associated with specific aspects of fatigue in PMR and RA.
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1.13.3 Measuring intracellular IL-6 production in PBMCs

Previous studies have looked at IL-6 production in one or two PBMC types, for example the monocytes (Roche et al., 1993, Alvarez-Rodriguez et al., 2010) or the T cells (Roche et al., 1993) and only one of these studies looked at intracellular IL-6 production at a single cell level (Alvarez-Rodriguez et al., 2010). Neither of these studies managed to locate the source of the elevated circulating IL-6 in PMR. Thus, in order to advance on these studies, the source of raised circulating IL-6 in PMR was explored within the five main PBMC types (T cells, monocytes, B cells, pDC and mDC), using intracellular staining and flow cytometry.

The study by Alvarez-Rodriguez and colleagues looked at IL-6 production in monocytes using similar techniques, however, the authors only presented data showing the percentage of IL-6 producing cells and not the intensity of IL-6 staining within each cell type, i.e. the MFI (Alvarez-Rodriguez et al., 2010). It is currently unclear whether the elevated concentrations of IL-6 may be coming from a large number of one cell type producing a small quantity of IL-6, or whether a small number of one cell type were producing an abnormally large quantity of IL-6. Thus, in order to account for both of these possible outcomes, both the percentage of IL-6-producing cells in each cell type and the intensity of the staining within all five cell types (MFI) were taken into consideration in the present study. Constitutive IL-6 secretion was measured in unstimulated PBMC, whereas the potential for these cells to secrete IL-6 was tested using a specific cell stimulant.

1.13.4 Measuring secreted IL-6 by PBMCs

A single method will not normally provide sufficient evidence to form a positive or negative conclusion, thus the aim was to accumulate data collected using different methods to strengthen the enquiry. Hence, as well as measuring circulating IL-6 concentrations in the plasma and serum, and measuring IL-6 production in individual PBMC types, the PBMC culture supernatants were collected after 24 hours in culture with or without additional stimulation, and secretory IL-6 measured using CBA. This gave an insight into whether large quantities of IL-6 were being secreted into the
extracellular matrix by the PBMC of PMR patients compared to the PBMC of healthy control and RA participants. If secretory IL-6 concentrations were significantly greater in PMR culture supernatants, it would confirm that one of the PBMC types within that culture were the source of spontaneous secretions of IL-6.

Secretary IL-6 from PBMCs of PMR patients has been measured in other studies, however, specific stimuli were used which were capable of activating only individual PBMC types. Roche and colleagues collected PBMC culture supernatants (isolated from PMR, GCA and HC blood samples) at 24, 48 and 72 hours post stimulation with immobilised anti-CD3 antibody (Roche et al., 1993). The concentration of secreted IL-6 in PMR supernatants was not significantly different from the concentration of secreted IL-6 in the healthy control supernatants at each time point (Roche et al., 1993). This contradicts their argument that the monocytes are the source of elevated IL-6 in PMR. The authors defend this finding by suggesting that the monocytes were not properly activated by the T cell specific stimuli (Roche et al., 1993). Therefore this requires re-testing with a stimulant specific for monocytes and the other cell types present in the cultures.

E. coli derived LPS is commonly used as a potent monocyte stimulator. LPS from other organisms can stimulate human B cells (Vaughan et al., 2010), however this has never been reported for E. coli LPS. Alvarez-Rodriguez and colleagues found that after 24 hours stimulation with LPS there was no significant difference between secreted IL-6 concentrations from PBMC in PMR patients and controls (Alvarez-Rodriguez et al., 2010). They do, however suggest that there is ‘a tendency’ for the PBMC in PMR patients to produce larger concentrations of IL-6, despite it not being statistically significant (Alvarez-Rodriguez et al., 2010). Previously, they suggest that monocytes are not the source of raised IL-6 in PMR, which implies that another PBMC type may be responsible for this ‘tendency’ to secrete high IL-6 concentrations in PMR patients.

Thus, these issues need resolving. The PBMC in this study were therefore cultured with stimulants capable of activating monocytes, lymphocytes and DCs in order to determine if any of these PBMC types are responsible for the elevated circulatory IL-6 reported in PMR patients.
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1.13.5 Looking at the circulating cytokine profile of PMR patients

Aside from IL-6, the other cytokines involved in PMR disease pathogenesis are not well characterised. Using CBA analysis, ten cytokines were measured in the serum and plasma of PMR patients, RA patients and healthy controls, in order to confirm which cytokines might be raised alongside IL-6 in PMR, and thus gain a deeper understanding of the pathology of the disease. Secreted cytokines in the PBMC culture supernatants were also measured using CBA, alongside IL-6.

1.14 Strengths of the study

1.14.1 Appropriate controls

Studies have shown that age (O’Mahony et al., 1998) may impact on clinical levels of IL-6 and thus is controlled for in this study. According to O’Mahony and colleagues, in a group of older subjects (62 years and above- mean age 73) there were significant increases in cytokine production in T cells compared with the younger subjects (20-40 years- mean age 29), in particular IL-6 and TNFα (O’Mahoney et al., 1998). It has also been reported that serum concentrations of IL-6 (and sIL-6r in women) increase naturally with age, and older individuals are often in a constant low-grade proinflammatory state, thought to be related to the increased risk of cardiovascular disease (Bruunsgard et al., 2003, Ferruci et al., 2005). It is now generally accepted that immunosenescence occurs as individuals get older, with ‘immune alterations’ evident even in apparently healthy 55-70 year olds (Hodkinson et al., 2006). Thus it was deemed necessary to age-match the healthy controls to the diseased patients as closely as possible in order to observe differences that were not related to immunosenescence.

Various studies have suggested a link exists between sex hormones such as oestrogen, progesterone and dehydroepiandrosterone and cytokine secretion by PBMCs, resulting in differential secretory cytokine profiles in men and women (Verthelyi and Klinman, 2000, Asai et al., 2001), so where possible gender-matched controls were obtained in this study.
One of the major strengths of this study is the inclusion not only of healthy controls of a similar age and gender distribution, but also the inclusion of a second disease control. There were initial concerns that circulating IL-6 in PMR patients might alter the biological activity of circulating PBMCs, confounding the interpretation of investigation results. In order to make allowances for this, patients with active RA were also included in this study as a comparator, as RA patients have similarly raised concentrations of circulating IL-6. Therefore, RA patients served as a control for any underlying general response of the PBMC to circulating IL-6, meaning that any differences in IL-6 production observed in PMR patients were specific to PMR disease pathogenesis and most likely due to abnormally functioning PBMCs. This additional control vastly increases the power of the enquiry.

The inclusion of a positive control cell line allowed the efficacy of the IL-6 antibody staining method to be validated, and was tested alongside each participant.

**1.14.2 Timing of sampling**

Knowing that IL-6 is strongly associated with a circadian fluctuation throughout the day, strict timing of blood sampling was adhered to, in order to control for this natural variation. The peak time for circulating IL-6 concentrations is 7:15am (Perry et al., 2009). Participant blood samples were taken between 9am and 11am, thus IL-6 concentrations would still be high, whilst the timing convenient for participants to visit the clinic to donate their sample.

**1.14.3 Blinding of study samples**

The study was performed blinded, with the disease status of the participant not revealed until after preliminary data analysis, meaning that the investigator could not form a biased interpretation of the data that best fitted the hypotheses. Thus, the power and reliability of the enquiry was further increased.
1.14.4 Optimisation of laboratory techniques

Prior to testing on study participant blood samples, the blood separation technique and in vitro stimulation methods were optimised on healthy donor blood samples at UWE, Bristol. Antibodies used for the detection of both surface markers and intracellular IL-6 were titrated in order to determine the optimal concentrations (i.e. the equivalence point, where both antibody and antigen are at an optimal concentration). The IL-6 antibody was titrated on a positive control cell line (Jurkat T cells), known for its ability to constitutively produce IL-6. Several cell stimulants were tested on healthy PBMC from blood donors at UWE, in order to determine which could stimulate IL-6 production in all five PBMC types. In their study, Roche and colleagues used a T-cell specific stimulus (immobilised anti-CD3 antibody), which was thought to indirectly activate the monocytes (Roche et al., 1993). Here, the T cell mitogens phytohemagglutinin (PHA) (Baran et al., 2001) or phorbol 12-myristate 13-acetate (PMA) and ionomycin (Touraine et al., 1977) were tested, alongside LPS and IFNγ (Hobbs et al., 1991, Verhasselt et al., 1997, Bueno et al., 2001) in order to try and activate the different PBMC types. The stimulation techniques which provided an all-round stimulation of all five PBMC types were selected for use in the assays.
1.15 Hypotheses

Several hypotheses were formed prior to carrying out the study, and are as follows:

1) The concentration of IL-6 will be significantly raised in the serum and plasma of PMR and RA patients compared to healthy controls of a similar age and gender distribution.

2) IL-6 concentrations in stored serum and plasma will demonstrate a positive correlation, but the values will not be exactly equivalent.

3) The IL-6 measured in the serum/plasma of PMR and RA patients will be biologically active, confirming a role for IL-6 in the pathogenesis of these diseases.

4) Using rigorously controlled timing of sampling, and the latest fatigue scoring system (BRAF-MDQ) there will be a strong correlation between all aspects of fatigue and IL-6 concentration in the plasma and serum of PMR and RA patients.

5) Within PBMC cultures, one of the PBMC subsets will be identified as the cellular source of IL-6 in patients with PMR but not RA (which is believed to be synovial-derived) nor healthy controls of a similar age and gender distribution.
1.16 Aims and objectives

Based on these hypotheses, the specific aims and objectives of this study were as follows:

1) To confirm that IL-6 is elevated in the serum and plasma of PMR and RA patients compared to healthy age-matched controls, using cytometric bead array (CBA) and sandwich ELISAs. The samples will be taken at a fixed specific time of day (9-11am) to control for circadian variation in circulating IL-6 levels, and thus to provide an accurate and consistent method of measuring IL-6.

2) To determine if a correlation exists between IL-6 concentrations in the serum and plasma, in order to determine whether these can be used interchangeably to measure cytokine concentrations in the blood, despite the difference in the method of extraction from the blood and the difference in composition.

3) To determine whether the elevated IL-6 in PMR is biologically active and thus capable of causing the symptoms described by PMR patients, using the IL-6-dependent hybridoma cell-line, B9 cells.

4) To determine if there is a correlation between fatigue and IL-6 concentrations in the blood using the latest BRAF-MDQ assessment questionnaire to assess different aspects of fatigue in each subject. As the samples will be taken at a fixed specific time of day (9-11am), this not only controls for circadian variation in circulating IL-6, but may also control for changes in PMR and RA symptoms such as fatigue, and thus makes it possible to examine more robustly whether there is a correlation between the circulating IL-6 concentration and fatigue.
5) To identify the cellular source of elevated circulating IL-6 within the PBMC of PMR patients using immunostaining of cell surface markers and intracellular IL-6, and flow cytometry techniques. RA patients and healthy controls will be tested alongside PMR patients to show specificity to PMR. Both constitutive and inducible IL-6 will be measured in five different cell types within the PBMC (T cells, monocytes, B cells, pDCs and mDCs), in order to observe differential cellular responses between control and disease groups and to determine the cellular potential for IL-6 production. Intracellular staining will be correlated with IL-6 secretion in PBMC cultures.

Additionally, this study aims to address the lack of knowledge of the cytokines involved in PMR pathogenesis, by characterising the serum/plasma cytokine profile for PMR, and comparing it to the serum/plasma cytokine profile of RA patients and healthy control patients.
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1.18 Summary

IL-6 is significantly raised in the bloodstream of PMR patients, implicating it as a major contributor to the pathogenesis of the disease. The significance of this finding is, however, still uncertain, with many questions and theories still requiring answers. Why is IL-6 raised in the circulation of PMR patients? Is this IL-6 biologically active, thus capable of causing the symptoms of PMR? If IL-6 is involved, then which symptoms is it specifically involved with and how? Does IL-6 work alone to cause these symptoms, or are there other cytokines which are involved? And perhaps the most crucial question of all; where does this IL-6 come from?

Despite being the subject of many years of research, the cellular source of IL-6 in PMR and the causative agent of its release are yet to be identified. A few isolated studies have shown limited evidence for the existence of a tissue component; however, collectively there is more evidence to suggest that there may be a peripheral source of IL-6 in PMR. This is demonstrated by the excess serum and plasma IL-6 concentrations (Roche et al., 1993, Straub et al., 2000, Cutolo et al., 2006, Alvarez-Rodriguez et al., 2010) and secretion of high levels of IL-6 in PBMC cultures (Roche et al., 1993). Studies have suggested that this IL-6 may (Roche et al., 1993) or may not (Alvarez-Rodriguez et al., 2010) be monocyte derived, but none have examined the other PBMC cell types. Weaknesses and discrepancies in these two studies have been discussed throughout this section. Here, evidence has been provided which demonstrates that IL-6 may be monocyte, dendritic cell, T cell or B cell derived. Hence, the principle aim of this study is to locate the source of IL-6 within these cell types. Subsequently, the cause of the dysfunction could potentially be unravelled and alternative targets for therapeutic intervention could be identified, reducing the need for harmful steroids.

Overall, this study aims to advance our current understanding of PMR, answering the questions posed in a systematic and exhaustive manner, in order to explore the overall immunopathology underlying polymyalgia rheumatica.
Chapter 2: Materials and Methods
Chapter 2- Materials and Methods

In this chapter the experimental procedures used to test the hypotheses are described in detail. First of all, the procedures used to screen and recruit participants and collect samples are outlined, along with the ethical practices put in place to maintain patient confidentiality and to abide by the rules of the Human Tissue Act (2004). Following this, the laboratory procedures are described in detail, including techniques used to culture cell lines and primary cells, the methods used to transport, separate and store blood samples, methods used to measure cytokines in serum, plasma and PBMC culture supernatants (CBA and/or ELISA), methods used to test the bioactivity of IL-6 in patient plasma samples (B9 cell proliferation assay) and the methods used for the detection and measurement of IL-6 at a single cell level within the PBMCs (using intracellular antibody staining and flow cytometry). Each of these methods required some level of optimisation, the methods for which are described prior to the description of each laboratory procedure. Finally, statistical methods are described at the end of the chapter.

2.1 Collection of blood samples

2.1.1 Recruitment and screening of study participants

Study participants were recruited at the University Bristol Hospitals, NHS Foundation Trust (UBHT), Rheumatology Centre. Those people with signs and symptoms of active PMR and RA and who fulfilled the inclusion criteria but not the exclusion criteria (Table 3 and 4) were provided with an information sheet about the study (Appendix B). Patients were given 24 hours to decide whether they would like to take part. Arrangements were then made to bring them back to the clinic the following day to complete the necessary screening and consent forms and provide a blood sample (Appendix A and B). Control participants were also recruited within the hospital setting. A blood test was used to confirm if control participants were suitable for the study, alongside the inclusion and exclusion criteria (Table 5).
Table 3: PMR patient screening: inclusion and exclusion criteria.

Inclusion criteria:

- Is the patient above 55 but below 85 years old?
- Does the patient meet 3 or more of the Bird criteria?

**Bird Criteria (Bird et al., 1979):**

- Bilateral shoulder pain/stiffness
- Duration of symptoms <2 weeks
- Initial ESR >40 mm/h (PV>1.72, CRP>5)
- Stiffness >1 hour
- Age >65 years
- Depression and/or weight loss
- Bilateral upper arm tenderness
- Currently active disease (CRP≥5, PV≥1.72 or ESR≥29)?

Exclusion criteria:

- On glucocorticoids (oral or parenteral) or biologic treatment in the last month?
- Inflammatory diseases such as inflammatory bowel disease, colitis, asthma?
- Giant cell arteritis?
- Other auto-immune diseases?
- Cancer?
- Infections, treatment with antibiotics within the past 6 weeks?
- Significant renal disease (creatinine >150 μmol/L and/or eGFR < 30 ml/min)?
- Significant hepatic impairment?
- Participation in a clinical trial within the past 30 days?
- Pregnancy and lactation?
- Working shift employee?
- Jet lag?
Table 4: RA patient screening: inclusion and exclusion criteria.

### Inclusion criteria:
- Is the patient above 55 but below 85 years old?
- Does the patient meet 4 or more of the ACR criteria?

**ACR Criteria (Arnett et al., 1988):**

- *Morning stiffness (≥1 hour)*
- *Swelling (soft tissue) of three or more joints*
- *Swelling (soft tissue) of hand joints (PIP, MCP, or wrist)*
- *Symmetrical swelling (soft tissue)*
- *Subcutaneous nodules*
- *Serum rheumatoid factor*
- *Erosions and/or periarticular osteopenia in hand or wrist joints seen on radiograph*

- Currently active disease (CRP≥5, PV≥1.72 or ESR≥29)?
- Are there at least 3 tender AND swollen joints?
- Is morning stiffness ≥ 45 minutes?

### Exclusion criteria:
- On glucocorticoids (oral or parenteral) or biologic treatment in the last month?
- Inflammatory diseases such as inflammatory bowel disease, colitis, asthma?
- Co-existent giant cell arteritis?
- Other auto-immune diseases?
- Cancer?
- Infections, treatment with antibiotics within the past 6 weeks?
- Significant renal disease (creatinine >150 μmol/L and/or eGFR < 30 ml/min)?
- Significant hepatic impairment?
- Participation in a clinical trial within the past 30 days?
- Pregnancy and lactation?
- Working shift employee?
- Jet lag?
Table 5: Healthy control screening: inclusion and exclusion criteria.

<table>
<thead>
<tr>
<th>Inclusion criteria:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Is the participant above 55 but below 85 years old?</td>
<td></td>
</tr>
<tr>
<td>Is there any inflammatory rheumatological illness?</td>
<td></td>
</tr>
<tr>
<td>Is CRP &lt; 5?</td>
<td></td>
</tr>
<tr>
<td>Is PV &lt; 1.75?</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Exclusion criteria:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>On glucocorticoids (oral or parenteral) or biologic treatment in the last month?</td>
<td></td>
</tr>
<tr>
<td>Inflammatory diseases such as inflammatory bowel disease, colitis, asthma?</td>
<td></td>
</tr>
<tr>
<td>Giant cell arteritis?</td>
<td></td>
</tr>
<tr>
<td>Other auto-immune diseases?</td>
<td></td>
</tr>
<tr>
<td>Cancer?</td>
<td></td>
</tr>
<tr>
<td>Infections, treatment with antibiotics within the past 6 weeks?</td>
<td></td>
</tr>
<tr>
<td>Significant renal disease (creatinine &gt;150 μmol/L and/or eGFR &lt; 30 ml/min)?</td>
<td></td>
</tr>
<tr>
<td>Significant hepatic impairment?</td>
<td></td>
</tr>
<tr>
<td>Participation in a clinical trial within the past 30 days?</td>
<td></td>
</tr>
<tr>
<td>Pregnancy and lactation?</td>
<td></td>
</tr>
<tr>
<td>Working shift employee?</td>
<td></td>
</tr>
<tr>
<td>Jet lag?</td>
<td></td>
</tr>
</tbody>
</table>

2.1.2 Sample collection and ethical approval

At the rheumatology clinic at UBHT, approximately 35 ml peripheral blood (PB) was taken from adults recently diagnosed with either PMR (n=7) or RA (n=6) who fitted the criteria outlined in the previous section. PB samples (approximately 35ml) were also taken from participants of a similar age and gender distribution (n=7) who did not have an inflammatory condition. C-reactive protein (CRP) and plasma viscosity (PV) levels were tested routinely at UBHT for PMR and RA patients in order to confirm the
diagnosis. A separate 5 ml blood sample was collected from healthy control subjects in order to similarly measure their CRP levels and thus confirm a lack of inflammatory disease.

To account for the fact that pronounced circadian variation of IL-6 occurs in both RA and PMR patients (Crofford et al., 1997, Perry et al., 2009), all blood samples were taken between 09:00am and 11:00am when IL-6 levels were still high. By rigorously controlling the timing of sampling, this allowed direct comparison of patient samples and provided a more accurate and consistent way of measuring IL-6 concentrations in the blood. Furthermore, as low intensity exercise has been reported to elevate cytokine IL-6 levels (Rosendal et al., 2005), participants were encouraged to arrive by pre-paid taxi when donating blood for the study.

The collection of PB complied with the relevant guidelines and institutional and ethical practices, as approved by the NRES Committee South West - Frenchay (formerly South West 5) (reference number 11/H0107/5) and local Research and Development department at UBHT (reference number ME/2011/3679). The samples were made anonymous by the research doctor at the clinic by the use of a coding system, prior to being transported to UWE by the researcher. This allowed unbiased blind testing of samples and ensured patient confidentiality.

To generate patient serum, an extra 4 ml of blood was collected into a plastic tube coated in 'silica act clot activator' (BD Biosciences). This was left at room temperature to clot for a minimum of one hour. The tube was then placed in a centrifuge (Capricorn, Hampshire) for ten minutes at 1600 g to ensure the complete separation of the serum from the rest of the blood. The top layer of serum was then removed using a plastic Pasteur pipette and stored in 1 or 2 aliquots of approximately 1ml in Eppendorf tubes. These aliquots were then frozen at -80°C within the hour (where possible). The remaining blood components were destroyed.

Simultaneously, 35 ml of blood was taken separately in glass tubes containing the anticoagulant sodium heparin (BD Biosciences) for transport to UWE. PBMC and plasma were separated as detailed below. In accordance with the Human Tissue Act,
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cells were in culture for no longer than one week, and all remaining sample constituents were destroyed at the end of the study. All samples taken to UWE were recorded in an electronic tissue ‘ledger’ in order to log the whereabouts of the human tissue sample at all times.

In order to optimise dual staining for surface markers and intracellular IL-6, 10-35 ml samples of peripheral blood (PB) were also taken from healthy donors at UWE. The collection of PB complied with guidelines and ethical practices set in place by UWE, as approved by the ethics committee and under the existing NRES approval. In compliance with the Human Tissue Act, the blood donors remained anonymous to the researcher, and again an electronic tissue ‘ledger’ was used to log the whereabouts of the human tissue sample at all times. Samples were rendered acellular (destroyed by autoclaving) within 5 days of extraction.

2.2 Materials

Unless otherwise stated, all chemicals and reagents were supplied by Sigma Aldrich (Dorset, UK), all plastics by Corning (The Netherlands) and all antibodies are listed in Table 6.
Table 6: List of monoclonal antibodies used in the flow cytometry assays. CD: cluster of differentiation, FITC: Fluorescein isothiocyanate, FL: fluorescence, PE: Phycoerythrin.

<table>
<thead>
<tr>
<th>Monoclonal Antibodies (mAbs)</th>
<th>Clone</th>
<th>Conjugate</th>
<th>Detection channel (FL)</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human CD3</td>
<td>UCHT1</td>
<td>PE</td>
<td>FL2</td>
<td>Ebioscience, San Diego.</td>
</tr>
<tr>
<td>Anti-human CD14</td>
<td>61D3</td>
<td>PE</td>
<td>FL2</td>
<td>Ebioscience, San Diego.</td>
</tr>
<tr>
<td>Anti-human CD19</td>
<td>HIB19</td>
<td>PE</td>
<td>FL2</td>
<td>Ebioscience, San Diego.</td>
</tr>
<tr>
<td>Anti-human CD123</td>
<td>6H6</td>
<td>PE</td>
<td>FL2</td>
<td>Ebioscience, San Diego.</td>
</tr>
<tr>
<td>Anti-human CD11c</td>
<td>3.9</td>
<td>PE</td>
<td>FL2</td>
<td>Ebioscience, San Diego.</td>
</tr>
<tr>
<td>Mouse IgG1 k Isotype control</td>
<td>P3.6.2.8.1</td>
<td>PE</td>
<td>FL2</td>
<td>Ebioscience, San Diego.</td>
</tr>
<tr>
<td>Anti-human CD45</td>
<td>-</td>
<td>FITC</td>
<td>FL1</td>
<td>Dako, Cambridge.</td>
</tr>
<tr>
<td>Anti-human IL-6</td>
<td>MQ2-13A5</td>
<td>FITC</td>
<td>FL1</td>
<td>BD Biosciences, Oxford.</td>
</tr>
<tr>
<td>Rat IgG1 k isotype control</td>
<td>R3-34</td>
<td>FITC</td>
<td>FL1</td>
<td>BD Biosciences, Oxford.</td>
</tr>
<tr>
<td>Fixable viability dye</td>
<td>-</td>
<td>Efluor780</td>
<td>FL4</td>
<td>Ebioscience, San Diego.</td>
</tr>
</tbody>
</table>

2.3 Cell culture, cell lines, primary cells and growth conditions

2.3.1 Culture and maintenance of B9 cells

B9 cells (ECCAC no.12121201) were maintained according to instructions provided by Leiniz-Institut Deutsche Sammlung Mikroorganism und Zellkulturen GmbH (DSMZ) (Brunswick, Germany) at purchase. B9 cells are a murine hybridoma B cell line, which are dependent on IL-6 for proliferation (Diamant et al., 1994). Cells were cultured at 5% CO₂ at 37°C in RPMI 1640 supplemented with: 2 mM L-glutamine (Lonza), 50 µM 2-mercaptoethanol, 50 pg/ml recombinant human IL-6 and 20% foetal...
bovine serum (FBS) (PAA). Cells were cultured in 24-well plates for approximately two to three days before being counted and re-seeded at 0.5 \( \times 10^6 \) cells/ml in complete media. Human recombinant IL-6 was added at 50 pg/ml every 1-2 days for the first week after the initial seeding to account for the higher metabolic rate of the cells (DSMZ, 2013), then every 2-3 days subsequently. B9 cells were starved of IL-6 for 48 hours before the cell proliferation assays, where approximately 5000 cells were added to each well of a 96-well plate.

2.3.2 Culture and maintenance of Mono Mac 6 cells

Mono Mac 6 (DSMZ cat no.ACC124), a human cell line which has many similar characteristics to mature blood monocytes (Ziegler-Heitbrock et al., 1994), were routinely grown in 5% CO\(_2\) at 37°C in RPMI 1640 supplemented with: 2 mM L-glutamine (Lonza); 200 U/ml penicillin; 200 µg/ml streptomycin (Invitrogen); 1 mM sodium pyruvate (Lonza); 1% non-essential amino acids (Lonza) and 10% heat-inactivated FBS (PAA). Cells were cultured in T75 tissue culture flasks for approximately three to four days before being counted and re-seeded at 2\( \times 10^5 \) cells in 20 ml complete media (as detailed above), or adjusted to 1\( \times 10^6 \) cells in 1 ml complete media and cultured overnight in non-stick polypropylene round-bottom tubes (BD Falcon). These cells were used in the flow cytometry assays as a positive control due to their reported ability to produce IL-6 when stimulated (Neustock et al., 1993).

2.3.3 Culture and maintenance of Jurkat cells

Jurkat E6.1 (ECCAC no.88042803), a human leukemic T cell lymphoblast cell line, were maintained at 5% CO\(_2\) at 37°C in RPMI 1640 supplemented with: 2 mM L-glutamine (Lonza), 200 U/ml penicillin, 200 µg/ml streptomycin (Invitrogen), and 10% foetal bovine serum (PAA). Cells were cultured in T75 tissue culture flasks for approximately two to three days before being counted and re-seeded at 2 \( \times 10^6 \) cells in 20 ml complete media. When needed cells were adjusted to 1\( \times 10^6 \) cells in 1ml complete media and cultured overnight in non-stick polypropylene round-bottom tubes for use in the flow cytometry assays as a positive control due to their ability to constitutively produce IL-6 (Khalaf et al., 2010).
2.3.4 Isolation and culture of PBMCs

Within 0.5 to 2 hours after sample collection, the PBMCs were separated from whole blood on Histopaque-1077® by density gradient centrifugation (Hettich Universal 320, USA) for 30 minutes at 400 g, room temperature (Hofman et al., 1982). Slow acceleration and slow deceleration were used in order to aid the gentle separation of the different blood components.

The overlying plasma (Figure 4) was removed using a Pasteur pipette and separated into aliquots. A minimum of four 2 ml cryovials (depending on the amount of plasma in the sample) were collected and stored at -80°C until ready for testing by IL-6 sandwich ELISA, CBA analysis and in the cell proliferation assays. Using a Pasteur pipette, the buffy coat layer of PBMC (Figure 4) was then transferred to a sterile 50 ml conical centrifuge tube.

The buffy coat layer was diluted in 40 ml RPMI 1640 and centrifuged at 400 g for 5 minutes at room temperature with moderate acceleration and deceleration to prevent the PBMC from getting stuck to the bottom of the tube. The supernatant was then discarded and the cells washed again in 40 ml RPMI 1640 and centrifuged at 400 g for 5 minutes at room temperature with moderate acceleration and deceleration. The supernatant was discarded and PBMC were re-suspended in 1 ml of complete RPMI 1640 containing 100 U/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen), 4 mM L-glutamine (Lonza) and 10 mM HEPES (Lonza). The cells were counted using a haemocytometer and viable cells were resuspended in complete media at a final concentration of 1x10^6 cells/ml ready for culture. When in culture, PBMCs were maintained at 5% CO₂, at 37°C.
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2.4 Cytometric Bead Array (CBA) analysis of cytokines in plasma, serum and culture supernatant samples

The cytometric bead array (CBA) human inflammatory cytokines kit and Th1/Th2/Th17 cytokines kit (BD Biosciences) were used in order to quantify a range of different cytokines including IL-6, IL-8, IL-10, TNF, IL-1β, IL-17, IFNγ, IL-2, IL-4, and IL-12p70 in plasma, serum and culture supernatant samples taken from the study participants, which had been frozen at -80°C for use in these assays.

