

# **The Role of Matrix Metalloproteinases in the Immunopathology of Cerebral Tuberculosis**

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**PhD Thesis**

“Those who look but do not see go away  
no wiser than when they came”

Frederick Horniman  
19<sup>th</sup> Century Tea Merchant

## Declaration of authenticity

I hereby declare that the following work presented in this thesis is my own:

A handwritten signature in black ink, appearing to read 'Justin Anton Green', with a large, stylized flourish at the end.

Justin Anton Green

## Abstract

*Introduction:* Central nervous system tuberculosis (CNS TB) has a high mortality. Microglial derived matrix metalloproteinases (MMPs) are implicated in the intense inflammatory response to *Mycobacterium tuberculosis* (*M.tb*) as the blood brain barrier is rich in MMP substrates.

*Methods:* In a cellular model of CNS TB human microglial cells were stimulated with *M.tb* and conditioned media from *M.tb* infected human monocytes (CoMTb). Gene expression of all 23 MMPs and the 4 tissue inhibitors of MMP (TIMPs) was analysed by real time RT-PCR and secretion by Luminex and Western blotting. MMP gene transcription regulation was studied by luciferase promoter reporter assays and NFkB/AP-1 ELISAs. MAP kinases were studied by Western blotting. Immunohistochemistry of CNS TB biopsies and ELISA analysis of cerebrospinal fluid (CSF) was performed.

*Results:* CoMTb up-regulated expression of MMP-1, -3 and -9, which was suppressed by dexamethasone. In contrast CoMTb did not alter TIMP-1 and reduced TIMP-2, -3 and -4 expression. *M.tb* up-regulated MMP-1 and -3 secretion. However, CoMTb drove MMP-1 and -3 secretion more potently than *M.tb* but suppressed MMP-2 secretion. Nuclear NFkB p50-p65 heterodimers and AP-1 cJun/FosB increased in CoMTb stimulated cells, with concomitant degradation of IkB $\alpha$ . Mutation of NFkB and AP-1 sites in the MMP-1 promoter abrogated CoMTb promoter activity. CoMTb drove early p38 and ERK MAP kinase phosphorylation. Chemical inhibition of both NFkB and p38 returned MMP-1 and 3 secretion to control levels but enhanced MMP-2 secretion that was also caspase 8 dependent. MMP/TIMP concentrations in CSF samples

from TBM patients were predictive of coma severity and outcome and demonstrated that dexamethasone preferentially suppressed MMP-9.

*Conclusions:* In CNS TB unopposed MMP-1 and -3 expression and secretion are driven by *M.tb*-infected monocyte networks and regulated by p38, NFκB and AP-1. CSF MMP/TIMP concentrations predict disease severity and offer a mechanism for the beneficial effect of dexamethasone therapy.

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## **Chapter 1 – Introduction**

The scourge of tuberculosis (TB) has been affecting humans since antiquity. Hippocrates considered TB (“phthisis”) as the most widespread disease of his time and the coughing of blood, associated with fever was considered almost always fatal. The ancient Greeks considered these symptoms due to an imbalance in the four humours. As it is now believed that tissue destruction caused by the host immune response to TB is responsible for many of the symptoms, signs and sequelae of infection the Greeks may well have been nearer the truth than they are given credit for.

### ***Worldwide Epidemiology of Tuberculosis***

TB is no less important today where 9 million new cases are thought to occur world-wide every year and 2 million die as a direct consequence of it [1, 2]. Rates of TB rose drastically in the late 1980s due to failing public health infrastructure and by 1993 the World Health Organisation (WHO) had declared TB a “global emergency” [3]. 13 years later, although incidence rates had started to level off it was still considered such a massive global problem that the “STOP TB” campaign was launched by WHO in 2006, setting ambitious targets in all aspects of TB diagnostics and treatment to be achieved within 9 years [4]. Halving TB prevalence and death rates by 2015 are also in the UN Millennium Development Goals.

Sub-Saharan Africa has the highest incidence rates of TB infection (290 per 100,000 population) but actual numbers of cases are higher in the more heavily populated Asian countries such as China and the Indian sub-continent, both of

whom report over a million new cases a year [5]. Vietnam, a country with a population of about 86 million people reported nearly 90,000 cases of smear positive TB in 2000 [6]. This puts it in the top 15 incidence countries in the world, yet its TB service is considered a success by WHO standards as number of cases put on short-course treatment have been over 70% since 1996 and reported cure rates are consistently over 85% [6].