2.4.1 Principles of CBA

CBA is a method of measuring particular analytes within a sample, using capture beads with unique fluorescence intensity characterisitics (BD Biosciences, 2010). Each bead was labelled with a different intensity of fluorochrome, designed to be resolved in
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the red channel (FL4), and then coated in capture antibodies specific to one cytokine (Figure 5). The participant sample was added, and cytokines with the correct specificity bound to antibodies coating the beads. Antibodies conjugated to a different fluorochrome, PE (resolved in channel FL2), were used to label the captured sample, forming sandwich complexes (antibody+antigen+antibody) (BD Biosciences, 2010). The brightness of the PE corresponded to the quantity of cytokine in the sample.

Figure 5: The principles of cytometric bead array (CBA). Beads with discrete fluorescence characteristics were coated in different capture antibodies. Cytokines in the sample with the correct specificity bound to the capture antibody. Captured cytokines were detected using antibody conjugated to fluorochrome (PE). Samples were then analysed using flow cytometry techniques. (Courtesy and © Becton, Dickinson and Company Reprinted with Permission)

The data was analysed using a flow cytometer, with either 6 or 7 bead arrays simultaneously resolved in channel FL4 (Figure 6a) and the fluorescence intensity corresponding to the amount of bound cytokine resolved in channel FL2 (Figure 6b). Five-polynomial standard curves were constructed, using a range of known concentrations of each cytokine, allowing the quantification of cytokines in participant samples.

Two different kits were used to measure the 10 cytokines of interest, with each kit measuring 6 or 7 cytokines simultaneously, though 3 of the cytokines were measured in both kits. The kits were used according to manufacturer’s instructions (BD Biosciences) and are now described in detail.
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Figure 6: Typical CBA plot from an inflammatory cytokine kit (BD Biosciences). Plot (a) demonstrates the negative control in which no sample and thus no cytokines were added. Plot (b) demonstrates an example of a stimulated PBMC culture supernatant sample with varying levels of cytokines. Each bead corresponds to a different cytokine.

2.4.2 Methods for CBA using the inflammatory cytokine kit

All lyophilized standards were transferred to a 25 ml centrifuge tube and reconstituted together in 2 ml assay diluent (a buffered protein solution provided in kit by BD Biosciences). The tube was gently agitated and then left to equilibrate for 15 minutes at room temperature before being gently mixed using a pipette. A set of serial 2-fold dilutions were then set up by adding 300 μl of the top standard to 300 μl assay diluent, gently mixing with a Gilson pipette and then removing 300 μl, which was then added to the next tube containing 300 μl assay diluent. Doubling dilutions continued until standards ranged from 5000 pg/ml to 2.5 pg/ml. A negative control containing only assay diluent was also prepared to serve as the 0 pg/ml standard.

To mix the cytokine capture beads, each tube containing the capture bead suspensions was vortexed vigorously for 3 to 5 seconds before use. Aliquots of 10 μl of each bead
mixture (per assay tube) were added to a single tube (for example, 30 assay tubes x 10 μl = 300 μl of each bead mixture). The tube containing all the aliquots was then mixed thoroughly by vortexing vigorously for 10 seconds.

The mixed capture beads were then centrifuged at 200 g for 2.5 minutes, and the supernatant carefully aspirated and discarded using a pipette. The mixed capture bead pellet was resuspended in ‘serum enhancement buffer’ (equal to the volume of liquid previously discarded) and vortexed thoroughly for 10 seconds to ensure that the pellet was thoroughly resuspended. The serum enhancement buffer was a protein solution provided by BD Biosciences which had the function of blocking endogenous human anti-mouse antibodies in the patient samples of plasma and serum that could potentially react with antibodies used in the assay, giving false-positive results. The mixed capture beads were then incubated for 30 minutes at RT, protected from the light to reduce fluorescence quenching.

The capture beads were mixed using the vortex and 50 μl of the bead suspension was added to all assay tubes. Next, 50 μl of the human inflammatory cytokine standard dilutions and negative control (assay diluent only) were added to the appropriate standard tubes. The unknown samples were added at a volume of 50 μl to the remaining tubes. The samples were incubated for 1.5 hours at RT, and protected from light.

Following the incubation, 1 ml of wash buffer (a PBS solution containing protein detergent, BD Biosciences) was added to each assay tube which were then centrifuged at 200 g for 2.5 minutes. The supernatant was carefully aspirated and discarded, leaving approximately 100 μl liquid in each assay tube. To this, 50 μl human inflammatory cytokine PE detection reagent was added. The tubes were then gently agitated to resuspend the pellet. The assay tubes were incubated for a further 1.5 hours at RT, protected from light, again to reduce quenching.

At this point, 1 ml of wash buffer was added to each assay tube and the tubes were centrifuged at 200 g for 2.5 minutes. The supernatant was then carefully aspirated and discarded from each assay tube. The bead pellet was resuspended in 200 μl 1%
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paraformaldehyde to kill any harmful bacteria or viruses that may be present in the biological samples, making them safe to analyse by flow cytometry. The samples were incubated at RT for 10 minutes. Following this, 800 μl wash buffer was added to each tube to dilute the paraformaldehyde, and the tubes were centrifuged at 200 g for 2.5 minutes. The supernatant was discarded appropriately and the pellet resuspended once more in 300 μl of wash buffer per assay tube, ready for acquisition.

Samples were acquired on an Accuri C6 flow cytometer, set to collect 2100 events on a medium flow rate to reduce the chance of missing dimly stained particles (BD Biosciences, 2010). Results were analysed using FCAP Array software (SoftFlow Inc., Hungary). Five parameter logistic (5PL) standard curves were constructed as recommended by BD Biosciences and cytokine concentrations were extrapolated accordingly. The theoretical limits of detection for the kits were as follows; IL-6: 2.5 pg/ml, IL-8: 3.6 pg/ml, IL-10: 4.5 pg/ml, IL-4: 4.9 pg/ml, TNFα: 3.8 pg/ml, IFNγ: 3.7 pg/ml, IL-2: 2.6 pg/ml, IL-12p70: 1.9 pg/ml, IL-17A: 18.9 pg/ml and IL-1β: 7.2 pg/ml.

2.4.3 Methods for CBA using the Th1/Th2/Th17 cytokine kit

The same methods were used as for the inflammatory cytokines except for the following difference:

- When plasma/serum or supernatant samples were added to the bead mixture, 50 μl human Th1/Th2/Th17 cytokine PE detection reagent was also added at the same time. These tubes were then incubated for 3 hours at RT, protected from light to reduce fluorescence quenching.
- Following this, the samples were washed, fixed and analysed as previously described for the inflammatory cytokines kit.

CBA was used to assess cytokine concentrations in serum, plasma, and PBMC culture supernatants. Due to the cost of the kits and limited volumes of sample, the CBA was run twice for the plasma samples (2x inflammatory cytokines kit and 2x Th1/Th2/Th17 cytokines kit) and only once for the serum and culture supernatants using each kit. As both kits measured TNF, IL-6 and IL-10, these three cytokines were measured four times in the plasma, and twice in the serum and culture supernatants. A geometric
mean was taken (of either two repeats or four repeats) which took into account the fact that the sensitivities of both kits differed slightly. Each value was logged (to the base 10), the mean calculated and then inverse $\text{LOG}_{10}$ was applied to the values.

2.5 Using sandwich enzyme-linked immunosorbent assays (ELISAs) to measure secreted analytes in serum, plasma and PBMC culture supernatants

The human IL-6 sandwich enzyme-linked immunosorbent assays (ELISAs) (R&D Systems) were performed in order to quantify the amount of IL-6 in the plasma, serum and PBMC culture supernatants from study participants 1-19 and the pilot sample. The kit was used according to the manufacturer’s instructions (R&D systems), with a few minor alterations which are detailed below.

A 96-well Maxisorp® immunoplate (Nunc) was coated in 100 µl mouse anti-human capture antibody (2.0 µg/ml) diluted in PBS without carrier protein. Plates were sealed with adhesive strips (R&D systems) and incubated at 4°C for a minimum of 48 hours for optimal binding. The plate was washed three times with 300-400 µl of wash buffer (0.05% Tween 20 in PBS) per well for each wash and incubated for 30 seconds per wash, in order to remove excess unbound antibody. Plates were blotted dry between each wash to ensure that any wash buffer or unbound antibody had been removed from the wells.

Following the wash step, 200 µl of reagent diluent (1% BSA in PBS) was then added to each well for one hour to block non-specific antibody binding prior to the samples being added. Standards and sample dilutions were prepared in reagent diluent during this time. Plasma and serum were not diluted as IL-6 levels were expected to be low and thus within range. Stimulated PBMC supernatants required a dilution of 1 in 20 in reagent diluent in order to fit within the limits of detection. The plate was thoroughly washed again three times with wash buffer (300-400 µl per well) and the samples were added to appropriate wells at 100 µl per well (Figure 7). The plate was then incubated overnight at 4°C to allow the antigen to bind with the immobilised capture antibody.
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The following day the plate was washed 3 times, again with 300-400 μl of wash buffer per well and per wash, to remove excess unbound sample. The detection antibody, biotinylated goat anti-human IL-6, was then diluted to a working concentration of 50 ng/ml in reagent diluent and added to the plate (100 μl per well). The plate was incubated at room temperature (RT) for 2 hours, to allow the secondary antibody to bind to the captured IL-6 antigen.

The plate was then washed again three times using 300-400 μl per well of wash buffer for each wash to remove any excess unbound detection antibody, and 100 μl of streptavidin conjugated horseradish-peroxidase (SHRP) was added to each well for 30 minutes at RT, avoiding exposure to quenching light (Figure 7). The plate was then washed once more with wash buffer (300-400 μl per well for each wash) and 100 μl of substrate solution (H₂O₂ and the chromogen Tetramethylbenzidine) was added to each well. The oxidation reaction between the SHRP and TMB (in the presence of H₂O₂) formed a blue product (Figure 7). The plate was incubated for a further 40 minutes in the dark at RT to allow the enzymatic colour reaction to develop. The reaction was stopped by the addition of 50μl 2NH₂SO₄ which turned the blue to yellow.

The optical density (OD) of the substrate colour change was determined at 450 nm on an Anthos HT11 plate reader (Anthos Labtech, Salzburg Austria). A reading at 540 nm was taken and then subtracted from the reading at 450 nm to correct for optical imperfections in the plate (R&D Systems, 2013). A four polynomial standard curve was generated on GraphPad Prism software, which Herman and colleagues suggest provides ‘more precise and less biased’ estimates for samples measured by sandwich ELISA than the 4-parameter logistic model (Herman et al., 2008). The amount of IL-6 in the samples was quantified by extrapolating values from the graph based on the OD of the sample, giving a final concentration of IL-6 in pg/ml.

Each sample was tested in triplicate, except for the serum ELISAs which were tested in duplicate due to the limited volume of sample. Plasma ELISAs were independently repeated three times, whilst serum was independently repeated twice, again due to limited volumes of the samples. Mean values of these repeats were taken for subsequent analysis.
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Figure 7: The main stages involved in the sandwich ELISA used to quantify IL-6 in the plasma and serum. (a) A 96-well plate is coated with IL-6 capture antibody (Ab) diluted in PBS. Following a wash, the sample or standards are added and IL-6 present in the sample binds to immobilised capture Ab. Materials that do not bind are washed away. (b) IL-6 detection Ab (conjugated to enzyme SHRP) is added, which binds to the captured IL-6. Detection Ab that did not bind is washed away. (c) Addition of the substrate solution (TMB and H$_2$O$_2$) results in the development of a blue colour. Colour development is proportional to the quantity of IL-6 present. The addition of H$_2$SO$_4$ prevents any further reaction and turns the blue colour to yellow. The absorbance is measured at 450 nm. (Courtesy and © R&D Systems Reprinted with Permission)
2.6 Testing the bioactivity of IL-6 in study participant plasma samples using the MTS cell proliferation assay

Whilst immunoassays such as CBA and ELISA are useful for the detection of cytokines in plasma and serum, they only indicate the presence of ‘immunologically reactive’ molecules, and not necessarily ‘biologically active’ molecules (Mire-Sluis and Thorpe, 1998). B cell proliferation assays have previously been conducted using IL-6-dependent B9 cells in order to detect IL-6 production in synoviocytes in RA patients, confirming the biological activity of the IL-6 present in the joints of these patients (Guerne et al., 1989, de Bendetti et al., 1991, Rosenbaum et al., 1992). The biological activity of elevated circulating IL-6 in PMR, however, has not previously been tested.

Thus, in order to test the biological activity of the IL-6 present in the study participant plasma samples, the ability to stimulate proliferation of the IL-6 dependent murine hybridoma cell line, B9 (Helle et al., 1988) was measured using CellTiter96® Aqueous One Solution (Promega). This colorimetric assay was centred around the bioreduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) by viable B9 cells into a coloured soluble formazan product which could be quantified by measuring the absorbance of the culture medium at 450 nm (Promega, 2012). The extent of the colour change was directly proportional to the amount of living cells in culture, and the amount of living cells in culture was directly proportional to the concentration of IL-6 in the samples.

A negative control of RPMI 1640 media containing 2 mM L-glutamine (Lonza) and 50 μM 2-ME was added to the first column (50 μl per well) and top row of a flat-bottomed 96-well plate (Nunc). The recombinant IL-6 was serially diluted (10-fold dilutions) in RPMI 1640 (containing 2 Mm L-glutamine and 50 μM 2-ME), from 50000 pg/ml to 0.5 pg/ml in Eppendorf tubes. The standards were then added in duplicate wells across the bottom two rows of the plate (Nunc) at 50 μl per well.

The plasma samples (from study participants 1-19) to be measured were heat inactivated in order to deactivate complement pathways, by placing the samples in a
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water bath at 56°C for 35 minutes (Madhok et al., 1993). Exactly 48 μl of each sample was added to the wells of a 96-well Maxisorp® immunoplate (Nunc). The samples were added in triplicate, with the first two wells also containing 2 μl of RPMI 1640, and the third well also containing 2 μl of the monoclonal anti-human interleukin 6 neutralising antibody (at a final concentration of 50 ng/ml). This was in order to subtract the levels of background stimulation, caused by other factors present in the plasma samples. The plate was equilibrated at 37°C in a humidified 5% CO₂ atmosphere for one hour so that the antibody had time to bind and neutralise the IL-6 in the sample, while the cells were harvested for use in the assay (Soman et al., 2009).

The B9 cells were washed twice in RPMI 1640 (containing 2 mM L-glutamine and 50 mM 2-ME) by centrifugation (Hettich Universal 320, USA) at 400 g for 5 minutes per wash. The cell number and viability were determined by trypan blue exclusion and cells were resuspended at a final concentration of 1x10⁵ cells/ml viable cells in RPMI 1640 with supplements but no IL-6 or FCS. Following this, 50 μl of the cell suspension (approximately 5000 cells) was dispensed into all wells of the plate, to make a final volume of 100 μl per well. The plate was then incubated for 72 hours at 37°C in a humidified 5% CO₂ atmosphere (Madhok et al., 1993).

The CellTiter96® Aqueous One Solution (Promega) was thawed in a water bath at 37°C for ten minutes. The plate was then collected and cells were mixed gently using a pipette in order to disrupt any clumps of cells. Precisely 20 μl of the MTS stock was pipetted into each well of the 96-well assay plate containing the samples and standards. This was then incubated for 4 hours in a humidified, 5% CO₂ incubator.

A multichannel pipette was used to gently mix the samples three times to ensure homogeneity of the colour and the absorbance was then read at 450 nm using an Anthos HT11 plate reader (Anthos Labtech, Salzburg Austria). The absorbance of the standards at 450 nm was plotted against the concentration of the recombinant IL-6 present and a four-parameter regression curve fitted. The unknown concentrations of biologically active IL-6 in the plasma samples were then interpolated from this standard curve. The mean value of the 2 duplicate wells was calculated for each sample, and the value from the well containing IL-6 neutralising antibody subtracted to
account for the fact that other cytokines and growth factors may be present in the plasma samples. Three independent repeats of the assay were carried out.

Titration of the neutralising antibody was performed prior to the assays to determine the optimal concentration required to neutralise the concentrations of IL-6 expected in the participant samples. For this, a range of concentrations of recombinant IL-6 (0.00025-25 ng/ml) were incubated with a range of concentrations of the IL-6 neutralising antibody (0.000-50 µg/ml). The inhibitory effects of the IL-6 neutralising antibody were observed and the concentration which reduced B9 cell growth for the longest and broadest range was chosen as the concentration to use in the B9 cell proliferation assays.

2.7 Fatigue analysis

At the time of blood sample extraction, patients and healthy controls were asked to complete the BRAF-MDQ, in order to assess levels of fatigue, a prominent feature of both RA and PMR. Each participant scored themselves on four individual aspects of fatigue, namely physical fatigue, fatigue associated with day-to-day living, cognitive fatigue, and emotional fatigue. These scores were then added together to give an overall fatigue score (Appendix A).

The scores for the individual aspects of fatigue and the overall fatigue scores were then plotted against the concentration of IL-6 measured in the plasma and serum by CBA to determine if there was any significant correlation between them. The correlation between various clinical measures of PMR and RA disease (such as CRP and PV levels) and the overall and individual aspects of fatigue was also investigated.

In order to determine whether other pro-inflammatory cytokines may be involved in fatigue, the correlation coefficients between plasma and serum concentrations of cytokines measured by CBA and the fatigue scores were also generated.
2.8 Preparation of PBMC and positive control cell line for immunostaining and flow cytometry

2.8.1 PBMC overnight culture

To prevent the known attachment of monocytes to plastic in culture, 1 ml PBMC volumes (containing $1 \times 10^6$ cells) were cultured in sterile round-bottom polypropylene tubes (BD Falcon) (Kelley et al., 1987). Cells were then stimulated as described below or they were cultured without stimulation in 5% CO$_2$ at 37°C, to see if the patients’ cells constitutively produced IL-6. In one tube, 50% of cells were heat-killed in a water bath (65°C for 1 minute, followed by 1 minute on ice) to function as a positive control for the viability dye, used later to exclude dead cells from the analysis.

2.8.2 Stimulation of PBMCs

Prior to testing patient samples, a range of stimulants were explored using PBMC isolated from healthy control blood from donors at UWE, Bristol. Phorbol 12-myristate 13-acetate (PMA) and Ionomycin (used simultaneously) and phytohemagglutinin (PHA) were tested for their ability to stimulate the populations within the PBMC to produce IL-6, using the same isolation and culture methods described previously. PMA and Ionomycin together make a potent T cell mitogen (Touraine et al., 1977), and were used at final concentrations of 10 and 100 ng/ml respectively. PHA, also considered to stimulate T cells and monocytes to their full cytokine-producing capacities (Baran et al., 2001), was used at a final concentration of 2.5 μg/ml. Lipopolysaccharide (LPS) from Escherichia coli serotype 0111:B4 and interferon gamma (IFNγ) were eventually selected to stimulate the PBMCs in the flow cytometry assays, due to their ability to promote both cell surface marker up-regulation and IL-6 production, in most or all cell types in healthy participants.

Lipopolysaccharide (LPS) was added to the appropriate tubes at a concentration of 1 μg/ml for the full 24 hours of culture. IFNγ was added to these same tubes for the final four hours of culture at a concentration of 100 ng/ml. Monensin (GolgiStop®, BD Biosciences) was also added to all tubes at 4 μM for the final four hours of culture to
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prevent Golgi export of cytokines and thus to enable intracellular detection of IL-6 (Pala et al., 2000). When added to PBMC cultures overnight, monensin had a toxic effect on the cells. Cells were also stimulated with LPS and IFNγ as above, but in the absence of monensin, to allow the concentration of secretory cytokines in the culture supernatant to be measured.

2.8.3 Positive control cell lines

Two cell lines were investigated as potential positive controls for the intracellular IL-6 staining; Mono Mac 6 (MM6) cells and Jurkat T cells. Initially, during assay optimisation, MM6 cells were stimulated with PHA at 10 µg/ml for 24 hours and stained for intracellular IL-6 alongside the PBMC. MM6 cells have many similar characteristics to mature blood monocytes and reportedly produce potent amounts of IL-6 both constitutively and following stimulation for 24 hours with PHA (Neustock et al., 1993). Results, however, were found to be extremely variable between assays (n=8) for reasons which were not fully determined, but may relate to the age of the cells and the high passage number. Infection, incubator CO₂ levels and temperature were investigated and consequently ruled out. As a result of this inconsistency, Jurkat cells were similarly tested and then chosen as the positive control for the IL-6 antibody staining to be run alongside the PBMC in each assay. The Jurkat cells were left unstimulated as they were known to constitutively produce IL-6 (Khalaf et al., 2010).

2.9 Immunohistochemistry and flow cytometric analysis of PBMC preparations

After 24 hours of incubation, samples were examined by intracellular staining for the presence of IL-6. At this point, the tubes containing cells which had not been treated with monensin were spun down in a centrifuge (DADE Immufuge 569) at 650 g for 2 minutes in order to pellet the cells. The culture supernatants were collected and stored in cryovials at -80°C until ready for testing by cytometric bead array (CBA) and sandwich ELISA.
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2.9.1 Staining the non-viable cells

PBMCs were washed twice in cold phosphate buffered saline (PBS) and re-suspended in 1 ml PBS ready for staining with the viability dye, eFluor780. Subsequently, 1 μl of the dye was added to each tube, except the control tubes with single stains (for colour compensation). The tubes were then incubated at 4°C for 30 minutes to allow the dye to bind, protected from light in order to prevent the fluorophore from quenching.

2.9.2 Cell surface marker staining

Following a wash in 1 ml PBS to remove excess dye, cells were resuspended in PBS containing 10% BSA for 15 minutes (5% CO₂, 37°C) in order to block non-specific Fc antibody (Ab) binding. Cells were washed and stained with mouse monoclonal PE-conjugated Abs against the cell surface markers (Davenport et al., 2003) including CD3, CD14, CD19, CD123 or CD11c or isotype control, diluted in PBS (containing 1% BSA and 1% sodium azide) and incubated for 45 mins at 4°C in the dark.

During optimisation of the assay a range of dilutions were made for each antibody to determine the concentration required for optimal staining (1.5:10- 1.25:100). The final antibody concentrations chosen for use in the flow cytometry assays were as follows: anti-human CD3: 5 μl, anti-human CD14: 5 μl, anti-human CD19: 10 μl, anti-human CD123: 5 μl and anti-human CD11c: 2.5 μl (per 100 μl cell suspension, containing 1x10⁶ cells).

Stained samples were washed twice with cold PBS to remove excess antibody, then fixed with 1% paraformaldehyde (PFA) for 20 minutes at 4°C.

2.9.3 Intracellular IL-6 staining

Cell membranes were permeabilised by treatment with saponin (0.1% in PBS) for 30 minutes at room temperature. Permeabilised cells were washed and stained with FITC-conjugated anti-human IL-6 antibody or matching isotype control antibody diluted in 0.1% saponin in cold PBS for 60 minutes at 4°C. The cells were washed twice in the
permeabilisation buffer to allow any internal unbound antibody to wash out of the cells, and then re-suspended in PBS containing 0.5% BSA for flow cytometric analysis.

2.9.4 Flow cytometric analysis

Cells were analysed on an Accuri C6 flow cytometer set to collect 10,000 events. PBMCs were gated based on forward scatter and side scatter properties. FITC labelled cell signals were resolved on channel FL1. PE labelled signals were resolved on channel FL2, and the viability dye, Efluor780 was resolved on channel FL4.

2.9.5 Gating strategy used to determine IL-6 positive and negative PBMC populations

Initial gating was based on excluding obvious dead cells and cell debris (Figure 8a). The viability dye, Efluor780, was used to stain dead cells within the gated population which had the potential to interfere with staining compensation. Cells stained with the dye were excluded from analysis (Figure 8b) as it is thought that dead cells are associated with ‘non-specific and erratic antibody binding’ (Pembroke et al., 2013) which may compromise the results.
Figure 8: Gating strategy used to exclude dead cells and cell debris from analysis. Plot (a) demonstrates a forward and side scatter plot of a PBMC sample. Dead cells and cell debris with very low FSC-A values (<~400,000) were not included in the collection gate and thus were not included in the analysis. 10,000 events were collected within the gate. Plot (b) demonstrates an example of positive control PBMCs (approx. 50% heat-killed), dyed with Efluor780. This allowed dead cells to be located within the gated population, which were subsequently excluded from further analysis.

Fluorescence compensation was then carried out using the brightest FITC and PE single stains as controls (CD3-PE and CD45-FITC) (Figure 9) to correct for ‘fluorescence spillover’ between channels (Maecker and Trotter, 2006).

Figure 9: Compensated single stains for (a) CD3-PE and (b) CD45-FITC. Single staining is shown with minimal (<1%) spill over into the regions highlighted (orange stripes)- achieved by subtracting a percentage of the fluorescence which is showing up in the neighbouring channel.
Quadrants to determine the percentage cell type and level of IL-6 staining above background were set based on the position of the FITC and PE isotype controls, leaving 1% in neighbouring quadrants as standard to enable detection of minor changes in fluorescence (Figure 10). Isotype controls are antibodies of identical class or isotype of immunoglobulin as the antibodies under study, however, these antibodies are generated against an antigen that is not present on the cells used in the study, thus act as a negative control for background or nonspecific staining (Hulspas et al., 2009).

Figure 10: Setting of quadrants using isotype controls.

Fluorescence minus one (FMO) controls were used to confirm the suitability of the gates set using the isotype control antibodies, as isotype controls have recently been questioned as suitable control measures. FMO controls are separate samples which are stained with all of the antibodies used in the assay, minus one (Hulspas et al., 2009). In this case, cells were labelled with PE conjugated antibodies and the viability dye, excluding the FITC-conjugated antibody, and then vice versa (FITC and Efluor780, but not PE). As samples are stained with all fluorochromes (minus one), they show the
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amount of fluorescence spill over from other channels in the particular channel of interest (Maecker and Trotter, 2006). Minimal spill over (<1%) was observed between fluorochromes using FMO controls (highlighted) and accorded with gates set using isotype controls (Figure 11a and b). Figure 11a demonstrates minimal spill-over (0.7%) into the FL1 channel when looking at PBMC stained with PE conjugated anti-CD3 antibodies (detected in FL2 channel) and the Efluor780 viability dye (detected in the FL4 channel). Figure 11b demonstrates minimal spill-over (0.2%) into the FL2 channel when looking at PBMC stained with FITC conjugated anti-CD45 antibodies (detected in the FL1 channel) and the Efluor780 viability dye (detected in the FL4 channel).

(a) CD3- PE + Efluor780 viability dye
(b) CD45- FITC + Efluor780 viability dye

Figure 11: Setting gates using ‘fluorescence minus one’ (FMO) controls.

2.9.6 Staining and acquisition of samples

After gating was set on negative controls, the quantity of IL-6 in each cell type (CD3+, CD14+, CD19+, CD123+ or CD11c+) was observed by looking at the percentage of cells which were positive for IL-6 and the median fluorescence intensity (MFI) of the
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IL-6 staining. As MFI measures the intensity of IL-6 staining within each cell population, and thus the relative amount of IL-6, it may be more important than looking at the percentage of positive cells, in terms of finding the cell type responsible for spontaneous production of IL-6 in PMR patients. MFI data for PMR has not been previously reported.

Cells which appeared in the top two quadrants (Figure 12a) were cells which were stained for the cell surface marker, and cells in the two right hand quadrants were considered to be cells stained for intracellular IL-6 (Figure 12b). Therefore, any cells in the top right-hand quadrant were considered to be the cell type of interest, stained for intracellular IL-6 (Figure 12c).

In total, twenty samples were tested, including one pilot sample which was not included in the flow cytometry results due to slight variations in the permeabilisation method and discrepancies with the timing of sampling. For each participant, the PBMCs were isolated and resuspended ready for culture within 3 hours of the sample being taken. All cells, including the positive control Jurkat cells, were cultured for 24 hours, with or without stimulation. The staining process was started immediately after this 24 hour culture period, and all cells were fixed within 3 hours of starting this process. Data was acquired on the flow cytometer within 24 hours of fixation. Controls were run alongside each sample and the gates and the quadrants were set specifically for each individual participant in order to account for slight differences in temperature or timings that may have affected the results.
Figure 12: IL-6 production in CD3+ T cells from a healthy individual.

2.9.7 Calculating the absolute numbers of specific PBMC types producing IL-6 in each subject

The absolute number of each cell type which were producing IL-6 in each subject was also calculated, using the percentage of each cell type that were positive for IL-6 in a
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resting state (measured using flow cytometry) and the total white cell counts (WCCs) performed on PMR and RA patient blood samples in the hospital laboratories at UBHT. The PMR WCCs were performed during the same week as the flow cytometry assays, whereas the RA WCCs were performed at different times (date not provided). The WCCs were not performed on the healthy control samples so normal reference ranges for healthy adults have been provided (Table 12).

Firstly, the total percentages of the five PBMC cell types in each subject were added together. The percentage of each cell type was then divided by the total percentage of all the cell types and multiplied by one hundred to give the proportion of each cell type in each patient. This proportion (represented as a percentage) was then multiplied by the total white cell count, to give the total amount of each cell type in each subject (Table 13).

The absolute number of each cell type which were positive for IL-6 was then calculated by multiplying the percentage of IL-6 positive cells (calculated previously using flow cytometry) by the total number of each cell type in each subject. These values were then compared to normal reference ranges. Unpaired T tests were used to determine if there were any significant differences between the number of IL-6 positive cells in PMR patients, RA patients and healthy control patients, for each cell type.

2.10 Statistical analysis

2.10.1 Statistical analysis plan

Where samples were found to be normally distributed (i.e. Gaussian), unpaired T tests were employed to detect whether the means of the different disease groups or healthy control group were significantly different. PMR or RA patient groups were compared with relevant controls, i.e. RA vs HC, PMR vs HC, and finally PMR vs RA. P-values of 0.05 or below were considered to be significant. When exploratory T-tests were
used to look for potential differences between groups (when null hypothesis not previously applied), the Bonferroni test was used to correct for multiple comparisons, i.e. the defined significance threshold (0.05) was divided by number of comparisons, and that value accepted as the significance threshold (Bland, 1995). If independent repeats gave similar patterns but very different values, the geometric mean of the data was taken in order to normalise the data.

When looking for correlation or a relationship between sets of data, the two sets of data were plotted against each other in a scatter plot and the Pearson correlation coefficient ($r$), R-squared value ($r^2$) and P-value (p) were determined. To confirm the correlation and judge whether results from different methods sufficiently agree, Bland-Altman plots were employed. In some cases, where determining Pearson correlation coefficients was not suitable, Spearman’s rank correlation coefficients were determined, then judged whether significant at a 5% probability level using predetermined critical values.

When these statistical tests were not appropriate, descriptive statistics were used to provide a simple summary of the overall pattern of the results.

### 2.10.2 Geometric mean

To work out the geometric mean, each value from each independent repeat was logged (to the base 10) and the mean of these values was calculated. The inverse $\text{LOG}_{10}$ was then applied to this value to give the geometric mean. If the initial values were 0 or were negative values, an arbitrary number was added to all scores before the initial log stage, then subtracted again from the final value.

### 2.10.3 Bland-Altman plots

The methods used to test clinical samples were compared using Bland-Altman plots. Here, the mean of the two values measured (x axis) was plotted against the difference between the two values measured (y axis). The level of agreement in methods could be visualised by determining the level of scatter about zero (zero being that there was no
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difference between values measured by the two methods). The direction of scatter gave an indication of bias in favour of one of the methods. By applying a regression line with 95% confidence bands the bias could be detected if it was not obvious by initial visualisation.

2.10.4 Graphical representation

Graphical representation and the majority of statistical analysis were performed in Microsoft Excel 2007/2010 and GraphPad Prism 5. CBA data was analysed using FCAP Array software (SoftFlow Inc., Hungary).

In order to present the growth kinetics of the B9 cells for the IL-6 bioactivity assays, 4-parameter log regression standard curves were constructed using GraphPad Prism 5. For the IL-6 ELISA standard curve, a 4-polynomial curve was constructed using the same software.

All values quoted represent mean ± standard error of the mean (SEM) of the sample unless stated otherwise.
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The results section is presented in the same order as the aims and objectives (Section 1.16), so as to address each one systematically. Where appropriate, results from the pre-optimisation assays are presented before the results of the clinical investigation.

3.1 IL-6 and the pathogenesis of PMR and RA

3.1.1 Participant demographics
The clinical data and demographics of the 20 study participants are set out in Table 7.

Table 7: Demographics of the PMR and RA patients and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>PMR patients (n=7)</th>
<th>RA patients (n=6)</th>
<th>Healthy controls (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of females/males</td>
<td>6/1</td>
<td>4/2</td>
<td>5/2</td>
</tr>
<tr>
<td>Age, mean (range) years</td>
<td>75 (65-88)</td>
<td>67 (55-75)</td>
<td>63 (59-71)</td>
</tr>
<tr>
<td>CRP, mean (range) mg/dL</td>
<td>33.4 (11-102)</td>
<td>13.5 (4-29)</td>
<td>2 (1-5)</td>
</tr>
<tr>
<td>Total white blood cell count (10³/µL)</td>
<td>7.9 (5.4-9.4)</td>
<td>7.6 (7.0-10.5)</td>
<td>-</td>
</tr>
</tbody>
</table>

3.1.2 IL-6 in the plasma and serum measured by CBA (refer to Aim 1 in Section 1.16)

Various studies have established that systemic IL-6 is raised in the plasma or serum of PMR (Cutolo et al., 2006) and RA (Arvidson et al., 1994) patients compared to healthy controls. In order to confirm this, the concentration of IL-6 was measured in the plasma of 7 PMR patients and 6 RA patients alongside 7 healthy controls of a similar age.
Figure 13: The concentration of IL-6 measured by CBA in the plasma and serum of RA and PMR patients alongside healthy control participants of a similar age. The concentration of IL-6 was measured in (a) the plasma and (b) the serum of each study participant using CBA. Each data point represents the geometric mean of cytokine concentration for each study participant (n=2 repeats for serum and n=4 repeats for plasma). The solid red line represents the mean of the patient or control group. The dotted red line represents the lower threshold limit of the CBA kit. *P< 0.05 **P< 0.01 NS=P> 0.05
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As hypothesised, IL-6 concentrations were significantly elevated in the plasma of PMR (P< 0.01) and RA (P< 0.05) patients compared to healthy controls of a similar age (Figure 13a) when measured using CBA. The mean plasma concentration of IL-6 in PMR patients was 12.8 pg/ml (range: 3.3-24.1 pg/ml), compared to 17.5 pg/ml (range: <2.5-41.2 pg/ml) in RA patients. In contrast, the concentration of IL-6 in all healthy controls fell below the sensitivity threshold for the assay (<2.5 pg/ml). There was no significant difference between the means of the plasma IL-6 concentration in PMR patients and RA patients.

To determine if there was a difference in the result when using patient plasma or serum, the concentration of IL-6 in the serum of study participants (extracted in the clinic) was also measured by CBA. Again, the concentration of IL-6 was significantly raised in the serum of PMR (P< 0.05) and RA (P< 0.05) patients compared to healthy controls of a similar age (Figure 13b). The mean serum concentration of IL-6 in PMR patients was 15.1 pg/ml (<2.5-35.2 pg/ml), compared to 25.7 pg/ml (<2.5-56.5 pg/ml) in RA patients, and again all healthy controls fell under the sensitivity threshold for the assay (<2.5 pg/ml). As for plasma, there was no significant difference between the mean of the serum IL-6 concentration in PMR patients and RA patients.

3.1.3 The correlation between IL-6 measured in the plasma and serum by CBA (refer to Aim 2 in Section 1.16)

The correlation between IL-6 measured in the plasma and serum (by CBA) was examined (Figure 14) in order to observe whether these different methods of sample preparation produced comparable results. The concentration of IL-6 in the plasma and serum of study participants correlated well, with a high correlation coefficient (r) of 0.961 and a P-value of less than 0.0001 (**). The angle of the regression line, however, is >45° demonstrating that serum-derived IL-6 concentrations were consistently higher than those derived from patient plasma samples.
Figure 14: The correlation between IL-6 measured in the plasma and serum of RA and PMR patients. Each data point represents the geometric mean of each study participant of n=2 repeats for serum and n=4 repeats for plasma. The correlation coefficient, $r = 0.961$, **P<0.0001.

Bland and Altman also provide a method of comparing clinical measurement, in order to judge whether results from different methods sufficiently concur, as the use of correlation can hide disagreement, especially at the lower end of the data (Bland and Altman, 1986). Figure 15 demonstrates the Bland Altman method of comparison of the plasma and serum values measured by CBA.
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Figure 15: Bland Altman plot comparing IL-6 concentrations measured in the plasma and serum of RA and PMR patients and healthy controls of a similar age. Each data point represents one participant.

By initial observation it can be seen that at low concentrations of IL-6 there is close agreement, i.e. the difference between the means is close to zero. All the values, however, are below zero suggesting a minimal amount of bias towards the serum measurements at the lower end. In contrast, at the higher end (above a mean IL-6 value of 20 pg/ml) there is more variation, and a clear bias towards the serum. This indicates that although the correlation coefficient was high (0.961) for the two sets of data, the IL-6 concentrations measured in the serum are higher than the IL-6 concentrations measured in the plasma i.e. the variation is much greater than the correlation graph suggests. The angle of the regression line also indicates a strong bias towards high serum measurements at high concentrations (Figure 15). Thus, based on this statistical method of comparison, the two methods could not be used interchangeably as they were not considered to sufficiently agree. In the rest of the study both serum and
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plasma were assessed, so that comparisons could be made with values seen in the literature, where values for either plasma or serum are specifically cited.

3.1.4 IL-6 measured in the plasma and serum by ELISA

Plasma and serum IL-6 concentrations were also measured by sandwich ELISA prior to being measured by CBA, in order to determine the range of IL-6 expected from the samples and the dilution factor required and to observe how similar these results were to the results from the CBA assays (Figure 16). Similar trends to those seen in the CBA assay were observed, with both the mean plasma and serum IL-6 values higher for PMR and RA patients than healthy controls. The mean IL-6 concentration in the plasma measured at 4.9 pg/ml (range: <4.7- 11.6) for healthy controls, 9.1 pg/ml (range: <4.7- 28.3) for RA patients and 10.8 pg/ml (range: <4.7- 19.6) for PMR patients. The mean serum IL-6 concentrations were 5.9 pg/ml (range: <4.7- 21.9) for healthy controls, 8.6 pg/ml (range: <4.7- 24.2) for RA patients and 12.4 pg/ml (range: <4.7- 28.8) for PMR patients. The lower threshold of sensitivity for the assay was 4.7 pg/ml (i.e. the lowest standard).

The actual values measured in the ELISA were lower in RA and PMR patients than the values measured in the CBA; so that the difference between the mean concentration of IL-6 in PMR and RA patients compared to healthy controls was not statistically significant. In both the plasma and serum ELISAs there were two healthy control subjects with much higher IL-6 concentrations than would be expected for a healthy individual. It was not, however, the same two individuals with raised concentrations of IL-6 in the plasma and in the serum, indicating an inconsistency in the results. Also, when measuring IL-6 in the plasma and serum of each participant, a large amount of variability was observed between independent repeats (3 independent repeats for plasma and 2 independent repeats for serum) of these healthy controls with a high mean concentration of IL-6. This variability between repeats was not observed in the CBA, where all healthy controls had undetectable concentrations of IL-6 as expected in both the plasma and serum in all repeats. Consequently, cytokine concentrations were measured in the plasma and serum of each participant by CBA for all further analysis.
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Figure 16: The concentration of IL-6 measured by ELISA in the plasma and serum of RA and PMR patients alongside healthy controls of a similar age. The concentration of IL-6 was measured in (a) the plasma and (b) the serum of each study participant using a sandwich ELISA. Each data point represents the mean of cytokine concentration for each study participant (n=3 repeats for plasma and n=2 repeats for serum). The solid red line represents the mean of the patient or control group. The dotted red line represents the lower threshold limit of the ELISA. *P< 0.05 **P< 0.01 NS=P> 0.05
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3.1.5. Determining the biological activity of circulating IL-6 in PMR and RA patients (refer to Aim 3 in Section 1.16)

3.1.5.1 IL-6-dependent B9 cell proliferation in response to IL-6

A B cell proliferation assay was conducted using IL-6-dependent B9 cells in order to test the bioactivity of the IL-6 previously detected in the plasma samples of the study participants. This colorimetric assay was centred around the bioreduction of MTS by B9 cells into a coloured formazan product which could be quantified by measuring the absorbance of culture medium at 450nm (Promega, 2013).

Figure 17: The range of IL-6 over which B9 cell growth was stimulated. B9 cells were incubated for 72 hours with a range of concentrations of recombinant IL-6, before MTS was added and the absorbance measured. A four parameter linear regression curve was applied. The red dotted lines indicate the range of IL-6 concentrations between which the B9 cell growth was stimulated. Data points represent the mean±SE of triplicate wells.
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The growth range of the B9 cells was observed in order to confirm whether the B9 cells would be responsive to the levels of IL-6 present in the study participant samples. When a four parameter log regression curve was applied, it was possible to see that the B9 cells were responsive to IL-6 between approximately 6.5 pg/ml and 850 pg/ml (Figure 17). This range was considered to be sufficient to test the biological activity of IL-6 in the study participant plasma samples, which had previously been found to contain up to 41 pg/ml of IL-6 in the CBA assays.

3.1.5.2 Titration of the anti-human IL-6 neutralising antibody

In order to confirm the IL-6 specificity of B9 cell proliferation, IL-6 neutralising antibodies were added to the plasma samples and proliferation was measured in parallel with untreated samples. The measured growth of the B9 cells with the plasma sample in the presence of IL-6 neutralising antibody was subtracted from the total measured B9 cell growth with plasma sample, in order to give the concentration of IL-6 which was actively causing the B9 cells to grow and proliferate.

In order to determine the optimal concentration of IL-6 neutralising antibody to use in the IL-6 bioactivity assays, different concentrations of the neutralising antibody (0.000-50 µg/ml) were added to a range of concentrations of recombinant IL-6 (0.00025-25 ng/ml) and then incubated with the IL-6-dependent B9 cells. The inhibitory effects of the neutralising antibody are illustrated in Figures 18 and 19.

When the IL-6 neutralising antibody was applied at a concentration of 0.005 µg/ml and 0.05 µg/ml, the growth of the B9 cells was not inhibited at any of the concentrations of recombinant IL-6 (Figure 18 and 19b). At a concentration of 0.5 µg/ml, the IL-6 neutralising antibody inhibits B9 cell growth when incubated with IL-6 at a concentration of 0.25 ng/ml, but not at any of the other IL-6 concentrations (Figure 19d). The neutralising antibody, however, had a potential stimulatory effect at the two highest IL-6 concentrations (2.5 and 25 ng/ml) (Figure 19d).
At a concentration of 5 µg/ml, the IL-6 neutralising antibody again had a clear inhibitory effect on B9 cell growth when stimulated with 0.25 µg/ml of IL-6 and a potentially stimulatory effect on the B9 cells when applied to the cells with 2.5 and 25 ng/ml of recombinant IL-6 as previously (Figure 19e).

At 50 µg/ml, the IL-6 neutralising antibody clearly inhibited B9 cell growth at 2.5 ng/ml and 0.25 ng/ml (Figure 19f). Thus this concentration of IL-6 neutralising antibody was chosen as the optimal concentration as it inhibited B9 cell growth for the longest and broadest range (Figure 18 and 19f).
Figure 19: The effect of adding varying concentrations of IL-6 neutralising antibody to varying concentrations of recombinant IL-6 on the growth of the IL-6-dependent B9 cell line. The IL-6-neutralising antibody was added to the recombinant IL-6 at a range of concentrations shown in (a) 0.000 µg/ml (b) 0.005 µg/ml (c) 0.05 µg/ml (d) 0.5 µg/ml (e) 5 µg/ml (f) 50 µg/ml. The recombinant IL-6 was at a range of concentrations as shown on each individual bar chart on the x axis, in order to determine the concentration of neutralising antibody at which the B9 cell proliferation was inhibited at the longest and broadest range. Representative experiment of 2 performed. The bars represent the mean±SE of duplicate wells.
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3.1.5.3 Testing the biological activity of the IL-6 in the study participant samples

In order to measure the bioactivity of IL-6 in the study participant plasma samples (SP1-19), the samples were incubated with and without the chosen concentration of IL-6 neutralising monoclonal antibody (50 μg/ml), and then the B9 cells were added for 72 hours. The proliferation of the B9 cells in response to incubation with study participant plasma samples was measured indirectly by measuring the absorbance of the samples at 450 nm in an MTS assay.

Standard curves were constructed for each independent repeat and represent the growth of the B9 cells following incubation with known concentrations of recombinant IL-6 (Figure 20). These standard curves illustrated in Figure 20, constructed using four parameter log regression, demonstrate the variability between the independent repeats of the assay.

Figure 20: The proliferation of B9 cells in response to standard concentrations of recombinant IL-6. The recombinant IL-6 was added to B9 cells at varying concentrations (x axis), 72 hours later the MTS assay was performed and the absorbance of wells measured at 450 nm. Data shown from three independent repeats, each performed in duplicate wells. Symbols represent mean ± SE.
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The IL-6 present in the plasma samples was found to be biologically active, determined by the proliferation of B9 cells in the presence of sample, minus B9 cells in the presence of sample and neutralising antibody (Figure 21b). The concentration of this biologically active IL-6 was measured by interpolating values from the standard curves generated for each individual assay repeat. The mean concentration (±SE) of biologically active IL-6 in each subject sample is demonstrated in Figure 21b.

Figure 21a demonstrates the IL-6 concentration measured in the plasma of study participants 1-19 by CBA. Despite slight differences in sensitivity between the assays, at initial observation the results appear to be very similar and thus both methods may be suitable for the measurement of IL-6 in human plasma, and the active IL-6 measured by the MTS assay is most likely to be the same IL-6 measured in the samples by CBA.

This ‘agreeability’ between assays was tested using the Bland-Altman principle (Figure 22) in order to confirm that the plasma IL-6 measured in both assays was the same. At the lower end of the scale, i.e. at concentrations of IL-6 <20 pg/ml, the measurements demonstrate some variation shown by the wide scatter, however, the points appear to be distributed randomly either side of zero suggesting that there is some broad agreement between the two assays. There are two major outliers, which suggests that there is some bias towards the MTS assay measurements at the higher end. This is, however, a biological system and thus some level of disagreement between assay measurements was expected, particularly at the higher end where the assays are not as sensitive. It was therefore accepted that the IL-6 measured in the plasma in the MTS assay was in fact the same IL-6 measured in the plasma by CBA, meaning that the IL-6 measured by CBA in PMR and RA patients was biologically active.
Figure 21: Biologically active IL-6 measured in study participant plasma samples. Graph (a) illustrates the geometric mean concentration of IL-6 in the plasma of study participants (1-19) as previously measured by CBA (n=4 repeats) showing the 95% confidence intervals. Graph (b) illustrates the mean concentration of IL-6 (±SE), measured in the same plasma samples using the B9 cell proliferation assay (n=3 repeats).
3.1.6 The relationship between IL-6 and fatigue in polymyalgia rheumatica and rheumatoid arthritis (refer to Aim 4 in Section 1.16)

The results of the CBA assays were compared with patient reported levels of fatigue, an important symptom of PMR and RA. Two studies in particular have confirmed a link between circulating IL-6 and fatigue in RA (Davis et al, 2008 and Helal et al, 2012). The aim of this part of the study was to confirm that this link between IL-6 and fatigue exists and to determine whether this link might also extend to PMR. Study
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Participants completed the BRAF-MDQ (Nicklin et al., 2010) at a fixed specific time of the day, i.e. 9am-11am when the blood sample was taken. Scores from the individual sections of the BRAF-MDQ were added and then a total score given. As previously described, IL-6 was measured in the serum and plasma of each subject by CBA (n=2 serum and n=4 plasma). The total fatigue scores and scores of the individual aspects of fatigue were directly compared in order to examine whether there was a correlation between IL-6 levels and fatigue in all participants. The results are illustrated below.

3.1.6.1 Fatigue vs. Plasma IL-6

The concentration of IL-6 in the plasma of study participants was plotted against the total fatigue score for each patient. There was a significant correlation between the data, evidenced by the P-value, which was below 0.05 (*) and the high Pearson’s correlation coefficient, 0.487 (Figure 23a).

The correlation between plasma IL-6 and the individual aspects of fatigue were also examined (Figure 23c-f). There was significant correlation between plasma IL-6 and the more somatic aspects of fatigue, i.e. physical fatigue and living fatigue, evidenced by high Pearson’s correlation coefficients (0.479 and 0.499 respectively) and low P-values (0.033 and 0.025 respectively). There was, however, no significant correlation between plasma IL-6 and the more psychological aspects of fatigue, i.e. cognitive fatigue and emotional fatigue (P> 0.05). This suggests that the link between increased IL-6 in the blood and fatigue may be due to the physical effects that IL-6 has on the individual.

Spearman’s rank correlation coefficients were also calculated in order to confirm the relationship between circulating IL-6 concentrations and fatigue. The distribution of points on the Pearson correlation graphs meant that calculating the Pearson correlation coefficients was not ideal, thus it was decided that ranking the data and using these ‘ranks’ instead of actual values was a more suitable way of testing the strength of the correlation between the IL-6 concentrations and fatigue scores. Table 8 shows the...
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Spearman’s rank correlation coefficients for plasma IL-6 vs total fatigue, as well as plasma IL-6 vs each individual aspect of fatigue. In agreement with the Pearson’s correlation coefficients, it was confirmed that total fatigue and physical and living fatigue showed significant positive correlation with plasma IL-6 concentrations in the participants. Again, emotional and cognitive fatigue did not show any significant correlations with circulating IL-6 concentrations.

Table 8: Spearman’s rank correlation coefficients calculated to test the strength of the relationship between IL-6 measured in participant plasma by CBA and the scores for the individual aspects of fatigue and total fatigue for the twenty study participants. The critical value at the 5% probability level was 0.378, thus anything higher than this value was taken to be significant.

<table>
<thead>
<tr>
<th>Plasma IL-6 concentration vs fatigue scores</th>
<th>Spearman’s rank correlation coefficient</th>
<th>Significant at 5% probability level?</th>
</tr>
</thead>
<tbody>
<tr>
<td>vs total fatigue score</td>
<td>0.543</td>
<td>Yes</td>
</tr>
<tr>
<td>vs physical fatigue score</td>
<td>0.673</td>
<td>Yes</td>
</tr>
<tr>
<td>vs living fatigue score</td>
<td>0.642</td>
<td>Yes</td>
</tr>
<tr>
<td>vs cognitive fatigue score</td>
<td>0.187</td>
<td>No</td>
</tr>
<tr>
<td>vs emotional fatigue score</td>
<td>0.334</td>
<td>No</td>
</tr>
</tbody>
</table>

3.1.6.2 Other cytokines and fatigue

The correlation between the other measured cytokines and total fatigue was also tested. No other measured cytokines displayed any significant correlation with fatigue.
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Figure 23: The correlation between the total fatigue score or score for individual aspect of fatigue (generated by the BRAF-MDQ) vs. plasma and serum IL-6 concentrations. Each symbol represents one subject; triangles represent healthy controls, squares represent RA patients and circles represent PMR patients. IL-6 concentrations that fell below the sensitivity threshold for the CBA kit were taken as 2.5 pg/ml (the highest those values could possibly be). Pearson’s ‘r’ indicated on each graph.
3.1.6.3 Fatigue vs other clinical measures of PMR and RA

The total fatigue score generated using the BRAF-MDQ was correlated with various clinical measures of disease activity, in order to observe whether a link existed between them. The CRP concentration was measured in the blood of all participants, as a measure of inflammation. This concentration was then plotted against the total fatigue score in order to determine whether a relationship existed between these two important measures of disease (Figure 24). Using Pearson’s correlation coefficient, no significant correlation was observed between these two diseases parameters, indicating that fatigue and the physiological mechanisms of inflammation occur as two unrelated disease events. However, when using rank order to test the relationship between these two disease measures, i.e. Spearman’s correlation coefficient, a significant positive correlation was evident (Table 9), indicating a relationship between CRP and fatigue in the participants.

Figure 24. The correlation between the CRP levels measured in the blood and the total fatigue score generated by the BRAF-MDQ. No significant correlation was observed when testing using the Pearson’s correlation coefficient ($r=0.404$). Each symbol represents one subject. The black line represents the line of best fit.
Table 9: Spearman’s rank correlation coefficient to test the strength of the relationship between CRP score and total fatigue score. Significance at 5% probability level indicated.

<table>
<thead>
<tr>
<th>C-reactive protein score vs total fatigue score</th>
<th>Spearman’s rank correlation coefficient</th>
<th>Significant at 5% probability level?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.661</td>
<td>Yes</td>
</tr>
</tbody>
</table>

In order to test the quality of the IL-6 assays, plasma IL-6 concentrations were plotted against CRP levels measured in the blood in order to observe if a correlation existed. As CRP is produced by hepatocytes in response to increased circulating concentrations of IL-6, it was predicted that there would be a strong correlation. When measured using Pearson’s correlation coefficient, no significant correlation was observed when all participants were included in the analysis (Figure 25a); however there was one participant with an extremely high CRP measurement, indicating that this might be an anomaly. When excluded from the analysis the correlation was highly significant (P<0.0001) (Figure 25b) indicating that the results from the IL-6 assays were reliable- as CRP levels corresponded with circulating IL-6 levels. Also, when tested using Spearman’s rank correlation coefficient, a high $r_s$ value of 0.789 indicating a strong correlation between CRP and IL-6- this value was significant at the 0.5% probability level.
Figure 25: The relationship between CRP levels measured in the blood and plasma IL-6 concentrations measured by CBA. No significant correlation was observed when using all participant data (a), however when participant 19 was omitted from the analysis (b), the correlation was highly significant. The black line represents the line of best fit. Each symbol represents one subject.
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3.1.7 Summary

Serum and plasma IL-6 concentrations in PMR and RA patients were found to be significantly elevated above healthy controls (P< 0.05), however IL-6 values were consistently higher in serum than plasma. Elevated plasma IL-6 in PMR and RA patients was found to be biologically active.

A significant correlation was observed for the first time between the total fatigue score and IL-6 concentrations in the plasma of both PMR and RA patients (r= 0.487). Two individual aspects of fatigue, namely ‘physical fatigue’ and ‘living fatigue’ also correlated very well with IL-6 concentrations in the plasma of both PMR and RA patients whereas ‘cognitive fatigue’ and ‘emotional fatigue’ did not. The total fatigue scores and CRP levels displayed a significant correlation, as did circulating IL-6 concentration with CRP levels, as expected.

3.2 Exploring the cellular source of elevated IL-6 in PMR patients (refer to Aim 5 in Section 1.16)

In order to identify the source of IL-6 in PMR patients, immunostaining and flow cytometry techniques were employed to detect IL-6 production by specific cell types in the blood. The percentage of cells which contained internal IL-6 was calculated for five PBMC types for each subject. The mean fluorescence intensity (MFI), a measure for the ‘intensity’ or concentration of the IL-6 staining within these cells, was also measured. Cell types included CD3+ T cells, CD14+ monocytes, CD19+ B cells, CD123+ pDCs and CD11c+ mDCs.

3.2.1 Optimising the staining procedure

Prior to measuring IL-6 within each cell type, optimisation assays were performed. This was to ensure that the optimal concentration of antibody was used (for identification of each cell type and intracellular IL-6), and also to find a suitable
stimulant which would activate each cell type. Other assays were performed to confirm the efficacy of the golgi-blocker (monensin, 4 μM) and permeabilisation buffer (0.1% saponin and 0.1% BSA in PBS) (data not included). The following section outlines the results obtained from the antibody titration and cell stimulant optimisation assays, tested on PBMC extracted from healthy blood, taken from donors at UWE, Bristol.

3.2.1.1 Titrating the antibodies used for surface staining PBMC types

The antibodies used for the identification of the different cell types were titrated on unstimulated PBMC from healthy donors, in order to find the dose at which the percentage of cells stained was at its peak, indicating the optimal concentration of antibody required for use in the following assays. Table 10 demonstrates the percentage of cells which were positively stained with antibodies against each of the five cell surface markers. The concentration which stained the highest percentage of each cell type was chosen as the optimal dose (highlighted).

Table 10: Antibody titration for each of the cell surface markers. Highlighted boxes indicate the greatest percentage of positively stained cells, i.e. the optimal concentration of antibody to use. Ab= antibody.

<table>
<thead>
<tr>
<th>Ab</th>
<th>Cell type</th>
<th>1.25</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human CD3</td>
<td>CD3+</td>
<td>44.6</td>
<td>54.6</td>
<td>58.7</td>
<td>55.5</td>
<td>49.4</td>
</tr>
<tr>
<td>Anti-human CD14</td>
<td>CD14+</td>
<td>6.2</td>
<td>5.6</td>
<td>6.4</td>
<td>5.9</td>
<td>5.0</td>
</tr>
<tr>
<td>Anti-human CD19</td>
<td>CD19+</td>
<td>4.3</td>
<td>1.7</td>
<td>4.4</td>
<td>5.0</td>
<td>3.7</td>
</tr>
<tr>
<td>Anti-human CD123</td>
<td>CD123+</td>
<td>7.5</td>
<td>13.1</td>
<td>15.2</td>
<td>13.2</td>
<td>13.7</td>
</tr>
<tr>
<td>Anti-human CD11c</td>
<td>CD11c+</td>
<td>3.3</td>
<td>4.3</td>
<td>3.6</td>
<td>3.1</td>
<td>3.1</td>
</tr>
</tbody>
</table>
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3.2.1.2 Titration of the anti-human IL-6 antibody

The Jurkat cell line constitutively produces IL-6 (Khalaf et al., 2010). Therefore the anti-human IL-6 antibody was titrated on the Jurkat cell line in order to find the optimal concentration for IL-6 detection in the PBMC. Cells were fixed and permeabilised prior to antibody staining in order to allow the antibody to bind intracellular IL-6. Figure 26a demonstrates the percentage of the Jurkat cells which were positively stained with the IL-6 antibody. Figure 26b demonstrates the mean fluorescence intensity of IL-6 staining in the Jurkat cell line at varying antibody concentrations. The optimal concentration was found to be 5 µl of antibody per 100 µl cell suspension containing 1x10^6 cells, evidenced by the largest percentage of IL-6-positive cells and greatest MFI of IL-6 staining at this concentration.

3.2.1.3 Testing cell stimulants

Prior to analysing study participant samples, various stimulants (PMA + Ionomycin, PHA, and LPS + IFNγ) were tested for their effectiveness in stimulating cellular responses and thus their ability to increase IL-6 production within all five PBMC cell types (CD3+, CD14+ CD19+, CD123+ and CD11c+) in blood from healthy donors. Isotype and FMO controls were used to set the gates.