## ***Tuberculosis in the UK***

### **Introduction**

In the UK TB notification is regulated under the Public Health (Control of Disease) Act 1984 and Public Health (Infectious Diseases) Regulations 1988 and co-ordinated by the Health Protection Agency (HPA), who produce an annual TB report from which the following is a summary of the most recent data [7].

### **Epidemiology**

8497 tuberculosis cases were notified to the HPA in 2006 in the UK, giving an overall incidence of 14/100,000. This represents a levelling off of TB incidence in the UK for the first time since the 1980s. The London region always accounts for the largest proportion of cases, 40% in 2006: an overall incidence of 45/100,000. The majority of cases occur in young adults (aged 15-44 years) and 73% of cases were non-UK born. 56% of patients had pulmonary disease.

### **Central nervous system TB in the UK 2002-2006**

No breakdown of the figures for extra-pulmonary TB was given in the HPA annual report to estimate central nervous system (CNS) TB numbers. However,

I have been able to obtain the most recent HPA CNS TB data from 2002-2006 (Table 1). This shows that there has been a 48% increase in the number of tuberculous meningitis (TBM) cases from 112 in 2002 to 166 in 2006 (the last year when data is available). In parallel a 46% increase in reported cases of “other CNS TB” (which equates to CNS tuberculomas as spinal TB is recorded as bone TB) over the same 5 year period. The number of cases of tuberculomas is lower than TBM: 48 rising to 70 cases over the same five year period. Therefore TBM accounts for a mean of 73% of all CNS TB when the 5 year data is averaged (694/948 cases). In 2006 CNS TB made up 2.9% of all TB cases and 6.5% of all extra-pulmonary TB.

**Table 1. Number of tuberculosis cases with CNS disease: England, Wales & Northern Ireland, 2002 – 2006.**

| Year        | TBM      | CNS other | Total* |
|-------------|----------|-----------|--------|
| <b>2002</b> | 112 (72) | 48 (28)   | 155    |
| <b>2003</b> | 116 (69) | 54 (31)   | 168    |
| <b>2004</b> | 127 (74) | 48 (26)   | 171    |
| <b>2005</b> | 173 (77) | 57 (23)   | 225    |
| <b>2006</b> | 166 (72) | 70 (28)   | 229    |

\*Some cases had site reported as both TBM and CNS other hence the total is less than the sum. Numbers presented are n (%).  
Source: Enhanced Tuberculosis Surveillance as at 14/08/08, Health Protection Agency Centre for Infections.

## **Ethnic make-up of TB cases in the UK and drug resistance patterns**

In 2006 tuberculosis incidence was 95/100,000 among the non-UK born population compared to 4/100,000 among UK born persons. However, only 20% of non-UK born cases arrived in the UK in the two years prior to their diagnosis, suggesting that port of entry screening for active disease alone will

not pick up enough cases to affect on-going transmission. Among the non-UK born population, most cases belonged to the South Asian group (45%), but the highest incidence rate was among those belonging to the black African ethnic group (395/100,000), which is higher than the WHO TB rate for sub-Saharan Africa. 7.7% of TB isolates were resistant to at least one first line drug and 6.9% were isoniazid resistant. Multi-drug resistance remained low at about 1.1%.

### **Death and TB in the UK**

Among cases with treatment outcome reported (88% of all cases from 2005 – the most recent data available) 453 patients with TB in any site died whilst on treatment, giving a death rate of 7%. However, most deaths were in the over 65 years age group (25% deaths) and it can be assumed that some patients were dying with, not because of, TB. Nevertheless the HPA considered that TB was causative of death in 31% of cases, equating to 140 deaths per year. A significant rise in the number of HIV-TB co-infected cases will have contributed to this [8].