3.2.1.4 PMA and Ionomycin upregulated IL-6 production in CD3+ cells only

Figure 27 (a-d) demonstrates four PBMC types stained with antibodies against each surface marker and intracellular IL-6, in both unstimulated and PMA- ionomycin stimulated cells.
Figure 26: Titration of the anti-human IL-6 antibody on the Jurkat cell line. Graph (a) demonstrates the percentage of Jurkat cells which were positively stained for IL-6 and (b) demonstrates the mean fluorescence intensity of IL-6 staining within Jurkat cells. The Jurkat cells were fixed, permeabilised and then stained with 5 different concentrations of the antibody, in 100 µl cell suspension containing 1x10^6 cells. Mean±SE of n=3 repeats.
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(a) Stimulated PBMC vs. Unstimulated PBMC
- CD3-PE
- IL-6-FITC

(b) CD14-PE
- IL-6-FITC

(c) CD19-PE
- IL-6-FITC
Figure 27: IL-6 production in PBMC subsets from a healthy individual following stimulation with PMA (10 ng/ml) and Ionomycin (100 ng/ml) for 24 hours. Plot (a) demonstrates the CD3+ T cells, (b) CD14+ monocytes, (c) CD19+ B cells, (d) CD123+ plasmacytoid DCs. 1x10^6 cells were stained using PE-conjugated antibodies against surface markers and FITC-conjugated IL-6 antibodies against intracellular IL-6. Analysed by Accuri C6 flow cytometer set to collect 10,000 events.

As expected, as a known T cell mitogen, PMA (with ionomycin) was successful in increasing the MFI of IL-6 staining in CD3+ T cells from 22,068 to 173,873 (Figure 27a). There was, however, evidence of cell death and debris (circled in blue, Figure 27a), which was not evident in the unstimulated culture, indicating that PMA is potentially detrimental to other cell types. In agreement with this, the monocyte population circled in red (Figure 27b) disappeared following stimulation with PMA and ionomycin, and cell death and debris were increased in this culture. The MFI of a small population of CD19+ B cells may have increased, indicating that some B cells may be activated by PMA and ionomycin in terms of IL-6 production (Figure 27c). There was, however, an overall decrease in the number of CD19+ B cells, indicating that this stimulation may be detrimental to surface expression of CD19. As with the CD14+ monocytes, the CD123+ pDC population disappeared following stimulation, suggesting that this method of stimulation is associated with cell death and/or a lack of cell surface expression of CD14, CD19 and CD123. Thus, this was considered not to be an ideal stimulatory technique for these assays.
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3.2.1.5 PHA did not induce IL-6 production in any of the PBMC

PHA was next used to stimulate PBMC cultures (Figure 28) which were then stained for cells surface markers and intracellular IL-6.

(a) Unstimulated PBMC

(b) Stimulated PBMC

IL-6- FITC

CD3- PE

CD14- PE

Unstimulated PBMC

Stimulated PBMC
Figure 28: IL-6 production in PBMC subsets from a healthy individual following stimulation with PHA (2.5 µg/ml) for 24 hours. Plot (a) demonstrates the CD3+ T cells, (b) CD14+ monocytes, (c) CD19+ B cells, (d) CD123+ pDCs and (e) CD11c+ mDCs. 1x10^6 cells were stained using PE-conjugated antibodies against surface markers and FITC conjugated IL-6 antibodies against intracellular IL-6. Analysed by Accuri C6 flow cytometer set to collect 10,000 events.
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PHA, also a known T cell mitogen, was tested for its ability to stimulate PBMC populations to increase IL-6 production as well as stimulating cell proliferation. A large, discrete CD3+ T cell population was observed in the unstimulated culture, however, following stimulation with PHA, the total amount of CD3+ T cells decreased by 37%, with only a small proportion of CD3+ cells demonstrating an increased MFI (i.e. an increase in IL-6 production) (Figure 28a). This indicated that PHA may have caused a reduction in the cell surface marker and did not successfully induce IL-6 production in CD3+ T cells. There was also some evidence of cell death and debris as demonstrated previously.

Following stimulation with PHA, discrete populations of CD14+ monocytes and CD19+ B cells disappeared, with a very small proportion of CD14+ cells demonstrating an increase in MFI (Figure 28b and c). Nevertheless, with more cell death evident in these plots, it was difficult to say whether these were monocytes or cell debris. The MFI of IL-6 staining did not increase in CD123+ pDC or CD11c+ mDC (Figure 28d and e). Furthermore, the percentage of IL-6 positive cells was reduced in CD11c+ mDC following stimulation with PHA (Figure 28e). In conclusion, PHA was not an ideal stimulant for IL-6 production in any of the PBMC cell types, despite its reputation as a potent T cell mitogen.

3.2.1.6 LPS and IFNγ induced IL-6 production in all PBMC

When LPS and IFNγ were added to healthy PBMC cultures, the percentage of IL-6 positive cells increased for every cell type (of the individual cell type, not the whole PBMC population), albeit a very minor increase for the CD14+ monocytes and CD123+ and CD11c+ dendritic cells (Figure 29b, d and e). The MFI, however, increased dramatically for all cell types (Figure 29a-e) indicating a large increase in IL-6 production by the cells involved, as well as an increase in the amount of each cell type producing IL-6. Thus, LPS and IFNγ were chosen as the stimulant for use in the assays, in order to test the IL-6 secreting potential of all 5 PBMC types.
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(a) Unstimulated PBMC

(b) Stimulated PBMC

(c) Unstimulated PBMC

(b) Stimulated PBMC

(c) Stimulated PBMC
Figure 29: IL-6 production in PBMC subsets from a healthy individual following stimulation with LPS (1 µg/ml) for 24 hours and IFNγ (100 ng/ml) for the final 4 hours. Plot (a) CD3+ T cells, (b) CD14+ monocytes, (c) CD19+ B cells, (d) CD123+ pDCs (e) CD11c+ mDCs. 1x10^6 cells were stained with PE-conjugated antibodies against surface markers and FITC-conjugated IL-6 antibodies against intracellular IL-6. Analysed on Accuri C6 flow cytometer set to collect 10,000 events.

3.2.2 Measuring constitutive IL-6 production in PBMC

3.2.2.1 The positive control cell line

As Jurkat cells had previously been shown to produce high levels of IL-6, this cell line was stained for intracellular IL-6 alongside the samples as a positive control, in order to confirm that the intracellular IL-6 antibody staining techniques had been successful.
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The dotplot in Figure 30a demonstrates an example of unstained Jurkat cells with the gating used to exclude dead cells and cell debris. Histograms shown in Figure 30 demonstrate unstained Jurkat cells (b), Jurkat cells stained with the IL-6 antibody (d), and Jurkat cells stained with the matching isotype control (Rat IgG1k) (e). Intracellular IL-6 was measured in the Jurkat cell line alongside every assay confirming that the staining and permeabilisation techniques were effective. The results displayed some variability from assay to assay, however, IL-6 was always measured within the Jurkat cells with the percentage of IL-6 positive cells ranging from 31% to 92% (mean 69%) (Table 11).

Table 11: The percentage of Jurkat cells which constitutively produced IL-6. Jurkat cells were used as a positive control for IL-6 staining for each sample (study participants 1-19).

<table>
<thead>
<tr>
<th>Study participant sample</th>
<th>The percentage (%) of IL-6 positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP1</td>
<td>53</td>
</tr>
<tr>
<td>SP2</td>
<td>81</td>
</tr>
<tr>
<td>SP3</td>
<td>92</td>
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<tr>
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<td>66</td>
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<td>SP7</td>
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<td>SP8</td>
<td>74</td>
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<td>SP9</td>
<td>72</td>
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<td>SP11</td>
<td>79</td>
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<td>SP12</td>
<td>76</td>
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<td>SP16</td>
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<td>SP18</td>
<td>88</td>
</tr>
<tr>
<td>SP19</td>
<td>77</td>
</tr>
</tbody>
</table>
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Figure 30: Constitutive intracellular IL-6 measured in Jurkat cells. Plot (a) demonstrates the gating used to exclude dead cells and cell debris, visible in the bottom left hand corner and up the left hand side, and histograms in (b),(c) and (d) demonstrate unstained Jurkats, Jurkats stained with the isotype control (Rat IgG1k) and Jurkats stained with the anti-human IL-6 antibody (respectively). The red marker is a second gate which was arbitrarily placed so that 2.5% of cells were positive when stained for the isotype control. Any fluorescence detected above this was taken as positive IL-6 staining.

(a) Unstained cells
(b) Unstained cells
(c) Cells stained with isotype control
(d) Cells stained with anti-IL-6 antibody
3.2.2.2 Measuring the percentage of PBMCs which constitutively produce IL-6

In healthy control subjects, between 87% and 100% of all unstimulated CD14+ monocytes and CD123+ plasmacytoid dendritic cells were positively stained for intracellular IL-6 (Figure 31a). This suggests that in normal healthy people nearly all monocytes and pDCs constitutively produce IL-6 when in a resting state. Between 61% and 90% of all CD11c+ myeloid dendritic cells were also found to be positive for intracellular IL-6. These cell types are all antigen presenting cells.

The lymphocyte populations (T and B cells) in healthy control patients were found to be more heterogeneous in a resting state with subjects displaying variable percentages of these cell types with internal IL-6 (Figure 31a). Between 26% and 100% of CD19+ B cells were found to be positively stained for intracellular IL-6 in a resting state, whilst between 10% and 75% of CD3+ T cells were positively stained for intracellular IL-6. The CD3+ T cells have a biphasic distribution, with 3 subjects clustered at around 75%- 80% IL-6 positive, and the remaining subjects below 40%, whereas the CD19+ B cells are more normally distributed (Figure 31a).

The percentage of IL-6 producing cells in RA and PMR patients was similarly measured (Figure 31b-c). Again, a high percentage (77%- 99%) of CD14+ monocytes was found to be positive for IL-6 in all RA subjects (Figure 31b). All RA subjects also had a high percentage of IL-6 positive CD123+ plasmacytoid dendritic cells (67%-98%). CD11c+ myeloid dendritic cells and CD19+ B cells were more heterogeneous cell populations with subjects displaying more variable levels of IL-6 positive cells. The percentage of IL-6 positive CD11c+ mDCs in RA patients ranges from 40%-91%, and 18%- 94% for the CD19+ B cells. The majority of RA subjects had a low percentage of IL-6 positive CD3+ T cells (<27%), except for one subject (SP6) in which 96% of their CD3+ T cells were positive for IL-6 (Figure 31b).

The PMR patients also had a high percentage of IL-6 producing antigen-presenting cells, with almost 100% of all CD14+ monocytes, CD11c+ dendritic cells and CD123+ dendritic cells positive for IL-6 except for one subject, SP8 (Figure 31c). Similarly to the HC and RA subjects, CD19+ B cells and CD3+ T cells were more varied in terms of the percentage of cells producing IL-6 (Figure 31c). CD19+ B cells had between 16%- 100% of IL-6 positive cells whilst in CD3+ T cells, the percentage of IL-6 positive cells varied between 5%- 94%, with a normal, evenly spread distribution (Figure 31c).
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There were no statistically significant differences between the mean percentage of IL-6 producing cells in RA, PMR and healthy controls for all cell types. It is therefore not obvious which of these five cell types (if any) are responsible for the over-production of IL-6 in PMR based on these results. What is clear is that all cell types in all subjects are capable of producing IL-6 in a resting state, some constitutively, with nearly 100% of all monocytes in all subjects (disease and controls) positive for IL-6, and a high percentage of the dendritic cells staining positive for IL-6 in all subjects. The percentage of lymphocytes, (CD3+ T cells and CD19+ B cells) which were positive for IL-6 was more varied between subjects; however there are no significant differences between the disease groups and healthy control group.
Figure 31: The percentage of each cell type within the PBMC containing constitutive intracellular IL-6. The cells were cultured for 24 hours prior to Ab staining. Values were determined by flow cytometry, with 10,000 events collected per participant. Graph (a) illustrates the percentage of IL-6 positive cells in healthy controls (b) in RA patients and (c) in PMR patients. Each symbol represents one participant.
Chapter 3 - Results

3.2.2.3 Calculating the number of IL-6 producing cells in each cell type

The actual number of IL-6 producing cells for each cell type was calculated in order to see if there were any significant differences between the disease groups and healthy control group. These calculations were based on previously measured percentages of each cell type at a resting state and the total white cell counts (WCCs) provided by the clinicians (Table 13), as the total number of cells in each sample was not recorded at the time of the cell count. The total WCCs used for the healthy controls were taken from published values normally seen in healthy adults (Longmore, 2010) (Table 12) as white cell counts were not performed on the healthy control blood samples taken.

The total WCCs fell within the normal range for all subjects, although the majority of PMR patients (71%) and RA patients (80%) had WCCs which fall in the top half of the normal expected range for a healthy adult (Table 13). One RA patient, SP7, had a particularly high WCC, at 10.5x 10⁶ per ml, which is very close to the top end of the normal range for a healthy adult.

Table 12: Published ‘normal’ ranges for a white cell count (WCC) in a healthy adult (Longmore, 2010).

<table>
<thead>
<tr>
<th>Normal range for healthy adult (x10⁶/ml):</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total white cell count:</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>4-11</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Lymphocytes (T and B cells):</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>1.5-4.5</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Monocytes:</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>0.2-0.8</td>
</tr>
</tbody>
</table>

Two subjects (SP9 and SP14) had very high T cell counts, outside of the normal range for a total lymphocyte count (Table 13). One of these subjects was a healthy control and the other was an RA patient. The monocyte counts were high for the majority of subjects (14 out of 19), and of the 5 subjects which fell within the normal range, 4 were healthy controls and one was a PMR patient (Table 13). No published values for normal dendritic cell counts were found.

Using the total WCC and the percentage of each cell type determined by flow cytometry, the number of IL-6 producing cells for each cell type could be calculated (Table 14). Unpaired T tests were performed to determine whether the mean number
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of IL-6-producing cells was different for healthy controls, RA patients and PMR patients in each of the five cell types, using the Bonferroni test to correct for multiple comparisons (meaning that any value equal to or below 0.01 was taken to be significant) (Bland, 1995). Using unpaired T tests, the mean number of IL-6-producing CD14+ monocytes was significantly higher (P= 0.01) for PMR and RA patients compared to healthy controls (Table 15). None of the other cell types were significantly different between the groups. Overall monocyte numbers were elevated in both disease patients but because they were not different between RA and PMR it suggests this may be a consequence of elevated circulating IL-6. It cannot account for the elevated circulatory IL-6 in PMR.
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Table 13: The absolute number of each cell type in each participant (x10⁶/ml). The number of each cell type (resting) in each participant was calculated using the percentages of each cell type measured by flow cytometry. The values in red are based on the median of the normal expected range for a healthy adult as WCCs were not performed on these subjects.

<table>
<thead>
<tr>
<th>Study participant</th>
<th>Diagnosis</th>
<th>CD3+</th>
<th>CD14+</th>
<th>CD19+</th>
<th>CD123+</th>
<th>CD11c+</th>
<th>Total white cell count (x10⁶ cells/ml)</th>
<th>CD3+</th>
<th>CD14+</th>
<th>CD19+</th>
<th>CD123+</th>
<th>CD11c+</th>
</tr>
</thead>
<tbody>
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<td>15</td>
<td>21</td>
<td>8.9</td>
<td>3.8</td>
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<td>1.3</td>
<td>1.9</td>
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<td>22</td>
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<td>0.3</td>
<td>0.9</td>
<td>1.7</td>
</tr>
<tr>
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<td>4</td>
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<td>21</td>
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<td>SP4</td>
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<td>6</td>
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</table>
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Table 14: The actual number of IL-6 producing cells (x10^6/ml) for each of the 5 cell types in resting PBMC. The values given for the healthy control patients are based on the median of the normal WCC count expected for a healthy adult.

<table>
<thead>
<tr>
<th>Study participant</th>
<th>CD3+</th>
<th>CD14+</th>
<th>CD19+</th>
<th>CD123+</th>
<th>CD11c+</th>
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<td>12</td>
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<td>1.4</td>
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</tr>
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<td>14</td>
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</tr>
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<td>1.6</td>
<td>0.6</td>
<td>1.1</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Table 15: The mean number of IL-6 producing cells (x10^6/ml) in healthy controls, RA patients and PMR patients. Unpaired student T tests were used to determine the statistical difference between the mean of the groups for each cell type. Values highlighted in yellow have a P-value that is less than or equal to 0.01, and are thus taken to be significant.

<table>
<thead>
<tr>
<th>Statistical testing</th>
<th>CD3+</th>
<th>CD14+</th>
<th>CD19+</th>
<th>CD123+</th>
<th>CD11c+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean no. of IL-6 producing cells in healthy controls</td>
<td>1.02</td>
<td>0.65</td>
<td>0.33</td>
<td>0.74</td>
<td>0.90</td>
</tr>
<tr>
<td>Mean no. of IL-6 producing cells in RA patients</td>
<td>1.12</td>
<td>1.24</td>
<td>0.46</td>
<td>1.14</td>
<td>1.15</td>
</tr>
<tr>
<td>Mean no. of IL-6 producing cells in PMR patients</td>
<td>1.53</td>
<td>1.32</td>
<td>0.25</td>
<td>1.05</td>
<td>1.59</td>
</tr>
<tr>
<td>T test RA vs HC</td>
<td>0.86</td>
<td>0.01</td>
<td>0.61</td>
<td>0.06</td>
<td>0.21</td>
</tr>
<tr>
<td>T test PMR vs HC</td>
<td>0.39</td>
<td>0.01</td>
<td>0.64</td>
<td>0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>T test: PMR vs RA</td>
<td>0.55</td>
<td>0.73</td>
<td>0.20</td>
<td>0.65</td>
<td>0.19</td>
</tr>
</tbody>
</table>
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3.2.2.4 Measuring the intensity of IL-6 staining within unstimulated cells

The mean fluorescence intensity (MFI) of the IL-6 antibody staining inside the unstimulated PBMCs was measured by flow cytometry in order to identify an individual cell type which may be producing greater amounts of IL-6 in patients with PMR, but not in RA patients or in healthy controls.

The MFI of IL-6 staining in CD3+ T cells was very low in all subjects in all groups (2,450-17,020) except for one RA subject (SP11) in which CD3+ T cells had a higher MFI of 44,568 (Figure 32). There were no significant differences between the means of the three groups. The MFI of IL-6 staining was also relatively low in CD14+ monocytes in the majority of subjects (4,611-40,494). There were, however, two subjects with significantly higher MFI values; one healthy control patient (SP9) with an MFI of 72,397 and one RA subject with an MFI of 151,313. Again there was no significant difference between the means of the groups.

The range of MFI in CD19+ B cells was greater in subjects with RA (2,391-114,817) and PMR (2,926-69,814) compared to healthy controls (4,443-41,420), however, there was no significant difference between the means of the groups. In the CD123+ pDCs and CD11C+ mDCs there was a greater range in MFI in the subjects with RA (3,033-43,990) and PMR (3,772-62,428) compared to healthy controls (7,516-26,096), but again, there were no statistically significant differences between the means of the diseased groups compared to healthy controls.

Based on these results, no single cell type was found to be constitutively producing high levels of IL-6 in PMR participants. Taken together, all cell types in all individuals were able to produce intracellular IL-6 but mostly at low or very modest levels. CD3+ T cells and CD19+ B cells were the most heterogeneous whilst DC and monocytes were homogeneous in their expression of IL-6, which demonstrated no elevation in PMR patients.
Figure 32: The mean fluorescence intensity (MFI) of intracellular IL-6 staining in the resting PBMCs of (a) healthy controls of a similar age (b) RA patients and (c) PMR patients. Blood samples were dual stained for cell type and IL-6. Samples were analysed by flow cytometry, with 10,000 events collected per sample. Each symbol represents one subject.
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3.2.2.5 IL-6 in the culture supernatant of resting PBMC

In order to determine if intracellular IL-6 staining reflected the secretory capacity of the cells, the supernatants from resting (i.e. unstimulated) PBMC were collected after 24 hours in culture and stored at -80°C before being analysed by CBA. The concentration of secreted IL-6 in the supernatants was measured.

Low levels of IL-6 were detected in the unstimulated PBMC culture supernatant of one PMR patient (SP1: 8.5 pg/ml) and one RA patient (SP4: 2.8 pg/ml) but not in the supernatant of any other subjects. This confirms that unstimulated PBMC from PMR patients do not constitutively produce higher quantities of IL-6 and thus are not responsible for the elevated levels of IL-6 in the circulation of these patients. Furthermore, the results suggest that despite the presence of low concentrations of intracellular IL-6 detectable within the PBMC, they do not seem to necessarily secrete it.

3.2.3 Measuring inducible IL-6 in PBMC

In order to determine if PBMC from PMR patients showed a greater responsiveness to stimulation, the PBMC were also cultured with LPS (1 μg/ml) for 24 hours and IFNγ (100 ng/ml) for the final four hours. This had been previously shown to stimulate cellular responses in all cell types in young healthy control PBMC.

3.2.3.1 The change in the percentage of IL-6 positive cells following stimulation

The actual percentage of IL-6 positive cells following stimulation is shown in Figure 33. The change in the percentage of IL-6-positive cells between unstimulated and stimulated PBMC are shown in Figure 34. For the majority of subjects in all groups (healthy controls, RA patients and PMR patients) the percentage of IL-6-positive CD14+ monocytes and CD123+ pDCs changed very little (<13%) following stimulation (Figure 34a-c). For most of these subjects the percentage of IL-6 positive
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CD14+ and CD123+ cells was between 85% and 100% prior to stimulation, thus making further stimulation difficult to assess. There were only minor increases and decreases in the percentage of IL-6 positive CD14+ monocytes and CD123+ pDCs for most subjects. One subject, SP6 (RA) experienced a relatively large increase in IL-6 positive CD123+ pDCs following stimulation, going from 67% to 99% IL-6 positive cells, a percentage change of 48% (Figure 34) and study participant 8 (PMR) experienced a percentage change of 27%. For these two cell types, only one subject, SP12 (HC) experienced a percentage decrease in the percentage of IL-6 positive cells of over 10% (Figure 34). There was no significant difference between the responses of the PMR patients, RA patients and healthy controls.

The responses of CD11c+ mDCs to stimulation were more varied than the responses of the CD14+ monocytes and the CD123+ pDCs (Figure 34a-c). Subjects experienced up to 24% change (increase or decrease) in the percentage of IL-6 positive cells. RA subject SP6 was most responsive to stimulation, with an increase of 57% following stimulation. There were no significant differences between the responses of the different groups in the CD11c+ mDCs.

The responses of the CD19+ B cells to stimulation were much more variable than in the monocytes and dendritic cells. There were a few ‘non-responders’ in all three groups (including healthy controls, RA patients and PMR patients); some of these were the subjects who already had a high percentage of IL-6 positive cells and thus were already responding at a maximum level so a large increase was not possible. Some subjects, however, had a low percentage of IL-6 positive CD19+ cells prior to stimulation and did not appear to respond at all to the stimulation (SP2, SP7 and SP18) (Figure 34a-c). In all three groups there were very high responders (SP4, SP8, SP10 and SP16 percentage increase by 91%, 102%, 77% and 176% respectively) (Figure 34a-c). In all groups there were subjects who experienced a large decrease in the percentage of IL-6 positive cells (SP1, SP7, SP11 and SP12, percentage decrease by 33%, 27%, 46% and 52% respectively). The responses were therefore much more variable in the CD19+ B cells than the other antigen presenting cells. Again, however, there were no significant differences between the IL-6 responses of the B cells in the different groups.
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The responses to stimulation in the CD3+ T cells were even more variable for all groups. Three healthy control subjects (SP3, SP9 and SP16) experienced very large percentage increases (up to 445%) in the percentage of IL-6 positive cells from unstimulated to stimulated T cells (Figure 34). Three RA subjects (SP4, SP11 and SP14) also experienced a very large increase (up to 1013%) in the percentage of IL-6 positive cells (Figure 34b). One PMR patient experienced a relatively large increase of 63% (Figure 34c) with other PMR subjects showing increases of up to 21%. Subjects in all three groups had large decreases (up to 82%) in the percentage of IL-6 positive CD3+ T cells following stimulation (Figure 34a-c). Again, some subjects in all groups had CD3+ T cells which were not very responsive to stimulation. There was a great deal of variation in the responses of CD3+ T cells to stimulation in all the groups, and some of the responses to stimulation were very extreme compared to the other cell types, perhaps due to the larger variation in percentage IL-6 positive cells prior to stimulation. There were no significant differences between the responses of the different groups.

In conclusion, there appears to be variable responses in all the cell types and in all the groups. The biggest changes appear to be in the CD3+ T cells and CD19+ B cells, however there was a greater capacity for change in these cells as the percentage of IL-6 positive cells in these cell types was more variable prior to stimulation. Many of the CD14+ monocytes, CD123+ pDCs and CD11c+ myeloid dendritic cells were already stimulated to their maximum capacity (i.e. approximately 80%-100% of cells were positive for IL-6) prior to stimulation and thus there was less capacity for change. There were also some cell types in some subjects which did not appear to be responsive at all, despite not being stimulated to maximum capacity prior to stimulation. The percentage of IL-6 positive cells decreased quite dramatically in some cell types (namely the CD3+ and CD19+ cells) after stimulation, suggesting that the stimulatory technique may be detrimental to IL-6 production in these cells.

Having tested the cellular responses to stimulation, i.e. the percentage change from unstimulated to stimulated values in each patient or control group, it was found that there were no significant differences between the mean percentage change in the percentage of IL-6 positive cells in PMR patients, RA patients and healthy control patients.
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Figure 33: The percentage of each cell type within the stimulated PBMC containing intracellular IL-6. The cells were stimulated for 24 hours with LPS (1 μg/ml) and IFNγ (100 ng/ml) for the final 4 hours prior to antibody staining. Values were determined by flow cytometry, with 10,000 events collected per participant. Graph (a) illustrates the percentage of IL-6 positive cells in healthy controls of a similar age, (b) in RA patients and (c) in PMR patients. Each symbol represents one participant.
Figure 3.4: The percentage of cells which were stained for intracellular IL-6 in unstimulated and stimulated PBMCs (joined by black line) in (a) healthy controls (b) RA patients and (c) PMR patients. Cells were stimulated with LPS (1 μg/ml) for 24 hours and IFNγ (100 ng/ml) for the final four hours.
3.2.3.2 Changes in intensity of intracellular IL-6 staining following PBMC stimulation

There were no significant differences between the mean intracellular IL-6 levels in stimulated PBMC between any of the groups (HC, RA and PMR). Thus the percentage change in the MFI between unstimulated and stimulated PBMC was examined (Figures 36a-c). Figure 35 shows the MFI of stimulated PBMC.

There was very little change in MFI from unstimulated to stimulated CD3+ T cells in all three groups (Figure 36), except for one RA subject (SP4) in which the MFI increased by a 1205%, one healthy control patient (SP16) which showed an increase of 240% and one PMR patient (SP17) which showed an increase of 110%. Ten out of the nineteen subjects displayed a decrease in MFI after stimulation, and three did not change, suggesting that these cells did not respond to LPS or IFNγ by producing IL-6 and the stimulation may have caused a down regulation in IL-6 production.

CD14+ monocytes displayed variable responses to stimulation in all three groups. In some subjects in all the groups the MFI increased dramatically, namely SP4 (988%), SP8 (258%), SP13 (370%), SP16 (230%) and SP18 (102%) (Figure 36). The MFI decreased in only 3 subjects and some did not respond at all to the stimulation, again this was in all the groups and therefore was not disease specific.

The responses of CD19+ B cells were variable in all groups. Again, SP4 (RA) showed a significant increase in MFI by 5033%, significantly greater than any other subject. SP10 and SP13 (healthy controls) also displayed large increases in MFI (1243% and 800% respectively). As with the CD3+ T cells and CD14+ monocytes, there were some subjects who experienced a decrease in MFI from unstimulated to stimulated cells and some subjects with non-responsive CD19+ B cells.

CD123+ pDCs and CD11c+ mDCs also displayed variable responses to stimulation in all groups. Generally there were smaller percentage increases or decreases in MFI (<100%) compared to the responses seen in the other cell types, suggesting that DCs were not stimulated by LPS and IFNγ to produce IL-6. The MFI, however, in RA
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subjects SP4, SP6 and SP11 and healthy control subjects SP12 and SP13 were significantly increased (>180%) following stimulation in both CD123+ pDCs and CD11c+ mDCs, and the MFI of healthy control subject SP16 was also significantly increased in the mDCs.

Overall, there were a group of subjects with highly responsive PBMCs in all three groups. There were, however, also subjects which responded negatively or did not respond at all to the stimulation in terms of IL-6 production in all three groups. Having tested the cellular responses to stimulation, i.e. the percentage change from unstimulated to stimulated values in each patient or control group, it was found that there were no significant differences between the mean percentage change in MFI in PMR patients, RA patients and healthy control patients.
Figure 35: The mean fluorescence intensity (MFI) of intracellular IL-6 staining in the stimulated PBMCs of (a) healthy controls (b) RA patients and (c) PMR patients. The cells were stimulated for 24 hours with LPS (1μg/ml) and IFNγ (100 ng/ml) for the final 4 hours. Blood samples were dual stained for cell type and IL-6. Samples were analysed by flow cytometry, with 10,000 events collected per sample. Each symbol represents one subject.
Figure 3: The mean fluorescence intensity of IL-6 staining in unstimulated and stimulated PBMCs (joined by a black line) in (a) healthy controls (b) RA patients and (c) PMR patients. Cells were stimulated with LPS (1 μg/ml) for 24 hours and IFNγ (100 ng/ml) for the final four hours.
3.2.3.3 IL-6 secreted in the supernatant of stimulated PBMC cultures

The supernatants from the stimulated PBMC were also collected after 24 hours in culture and stored at -80°C before being analysed by CBA as previously described. IL-6 was measured in these supernatant samples.

Concentrations of IL-6 were significantly higher in the stimulated supernatants compared to unstimulated PBMC in all the groups (Figure 37). In healthy controls the mean concentration of IL-6 measured in stimulated supernatants was 3926.1 pg/ml, compared to 8182.3 pg/ml in RA patients, and 5301.2 pg/ml in PMR patients. In the unstimulated PBMC supernatants, only two subjects (one PMR and one RA) had concentrations of IL-6 which were detectable above the sensitivity threshold for the CBA kit (above 2.5 pg/ml).

These results suggest that at least one cell type in every participant sample was stimulated to secrete very high concentrations of IL-6 (>1000 pg/ml) when stimulated by LPS and IFNγ over 24 hours. There was, however, no significant difference between the mean of the IL-6 concentration measured in the stimulated PBMC supernatants between RA patients, PMR patients and healthy controls with these sample sizes.

Secreted IL-6 values did not reflect the results observed in the flow cytometry assays following cell stimulation. None of the cell types measured by flow cytometry were found to have particularly high MFI stains suggestive of large amounts of IL-6 in any of the participant groups, and responses to stimulation were very variable. Reasons for this discrepancy are considered in the discussion.
Figure 37: The IL-6 concentration measured in the supernatants taken from PBMC cultured for 24 hours with LPS (1 µg/ml) and IFNγ (100 ng/ml) for the final 4 hours. Each symbol represents an individual study participant. The red line represents the mean for the group. NS= not significant.

### 3.2.4 Summary

CD3+, CD14+, CD19+, CD123+ and CD11c+ PBMC in all subjects constitutively produced low to modest levels of intracellular IL-6. This corresponded with very low concentrations of secreted IL-6 in parallel cultures (<8.5 pg/ml). Stimulation of PBMC with IFNγ/LPS induced strong intracellular IL-6 responses in all cell types of some samples. Overall, however, responses were highly variable and no significant differences in cell responsiveness were observed between PMR, RA and control samples. In contrast, secreted IL-6 increased dramatically following stimulation of all cultures (>1000 pg/ml) with LPS and IFNγ, suggesting intracellular staining may not reflect the secretory capacity of these cells, but also confirming no differences between PBMC responses of PMR RA and control groups.
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3.3 The cytokine profile of PMR patients, RA patients and healthy controls (refer to Aim 6 in Section 1.16)

3.3.1 Measurements of other inflammatory cytokines in the plasma

A panel of other cytokines (IL-10, TNFα, IL-12p70, IL-1β, IL-2, IL-4, IL-8, 1L-17A and IFNγ) were measured in the plasma of the PMR patients, RA patients and healthy controls in order to examine the disease pathologies more broadly (Figure 38).

All of the cytokines measured in healthy controls fell below the sensitivity threshold for the CBA assay and were therefore not detectable, except for IL-8 in all subjects, and IL-12p70 in one subject (Figure 38a). Concentrations of IL-8 in healthy controls ranged from 3.6 to 7.1 pg/ml, with a mean of 5.2 pg/ml (Figure 39).

The same cytokine panel was measured in the plasma of RA patients by CBA (Figure 38b). Concentrations of IL-17A, IFNγ and IL-1β were not detectable in any participants as they fell under the sensitivity threshold of the CBA kit. Two out of six RA patients, however, had significantly elevated concentrations of five other cytokines, IL-10, TNFα, IL-12p70, IL-2 and IL-4 (Figure 38b). This suggests a potential difference in disease pathology in these RA patients. Plasma IL-8 was detectable in all RA subjects (range; 6.3-32.0 pg/ml, mean; 12.1 pg/ml) (Figure 39), however, the mean was not statistically different to the mean of the IL-8 concentration in healthy controls (P= 0.100).

This panel of cytokines was also measured in the plasma of PMR patients by CBA (Figure 38c). In contrast to RA and similarly to the healthy controls, all cytokines were below the lower threshold of detection, except for IL-6 and IL-8 (range; 6.3-32.0 pg/ml, mean; 8.1 pg/ml). Again, although the mean concentration of IL-8 was visibly higher than the mean IL-8 concentration in healthy controls, the P value was not significant (P= 0.103) (Figure 39).
Figure 38: The concentration of ten cytokines measured in the plasma of RA (b) and PMR (c) patients and healthy controls (a) of a similar age. Each symbol represents the geometric mean of n=4 repeats for IL-6, IL-10 and TNFα and n=2 repeats for all other cytokines for each participant. The dotted red line represents the lowest threshold level of detection as stated on the CBA kits.
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3.3.2 Measurements of other inflammatory cytokines in the serum

The concentrations of these cytokines were also measured in the serum by CBA (Figure 41), which demonstrated similar results but perhaps greater sensitivity. For example, in healthy control patients and PMR patients only IL-8 was detectable above the threshold limit of the kits, except for one control subject who had raised IL-4 and IL-2 levels. In the serum of the same two RA patients (SP7 and SP14), levels of IL-10, TNFα, IL-12p70, IL-2, IL-4 and IL-8 were raised to a detectable level, with a third RA subject (SP11) also having detectable levels of TNFα, IL-2 and IL-4 (Figure 41b).

A Bland-Altman plot comparing IL-8 concentrations in the plasma and serum showed a cluster of patients with a very small difference in the mean measurements (close to zero) at the lower end, suggesting that the measurements for plasma and serum were agreeable (Figure 40). As with IL-6, there is, however, a clear bias in favour of the serum measurements particularly at the higher end (mean IL-8 concentration 10 pg/ml...
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and above), also evidenced by the fact that nearly all data points are below zero. This suggests that the concentrations of both cytokines are higher in the serum than in the plasma, particularly at the higher end of the measurements where there is more variation.

Figure 40: Bland Altman plot comparing IL-8 concentrations measured in the plasma and serum of RA and PMR patients and healthy controls of a similar age. Each data point represents one participant.

3.3.3 Summary

Looking more broadly at cytokine responses in the serum and plasma of our participants, a small number of RA patients (2 out of 6) were found to have raised levels of several cytokines together including IL-4, TNFα, IL-10, IL-12p70 and IL-2. This was not observed in PMR patients or healthy controls, and may indicate that different disease pathologies exist within RA that are not present in PMR, although has to be applied in interpretation due to the small sample numbers.
Figure 41: The concentration of ten cytokines measured in the serum of RA (b) and PMR (c) patients and healthy controls (a) of a similar age. Cytokines were measured by CBA. Each symbol represents the geometric mean of n=2 repeats for IL-6, IL-10 and TNF and n=1 repeat for all other cytokines for each participant. The dotted red lines represent the threshold levels of detection for the CBA kits.
3.3.4 Cytokines in the culture supernatant of resting (unstimulated) PBMC

The same panel of nine cytokines (as well as IL-6) were measured in the PBMC culture supernatants of unstimulated PBMC (Figure 42a-c). Only secreted IL-8 was detectable in all three patient groups. The concentration of secreted IL-8 in the culture supernatants was around 100 times greater than the concentration of IL-8 measured in the serum. Results suggest that a high concentration of IL-8 is constitutively secreted by one, a few, or all of the PBMC types when in a resting state in all the subjects, including the healthy controls. There was no significant difference between the mean IL-8 concentration of the healthy controls (1082.4 pg/ml (range: 515.3-2274.0)), RA patients (2116.6 pg/ml (range: 410.8-2269.0)) and PMR patients (1946.4 pg/ml (range: 622.5-4577.7) (Figure 43), although the range for PMR was notably higher.

TNF was detected in the unstimulated PBMC culture supernatants of three healthy controls, three RA patients, and one PMR patient only, at a maximum concentration of 26.1 pg/ml. IL-1β was measured at a concentration of 181.7 pg/ml in one PMR patient. One other PMR patient, one RA patient and one healthy control also had detectable levels of IL-1β, however the maximum concentration for these other subjects was 14.2 pg/ml. IL-17A was measured in one healthy control at a concentration of 21.5 pg/ml. The concentration of IL-17A in all other subjects fell below the high sensitivity threshold for the CBA assay (18.9 pg/ml). IL-12p70, IFNγ, IL-10, IL-4 and IL-2 were not detectable in the unstimulated PBMC culture supernatants in any of the subjects.

Taken together, the culture supernatants from unstimulated PBMC do not reflect the cytokine profiles shown in the participants’ serum or plasma. It is possible that substances in their own serum or in vivo which are not present in the culture are required to stimulate the cells, or alternatively, the PBMC are not the predominant source of these cytokines.
Figure 42: The concentration of ten cytokines measured in the culture supernatants of unstimulated PBMC from RA (b) and PMR (c) patients and healthy controls of a similar age (a). Cytokines were measured in the culture supernatants using CBA kits. Each symbol represents one participant. The red dotted lines represent the lowest threshold limit of detection for the kit.
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3.3.5 Cytokines secreted in the supernatant of stimulated PBMC cultures

The supernatants from the stimulated PBMC were also collected after 24 hours in culture and stored at -80°C before being analysed by CBA as previously described. The same panel of cytokines were measured in these supernatant samples.

As with the unstimulated PBMC supernatants, IL-8 was present at the highest concentrations of all the measured cytokines in stimulated culture supernatants. Significantly greater (100-500 fold) concentrations were detected in stimulated PBMC culture supernatants (39,190-126,529 pg/ml) compared with unstimulated PBMC culture supernatants (229-6,534 pg/ml) (Figure 44a). This indicates that there are one or more PBMC cell types which are strongly stimulated by LPS and/or IFNγ to produce IL-8. The mean concentration of IL-8 in stimulated PBMC supernatants was greater for PMR than RA, and for RA than healthy controls, but this was not found to be significant using unpaired T tests (Figure 44a) for these sample sizes.

Figure 43: The concentration of IL-8 in the unstimulated PBMC culture supernatants taken from RA and PMR patients and healthy controls of a similar age. The concentration of IL-8 was measured using a CBA kit. Each symbol represents one participant. The red line represents the mean of each group. NS= not significant.
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The IL-1β concentration in the supernatants of stimulated PBMC was significantly higher than the concentration in the unstimulated PBMC supernatants for all subjects. The concentration of IL-1β in stimulated PBMC supernatants ranged from 192.1 pg/ml to 4802.9 pg/ml, compared to the concentration in unstimulated PBMC supernatants; in which only 4 subjects had detectable levels of IL-1β (i.e. only 4 subjects had levels of IL-1β over 7.2 pg/ml). Again, as with IL-6 and IL-8, there was no significant difference between the mean IL-1β concentrations in the 3 groups (Figure 44b) indicating that PBMC secretion of IL-1β is not linked to PMR or RA disease, but is a typical inflammatory reaction to LPS and IFNγ stimulation.

The concentration of TNF in the stimulated PBMC supernatants was also significantly increased above the measured concentration in the unstimulated PBMC supernatants. TNF was only detectable in the unstimulated PBMC supernatant of seven subjects (3 HC, 3 RA and 1 PMR), where TNF levels ranged from 3.8 pg/ml (the threshold of detection) to 14.5 pg/ml. TNF levels were detectable in the stimulated PBMC supernatants in all subjects, with levels ranging from 11.7 pg/ml up to 479.7 pg/ml (Figure 44c). Again, no statistically significant difference between the means of the three groups was observed for these sample sizes, indicating that this is the ‘normal’ PBMC response to LPS and IFNγ stimulation.

IL-10 was not detectable in any of the unstimulated PBMC culture supernatants, however when stimulated with LPS and IFNγ, three RA patients (SP4, SP6 and SP14) and two healthy controls (SP5 and SP16) had detectable levels of IL-10. The maximum IL-10 concentration in the healthy controls was 9.8 pg/ml whereas the maximum concentration in RA subjects was 32.6 pg/ml. There was no detectable IL-10 in PMR subjects.

IL-12p70 was not detectable in the unstimulated PBMC supernatants in any subjects, however it was detectable at very low levels in one RA patient (SP7) and one healthy control subject (SP16). IL-12p70 was not detectable in the stimulated PBMC supernatants in any of the PMR patients.

IL-17 was only detectable in the unstimulated PBMC supernatant of one healthy control subject (SP12) and no RA or PMR subjects. When stimulated with LPS and
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IFN\(\gamma\), however, one healthy control subject had a raised concentration of IL-17 (SP5-57.5 pg/ml) in the PBMC supernatant, and two RA patients had elevated IL-17 concentrations (SP4-85.7 and SP6-104.9 pg/ml). No PMR patients had raised levels of IL-17 in the stimulated PBMC supernatant, this time suggesting that IL-17 may be linked to RA but not to PMR. A larger patient cohort would be required to confirm this.

IL-2 was only detectable in the stimulated PBMC supernatant of two RA patients (SP4 and SP6) and no other subjects. IL-4 was detectable in the stimulated PBMC supernatant of only one RA patient (SP4) and no other subjects. IFN\(\gamma\) was added to the stimulated PBMC for the final four hours of culture in order to stimulate certain cell types, thus analysing the levels of secretory IFN\(\gamma\) in these samples was not possible.

3.3.6 Summary

When left unstimulated, PBMC did not consistently secrete significant concentrations of any cytokine, except IL-8. No significant differences between the IL-8 secreted by disease groups and control group were observed. Upon stimulation with IFN\(\gamma\)/LPS, increased secretion of IL-8, TNF\(\alpha\) and IL-1\(\beta\) from PBMC was observed. Nonetheless, there were no significant differences between cytokines secreted by PBMC in disease groups and the control group.
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Figure 44: The concentration of secreted (a) IL-8 (b) IL-1β (c) TNF in culture supernatants taken from PBMC stimulated for 24 hours with LPS (1 μg/ml) and IFNγ (100 ng/ml) for the final 4 hours. Each symbol represents one participant. Concentrations were measured using CBA kits. The red lines represent the mean for the group. NS= not significant.
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Previous studies have looked for the source of IL-6 specifically in peripheral blood monocytes in PMR, with varying results and no clear-cut answers. Roche and colleagues suggested that monocytes were the source, using PCR methods to determine that monocytes contained IL-6 mRNA, whereas T cells did not (Roche et al., 1993). There was, however, no difference between PMR patients and healthy controls, and just because the monocytes contained IL-6 mRNA did not prove that they secrete it or are the major source. Alvarez-Rodriguez and co-workers, however, concluded that monocytes were not the major source of IL-6 in PMR, using intracellular staining and flow cytometry methods (Alvarez-Rodriguez et al., 2010). They did not, however, present a complete set of data, only taking into account the percentage of IL-6 positive cells and not the MFI of IL-6 staining. Moreover, almost 100% of monocytes were stimulated in both PMR patients and controls, making it difficult to observe increases in IL-6 production in the patients with PMR. Furthermore, this study did not include a disease control, thus it was not possible to control for the effects of raised circulating IL-6 concentrations on IL-6 responses in the monocytes.

This study aimed to resolve these issues, ensuring that laboratory work was completed with appropriate controls and an exhaustive approach to explore the PBMC as a possible source of IL-6 in PMR. Two types of negative control were included in the present study, one group had no sign of inflammatory disease and were thus deemed the ‘healthy controls’, and the other group were patients with active RA and thus had raised circulating concentrations of IL-6. This meant that any general effects caused by this circulating IL-6 had been controlled for, and any differences seen in PMR PBMCs were related specifically to PMR disease pathogenesis.

This section will discuss the findings, limitations, and implications of the present study, answering some of the questions relating to the pathogenesis of PMR in the same order as the methods and results. Secondary findings relating to RA are also discussed here, with an emphasis on comparing the pathogenesis of these two inflammatory diseases.
4.1 The significance of IL-6 in PMR disease pathogenesis

4.1.1 Circulating IL-6 in PMR and RA (refer to Hypothesis 1 in Section 1.15, Aim 1 in Section 1.16)

In agreement with previous studies (Straub et al., 2000, Cutolo et al., 2006), it was confirmed that mean IL-6 concentrations are significantly elevated in the plasma and serum of PMR and RA patients compared to healthy controls of a similar age and gender distribution (Section 3.1.2). Unlike previous studies, the timing of the blood sampling and processing was strict, in order to account for the circadian variation associated with IL-6 (Crofford et al., 1997, Perry et al., 2009). Age and gender boundaries were also adhered to with regards to recruitment of the patients, confirming that the differences seen here are relevant to PMR and RA, not due to natural immunosenescence or hormonal differences.

As IL-6 concentrations are consistently and significantly raised in PMR and RA patients compared to healthy controls, IL-6 could potentially be a good bio-marker for these diseases. If a quick and economically viable method of measurement became available then measuring circulating IL-6 could become an important tool for clinicians in terms of diagnosing both PMR and RA. Nevertheless, serum and plasma IL-6 is raised in other inflammatory conditions so existing disease criteria would also need to be used alongside the measurement of this clinical disease marker.

4.1.2 IL-6 measurement: plasma vs serum (refer to Hypothesis 2 in Section 1.15, Aim 2 in Section 1.16)

According to the literature, there is no agreement as to whether plasma or serum should be used to analyse cytokine profiles. Thus in this study, IL-6 was measured in both the plasma and the serum in order to observe if there were any major differences in IL-6 concentrations observed. The serum was separated from the rest of the blood constituents at the clinic within one hour of being extracted and the plasma was separated within two hours, after whole blood was transported from the clinic to the
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UWE laboratories (30-40 minute journey), meaning that the timing of the separation of plasma and serum from other blood constituents, and the conditions of storage varied slightly between plasma and serum. Therefore this needs to be taken into consideration when comparing cytokine concentrations in these two blood components. The composition of plasma and serum also varies considerably. Serum is separated from fibrin in clotted blood, as well as blood cells and related coagulation factors by centrifugation, whereas plasma is separated from blood cells only, due to the addition of an anticoagulant prior to centrifugation (Yu et al., 2011). This means plasma still contains clotting factors which could affect cytokine measurement. Serum on the other hand is reported to contain RF, which may also interfere with cytokine measurement (Bartels et al., 2011, Churchman et al., 2012, Bartels and Madsen, 2013).

A correlation between the concentration of IL-6 measured in the plasma and serum of participants was observed (Section 3.1.3). Plasma and serum IL-6 concentrations correlated very well with a high correlation coefficient of 0.961 (P< 0.0001), however, there was a clear bias towards the serum measurements highlighted by the >45° angle of the regression line (Figure 14). A Bland and Altman plot confirmed this bias towards the serum measurements. This bias towards the serum measurements was similar for IL-8, which was also measured in the plasma and serum by CBA (Figure 38). This indicates that although the values correlated well and thus were roughly equivalent, the values were not exactly the same. Serum values were consistently higher than the plasma values, which could mean that the preparation method for serum may be more suitable for cytokine measurement, however, it could alternatively mean the shorter time to freezing of the serum preserves the higher concentrations of cytokines, which appear to be more quickly degraded in plasma.

Future studies should therefore consider carefully which blood component to use when studying cytokine profiles and also how to prepare and store samples in order to reduce degradation of cytokines. There are a number of factors which may have affected cytokine concentrations in the plasma and serum and these are discussed in the following section.
4.1.3 Biological activity of raised IL-6 (refer to Hypothesis 3 in Section 1.15, Aim 3 in Section 1.16)

Having confirmed that IL-6 was elevated in the circulation of PMR patients, it was then necessary to determine whether this IL-6 was active and thus capable of causing the pathological symptoms experienced by PMR patients. Results showed for the first time, that the IL-6 present in the plasma of PMR patients is biologically active, determined by the proliferation of IL-6-dependent B9 cells following incubation with the participant plasma samples (Section 3.1.5). It was confirmed that the IL-6 measured in the plasma of RA patients (most likely to be derived from the synovium) was also biologically active. This confirms a crucial role for IL-6 in the pathogenesis of these diseases.

Biologically active concentrations of IL-6 in the plasma measured by the B9 bioassay were actually higher than the IL-6 concentrations measured by CBA, at the higher values. According to Mire-Sluis and colleagues, immunoassays (such as ELISA and CBA) provide very ‘variable estimates’ of cytokine concentration in the sample, whereas bioassays may provide a more accurate estimate of the concentration of active cytokine in the sample (Mire-Sluis et al., 1995). Therefore the results from bioassays provide a more accurate representation of the action of IL-6 in vivo. The B9 bioassay, for example, takes into account the action of the IL-6 when free and when bound to its soluble receptor. The IL-6 soluble receptor has also been shown to enhance the sensitivity of B9 cells in a dose-dependent manner (Diamant et al., 1994). Concentrations of the soluble IL-6 receptor have been shown to be increased in RA patients compared to healthy controls (Robak et al., 1998), which may have enhanced the B9 cell proliferation and could explain the higher concentrations of IL-6 measured in the B9 bioassays compared to the CBA assays. The concentration of soluble IL-6 receptor has not yet been quantified in PMR patients. Jones and colleagues suggest that the soluble IL-6 receptor itself may actually contribute to the pathology of diseases such as RA, where it has such a crucial role in controlling IL-6-mediated events (Jones et al., 2001).
4.1.4 IL-6 and fatigue (refer to Hypothesis 4 in Section 1.15, Aim 4 in Section 1.16)

Until recently, researchers have looked for correlation between fatigue and IL-6 in PMR but found unexpectedly poor correlation, despite a clear temporal correlation in early morning IL-6 levels and fatigue symptoms. Thus, the following step was to determine whether IL-6 was directly associated with this major and highly debilitating symptom of PMR and RA. The recently devised BRAF-MDQ has given clinicians a deeper insight into the different aspects associated with fatigue (Hewlett et al., 2005). With the development of this questionnaire, it was possible to separate fatigue into four different groupings, two with a more somatic aspect: physical fatigue and living fatigue, and two with a more psychological aspect: cognitive fatigue and emotional fatigue. Other researchers have considered a potential link between IL-6 and fatigue as a whole in RA, with one study recently using the BRAF-MDQ to find a correlation between serum IL-6 and total fatigue (Helal et al., 2012). Until now, however, no study has looked at the individual aspects of fatigue to see whether IL-6 has a particular association with certain aspects, and no study has included PMR patients, who also suffer from fatigue.

Here, it is shown for the first time using the BRAF-MDQ, that IL-6 concentrations in the plasma of PMR and RA patients and total fatigue score demonstrate an unequivocal positive correlation ($r= 0.487$, $P= 0.030$), indicating a significant association between IL-6 and fatigue (Section 3.1.6.1). Furthermore, when individual aspects of fatigue were plotted against plasma IL-6 concentrations, physical fatigue ($r= 0.479$) and living fatigue ($r= 0.499$) correlated well. Cognitive fatigue ($r= 0.067$) and emotional fatigue ($r= 0.311$), in contrast, showed no correlation. Overall this suggests that the more physiological aspects of fatigue are linked to IL-6, whereas the psychological aspects of fatigue are not directly related to IL-6, but may be a consequence of the physical aspects.

Studies have shown that there is a strong relationship between the circadian fluctuation of IL-6, activity of the HPA axis, and production of adrenocorticotropic hormone.
(ACTH) and cortisol, with cortisol acting to suppress inflammation and cytokine secretion in a negative feedback control loop under normal circumstances (Figure 45) (Crofford et al., 1997, Morand and Leech, 2001, Perry et al., 2009). In RA, with known circadian fluctuation of IL-6, synovial fluid IL-6 concentrations were shown to increase around 5.75 hours before increasing in the blood, with plasma cortisol concentrations rising 3 hours later in order to suppress and resolve inflammation (Perry, 2008). There are, however, reports suggesting that the HPA-axis response is defective in PMR and RA patients, resulting in inadequate cortisol and ACTH levels in relation to the high circulating levels of cytokines such as IL-6 (Gudbjörnsson et al., 1996, Straub et al., 2000, Straub et al., 2002). Furthermore, increased circulating pro-inflammatory cytokine concentrations (mainly IL-6) are believed to play a direct role in the dysfunction of the HPA-axis (Choy, 2012).

Figure 45: Illustration adapted from Perry (2008), showing the regular HPA axis control loop (dotted line on left) and the hypothesised cortisol/IL-6 negative feedback loop (dotted line on right). In RA and PMR inadequate HPA axis mechanisms (red arrow) in relation to circulating IL-6 levels may lead to fatigue symptoms. Narrow solid lines indicate positive feedback and narrow dotted lines indicate negative feedback.
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This malfunction of the HPA-axis has been linked to the development of fatigue (Tsigos and Chrousos, 2002 and Sriringan and Choy, 2010), although the exact mechanisms involved are yet to be resolved (Figure 45). Nonetheless, this link is apparent in the results of the present study, where increased concentrations of IL-6 in both RA and PMR patients correlated well with the physical aspects of fatigue. Thus, the existence of a relationship between circulating IL-6 concentrations and fatigue seems very likely, and should form the basis for new research into the physiological mechanisms behind this relationship.

Moreover, a link between the CRP levels of PMR and RA patients and fatigue was explored. As expected, a significant correlation was observed between this secondary measure of inflammation and fatigue when using Spearman’s rank correlation coefficient ($r_s = 0.661$). Furthermore, the quality of IL-6 assays was confirmed by testing the strength of the correlation between plasma IL-6 concentrations and CRP levels. CRP is raised in response to raised circulating IL-6 levels. When tested using Spearman’s rank correlation coefficient, a strong correlation between CRP and IL-6 levels was found at the 0.5% probability level, suggesting that the results from the IL-6 assays were accurate as they corresponded well with this secondary measure of inflammation.

4.2 Exploring the cellular source of IL-6 in PMR (refer to Hypothesis 5 in Section 1.15, Aim 5 in Section 1.16)

With IL-6 confirmed as having a role in the pathogenesis of PMR, and in particular having a potential role in the onset of fatigue, the crucial next step was to explore the cellular source of the elevated circulating concentrations of IL-6. It was hypothesised that one of the PBMC types (T cells, monocytes, B cells or DCs) may be responsible for the significant quantities of IL-6 measured in the plasma and serum of PMR patients. RA patients whose circulating IL-6 is derived from synovium in the joints, were used as an independent control for the downstream activation of peripheral blood cells by high levels of circulating IL-6.
4.2.1 IL-6 production by PBMC in ‘a resting state’

It was demonstrated that all cell types in all participants were capable of producing IL-6 in ‘a resting state’, but mostly at low or very modest levels (Section 3.2.2). Some cell types constitutively produced IL-6; with nearly all monocytes in all subjects (disease and controls) staining positively for IL-6 (70-100%), and a high percentage of the two types of dendritic cells staining positively for IL-6 (35-100%) in all subjects. The percentage of lymphocytes (CD3+ T cells and CD19+ B cells) which were positive for IL-6 was more varied (4-99% and 13-100% respectively). When looking at the MFI of IL-6 staining in the same cells, however, it became clear that only low or modest concentrations of IL-6 were present in all of the cells.

Crucially, there were no significant differences between the percentage of IL-6 positive cells or MFI in the PMR patients, RA patients and healthy controls in the unstimulated PBMC. This was confirmed in parallel cultures, where very low concentrations of secreted IL-6 were measured in culture supernatants from resting PBMC (<9 pg/ml), with no difference between the patient groups and healthy control group. Thus without additional stimulation, the PBMC from patients with active disease (and no treatment at the time of sampling) were not the source of elevated concentrations of circulating IL-6 in PMR patients.

When calculating the absolute number of IL-6 producing cells for each PBMC cell type using data from the flow cytometry assays (i.e. the percentage of IL-6 positive cells) and the WCCs provided by the clinicians (Section 3.2.2.3), it was found that the mean number of IL-6 producing CD14+ monocytes was significantly higher (P< 0.05) in PMR patients and RA patients compared to healthy controls. This may suggest that monocytes produce large quantities of IL-6 in inflammatory diseases. There was, however, no significant difference between PMR and RA samples, and no single cell type which produced elevated concentrations of IL-6 in PMR patients alone, confirming that the PBMC are not the source of IL-6 in PMR patients. Actual WCCs were not performed on healthy control samples, so published ‘normal’ values were used to calculate absolute numbers of IL-6 producing cells in healthy controls. With
the values for healthy controls based entirely on estimated total white cell counts, these results must be interpreted with caution, but they do substantiate the previous findings.

4.2.2 Evidence to support findings

There is strong evidence in the literature which demonstrates that the five cell types under study are capable of producing IL-6 (Section 1.11). Furthermore, a study by Kashipaz and colleagues demonstrates that PBMC in normal subjects constitutively produce low levels of IL-6 when in a resting state (Kashipaz et al., 2003), confirming observations in this study. Kashipaz and colleagues also found that there were a higher percentage of IL-6 positive CD14+ cells than CD14- cells, i.e. monocytes produce more IL-6 than the other PBMC (Kashipaz et al., 2003), again corroborating the findings from the present study, where almost all monocytes in all patients were found to constitutively produce IL-6.

In contrast to the present study, Alvarez-Rodriguez and colleagues measured the percentage of unstimulated monocytes containing intracellular IL-6 in PMR patients and healthy controls, and found that a mean of approximately 30% were positive for IL-6 in both groups (Alvarez-Rodriguez et al., 2010). This was much lower than the percentage of IL-6 positive monocytes measured in this study. The PBMC were, however, treated very differently. In the study by Alvarez-Rodriguez and colleagues, whole blood samples were cultured for four hours prior to surface staining (Alvarez-Rodriguez et al., 2010). PBMC were subsequently separated by lysing the red blood cells (RBCs) and the PBMC were then fixed, permeabilised and intracellularly stained (Alvarez-Rodriguez et al., 2010). In the present study PBMC were separated on Histopaque 1077 and cultured for 24 hours both of which may have stimulated further IL-6 production from the monocytes.

In summary, PBMC from RA, PMR and HC groups all constitutively contain low levels of IL-6, including the majority of monocytes and DC, and a proportion of T and B cells. No significant differences were observed between patient samples and controls that could account for elevated IL-6 in PMR, despite the fact that samples were taken from patients with currently active and untreated disease at the time of sampling.
4.2.3 PBMC response to *in vitro* stimulation

Having demonstrated that PBMC from PMR patients do not constitutively contain or produce IL-6, it was hypothesised that PBMC in the PMR patients may have an altered or enhanced IL-6 response to factors present *in vivo*, causing an overproduction of IL-6. To test this theory, the PBMC were subjected to stimulation with LPS and IFNγ which was shown to stimulate all cell types in pilot studies using PBMC from healthy controls.