#### *CNS TB and death*

Unpublished data from the treatment outcome database was also obtained from the HPA to give crude CNS TB related death rates (Table 2). CNS TB accounted for 15% (21/140) of TB deaths in 2005. Although the number of deaths is small ranging between 11 and 21 per year the overall 5 year averaged death rate from CNS TB in the UK is still high at 16% (99/618). Rates of death from TBM are lower than those reported from the developing world literature, which probably reflects better access to intensive care, radiological and neurosurgical services.

Tuberculomas appear to run a more benign clinical course with an 11% total death rate (19/174), which is lower than the TBM rate of 18% (83/457,  $p=0.03$  for group comparison by  $\chi^2$  test). No data on HIV status, severity of disease at presentation or time to diagnosis were available to assess either the impact of HIV or whether UK doctors are performing well at diagnosing CNS TB.

**Table 2. Number of deaths among tuberculosis cases with CNS disease: England, Wales & Northern Ireland, 2002 - 2006#.**

| Year        | TBM     |              | CNS other |              | Total*  |              |
|-------------|---------|--------------|-----------|--------------|---------|--------------|
|             | Total # | Deaths (%)** | Total #   | Deaths (%)** | Total # | Deaths (%)** |
| <b>2002</b> | 101     | 12 (12)      | 37        | 6 (16)       | 134     | 16 (12)      |
| <b>2003</b> | 107     | 26 (24)      | 45        | 6 (13)       | 150     | 32 (21)      |
| <b>2004</b> | 107     | 27 (25)      | 40        | 4 (10)       | 143     | 30 (21)      |
| <b>2005</b> | 142     | 18 (13)      | 52        | 3 (6)        | 191     | 21 (11)      |

# among cases with treatment outcome reported (hence numbers less than table 1).

\* some cases had site reported as both CNS meningitis and CNS other.

\*\* died before completing TB treatment.

Source: Enhanced Tuberculosis Surveillance as at 14/08/08, Health Protection Agency Centre for Infections.

### ***The HIV epidemic and TB – global perspectives***

The onset of the HIV epidemic has further fuelled the rise in TB incidence as rates of TB mimic those of HIV and HIV is the strongest risk factor known for the reactivation of latent TB disease [9]. Without anti-retroviral therapy immunosuppressed HIV positive patients have a 10% *annual* risk of reactivating latent disease, whereas HIV negative counterparts carry a 10% *lifetime* risk [10]. It is estimated that about 10% of all new TB and 12% of TB deaths are attributable to HIV co-infection. This is quite geographically biased though as in Africa it is over 30% but in most developed nations well below 10% where antiretroviral medication is more readily available. TB treatment success rates are similar between HIV positive and negative patients, but the former are at a

higher risk of reinfection and death. For example in a cohort of South African miners 13.7% of HIV positive patients with active TB died, compared to only 0.5% of those HIV negative patients [11].

### ***TB Pathogenesis***

*Mycobacterium tuberculosis* (*M.tb*) is an aerobic, non-spore forming acid fast bacillus that principally infects humans. Spread from person to person is due to inhalation of airborne particles containing bacilli coughed from the lungs of an infectious person. They are small (1-5  $\mu\text{m}$ ) so they can remain airborne far longer than droplets that spread other respiratory illnesses such as influenza. The initial infection is thus pulmonary, which can present with a productive cough, fever, weight loss and sometimes haemoptysis (“primary progressive TB”). However, initial infection is usually asymptomatic leading to latent infection. It is during this early asymptomatic phase that a transient low level bacteraemia leads to dissemination of bacilli around the body.

Latent infection occurs when the infection is self-contained by complex immune mechanisms. Once alveolar macrophages phagocytose *M.tb* the bacilli can inhibit IFN- $\gamma$  signalling and arrest the formation of phago-lysosomes.

Phagosome acidification is the final step to successful intracellular killing of pathogens. Thus infection can remain dormant for many years, with viable non-replicating bacilli remaining within macrophages. Recent evidence suggests this may be mediated by Rv3671c, a pathogen gene encoding a membrane-associated protein of previously unknown function. *M.tb* mutants with this gene deleted were unable to grow in acidified broth and failed to maintain intrabacterial pH in IFN- $\gamma$  activated macrophages and in mice [12]. In normal

circumstances infected macrophages process *M.tb* peptides and present them on cell membrane expressed class I and II major histocompatibility complex (MHC) molecules. However, it is believed mycobacteria remain in phagosomes and do not gain access to the cytosolic MHC-I presentation pathway. In addition *M.tb* drives apoptosis in adjacent non-infected macrophages, thereby reducing antigen presentation [13]. So how is efficient T-cell cross-priming achieved?