Despite good responses to LPS/IFNγ in healthy controls, the overall responses to stimulation *in vitro* were hugely variable, with some cells responding strongly, some proving unresponsive and some being negatively affected by stimulation in all groups (whether examining intensity or percentage of IL-6 positive cells). The type of response to stimulation appears to be person-specific. Overall PBMC from PMR patients were not found to differ in their responsiveness to stimulation *in vitro* compared to RA or healthy controls, with both responders and non-responders evident in all groups.

4.2.4 Secreted IL-6 in PBMC culture supernatants

In contrast, stimulation of PBMCs with LPS/IFNγ induced dramatic secretion of IL-6 in all samples as quantities greater than 1019 pg/ml were measured in PBMC culture supernatants, compared to less than 9 pg/ml in unstimulated PBMC culture supernatants (*Section 3.2.3.3*). There were, however, no significant differences between the mean concentrations of secreted IL-6 in PMR patients, RA patients and healthy controls. This confirms that although all these cell types are capable of IL-6 production (specifically in response to 24 hour LPS/ 4 hour IFNγ stimulation), the PBMC cell types in PMR patients were not behaving any differently to those in RA patients and healthy controls, and one, a few, or all cell types secreted large quantities of IL-6 in response to stimulation. This substantiates the previous evidence that the PBMC are not the source of the significantly raised serum/plasma IL-6 in PMR.
4.2.5 Evidence to support findings

Roche and colleagues similarly found that there was no difference in IL-6 secretion by PBMC cultured from PMR patients, GCA patients and normal healthy controls in response to anti-CD3 stimulation (Roche et al., 1993). After 24 hours in culture, IL-6 was produced by the PBMC from PMR patients (mean 42 pg/ml), GCA patients (mean 99 pg/ml), and normal healthy controls (mean 74 pg/ml) at ten-fold lower levels than this study, which may reflect the use of different stimulants. Roche and colleagues provided weak evidence that the monocytes may be the source of the spontaneous IL-6 secretion in PMR, and attribute the fact that there was no difference in responsiveness between the groups to the fact that they did not use a monocyte-specific stimulus (Roche et al., 1993). LPS, used in the present study, has the ability to stimulate IL-6 production in monocytes (Rossol et al., 2011) alongside IFNγ (Delneste et al., 2003). Thus, even when a monocyte-specific stimulant was applied, there was no evidence to suggest that monocytes are the source of elevated IL-6 in PMR. This concurs with the findings of Alvarez-Rodriguez and colleagues who also examined ex vivo CD14+ cell responses to LPS stimulation (Alvarez-Rodriguez et al., 2010). This study extends their work by examining the intensity of staining within the monocyte population, and concludes that monocytes are not the source of elevated circulating IL-6 in PMR patients.

For the first time other cells within the PBMC population, namely T cells, B cells and DCs, were examined as the potential source of IL-6 in PMR. Using IFNγ and LPS stimulants capable of triggering IL-6 production from monocytes and all of the other PBMC, no differences were observed between the mean concentrations of IL-6 produced by PMR patients, RA patients and healthy controls in the present study. These PBMC are thus not the likely source of spontaneous IL-6 production in PMR.
4.2.6 Another cell type within PBMC cultures responsible for IL-6 production?

It is possible that there may be other cell types, like natural killer (NK) cells, within the PBMC cultures that may be capable of secreting high levels of IL-6, but which were not identified by flow cytometry due to it not expressing one of the five surface markers which the cells were stained for (CD3, CD14, CD19, CD123 or CD11c).

NK cells are a potent source of IL-6 (Al-Tae, 2012) and are stimulated by LPS (Conti et al., 1991). NK cells are likely to have been present in the cultures and they do not possess surface CD3 (Anderson et al., 1989) or any of the other surface markers which were stained for, meaning that they would not have been identified by flow cytometry. Distinguishing NK cells is problematic as they lack an NK-specific cell surface marker (Milush et al., 2009), but with NK cells making up approximately 10–20% of the PBMC (Zamai et al., 2007), this does make them a potential source of the IL-6 found in the stimulated PBMC culture supernatant samples. Research, however, has suggested that patients with active untreated PMR have significantly lower numbers and relative percentages of lymphocytes with natural killer cell activity than healthy controls (Uddhammer et al., 1995), meaning that they would be an unlikely source of the IL-6 in PMR patients. Furthermore, even if NK cells are capable of producing IL-6, there were no significant differences between the IL-6 concentrations measured in the PBMC supernatants of PMR patients, RA patients and healthy controls, indicating that NK cells are not likely to be critically involved in the pathogenesis of PMR.

Alternatively, some peripheral blood cell types may have been excluded, like neutrophils, due to the PBMC isolation process. Neutrophils are polymorphonuclear rather than mononuclear and were therefore not isolated alongside the other cells types, so could not account for the elevated IL-6 in the stimulated PBMC culture supernatants. They could, however, be the source of elevated circulating IL-6 in PMR patients, as they are known to be capable of producing IL-6 (Kaplanski et al., 2003). Alvarez-Rodriguez and colleagues recently showed that effector functions of phagocytes, are deregulated in PMR, GCA and elderly onset RA patients and have suggested that that neutrophils may have a pathogenic role in these inflammatory diseases (Alvarez-Rodriguez et al., 2013); however, they did not look specifically at
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IL-6 production in these cells. Hence, this requires further exploration in order to confirm whether neutrophils may be the source of IL-6 in PMR.

4.2.7 Summary

Taken together, using different methods to test the hypothesis, PBMC were consistently found not to be the source of elevated plasma/serum IL-6 in PMR patients. Using a highly sensitive method to look at intracellular IL-6 production at a single cell level in PMR patients, RA patients and healthy controls, as well as measuring secretory IL-6 concentrations in the serum and plasma and in parallel PBMC supernatant cultures, it has been confirmed that there is no difference in the production or secretion of IL-6 between unstimulated and stimulated PBMC of PMR patients, RA patients and healthy controls. Thus, it is concluded that PBMCs are not the source of raised IL-6 in PMR.

4.3 Other circulating cytokines in PMR and RA (refer to Hypothesis 6 in Section 1.15, Aim 6 in Section 1.16)

A panel of nine cytokines (excluding IL-6) were quantified in the plasma and serum of the PMR and RA patients alongside the healthy controls in order to determine whether any other cytokines play a role in the pathogenesis of PMR and to examine the cytokine profile of RA more broadly (Section 2.4).

4.3.1 IL-8

The proinflammatory chemokine, IL-8 was detectable in the plasma and serum of almost all RA patients (plasma: 6-32 pg/ml, serum: 11-36 pg/ml), all PMR patients (plasma: 5-16 pg/ml, serum: 6-18 pg/ml) and all healthy controls (plasma: 4-7 pg/ml, serum: 4-23 pg/ml) (Section 3.3). Visually there appears to be a trend towards higher
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IL-8 concentrations in the plasma and serum of RA patients and PMR patients than the healthy controls. There were, however, no significant differences between the mean plasma and serum IL-8 concentrations of the three groups. This indicates that circulating concentrations of IL-8 are naturally higher than the other cytokines measured in the plasma and serum, however, based on this data, IL-8 is not likely to have a role in the pathogenesis of RA or PMR.

In contrast, in a much larger study, Alvarez-Rodriguez and colleagues found that circulating serum IL-8 concentrations were significantly elevated in patients with elderly-onset RA (EORA) (40 subjects) and active PMR (95 subjects) compared to healthy controls (25 subjects), even after GC treatment (Alvarez-Rodriguez et al., 2013). No difference was observed between PMR and EORA serum IL-8 concentrations (Alvarez-Rodriguez et al., 2013). Reasons for elevated serum concentrations of IL-8 in these conditions were considered. Circulating neutrophils in patients with EORA demonstrated an ‘increased migratory capacity’, possibly linked to increased circulating IL-8 concentrations; however this was only apparent in EORA patients. Reasons for increased circulating IL-8 in PMR were unclear, however it was suggested that again there may be a link between IL-8 and dysfunctioning neutrophils. As there was no significant difference between the mean serum concentration of IL-8 in RA patients, PMR patients and healthy controls in the present study, it seems that the role of IL-8 in the pathogenesis of these diseases may not be as important as the role of IL-6, which was significantly raised in the disease groups even with the small patient sample numbers.

4.3.2 IL-17

Previous studies have indicated a key role for IL-17 in the pathogenesis of RA (Hwang et al., 2004, Oukka, 2008). Thus, it was predicted that IL-17 would be raised in the serum and plasma of the RA patients in this study. IL-17 concentrations in the serum and plasma were, however, undetectable in all RA subjects. Evidence suggests that IL-17 acts locally at the site of the inflammation (the synovium), with high levels expressed in the SF of RA patients (Chabaud et al., 1999, Harrington et al., 2005) and a direct involvement in the destruction of joint tissue (Oukka, 2008). With such a
specific site of action, this could be why elevated levels of IL-17 were not detected in the blood.

A recent study showed that Th17 cell numbers are increased in PMR patients, suggesting that IL-17 might be elevated and may, therefore, be contributing to PMR disease (Samson et al., 2012). As IL-17 was not raised in the serum and plasma of PMR patients, and these patients do not exhibit symptoms of joint inflammation and destruction, it does not seem likely that IL-17 has a critical role in PMR disease pathogenesis.

4.3.3 Other circulating cytokines in PMR and RA- current knowledge and new evidence

In a previous study by Clarke and colleagues, it was demonstrated that the cytokines TNFα, IL-1β and IL-4 were elevated in the plasma of a sub group of RA patients (3 out of 9 individuals), which they proposed may be linked to the fact that these patients were also unresponsive to GC therapy (Clarke et al., 2011). This was, however, only a small-scale study and the real significance of this finding was unclear. To add weight to this finding, in the present study it was also found that cytokines TNFα and IL-4 were raised in the serum and plasma of a sub-group (2 out of 6) of RA patients, but not PMR patients or healthy controls (Figure 37 and 40). In contrast to the study by Clarke and co-workers, plasma and serum IL-1β concentrations were not detectable in any of the participants. The detection limit of the CBA kit for IL-1β (7.2 pg/ml), however, was higher than that for TNFα (3.8 pg/ml) and IL-4 (4.9 pg/ml), so the kit may not have had ‘sufficiently robust’ sensitivity (Churchman et al., 2012) to detect low levels of IL-1β that may have been present in the samples.

Knowing the key role of TNFα in inducing synovitis and inflammatory cytokine production in RA (Section 1.5.1), it was not surprising that this cytokine was elevated in the serum and plasma of some RA patients in this study. In line with the present study, Cicuttini and colleagues demonstrated that IL-4 was raised in a sub group of patients with RA (12 out of 140 patients), as well as sub-groups of patients with systemic lupus erythematosus (2 out of 19 patients), psoriatic arthritis (2 out of 24
patients) and Behçet’s syndrome (1 out of 5 patients), whilst no IL-4 was detected in any PMR patients tested (6 patients) (Cicuttini et al., 1995). It remains unclear as to why this cytokine is raised in only a subset of these patients, but it seems that it may not be crucial to the onset of these diseases; otherwise all patients would be exhibiting high levels of the cytokine. Hence, it seems that IL-4 is likely to be elevated as a consequence of the disease process, as opposed to being a direct contributor to RA pathogenesis.

4.3.4 Further raised cytokines in same RA sub-group

As well as confirming that cytokines TNFα and IL-4 were raised in the serum and plasma of a subset of RA patients, it has been shown for the first time that there are a range of other cytokines, namely IL-2, IL-12p70 and IL-10 which are also elevated in the same subset of RA patients (Figure 37 and 40). As with TNFα, IL-4 and IL-1β, these cytokines were not raised in PMR patients compared to healthy controls.

Furthermore, using data taken from the MD thesis of Dr Mark Perry, where a range of cytokines were measured in the plasma of 16 RA patients using a Luminex100®, it is possible to demonstrate that these same cytokines are also raised in a sub-group (50%) of RA patients (Perry, 2008) (Table 16). This finding was not identified or discussed by the author.

Using the data from Perry, in order to prove that statistically two sub groups exist within RA, with either 5 raised cytokines or no raised cytokines, a system was developed whereby the median concentration for each group of cytokines was identified, and then the cytokine concentrations for each patient examined to see whether it fell above or below the median value (Perry, 2008, Kirwan, J., personal communication, 2013). The number of cytokines which were above the median for the group was recorded (Table 17) and a Chi-Square test used to determine whether the distribution of the number of cytokines above the median was different from what would normally be expected (Table 17) in a Gaussian distribution.
In agreement with the present study, there was a sub-group of RA patients (8 out of 16 patients) who had raised concentrations of several cytokines namely; TNFα, IL-4, IL-2, IL-10 and IL-6 (Table 16). There are two distinct groups of patients, those with no or one cytokine raised above the median concentration, and those with 3, 4 or all 5 cytokines above the median concentration. The distribution was statistically different from the expected Gaussian distribution (P< 0.0001), confirming the findings from the present study, which indicate that there are two sub-groups of RA patients with very different cytokine profiles.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>IL-6</th>
<th>TNFα</th>
<th>IL-1β</th>
<th>IL-4</th>
<th>IL-10</th>
<th>No. of cytokines above median</th>
</tr>
</thead>
<tbody>
<tr>
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<td>80</td>
<td></td>
<td></td>
<td>*</td>
<td>*</td>
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<td>2</td>
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<td>10</td>
<td>83</td>
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<td>3</td>
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<td>452</td>
<td>16</td>
<td>460</td>
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Median: 63, 69, 9, 84, 549
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Table 17: Chi-Square test to confirm the existence of two sub-groups of RA patients: one with low concentrations of circulating cytokines, and one with high concentrations of several circulating cytokines.

<table>
<thead>
<tr>
<th>No. of cytokines above median</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
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<tbody>
<tr>
<td>Observed</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Expected</td>
<td>0.5</td>
<td>2.5</td>
<td>5</td>
<td>5</td>
<td>2.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Deviation (O-E)</td>
<td>5.5</td>
<td>-0.5</td>
<td>-5</td>
<td>-1</td>
<td>-1.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Deviation squared (O-E)^2</td>
<td>30.25</td>
<td>0.25</td>
<td>25</td>
<td>1</td>
<td>2.25</td>
<td>6.25</td>
</tr>
<tr>
<td>(O-E)^2/E</td>
<td>60.5</td>
<td>0.1</td>
<td>5</td>
<td>0.2</td>
<td>0.9</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Chi-square value higher than critical value- therefore statistically there is a difference between observed and expected values (P< 0.0001)

Degrees of freedom 5

Chi-square 79.2
Critical value 11.07
Chi-squared P 1.234E-15

4.3.5 What are the implications of this finding?

The Th1 immune response is predominant in RA, thus, finding increased concentrations of Th1 cytokines such as IL-12p70 in the serum and plasma of some RA patients was not overly surprising, especially in the subset of patients who have raised levels of a number of other predominantly Th1 cytokines. IL-4, however, strongly promotes a Th2 response (Trinchieri, 1995). Studies have shown that IL-4 suppresses IL-12-induced IFNγ production (D’Andrea et al., 1993), which is why IL-4 may have been raised in these RA patients alongside IL-12p70. IL-10 was raised in the plasma and serum of the same sub-group of RA patients, thus reinforcing the theory that IL-10 is over-produced in order to down-regulate the production of pro-inflammatory cytokines such as TNFα.

As there are only 2 RA patients out of 6 with increased cytokine concentrations in the present study it is difficult to make any proper assumptions about what might be happening with these patients. Nonetheless, these two patients do stand out from the others, and these results confirm what has been shown in other studies (Cicuttini et al., 1995, Perry, 2008, Clarke et al., 2011), thus it is reasonable to consider that there could be a specific disease mechanism involved. If this were true, there might be clinical implications for RA patients. Overall, there is compelling evidence for the
existence of two pathologies within RA (and possibly in other immune-mediated inflammatory diseases with a synovitis component) with very different cytokine profiles, which may require differential diagnosis and treatment. A study with a much larger cohort of RA patients (100+) would be necessary to confirm this finding.

4.3.6 Secreted cytokines in the PBMC culture supernatants

Contrary to what was observed in the serum and plasma, where a subset of RA patients were found to have raised concentrations of IL-2, IL-4, IL-10, and IL-12p70, these same cytokines were not secreted at detectable levels by PBMC in any of the supernatants after 24 hours culture without stimulation (Section 3.3.4). Thus the increased concentrations of these cytokines in the serum and plasma must have come from an alternative, more localised source, such as the synovial cells, not the PBMC. Secreted TNF was detectable in 5 participants, but there were no significant differences between RA patients, PMR patients and healthy controls. Secreted IL-1β was detected in only 4 participants, with no significant differences between groups, and secreted IL-17 was detectable in one healthy control participant. These results confirm the idea that in RA, these cytokines are predominantly produced locally by cells at the site of inflammation, not by circulating PBMC.

IL-8 was measured in the unstimulated PBMC culture supernatants of nearly all study participants, and was secreted at 200-2000 fold higher quantities (229 pg/ml- 2456 pg/ml) than the other cytokines measured. IL-8 concentrations in the culture supernatants were approximately 100 times greater than the concentration of IL-8 measured in the serum, suggesting that the PBMC are a major source of IL-8 and that the culture conditions may have strongly stimulated the cells responsible. There were, however, no significant differences between the unstimulated mean IL-8 concentrations for the three groups (PMR, RA and healthy controls); again suggesting that IL-8 does not play a major role in the pathogenesis of these diseases (Figure 42).

Stimulation with LPS and IFNγ increased secretory IL-8 concentrations by 100-500 fold, and as with the plasma and serum data, there is a visible trend towards higher secretory IL-8 concentrations in the disease groups, however the means are not
significantly different. A larger patient sample would need to be tested in order to confirm any role for IL-8 in PMR. As mentioned, Alvarez-Rodriguez and others have suggested that circulating IL-8 is raised in RA and PMR patients and may be due to dysfunctioning neutrophils (Kreiner et al., 2010, Alvarez-Rodriguez et al., 2013).

Following stimulation with LPS and IFNγ, secretory IL-1β and TNF concentrations also increased in all participants (100- 5000 fold and 10- 200 fold respectively) (Figure 43). Again, there were no significant differences between disease and control groups, thus suggesting that these are ‘normal’ PBMC responses and thus are not related to PMR or RA disease.

4.4 Overall discussion of findings

Having determined that there is a sub-group of RA patients with raised cytokines in the plasma and serum, and provided speculative reasons as to why biologically they might be raised, reasons why these cytokines are raised in only a small sub-group of RA patients but not all patients were considered, as well as what this tells us about RA and PMR, which have previously been considered as diseases with similar pathologies.

Clarke and colleagues suggested a link between the subgroup with raised cytokines and a lack of responsiveness to GC therapy. This has not been tested here but is an idea which requires further exploration. There is some weak evidence to suggest that the cytokine profile reflects the different stages of disease. According to a study by Raza and colleagues, cytokines IL-2, IL-4, IL-13, IL-17 and IL-15 were significantly raised only in the sub-group of patients in the very early stages of RA disease, which they demonstrated was no longer present in patients with long-term, established disease (Raza et al., 2005). Within the data in the present study and the data taken from Perry’s thesis there is no evidence of a link between the cytokine profile of the patient and the different disease ‘stages’ in RA or PMR patients (Perry, 2008). For example, of the 8 patients with raised cytokines in Perry’s study, the disease duration spans from 4 years to 30 years, and those with all 5 cytokines raised have disease durations of 7, 18 and 5
years. Despite the small sample of patients, this does not concur with the theory that the cytokines might be raised in the patients only in the very early stages of RA.

It was also considered that the sub group of RA patients with raised cytokine concentrations may display different symptoms or levels of clinical markers. This did not appear to be the case based on the data from Perry’s thesis (Perry, 2008). As with the present study, all patients were selected because they had clinically active disease. Concentrations of clinical markers such as PV and CRP did not correlate with the number of cytokines above the median. Notably, however, all patients in the sub-group with raised cytokines were positive for RF, whereas those without raised cytokines were not all positive for RF. The significance of this finding is unclear at this stage.

The fact that there are two such different and distinctive cytokine profiles within RA patients could indicate that there are two different pathologies within RA disease. This also raises the question as to whether the circulating cytokine profile of RA patients is acquired or inherent. Only a subset of RA patients displayed these elevated levels of particular cytokines, yet all RA patients displayed similar symptoms, thus it could be that this cytokine profile may be a consequence of the disease process as opposed to being the cause of the disease. One theory is that there may be two types of RA, one more immunologically aggressive, which requires higher titres of cytokines in order to reduce the inflammation.

The fact that there was no sub-group with raised cytokines in PMR suggests that RA and PMR have very different pathologies. The symptoms of PMR appear to be associated entirely with IL-6, of which the source is still unknown, despite these extensive investigations. RA patients, on the other hand, are associated with having a cascade of different inflammatory cytokines, produced predominantly in the synovium, perhaps as a result of the disease process. Thus, despite presenting similar symptoms of inflammation and fatigue, there may be two very different underlying causes for these diseases.

Despite demonstrating very similar results, all three studies (Perry, 2008, Clarke et al., 2011, and the present study) only have very limited patient numbers. A larger scale study with a greater number of patients, looking at a larger panel of cytokines, and
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looking further in depth at the clinical symptoms and clinical markers of disease would be required to test these theories and to find out more about the underlying pathologies of PMR and RA.

This brings about the question as to whether PMR may even be called an ‘inflammatory disease’. This label has been applied to PMR, and perhaps rightly so, as increased IL-6 in the blood brings about an APR in all PMR patients (if an APR is not present then the diagnosis of PMR is considered incorrect) (Kirwan, J., personal communication, 2013), which is a key feature of an inflammatory response. In spite of this, with no clear and isolated site of inflammation, the other features of chronic inflammation appear to be absent. Inflammation is usually brought about in response to tissue damage or infection, with appropriate leukocytes recruited to a specific and localised area (Punchard et al., 2004). With no known site of inflammation, however, this typical inflammatory response is not observed. The classic signs of inflammation are made up of four components; pain (dolor), heat (calor), redness (rubor) and swelling (tumour) (Punchard et al., 2004); all of which are present in affected joints in RA, but appear to be absent in PMR (but may be manifesting as more systemic symptoms). There is a wealth of evidence pointing to the fact that IL-6 is the main cause of the symptoms of PMR, however it appears to have a very solitary role in the disease, with the other signs of inflammation lacking. This is unless, however, there is an inflammatory site that is yet to be discovered.

4.5 Discussion of methodologies

4.5.1 Limitations of the study

4.5.1.1 Difficulties with participant recruitment

Although the target sample numbers for the three participant groups were reached (the aim was to recruit 6-10 per group), the number of participants involved in this study was still relatively small (7 PMR patients, 6 RA patients and 7 healthy controls). The sample number reflected the aim of finding large and obvious differences between PMR patients, RA patients and controls, which could then be confirmed later in a larger more comprehensive study. If differences between diseased patients and healthy
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controls were not significant, then it was considered unlikely that they would be relevant to our understanding of the pathology of the diseases.

Other studies in the field have tended toward relatively small to modest sample numbers, for example Roche and colleagues included 13 PMR patients and 20 healthy controls in a similar study and Alvarez-Rodriguez and colleagues included 34 PMR patients and 17 healthy controls in their study into circulating cytokines in PMR (Roche et al., 1993, Alvarez-Rodriguez et al., 2010). It suggests that other research groups may have struggled with the recruitment of participants, where the inclusion criteria for participants are very specific (requiring no current treatment) and a certain level of commitment is involved for all parties.

4.5.1.2 Difficulties finding patients to fit study criteria

Difficulties were encountered in getting general practitioners to recruit patients for the study, and further difficulties were experienced in the recruitment of patients who had not already been put on GC treatment. As a consequence, collecting enough participant samples to reach the initial targets took much longer than expected.

4.5.1.3 Commitment for participant

Although not a huge commitment, patients were required to return to the clinic for 9am, 48 hours after their initial appointment, and thus were asked to delay their treatment for a further two days. This may have been off-putting for those in pain and discomfort looking for immediate relief from symptoms.
4.5.2 Sample handling

4.5.2.1 Timing and transportation of blood samples

The way in which the plasma and serum were separated and handled prior to testing may have affected the concentration of IL-6 present in the sample. The serum was usually separated from the cellular elements within an hour of being taken from the participant, and this sample was subsequently frozen at -80°C as soon as possible (Section 2.1.2). This protocol, however, was not always adhered to as the research doctors involved were often busy seeing other patients in the rheumatology clinic and the process was delayed by variable lengths of time (exact times not recorded). The plasma, conversely, was transported from the rheumatology clinic to the UWE laboratories (a journey of approximately 30-40 minutes), and then separated from the cellular constituents approximately 1-1.5 hours after the blood sample was extracted from the study participant. The timing of this was relatively consistent from sample to sample.

De Jager and colleagues recommend that serum or plasma are separated from whole blood immediately in order to maintain ‘optimal analyte stability’, or within one hour if immediate separation is not possible (de Jager et al., 2009). It has also been suggested that a delay in sample processing could lead to altered cytokine expression as a result of degradation, absorption or increased cellular production of cytokines by the blood cells present in the sample (Duvigneau et al., 2003).

The conditions within the blood collection tube have also been shown to trigger the release of cytokines from blood cells, as well as the anticoagulant (sodium heparin in this study), lack of nutrients, cell death and external temperature (Jackman et al., 2011). Duvigneau and colleagues reported that in porcine blood samples, a delay in sample processing (of over an hour) and storing the sample either at RT or at 4°C resulted in increases of IL-6, IL-10 and IL-1α production, indicating that monocytes in particular may be particularly susceptible to environmental changes (Duvigneau et al., 2003). Furthermore, Exley and colleagues recommend that blood samples are stored at
4°C for a maximum of 30 minutes prior to separation to ensure the optimal measurement of TNFα (Exley et al., 1990).

Thus the delay in serum and plasma separation (due to busy schedules in the clinic and transportation respectively) meant that these samples were not always frozen down immediately or within the hour, therefore cytokine concentrations may have been affected. The time delay in separating plasma from the whole blood sample was consistent for each sample, thus controlling for these effects to some extent. Nonetheless, direct comparisons with other studies where the time delay before processing is not stated would not be appropriate. External temperatures would have differed for each sample as the blood samples were kept at atmospheric temperature during the journey- and as the samples were collected over the period of a year, the temperature conditions would have varied dramatically from the winter months to the summer months.

4.5.2.2 The blood separation process

The separation processes involved in extracting plasma and serum may in fact trigger cytokine production in blood cells. According to Whiteside and colleagues, the clotting process involved in separating serum from the blood activates leukocytes within the sample to produce cytokines, so for this reason it may be better to quantitate cytokines in the plasma instead to avoid this clotting process and thus avoid leukocyte activation (Whiteside et al., 1994). This could explain the higher cytokine titres in the serum, which contrary to previous assumptions, may not be the ideal outcome.

4.5.2.3 Sample storage

Long-term storage is also known to affect cytokine stability. According to a study by de Jager and co-workers, however, within the short time frame that the samples were stored for in this study (<1.5 years), out of the ten cytokines measured, only degradation of IL-17 may have occurred (de Jager et al., 2009). As IL-17 was not detected in any of the plasma and serum samples and was detected in only one unstimulated culture supernatant sample and three stimulated culture supernatant
samples, this could be a valid reason. The exact half-life of many cytokines, however, has not yet been elucidated, and may vary between cytokines (Bartels and Madsen, 2013). In the de Jager study IL-6, IL-1β and IL-10 degraded up to 50% of their original concentration within 2-3 years in storage, and IL-2, IL-4 and IL-12 began to degrade within 3 years in storage (de Jager et al., 2009). This should not have been an issue with the short time frame that the samples were stored for, however the lower study participant numbers were stored for longer than the higher participant numbers (up to 1.5 years compared to 1 month) prior to laboratory analysis.

4.5.2.4 Anticoagulant

Sodium heparin coated the glass blood collection tubes in order to stop the blood coagulating. According to a study by de Jager and colleagues, out of three anticoagulants tested (sodium heparin, ethylenediaminetetraacetic acid (EDTA) and sodium citrate), plasma collected in sodium heparin coated tubes demonstrated the most consistent recovery for the range of cytokines that they looked at, including IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, TNF and IFNγ (de Jager et al., 2009). Consequently they concluded that sodium heparin is the ‘preferred anticoagulant’ for measuring cytokines in multiplex assays (de Jager et al., 2009) and endorses its use in this study. Alternatively, Riches and colleagues suggest that in order to reflect in vivo cytokine measurements, it does not matter which anticoagulant is used, as long as it is endotoxin-free (Riches et al., 1992).

4.5.2.5 Sample freeze-thawing

The process of freeze-thawing a plasma or serum sample is believed to affect the stability of cytokines. Each test (ELISA, CBA, IL-6 bioassay) was performed using a fresh aliquot, with any repeats performed on the same sample which had been re-frozen and re-thawed. Out of interest, the IL-6 in the same plasma samples was tested three times by ELISA, re-freezing the sample between each test, in order to determine whether the freeze-thawing process made any significant difference to the stability of the cytokine (data not shown). No significant differences between the repeats were observed, and thus it was accepted that the freeze-thawing process was not detrimental
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to the stability of IL-6. This is corroborated by de Jager and co-workers who found that IL-6 was particularly stable throughout multiple freeze-thawing cycles and during short-term storage (de Jager et al., 2009).

In contrast, in this same study by de Jager and co-workers, the stability of cytokines IL-4, IL-17, TNFα and IFNγ were particularly affected by multiple freeze-thawing cycles (de Jager et al., 2009). There was no evidence of cytokine degradation within the CBA results of the current study, where only very low concentrations of these cytokines were measured in the serum and plasma of some participants, and there were no significant differences between repeats using the same CBA kits.

4.5.3 The B9 bioassay

4.5.3.1 B9 sensitivity

B9 cells are very sensitive to IL-6 (Guerne et al., 1989); however, a crucial problem with cell lines is that they are increasingly being shown to be able to respond to a range of other cytokines (Mire-Sluis et al., 1995). Studies have demonstrated that B9 cells may proliferate in response to IL-4 (Helle et al., 1988), IL-11 and IL-2 (Diamant et al., 1994). Hence IL-6-neutralising antibodies were used in this study to be able to confirm that the B9 cell proliferation was specifically due to IL-6 present in the samples.

4.5.3.2 MTS assay vs DNA tritiated thymidine incorporation assay

The incorporation of [3H] thymidine into cell DNA is a routine method used to measure DNA synthesis and hence cell proliferation in B9 cells (and other cell lines) in response to IL-6 (Guerne et al., 1989, de Benedetti et al., 1991, Rosenbaum et al., 1992, Diamant et al., 1994). Nonetheless, a colorimetric assay based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to MTS was used in this study due its speed and low-cost compared to the [3H] thymidine incorporation assay. According to Gieni and colleagues, the MTS assay may be a more
sensitive method than $[^3]H$ thymidine incorporation as MTS assays were capable of detecting ‘2-16 fold’ lower cytokine concentrations in their study (Gieni et al., 1995).

4.5.4 ELISA and CBA

4.5.4.1 Problems associated with cytokine assays

There is large variation between the IL-6 concentrations measured in the serum and plasma in different studies, largely because there is no standard method for the measurement of cytokines in clinical material. The majority of studies which have quoted values for serum and plasma IL-6 have measured the cytokine by ELISA, which is thought to be the most commonly used and ‘best validated’ method of cytokine measurement (Leng et al., 2008). Multiplex cytokine assays such as cytometric bead array (CBA) have become a more widely used technique as they have the advantage of being able to detect multiple cytokines in a smaller volume of sample, however, according to Breen and colleagues, they may not provide sufficiently reproducible results when carrying out repeat testing (Breen et al., 2011). This was not observed here. CBA trends were highly reproducible, but actual values did vary greatly between the CBA kits, necessitating the use of geometric means to normalise the data. In contrast, the ELISA was found more likely to produce false positive results and inconsistent results. The reasons for this are not clear, although RF (discussed below) may be a factor.

4.5.4.2 Endogenous plasma proteins

Concerns have been raised about the suitability of cytokine assays for use in rheumatology. Endogenous plasma proteins, in particular RF, are believed to interfere with binding by providing competition for binding sites, giving false positive results (Bartels et al., 2011, Churchman et al., 2012, Bartels and Madsen, 2013). RFs are human autoantibodies (normally of IgM class, but can be IgA, IgD, IgE or IgG) which target the Fc region of IgG antibodies, and are present in 70-80% of RA patients, and up to 10% of healthy individuals (Mewar and Wilson, 2006). Bartels and colleagues suggest that RFs interfere particularly with ELISAs and multiplex assays such as CBA,
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but can be removed by precipitating the sample prior to conducting the assay (Bartels et al., 2011). In a recent study, however, it was found that cytokine concentrations (including IL-6) in the serum samples from RA patients were not largely affected by the removal of RF when measured by CBA, which indicated that there was no interference from RF or false positivity when using CBA to measure cytokines in individuals with inflammatory diseases such as RA (Churchman et al., 2012). Cytokine measurement by Luminex bead assays, on the other hand, was particularly affected by interference with RF (Churchman et al., 2012), suggesting that CBA was the better choice of multiplex assay for the measurement of cytokines in rheumatoid arthritis patients in this study. Nevertheless, cytokine results from the Perry study may have been affected by this, as a Luminex 100® was used to measure cytokines in RA patient plasma (Perry, 2008).

4.5.4.3 Should cytokine measurement methods be standardised?

There is accumulating evidence which highlights a need to standardise the way cytokines are measured in order to be able to draw direct comparisons between and within studies. This issue was raised over 20 years ago at the European workshop for Rheumatology Research, when several European research groups conducted immunoassays (and bioassays) to detect a range of inflammatory cytokines in human synovial fluid and serum in different laboratories and compared the results (Roux-Lombard and Steiner, 1992). Laboratories testing the same samples with the same ELISA kits produced similar results, but different ELISA kits produced different values, albeit with similar patterns (Roux-Lombard and Steiner, 1992). Another study showed contrasting results from different lots of ELISA kits from the same manufacturer- with some results differing by up to 50% (Aziz et al., 1999). Based on the current study, other aspects of these assays require standardising in order for direct comparisons between studies to be drawn, including standardisation of the use of serum or plasma, storage conditions, use of controls, use of terminology (i.e. quoting means, medians, range, SEM or standard deviation) and methods used to normalise data.
4.5.4.4 Threshold limits of CBA kit

The CBA kit may not have been sufficiently sensitive to measure serum/plasma IL-17. The lowest threshold limit of detection was 18.9 pg/ml. Thus, a more sensitive approach to measuring this cytokine may be required in order to determine whether IL-17 is raised in PMR and thus important to its pathogenesis. For future studies a highly sensitive ELISA might be more effective. The lowest threshold limit of detection for IL-1β was also relatively high (7.2 pg/ml), so again, this lack of sensitivity may have resulted in IL-1β being undetected in any of the samples, and thus differences between the disease groups and control group could not be observed.

4.5.4.5 Serum/plasma- an indirect way of measuring cytokine production

The advantage of looking at the cytokine profile in blood samples is that they are far easier to obtain than synovial fluid, or biopsies. Bartels and Madsen (2013), however, recently made the point that only a very substantial cytokine concentration would be reflected in the blood, which could be why so many cytokines were ‘undetectable’ in this study. Cytokines function at a local or ‘microenvironment level’ (Alvarez-Rodriguez et al., 2010), meaning that cytokines involved in the pathogenesis of RA would most likely be functioning at the site of inflammation, and would therefore not be detectable in the circulation in any great concentrations. The cytokine profile of the plasma or serum therefore may not be the most accurate representation of what is happening in vivo.

4.5.5 Flow cytometry

4.5.5.1 Background stimulation

One concern with measuring intracellular IL-6 in the PBMC by immunostaining and flow cytometry was that the cells may have been subjected to some background stimulation, causing the cells to produce low levels of IL-6, and thus not showing an accurate representation of the cell behaviour in vivo. This has been noted in another
study by Kashipaz and co-workers, where a low percentage of unstimulated PBMC were found to produce IL-6 in the absence of additional stimulation (Kashipaz et al., 2003). IL-6 production was considerably reduced in the presence of polymyxin B (Kashipaz et al., 2003), indicating that bacterial endotoxin may have been present in the samples causing low-level background stimulation.

There are also a number of other ways by which the PBMC may have been unintentionally stimulated to produce IL-6, including the invasive process involved in collecting the blood sample (Bartels and Madsen, 2013), a delay in sample processing or sample storage (Duvigneau et al., 2003), the process involved in the blood separation (Whiteside et al., 1994), or even just the action of gently pipetting the sample (Mcleod, J., personal communication, 2011). This prompts the question as to whether the cells were ever actually in ‘a resting state’. The cells would have been under constant stress during the collection, separation and staining process, so again, perhaps the results here may not be indicative of what is happening in vivo. Steps were taken to reduce background stimulation, including not culturing the PBMC in FCS, a process which has previously been shown to increase IL-6 mRNA expression in synoviocytes (Rosenbaum et al., 1992); however some potentially stimulatory actions (such as pipetting) could not be avoided.

4.5.5.2 Do the flow cytometry results reflect the CBA data?

The dramatic differences in the IL-6 concentrations measured in the supernatants of unstimulated and stimulated PBMC do not reflect the minor and variable changes seen in the flow cytometry data from unstimulated to stimulated cells. Reasons for this are considered here.

Timing of IL-6 release from cells

Firstly, the timing of IL-6 release from cells may explain why generally only small changes in intracellular IL-6 quantities from unstimulated to stimulated PBMC were observed by flow cytometry. The staining process was started after the PBMC had been in culture for 24 hours, with a golgi-blocker (monensin) added for the last 4 hours
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of this period. Research suggests that some cells (leukocytes, glial cells and a macrophage cell line) secrete large quantities of IL-6 between 4 and 6 hours post LPS stimulation, which then decreases dramatically and subsequently plateaus (De Forge and Remick, 1991, Minogue et al., 2012). This could explain why large quantities of IL-6 were measured in the stimulated PBMC supernatants at 24 hours but no longer intracellularly.

DeForge and Remick found that following LPS stimulation (10 µg/ml) of whole blood, leukocyte IL-6 mRNA induction occurred after 1 hour with levels peaking at 4 hours and then plateauing again at approximately 6 hours to less than 10% of the maximum mRNA up until 24 hours when they stopped the assay (DeForge and Remick, 1991). The presence of IL-6 mRNA obviously does not confirm its production or release from these cells, however, IL-6 concentrations were also measured in the plasma taken from these stimulated cultures at time points 4, 6 and 24 hours. It was found that the mean IL-6 concentration in the plasma increased by 1900 pg/ml from 4 to 6 hours, and by only 1300 pg/ml from 6 to 24 hours (DeForge and Remick, 1991). This strongly suggests that the majority of IL-6 was released from cells long before the 20 hour time point where, in the present study, monensin was added to the cells in order to block the golgi from releasing IL-6. Minogue and colleagues demonstrated that following LPS-stimulation (100 ng/ml) IL-6 mRNA expression in glial cells increased at 2 hours but IL-6 was released from cells at 4 hours, which they concluded after measuring IL-6 in the supernatants from the stimulated glial cultures (Minogue et al., 2012). They also suggested that the slight delay in the induction of IL-6 mRNA in glial cells is due to the requirement of TNFα (which is released at 1 hour post LPS stimulation) to activate JAK2/STAT1 signalling pathways, triggering the transcription of IL-6 mRNA (Minogue et al., 2012). A study by Manderson and co-workers demonstrated that in the macrophage RAW264.7 cell line, following stimulation with 100 ng/ml LPS and 500 pg/ml IFNγ, IL-6 was detected in the supernatant at 4 hours post stimulation (approx. 900 pg/ml), and concentrations dramatically increased in the supernatant at 6 hours post stimulation (approx. 2500 pg/ml) (Manderson et al., 2007). Using fluorescence imaging techniques, at 6 hours, IL-6 was visible in the golgi complex where it was accumulating prior to release via recycling endosomes through the constitutive secretory pathway (Manderson et al., 2007).
Thus, there is a large body of evidence to suggest that PBMC begin synthesising IL-6 at around 1 hour post-stimulation, and subsequently release the IL-6 from the cell at approximately 6 hours post stimulation (De Forge and Remick, 1991, Manderson et al., 2007). So, in this study, at the time the PBMC were stained (following 24 hours in culture with the golgi-blocker added for the final 4 hours) intracellular IL-6 concentrations may have been as low as 10% of what they would have been after 6 hours in culture. Thus, although large quantities of IL-6 were released by the PBMC into the culture supernatant in all participants, only minimal quantities were measured intracellularly by flow cytometry. Prior to the study, healthy control PBMCs taken from donors at UWE, Bristol were tested for intracellular IL-6 after 4 and 24 hours stimulation with LPS and IFNγ, however it was found that both were similar with respect to intracellular IL-6 (MFI) values and the percentage of IL-6 positive cells. Again, the peak time for IL-6 production and secretion is most likely to have been approximately 4-6 hours post stimulation, so peak timing for IL-6 production and accumulation within the cells may have been missed. In future studies, if this were to be repeated, or IL-6 was to be measured intracellularly, then cells should be stained approximately 6 hours post LPS-stimulation in order to be able to measure intracellular IL-6 effectively.

Nonetheless, in the present study there were no significant differences between the secreted concentrations of IL-6 in the PBMC culture supernatants of PMR patients, RA patients and healthy controls. Thus, even if the peak time for antibody staining had been missed, the PBMC of PMR patients were not secreting greater quantities of IL-6 than the PBMC of RA patients or healthy controls. This demonstrates the power of measuring IL-6 production using different methods.

**Understanding IL-6 trafficking and release from cells**

Using flow cytometry, low levels of intracellular IL-6 were measured in a percentage of every PBMC type in every participant in the absence of stimulation. This, however, did not reflect the results of the CBA measurement of IL-6 in the unstimulated PBMC culture supernatants, where IL-6 was barely detectable (<9 pg/ml). Reasons for this are considered.
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According to various studies, low levels of cytokines may be produced constitutively and stored within cells, meaning additional stimulation is not required to trigger production of the cytokine, although receptor-mediated stimulation may be required to trigger release of the cytokine into the extracellular environment (Stow et al., 2009, Lacy and Stow, 2011). It seems that flow cytometry was a sensitive enough technique to detect these low levels of stored IL-6 within the PBMC. In particular, it has been shown that neutrophils and macrophages store IL-6 within secretory granules and lysosome-related organelles (respectively) which they continuously release in very small quantities through a process known as ‘constitutive exocytosis’, which can then be up-regulated in response to stimulation (Stow et al., 2009, Lacy and Stow, 2011). According to Lacy and Stow, the advantage of storing pre-formed cytokines in granules inside the cell is that they can be released within minutes following receptor-mediated cell stimulation (Lacy and Stow, 2011). If this was the case, and IL-6 was produced constitutively at low levels and stored within the cells, then detecting differences between unstimulated and stimulated IL-6 production within cell types using flow cytometry would have been extremely difficult- as demonstrated in the results of the present study.

This question of whether intracellular staining accurately represents the secretory ability of cells has been raised in other studies (Alvarez-Rodriguez et al., 2010). The results of the present study confirm that the intracellular levels and secretory levels may not necessarily correspond. The processes involved in cytokine secretion appear to be very complex and the trafficking pathways involved and timing of release are dependent on the type of cell. Thus further research into the way IL-6 is trafficked and released from the different PBMC types would be necessary to gain a deeper understanding of the flow cytometry results.

In conclusion, both the timing and mechanisms of cytokine release are crucially important to the measurement of intracellular IL-6. Having seen the biological variability between responses to stimulation, it is also clear that not every person or every cell will respond in the same way with regards to cytokine production and release, so perhaps this method is not ideal for detecting differences between IL-6 production in PMR, RA and healthy control PBMCs.
4.6 Implications and future considerations

4.6.1. Standardise methodologies and data reporting

One of the main issues to stand out from this study is the need for many of the techniques to be standardised, in order to achieve some sort of consistency within the reporting of data. This is vital for studies measuring cytokines in order to be able to draw direct comparisons between and within studies. In cytokine-based assays such as this, future studies should carefully consider whether to use serum or plasma, as there are unequivocal differences between the results obtained from these two blood components.

4.6.2 Find cytokine assay with sufficient accuracy and reproducibility

Despite displaying similar trends, the results from different CBA kits gave very different values. Future studies should consider performing the assay in triplicate in order to gain a more accurate idea of the concentration of cytokines in a sample, if financially viable. Future studies should also move towards using assays which measure both free and bound cytokine to gain a more accurate estimation of the concentration of active cytokine in a sample.

4.6.3 Sample collection, handling, processing and storage

Future studies should strongly consider the optimal method to collect, separate, process and store blood samples when carrying out cytokine-based assays, taking into account which cytokines are to be analysed and their level of resilience to degradation, as well as the cells’ susceptibility to environmental and physical alterations.
4.6.4 Larger cytokine studies

The results from three independent studies, including this one, have shown that there is a sub-group of RA patients (33-50%) with raised circulating concentrations of several cytokines (Clarke et al., 2011, Perry, 2008). Nonetheless, the sample sizes in all three studies were relatively small, and thus the significance of this finding is still unclear.

A study looking at a larger panel of cytokines in a larger number of RA patients would confirm which cytokines are raised and what proportion of RA patients they are raised in. It would be useful to look at the cytokine profile of patients at all stages of the disease, and in those with active and non-active disease to see if any correlations or trends exist. Additionally, the usual clinical measures of RA should be tested (i.e. CRP and PV levels, presence or absence of RF), and questionnaires such as the BRAF-MDQ performed, to see if a link exists between specific clinical symptoms and high levels of cytokines. Clarke and colleagues suggested that there may be a link between the patients with raised cytokines and the fact that they are resistant to glucocorticoid therapy (Clarke et al., 2011). This theory could also be tested using a much larger cohort of RA patients.

4.6.5 Measuring intracellular cytokines by flow cytometry

Research into the way individual cytokines are trafficked and released from different cell types would be highly valuable for future studies involving the measurement of intracellular cytokines, as well as time-course studies to detect when IL-6 is released from each cell type following stimulation. With this knowledge, it would be easier to ensure that cytokines are measured when concentrations are at their peak within the cell prior to release into the extracellular environment.
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4.6.6 A relationship between IL-6 and fatigue

The existence of a relationship between circulating IL-6 concentrations and fatigue seems extremely likely based on the results of the present study. Further investigations into the relationship between IL-6, the HPA-axis and the onset of the physical aspects of fatigue could be investigated in order to find a way to reduce this symptom.

4.6.7 Exploring other potential sources of IL-6 in PMR

PMR is considered an inflammatory disease purely due to increased concentrations of circulating IL-6. No other cytokines are raised in the circulation of PMR patients. At any other time you would consider inflammation to be an influx of cells and cascade of inflammatory cytokines at a particular site in the body, so with the original hypothesis disproved, there may be a specific site of inflammation that is yet to be discovered. This section suggests potential sources of IL-6 or inflammatory sites in PMR.

Neutrophils?

As mentioned previously, neutrophils were not isolated or tested in this study, however neutrophils are capable of producing large quantities of IL-6 (Kaplanski et al., 2003), and thus may be the source of circulating IL-6 in PMR patients. This requires further testing, using alternative density gradient centrifugation methods (sodium metrizoate and Dextran 500) to isolate the neutrophils (Oh et al., 2008, Axis-Shield, 2011), in order to identify whether these cells produce significantly greater quantities of IL-6 in PMR patients compared to RA patients and healthy controls.

Vasculitis?

Activated vascular endothelial cells are known to be capable of producing large quantities of IL-6 (Dasgupta and Panayi, 1990) over an extended period of time, in response to LPS stimulation (Loppnow et al., 1989). Thus, in future studies it may be beneficial to look at the vascular endothelium as a potential source of the elevated circulating IL-6 in PMR patients. This could be observed by taking biopsies of blood vessels from PMR patients and healthy controls and directly testing endothelial cells.
for IL-6 production. Alternatively, as this procedure is highly invasive, there may be other less direct ways of finding out whether the endothelium might be involved prior to taking biopsies.

Various biomarkers in the bloodstream may indicate that endothelial cells are activated, and hence more likely to be the source of elevated IL-6 in PMR. Soluble inter cellular adhesion molecule-1 (sICAM-1) is constitutively expressed on endothelial cells, but is upregulated by proinflammatory cytokines and is thought to have a direct contribution to inflammatory responses in the blood vessel wall (Lawson and Wolf, 2009). This soluble ICAM-1 is shed from the membrane during inflammation as a result of proteolysis (Newby, 2005). According to a study by Macchioni and colleagues, not only were levels of sICAM-1 significantly increased in the serum of patients with PMR vs healthy controls (measured by ELISA), these levels also corresponded to the level of disease activity in both PMR and GCA, based on complete clinical and laboratory investigations carried out before and after treatment (Macchioni et al., 1994).

It has also been suggested that vascular endothelial growth factor (VEGF) causes endothelial cells to secrete IL-6 (Dankbar et al., 2000). Meliconi and co-workers postulate that this may occur as a result of a positive feedback loop, with mononuclear cells producing VEGF, which induces the release of IL-6 from endothelial cells, bringing about an inflammatory immune response (Meliconi et al., 2000). In PMR patients, serum concentrations of VEGF are significantly elevated compared to normal controls (Meliconi et al., 2000). If VEGF does induce IL-6 secretion from endothelial cells, then the endothelium could potentially be a major source of IL-6 in PMR. In RA, increased VEGF expression (as a result of elevated levels of IL-6/sIL-6R) has been found to stimulate pannus (fibrovascular tissue) development (Ballara et al., 2001). Thus it would be interesting to observe if there was a correlation between specific disease symptoms, IL-6 and VEGF concentrations in the blood of both PMR and RA patients.

Aortitis?

Although apparently uncommon in PMR, limited evidence suggests that PMR is associated with subclinical aortitis (Ashraf et al., 2013), i.e. inflammation of the aortic
Chapter 4 - Discussion

wall (Gornik and Creager, 2008). GCA and Takayasu arteritis are the most common causes of aortitis but it is also associated with other rheumatic diseases such as RA, where rheumatoid nodules form in the aortic wall (Gornik and Creager, 2008). Previously, if PMR patients were found to have signs of vasculitis then the diagnosis would change to GCA (Weyand and Goronzy, 2003). A number of more recent reports, however, have suggested that aortitis may actually be a rare symptom of PMR.

Narváez and colleagues discuss MRI as a useful, non-invasive technique for diagnosing aortitis in GCA and PMR patients (Narváez et al., 2005). Nevertheless, the GCA and PMR patients in their study presented with very similar symptoms so it seems highly likely that all the patients actually had co-existing GCA where vasculitis is one of the key features. Katoake and colleagues also reported two incidences where supposed PMR patients had aortitis without stenosis (narrowing of the blood vessels) and no evidence of GCA, leading them to believe that aortitis might be a feature of PMR (Katoake et al., 2008). Again, the diagnoses are uncertain, and based on their description of patient symptoms it seems possible that these PMR patients had co-existing GCA. Milchert and Brzosko agree that aortitis is ‘difficult to classify’ in patients with symptoms of PMR (Milchert and Brzosko, 2008).

Furthermore, a case of ‘life-threatening PMR’ with aortitis in the absence of GCA symptoms has recently been reported (Ashraf et al., 2013). The patient did not respond to GC treatment, (a sign that perhaps this patient did not have PMR) however anti-IL-6 monoclonal antibody therapy (tocilizumab) and aortic reconstruction was apparently successful, thus suggesting a link between PMR, vasculitis and IL-6. Again, the diagnosis of the patient is questionable, and it is likely that although not exhibiting all the symptoms of large vessel vasculitides, there may have been another underlying cause.

Thus, there is weak evidence for the existence of aortic inflammation in PMR, suggesting that the aorta may be the source of raised IL-6 in PMR. The diagnoses of PMR in nearly all cases, however, are unconvincing. Moreover, this vasculitis/aortitis is apparent in only a small sub-group of PMR patients, yet all PMR patients exhibit high circulating concentrations of IL-6, making it unlikely that an inflammatory site within the aorta is the source of elevated concentrations of IL-6. To test this theory, non-invasive methods of testing for aortitis, such as MRI, could be adopted for a large sample of PMR patients, in order to see the exact prevalence of this symptom within a
Chapter 4 - Discussion

PMR population and whether it could be the source of increased concentrations of IL-6.

Synovitis?
There is also weak evidence to suggest that a synovitis component exists in PMR (Meliconi et al., 1996, Frediani et al., 2002) despite the lack of localised inflammation or swelling. Other studies have suggested that muscular tissue may be the source of elevated IL-6 in PMR (Kreiner et al., 2010), or the bursae (Salvarani et al., 1997) or even ligament attachment sites (Kotani et al., 2011) (refer to Section 1.12). Thus, future studies could consider that a tissue component may exist, and may require more invasive techniques, such as muscle biopsies of affected and unaffected sites within the same patient (internal controls) to further investigate this theory.

4.7 Conclusions

4.7.1 Circulating IL-6 in the plasma and serum is raised in the circulation of PMR and RA patients compared to healthy age and gender-matched controls (P<0.05)

IL-6 appears to play a direct role in the pathogenesis of PMR and RA and may be a useful bio-marker for these diseases as it is consistently and significantly elevated in the blood of these patients above healthy controls. If a cheap, quick and reproducible test to measure this cytokine could be developed, this may give a more direct reflection on the patients’ disease state than measuring secondary disease events such as CRP or PV levels, which are used currently for diagnosis.

4.7.2 Serum and plasma IL-6 concentrations correlated very well, but IL-6 concentrations are higher in serum than in plasma

Plasma and serum IL-6 concentrations correlated very well with a high correlation coefficient of 0.961 (P< 0.0001). There was, however, a clear bias towards the serum measurements, particularly at the higher concentrations of IL-6. This indicates that
there are significant differences between cytokine concentrations in the plasma and serum, thus deciding which blood component to test should be considered carefully. Sample collection, processing, handling and storage prior to analysis may be of upmost importance in reducing degradation of cytokines, although IL-6 is relatively stable. Further testing is required to determine the optimal conditions for cytokine analysis in blood samples.

4.7.3 Raised circulating IL-6 in PMR and RA patients is biologically active

For the first time IL-6 in the plasma of PMR (and RA patients) caused IL-6-dependent B9 cells to proliferate, which was largely neutralised in the presence of a neutralising IL-6 antibody. Deemed ‘biologically active’, this raised IL-6 is therefore highly likely to be involved in PMR and RA pathogenesis.

4.7.4 Fatigue, and in particular the somatic aspects of fatigue (living fatigue and physical fatigue) strongly correlate with circulating concentrations of IL-6

For the first time, using the BRAF-MDQ, plasma IL-6 levels in both PMR and RA patients have been shown to strongly correlate with fatigue ($r = 0.487$). Two individual aspects of fatigue namely ‘physical fatigue’ and ‘living fatigue’ in particular correlated very well with IL-6 concentrations in the plasma ($r = 0.479$ and $r = 0.499$ respectively), whereas ‘cognitive fatigue’ and ‘emotional fatigue’ did not ($r = 0.067$ and $r = 0.311$ respectively). This indicates that there is a relationship between circulating concentrations of IL-6 and the physical aspects of fatigue. Thus, reducing circulating IL-6 concentrations could have a direct effect on a patient’s physical well-being and ability to cope with day-to-day life. Cognitive and emotional fatigue may not have a physiological trigger, but may occur as a consequence of the physical aspects.
4.7.5 The circulating lymphocytes and antigen-presenting cells are not the source of elevated plasma/serum IL-6 in PMR patients

CD3+ T cells, CD14+ monocytes, CD19+ B cells, CD123+ pDCs and CD11c+ mDCs in all subjects constitutively produced low to modest levels of intracellular IL-6. This corresponded with very low concentrations of secreted IL-6 in parallel cultures (<9 pg/ml), but there were no significant differences in secreted IL-6 concentrations in unstimulated cells from PMR, RA and HC samples.

Stimulation of PBMC induced strong intracellular responses in all cell types of some samples. Overall, however, responses were highly variable and no significant difference in cell responsiveness was observed between PMR, RA and healthy control samples. In contrast, secreted IL-6 increased dramatically in all participants following stimulation (>1000 pg/ml) suggesting that intracellular staining may not reflect the secretory capability of these cells, but also confirming no differences between PBMC responses of PMR, RA and control groups.

Taken together, using different methods to test the hypothesis, the PBMCs tested were not found to be the source of elevated concentrations of IL-6 in PMR, suggesting that the neutrophils, vascular endothelium, joints, muscular tissue or aorta could be potential sources.

4.7.6 There may be a subset of RA patients with raised circulating concentrations of several cytokines

Looking more broadly at the cytokine profiles in the serum and plasma of the participants, a subgroup of RA patients were found to have raised levels of several cytokines including IL-4, TNFα, IL-10, IL-12p70 and IL-2. This was not observed in PMR patients or healthy controls. This could be an indicator that different disease pathologies exist within RA, that are not present in PMR. As all RA patients exhibit similar symptoms, this could indicate that cytokines are raised in some patients as a consequence of a more immunologically aggressive form of the disease.
4.7.7 PMR is associated with raised circulating IL-6 only

IL-6 is responsible for the symptoms observed in PMR and thus is a major part of the pathogenesis of the disease. No other cytokines measured were raised in the circulation of PMR patients and thus no other cytokines appear to be involved with the pathogenesis of the disease. This indicates that PMR and RA are very different in terms of disease pathology, despite sharing similar symptoms.
4.8 Overall summary & conclusions

This study has confirmed that IL-6 concentrations in the serum and plasma are significantly raised in PMR and RA patients compared to healthy controls, whilst demonstrating that IL-6 concentrations were consistently higher in serum than in plasma. This IL-6 was found to be biologically active, thus capable of contributing to the pathogenesis of both these diseases. A significant correlation was observed between the total fatigue score and circulating IL-6 concentrations in the plasma of PMR and RA patients, with two individual aspects of fatigue; physical fatigue and living fatigue, correlating very well with plasma IL-6 concentrations.

Furthermore, using a variety of methods to test the hypothesis, this study has ruled out the PBMCs as a potential source of IL-6 in PMR, increasing the likelihood that either the vascular endothelium or muscle tissue may be involved. Looking more broadly at the circulating cytokine profiles of PMR and RA patients, IL-6 was the only cytokine to be significantly raised in the circulation of PMR patients compared to healthy controls. A small sub-group of RA patients, however, had raised circulating concentrations of IL-4, TNFα, IL-10, IL-12p70 and IL-2, which may indicate that different disease pathologies exist within RA, that are not present in PMR.

Methodologies involved in cytokine analysis and the reporting of this analysis requires standardisation, in order for researchers to be able to draw direct comparisons between studies, enabling research to move forward more quickly and efficiently.

Future investigations should consider the clinical implications for raised IL-6, look deeper into the relationship between IL-6 and fatigue onset, carry out a larger more comprehensive study of the circulating cytokines in RA, establish a more standardised approach to cytokine analysis, and crucially, continue exploring the cellular source of IL-6 in polymyalgia rheumatica.
References


References


References


References

in rheumatoid synovial fluid, synovial tissue and peripheral blood. *Clinical and Experimental Immunology.* 59 (3): 520-528.


References


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References


References


References


References


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References


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References


References


References


Xu, W-D., Zhang, M., Feng, C-C., Yang, X-K., Pan, H-F., Ye, D-Q. (2013) IL-32 with potential insights into rheumatoid arthritis. *Clinical Immunology.* 147 (2): 89-94.


Appendices
Appendices

Appendix A: Questionnaires and case report forms (CRF) used for the recruitment of patients and controls

Screening for recruitment of controls

Date of Birth: ....................................

Gender: Male □  Female □

Inclusion Criteria

Is the patient above 55 but below 85 years old? Yes □  No □
Is there any inflammatory rheumatological illness? Yes □  No □
Is CRP < 5? Yes □  No □
Is PV < 1.75? Yes □  No □

Exclusion Criteria

On glucocorticoids (oral or parenteral) or biologic treatment in the last month? Yes □  No □
Inflammatory diseases such as inflammatory bowel disease, colitis, asthma? Yes □  No □
Giant cell arteritis? Yes □  No □
Other auto-immune diseases? Yes □  No □
Cancer? Yes □  No □
Infections, treatment with antibiotics within the past 6 weeks? Yes □  No □
Significant renal disease (creatinine >150 μmol/L and/or eGFR < 30 ml/min)? Yes □  No □
Significant hepatic impairment? Yes □  No □
Participation in a clinical trial within the past 30 days? Yes □  No □
Pregnancy and lactation? Yes □  No □
NA □
Working shift employee? Yes □  No □
Jet lag? Yes □  No □

CRP =...............................  
PV=...............................  
Participant (control) consented on.............................
Screening for the recruitment of PMR patients

Date of Birth: ....................................

Gender: Male ☐   Female ☐

**Inclusion Criteria**

- Is the patient above 55 but below 85 years old? Yes ☐ No ☐
- Does the patient meet 3 or more of Bird criteria? Yes ☐ No ☐
  
  **Bird Criteria:**
  - Bilateral shoulder pain/stiffness Yes ☐ No ☐
  - Duration of symptoms <2 weeks Yes ☐ No ☐
  - Initial ESR >40 mm/h (PV>1.72, CRP>5) Yes ☐ No ☐
  - Stiffness >1 hour Yes ☐ No ☐
  - Age >65 years Yes ☐ No ☐
  - Depression and/or weight loss Yes ☐ No ☐
  - Bilateral upper arm tenderness Yes ☐ No ☐

  **Number of Bird criteria met:** ...................................

- Currently active disease (CRP≥5, PV≥1.72 or ESR≥29)? Yes ☐ No ☐

**Exclusion Criteria**

- On glucocorticoids (oral or parenteral) or biologic treatment in the last month? Yes ☐ No ☐
- Inflammatory diseases such as inflammatory bowel disease, colitis, asthma? Yes ☐ No ☐
- Giant cell arteritis? Yes ☐ No ☐
- Other auto-immune diseases? Yes ☐ No ☐
- Cancer? Yes ☐ No ☐
- Infections, treatment with antibiotics within the past 6 weeks? Yes ☐ No ☐
- Significant renal disease (creatinine >150 μmol/L and/or eGFR < 30 ml/min)? Yes ☐ No ☐
- Significant hepatic impairment? Yes ☐ No ☐
- Participation in a clinical trial within the past 30 days? Yes ☐ No ☐
- Pregnancy and lactation? NA ☐
- Working shift employee? Yes ☐ No ☐
- Jet lag? Yes ☐ No ☐

**PMR diagnosis made on**……………………………..

**Patient consented on**……………………………..
Screening for the recruitment of RA patients

Date of Birth: ....................................

Gender: Male ☐ Female ☐

**Inclusion Criteria**

Is the patient above 55 but below 85 years old? Yes ☐ No ☐

Does the patient meet 4 or more of the ACR criteria? Yes ☐ No ☐

**ACR Criteria:**

Morning stiffness (≥1 hour) Yes ☐ No ☐

Swelling (soft tissue) of three or more joints Yes ☐ No ☐

Swelling (soft tissue) of hand joints (PIP, MCP, or wrist) Yes ☐ No ☐

Symmetrical swelling (soft tissue) Yes ☐ No ☐

Subcutaneous nodules Yes ☐ No ☐

Serum rheumatoid factor Yes ☐ No ☐

Erosions and/or periarticular osteopenia in hand or wrist joints seen on radiograph Yes ☐ No ☐

**Number of ACR criteria met:** ..........................................

...  

Currently active disease (CRP≥5, PV≥1.72 or ESR≥29)? Yes ☐ No ☐

Are there at least 3 tender AND swollen joints? Yes ☐ No ☐

Is morning stiffness ≥ 45 minutes? Yes ☐ No ☐

**Exclusion Criteria**

On glucocorticoids (oral or parenteral) or biologic treatment in the last month? Yes ☐ No ☐

Inflammatory diseases such as inflammatory bowel disease, colitis, asthma? Yes ☐ No ☐

Co-existent giant cell arteritis? Yes ☐ No ☐

Other auto-immune diseases? Yes ☐ No ☐

Cancer? Yes ☐ No ☐

Infections, treatment with antibiotics within the past 6 weeks? Yes ☐ No ☐

Significant renal disease (creatinine >150 μmol/L and/or eGFR < 30 ml/min)? Yes ☐ No ☐

Significant hepatic impairment? Yes ☐ No ☐

Participation in a clinical trial within the past 30 days? Yes ☐ No ☐

Pregnancy and lactation? NA ☐ Yes ☐ No ☐

Working shift employee? Yes ☐ No ☐

Jet lag? Yes ☐ No ☐

**Patient consented on**…………………………..
Appendices

Clinical Summary sheet

Past Medical History
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Allergies
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Social History
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Clinical Examination
BP.................... Temp.................... Body Weight....................
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Sats.................... Pulse....................
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Bristol Rheumatoid Arthritis Fatigue- Multi Dimensional Questionnaire (BRAF-MDQ)

We would like to know how fatigue has affected you in the past 7 days. Please answer all of the questions. Don’t think too long and hard, just give your first reaction - there are no right or wrong answers!

1 Please circle the number that shows your average level of fatigue during the past 7 days.

No Fatigue 0 1 2 3 4 5 6 7 8 9 10 Totally Exhausted

************

For each of the following questions, please tick one answer that best applies to you

2 How many days did you experience fatigue during the past week (7 days)?

0 □ 4 □
1 □ 5 □
2 □ 6 □
3 □ Every day □

3 How long, on average, has each episode of fatigue lasted during the last 7 days?

Less than one hour □
Several hours □
All day □
<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Not at all</th>
<th>A little</th>
<th>Quite a bit</th>
<th>Very much</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Have you lacked physical energy because of fatigue?</td>
<td>-----</td>
<td>-----</td>
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<tr>
<td>5</td>
<td>Has fatigue made it difficult to bath or shower?</td>
<td>-----</td>
<td>-----</td>
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</tr>
<tr>
<td>6</td>
<td>Has fatigue made it difficult to dress yourself?</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
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<tr>
<td>7</td>
<td>Has fatigue made it difficult to do your work or other daily activities?</td>
<td>-----</td>
<td>-----</td>
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</tr>
<tr>
<td>8</td>
<td>Have you avoided making plans because of fatigue? e.g. plans to go out, or do jobs around the home or garden</td>
<td>-----</td>
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<tr>
<td>9</td>
<td>Has fatigue affected your social life?</td>
<td>-----</td>
<td>-----</td>
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<tr>
<td>10</td>
<td>Have you cancelled plans because of fatigue? e.g. plans to go out, or do jobs around the home or garden</td>
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<tr>
<td>11</td>
<td>Have you refused invitations because of fatigue? e.g. meeting up with a friend</td>
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<tr>
<td>12</td>
<td>Have you lacked mental energy because of fatigue?</td>
<td>-----</td>
<td>-----</td>
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</tr>
<tr>
<td>13</td>
<td>Have you forgotten things because of fatigue?</td>
<td>-----</td>
<td>-----</td>
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<td>-----</td>
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<tr>
<td>14</td>
<td>Has fatigue made it difficult to think clearly?</td>
<td>-----</td>
<td>-----</td>
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<td>-----</td>
</tr>
<tr>
<td>15</td>
<td>Has fatigue made it difficult to concentrate?</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>16</td>
<td>Have you made mistakes because of fatigue?</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>17</td>
<td>Have you felt you have less control in areas of your life because of fatigue?</td>
<td>-----</td>
<td>-----</td>
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</tr>
<tr>
<td>18</td>
<td>Have you felt embarrassed because of fatigue?</td>
<td>-----</td>
<td>-----</td>
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<td>-----</td>
</tr>
<tr>
<td>19</td>
<td>Has being fatigued upset you?</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>20</td>
<td>Have you felt down or depressed because of fatigue?</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
</tbody>
</table>
**Health Assessment Questionnaire (HAQ)**

Please tick the one response which best describes your usual abilities over the past week.

<table>
<thead>
<tr>
<th>1. DRESSING AND GROOMING</th>
<th>Without ANY difficulty</th>
<th>With SOME difficulty</th>
<th>With MUCH difficulty</th>
<th>UNABLE to do</th>
</tr>
</thead>
</table>

Are you able to:

a. Dress yourself, including tying shoelaces and doing buttons?

b. Shampoo your hair?

<table>
<thead>
<tr>
<th>2. RISING</th>
<th>Without ANY difficulty</th>
<th>With SOME difficulty</th>
<th>With MUCH difficulty</th>
<th>UNABLE to do</th>
</tr>
</thead>
</table>

Are you able to:

a. Stand up from an armless straight chair?

b. Get in and out of bed?

<table>
<thead>
<tr>
<th>3. EATING</th>
<th>Without ANY difficulty</th>
<th>With SOME difficulty</th>
<th>With MUCH difficulty</th>
<th>UNABLE to do</th>
</tr>
</thead>
</table>

Are you able to:

a. Cut your meat?

b. Lift a full cup or glass to your mouth?

c. Open a new carton of milk (or soap powder)?

<table>
<thead>
<tr>
<th>4. WALKING</th>
<th>Without ANY difficulty</th>
<th>With SOME difficulty</th>
<th>With MUCH difficulty</th>
<th>UNABLE to do</th>
</tr>
</thead>
</table>

Are you able to:

a. Walk outdoors on the flat ground?

b. Climb up five steps?

**PLEASE TICK ANY AIDS OR DEVICES THAT YOU USUALLY USE FOR ANY OF THESE ACTIVITIES:**

- Cane (W)
- Walking frame (W)
- Built up or special utensils (E)
- Crutches (W)
- Wheelchair (W)
- Special or built up chair (A)

Devices used for dressing (button hooks, zipper pull, shoe horn)

Other (specify)..........................................................

**PLEASE TICK ANY CATEGORIES FOR WHICH YOU USUALLY NEED HELP FROM ANOTHER PERSON:**

- Dressing and Grooming
- Eating
- Rising
- Walking
### 5. HYGEINE

Are you able to:

<table>
<thead>
<tr>
<th>Without ANY difficulty</th>
<th>With SOME difficulty</th>
<th>With MUCH difficulty</th>
<th>UNABLE to do</th>
</tr>
</thead>
<tbody>
<tr>
<td>b. Take a bath</td>
<td>..........</td>
<td>..........</td>
<td>..........</td>
</tr>
</tbody>
</table>

### 6. REACH

Are you able to:

<table>
<thead>
<tr>
<th>Without ANY difficulty</th>
<th>With SOME difficulty</th>
<th>With MUCH difficulty</th>
<th>UNABLE to do</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Reach and get down a 5 lb object (e.g. bag of potatoes) from just above your head?</td>
<td>..........</td>
<td>..........</td>
<td>..........</td>
</tr>
<tr>
<td>b. Bend down to pick up clothing off the floor?</td>
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<td>..........</td>
</tr>
</tbody>
</table>

### 7. GRIP

Are you able to:

<table>
<thead>
<tr>
<th>Without ANY difficulty</th>
<th>With SOME difficulty</th>
<th>With MUCH difficulty</th>
<th>UNABLE to do</th>
</tr>
</thead>
<tbody>
<tr>
<td>b. Open jars which have been previously opened?</td>
<td>..........</td>
<td>..........</td>
<td>..........</td>
</tr>
</tbody>
</table>

### 8. ACTIVITIES

Are you able to:

<table>
<thead>
<tr>
<th>Without ANY difficulty</th>
<th>With SOME difficulty</th>
<th>With MUCH difficulty</th>
<th>UNABLE to do</th>
</tr>
</thead>
<tbody>
<tr>
<td>c. Do chores such as vacuuming, housework or light gardening?</td>
<td>..........</td>
<td>..........</td>
<td>..........</td>
</tr>
</tbody>
</table>

**PLEASE TICK ANY AIDS OR DEVICES THAT YOU USUALLY USE FOR ANY OF THESE ACTIVITIES:**

- Raised toilet seat (H)
- Bath seat (H)
- Bath rail (H)
- Long handles appliances for reach (R)
- Jar opener (for jars previously opened) (G)
- Other (specify): ...........................................................................................................

**PLEASE TICK ANY CATEGORIES FOR WHICH YOU USUALLY NEED HELP FROM ANOTHER PERSON:**

- Hygiene
- Gripping and opening things
- Reach
- Errands and housework
Rheumatoid Arthritis Disease Activity Score (DAS)

No of swollen joints: ..........   No of tender joints: ..........

CRP =..........

Patient’s global health:
Considering all the ways your arthritis affects you, please mark on the line how well you think you are doing:

Very well -------------------- Very badly

DAS-28 =.........
Appendices

Additional notes on blood sampling

Whole Blood sample taken at: .............................................

Amount withdrawn: ............................................................

Further notes on blood sampling:
..............................................................................................
..............................................................................................
..............................................................................................
..............................................................................................
..............................................................................................

Notes on individual samples:

<table>
<thead>
<tr>
<th>Actual amount</th>
<th>Small sample (4ml plasma)</th>
<th>Large sample (35ml)</th>
</tr>
</thead>
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<td>Outcome</td>
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-: no haemolysis
±: very minimal
+: minimal
++: moderate
+++: severe
Score sheet for answers generated from questionnaires

**BRAF-MDQ**

<table>
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<td>Living total (0-21), Q 5-11</td>
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<td>Emotion total (0-12), Q 17-20</td>
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<td><strong>Total score (0-70)</strong></td>
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**HAQ**

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**HAD**

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<tbody>
<tr>
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Appendices

Appendix B: Patient information sheet and consent form

The cellular source of Interleukin-6 in Polymyalgia Rheumatica

Patient Information Sheet
(Version 1.3 Date 25 March 2012)

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

Polymyalgia rheumatica causes pain and stiffness around shoulder and hip joints. We think that these symptoms are related to various “chemical messengers” (also called cytokines). We are particularly interested in studying one of these cytokines which is called interleukin-6 or for short, IL-6. We already have some limited information about its role in polymyalgia rheumatica but we would like to know more and find out its source. In other words, we would like to know what cells are responsible for secreting it.

We have also found out from our previous work in rheumatoid arthritis that IL-6 levels are elevated in this disease. Rheumatoid arthritis is a disease that causes pain, swelling and stiffness of joints across the body. In both polymyalgia rheumatica and rheumatoid arthritis, patients wake up in the morning with profound stiffness. However in polymyalgia rheumatica, IL-6 levels are higher than those detected in rheumatoid arthritis.

We aim towards recruiting 6-10 patients with polymyalgia rheumatica, 6-10 with rheumatoid arthritis and 6-10 controls who do not have any disease that causes inflammation. By doing so, we will be able to compare between the three groups and add more power to our study.
Appendices

The information we are going to get from you and other participants will greatly help us set up a bigger study in the future.

**Why have I been chosen?**

You have been chosen to take part in this study because you fall into one of the following groups:

1) You have recently been diagnosed with polymyalgia rheumatica
2) You have recently been diagnosed with rheumatoid arthritis
3) You belong to the control group*

*The control group gives us a comparison for these other inflammatory conditions.

**What will I be asked to do if I take part?**

One or two days after reading this leaflet, the research doctor will telephone you to know about your decision. If you have agreed to take part, we will be sending you a taxi to bring you to our Rheumatology Centre where we first saw you and talked to you about the study.

The doctor will ask you to read and sign a consent form. You will get a copy of the signed consent form to take with you as well. The doctor will then perform clinical assessment on you and ask you to complete a few questionnaires. Next, one blood sample will be taken from you through a needle prick. We would like to take 35mls of blood. This amount of blood equates to 3-4 tablespoons approximately.

After giving blood, you will be free to go home through our transport. Please feel free to help yourself with any coffee or tea whilst in the department. The taxi will pick you up back home at your convenience. We estimate the total duration of your stay in the department for the study will be around one hour.

Blood taken from you will be processed immediately and then transported in a special container without any personal identifiers to the University of the West of England where further analysis will take place. Your confidentiality will be highly respected and secured throughout the study. After performing the required tests on your sample, all blood materials left will be destroyed.

You will be later invited to attend a short meeting with the other patients in the study. You do not have to attend this meeting. If you do come it will give you the chance to tell us about any ideas that you have, so we can improve future studies. We welcome your suggestions to help us to improve how we look after patients involved in research at any time.

**Do I have to take part?**
Appendices

No, taking part is voluntary. It is up to you to decide whether or not to take part. If you decide to take part, you are still free to withdraw at any time. If you decide not to take part, you do not have to give a reason, nobody will be upset and the standard of care you receive will not be affected in any way.

What are the possible risks of taking part?

There are hardly any real risks from taking part in this study. However, and as you may be aware of, this study involves taking one blood sample from you. This means that you may experience some pain at the site of needle prick. A bruise may also appear on this site later. This bruise does not last for long and normally fades away within a few days.

What are the possible benefits of taking part?

You will not be paid for taking part. The information that we get from this study may help us understand polymyalgia rheumatica better and therefore, improve the way this disease is managed in the future.

Is my doctor being paid for including me in the study?

No.

Are there any restrictions on what I might eat or do?

No.

What if something goes wrong?

Considering how our study is conducted, we believe that this is very unlikely to happen. However, if anything goes wrong for any reason, our research team will stay with you and try to help and sort out any problem.

After you have gone home, if you still have a question, you can always contact the research doctor at work on 0117 342 2515 or 0117 342 2902. During out of hours, the doctor will be happy to take your call on his own mobile phone which is 0753 320 4380. In the unlikely event of an emergency, you will need to go through the normal route by contacting the Accident and Emergency Department.

If you have concerns about any aspect of the way you have been approached during the course of this study you may wish to contact the hospital’s Patient Advice and Liaison Service (PALS) on 0117 928 3571, Mincom number 0117 934 9261, or write to PALS, Bristol Royal Infirmary, Main BRI Front Entrance, Queens Building, Bristol, BS2 8HW. If you wish to make a formal complaint please write to Dr, Chief Executive U.H. Bristol Headquarters, Marlborough Street, Bristol, BS1 3NU or telephone Patient Complaints Manager on 0117 928 3604.
Confidentiality - who will know I am taking part in the study?

All information we collect about you during the course of the research will be kept strictly confidential. Any information about you that leaves the hospital (including any samples sent to the University of West of England) will be made anonymous so that it cannot be used to identify you. Your medical records may be looked at by people from the hospital’s Research Office or the regulatory authorities to check that the study is being carried out correctly. Your name, however, will not be disclosed outside the hospital.

Do I need to inform my GP about my participation in the study?

As our study does not involve any new treatments or changes to your medical plans, there is no need to inform your GP. However if you would like to discuss it with your GP, then feel free to do so.

Who has reviewed the study?

This research study has been reviewed and approved by the Ethics Committee.

Contact Information:

<table>
<thead>
<tr>
<th>University of Bristol</th>
<th>University of the West of England</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Professor John Kirwan</strong></td>
<td><strong>Miss Hannah Bazzard</strong></td>
</tr>
<tr>
<td>Consultant Rheumatologist and Professor of Rheumatic Diseases</td>
<td>PhD Student</td>
</tr>
<tr>
<td>0117 342 2904</td>
<td>0117 328 3812</td>
</tr>
<tr>
<td><strong>Dr Vanessa Quick and Dr Matt Roy</strong></td>
<td><strong>Dr Victoria Davenport</strong></td>
</tr>
<tr>
<td>Clinical Research Fellows</td>
<td>Academic Supervisor</td>
</tr>
<tr>
<td></td>
<td>0117 928 2178</td>
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</table>

A copy of this Patient Information Sheet and the signed Informed Consent Form will be provided to the patient to keep.
CONSENT FORM

Title of project: The cellular source of Interleukin-6 in Polymyalgia Rheumatica

Name of Researchers: Professor John Kirwan, Consultant Rheumatologist and Professor of Rheumatic Diseases
Dr Vanessa Quick, Clinical Research Fellow
Dr Matt Roy, Clinical Research Fellow
Miss Hannah Bazzard, PhD student
Dr Victoria Davenport, Academic Supervisor

Please initial box

1. I confirm that I have read and understand the information sheet dated 25 March 2012 (version 1.3) for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time; without giving any reason, without my medical care or legal rights being affected.

3. I understand that my medical notes will be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

4. I understand that the samples may be transferred to another place for the research to be performed, and that any samples that leave the hospital will be coded so that it is not possible to use them to identify me.
5. I understand that my samples will be destroyed after performing the required analyses on them.

6. I understand that the University of Bristol and the University of the West of England will have ownership of the research results and may file patents or otherwise protect and commercialise research results. I understand that there will be no financial benefit or direct personal benefit to me, either as a result of commercialisation of the research results or in respect of the original samples donated.

7. I agree to take part in the above study.

After you have initialled all the above boxes, please complete below by printing your name, today’s date and your signature.

________________________  ____________________
Name of Patient            Date                        Signature

________________________  ____________________
Name of Person taking consent (If different from researcher) Date                        Signature

________________________  ____________________
Researcher                       Date                        Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes
## Appendix C: Raw data

### Patient demographics

| Participant number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | Pilot |
|--------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|-------|
| Gender             | F | F | F | M | F | F | F | M | F | M | F | M | F | F | F | F | F | F | F    |
| Age (years)        | 75| 72| 63| 62| 72| 73| 79| 59| 65| 68| 60| 71| 60| 73| 60| 88| 76| 65| 75    |
| Diagnosis          | PMR| PMR| Control| RA| Control| RA| RA| PMR| Control| Control| RA| PMR| Control| PMR| PMR| PMR| PMR| RA    |
| C-Reactive Protein (mg/L) | 11| 15| 2 | 4 | 5 | 29| 11| 15| 1 | 1 | 11| 2 | 1 | 7 | 38| 2 | 25| 28| 102| 19   |
| RA DAS-28 (score)  | 11.5| 5.65| 4.79 | 5.1 | 6.9 | 6.64 | 4.84 | 23.2 | 34.2 | 43.2 | 57 | 72.9 | 69.8 | 132.1 | 0.375 | 1.5 | 0.25 | 2 | 0 | 1.63 | 2.13 | 1.75 | 0 | 0.875 | 1.25 | 0 | 2.125 | 1.143 | 0 | 1.75 | 2 | 1.75 | 1.25 |

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<td>PMR</td>
<td>PMR</td>
<td>RA</td>
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<td>12</td>
<td>18</td>
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### ELISA data

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### Appendix

#### B9 bioassay data

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#### CBA cytokine data

**Plasma**

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**Serum**

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### Appendix

#### Unstimulated PBMC culture supernatant

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<td>PMR</td>
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<td>*</td>
</tr>
<tr>
<td>TNFα (pg/ml)</td>
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<tr>
<td>IL-10 (pg/ml)</td>
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<tr>
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<td>IL-1β (pg/ml)</td>
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<td>IFNγ (pg/ml)</td>
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<tr>
<td>IL-4 (pg/ml)</td>
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<td>IL-2 (pg/ml)</td>
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#### Stimulated PBMC culture supernatant

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<td>PMR</td>
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Flow cytometry data

| Participant number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | Pilot |
|--------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|-----|
| % IL-6 positive cells | Unstimulated or Stimulated | Cell type/ Diagnosis | PMR | PMR | Control | RA | Control | RA | RA | PMR | Control | RA | Control | RA | PMR | Control | RA | PMR | RA |
| % IL-6 pos | Unstimulated | T cells | 94 | 28 | 30 | 23 | 75 | 96 | 22 | 4 | 15 | 10 | 27 | 72 | 70 | 7 | 41 | 18 | 51 | 22 | 81 |
| % IL-6 pos | Unstimulated | Monocytes | 97 | 93 | 99 | 98 | 93 | 99 | 77 | 76 | 88 | 90 | 95 | 95 | 99 | 93 | 98 | 98 | 98 | 94 | 100 |
| % IL-6 pos | Unstimulated | B cells | 93 | 40 | 100 | 52 | 85 | 94 | 18 | 16 | 51 | 26 | 94 | 72 | 95 | 63 | 100 | 34 | 66 | 26 | 100 |
| % IL-6 pos | Unstimulated | pDC | 89 | 97 | 100 | 98 | 93 | 67 | 67 | 63 | 88 | 87 | 93 | 97 | 97 | 81 | 99 | 94 | 99 | 90 | 99 |
| % IL-6 pos | Unstimulated | mDC | 95 | 76 | 89 | 91 | 88 | 61 | 39 | 41 | 61 | 77 | 74 | 79 | 86 | 73 | 94 | 90 | 93 | 95 | 97 |
| % IL-6 pos | Stimulated | T cells | 24 | 5 | 70 | 99 | 51 | 90 | 4 | 5 | 40 | 10 | 67 | 16 | 76 | 82 | 49 | 97 | 83 | 14 | 68 |
| % IL-6 pos | Stimulated | Monocytes | 99 | 94 | 100 | 100 | 94 | 99 | 70 | 85 | 93 | 99 | 90 | 72 | 100 | 96 | 98 | 96 | 92 | 92 |
| % IL-6 pos | Stimulated | B cells | 63 | 34 | 93 | 100 | 87 | 94 | 13 | 33 | 52 | 47 | 51 | 35 | 100 | 68 | 98 | 93 | 81 | 27 | 100 |
| % IL-6 pos | Stimulated | pDC | 86 | 98 | 100 | 99 | 92 | 99 | 64 | 80 | 91 | 98 | 94 | 89 | 100 | 92 | 100 | 90 | 97 | 88 | 100 |
| % IL-6 pos | Stimulated | mDC | 89 | 69 | 68 | 100 | 93 | 96 | 35 | 50 | 73 | 93 | 62 | 70 | 100 | 84 | 96 | 95 | 85 | 90 | 98 |

| Participant number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | Pilot |
|--------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|-----|
| Mean fluorescence intensity (MFI) | Unstimulated or Stimulated | Cell type/ Diagnosis | PMR | PMR | Control | RA | Control | RA | RA | PMR | Control | RA | Control | RA | PMR | Control | RA | PMR | RA |
| MFI | Unstimulated | T cells | 5617 | 3383 | 6954 | 3477 | 5270 | 13840 | 2791 | 2450 | 5030 | 10945 | 44658 | 5894 | 9962 | 5068 | 9100 | 4302 | 4478 | 15648 | 17820 |
| MFI | Unstimulated | Monocytes | 9672 | 13252 | 10455 | 8567 | 15505 | 23186 | 4611 | 5268 | 72397 | 8498 | 151313 | 29346 | 13443 | 16199 | 22680 | 11785 | 10684 | 10150 | 40494 |
| MFI | Unstimulated | B cells | 8663 | 4979 | 33998 | 4134 | 9668 | 56067 | 2391 | 2926 | 36305 | 4443 | 114817 | 41420 | 13685 | 38000 | 48359 | 6570 | 12707 | 69814 | 66495 |
| MFI | Unstimulated | pDC | 5783 | 15732 | 27079 | 8389 | 15604 | 5420 | 3887 | 4958 | 17895 | 27385 | 43990 | 13441 | 9290 | 27724 | 26712 | 8549 | 12033 | 18613 | 52462 |
| MFI | Unstimulated | mDC | 9426 | 5482 | 14049 | 6703 | 12041 | 4763 | 3053 | 3772 | 18795 | 26096 | 13231 | 10058 | 14621 | 34560 | 20724 | 7516 | 10072 | 39443 | 62428 |
| MFI | Stimulated | T cells | 2730 | 2382 | 8543 | 45359 | 3884 | 10924 | 1983 | 2537 | 3879 | 5971 | 35264 | 5772 | 10191 | 5268 | 10536 | 14302 | 9418 | 5225 | 20373 |
| MFI | Stimulated | Monocytes | 13816 | 7889 | 25128 | 93187 | 15965 | 41788 | 4808 | 18682 | 7859 | 15621 | 288654 | 9375 | 61033 | 23491 | 37355 | 38918 | 20636 | 20058 | 42814 |
| MFI | Stimulated | B cells | 8654 | 27751 | 24954 | 233699 | 9421 | 36990 | 2454 | 4187 | 8024 | 59651 | 128455 | 8975 | 123171 | 39128 | 66299 | 24780 | 22831 | 14369 | 85433 |
| MFI | Stimulated | pDC | 6398 | 11951 | 18846 | 87081 | 22486 | 38883 | 4967 | 6420 | 9102 | 40474 | 123918 | 79458 | 44000 | 22347 | 50681 | 11628 | 18091 | 15106 | 84431 |
| MFI | Stimulated | mDC | 6818 | 6653 | 10879 | 137693 | 12234 | 26420 | 3370 | 4948 | 14965 | 17126 | 124793 | 57746 | 44759 | 28758 | 41194 | 33958 | 13533 | 17525 | 59612 |