Some authors believe non-infected macrophages and dendritic cells (DC) ingest the apoptotic remnants (vesicles) of dead cells and after endosomal processing presentation of antigen to T-cells occurs [14]. Certainly these apoptotic vesicles are immunogenic and can be used to “vaccinate” mice protecting them against subsequent infection [15]. Additionally this process leads to presentation of mycobacterial lipid to CD1 restricted T-cells via CD1b molecules (expressed by DCs but not macrophages), an important further source of a Th-1-like T-cell response. Others, however, consider that autophagy occurs. This innate immune response to intracellular pathogens is induced *in vivo* by IFN- $\gamma$  and *in vitro* by drugs like rapamycin. It allows the host to overcome mycobacterial phagosome maturation block, leading to cytosolic processing of mycobacterial antigen and successful presentation of antigen on MHC molecules [16].

Either way cytolytic CD8<sup>+</sup> and Th-1 polarised CD4<sup>+</sup> T-cells are stimulated by *M.tb* infected macrophages and DCs. CD8<sup>+</sup> T-cells then lyse and kill some of the infected macrophages by inducing apoptosis and using molecules like granulysin. Both types of T-cell secrete IFN- $\gamma$  in response to engagement of TB antigen/MHC to the appropriate T-cell receptor and are co-stimulated by

macrophage derived IL-12 and -18. As this system does not eliminate all viable bacilli a granuloma forms, with central coalesced monocytes (multinucleate giant cells) surrounded by a mixed T-cell population and a fibrous cap of stromal cells. Macrophages are sustained in the granuloma by a complex cell mediated immune (CMI) response involving secreted chemokines, IL-2, TNF- $\alpha$  and IFN- $\gamma$  [17]. If the centre of the granuloma becomes necrotic then caseation is said to have occurred as the material has a cheese-like appearance. It is this process that drives local tissue destruction.

Reactivation can take place at anytime in a person's life once they are latently infected due to defects developing in the innate and adaptive immune response. About 5-10% of those latently infected will reactivate disease at some time in their life. Well described immune defects include HIV infection, corticosteroid therapy, poorly controlled diabetes mellitus, cancer, renal failure, vitamin deficiencies, anti-TNF- $\alpha$  therapies (increasingly used in the treatment of connective tissue diseases, such as rheumatoid arthritis) and other inheritable abnormalities in both cytokine production and receptor function (e.g. involving IFN- $\gamma$  and IL-12) [18-23]. Geographical genetic dispositions exist too, such as polymorphisms in the zinc transporter solute carrier family 11 member 1 (SLC11A1: formally NRAMP1, natural resistance-associated macrophage protein-1) [24].

### ***Clinical features of active disease***

TB presents in a myriad of ways but the hallmark is one of tissue destruction, often in excess of bacillary load. In about 50% of cases recurrent pulmonary infection occurs, often with destructive cavitary lesions in the lungs where

*M.tb* can freely replicate leading to cough and haemoptysis. Cervical lymphadenopathy can breakdown leading to scrofula, more common in children and young women. Pleural disease presents as breathlessness caused by an effusion. Fever of unknown origin associated with disseminated infection, or “miliary TB”, is so named because the pulmonary infiltrates look like millet seed [25]. Disease of the central nervous system (CNS) is considered below and is more common in children and HIV patients. TB of the spine (Pott’s disease), which can lead to paraplegia, is the commonest form of bony involvement. However, TB appears to be able to infect almost any part of the body as the vast back catalogue of case reports atone to [26-33].

Diagnosis is often based upon clinical suspicion as culture (where available) takes several weeks and smear is insensitive. The tuberculin skin test (injection of a bleb of purified protein derivative intradermally) can be helpful if strongly positive but can be negative in active disease and is confounded by previous BCG vaccination or exposure to environmental mycobacteria. Interferon- $\gamma$  release assays (IGRA) are based on stimulating whole blood with *M.tb* specific peptides (early secretory antigenic target, ESAT-6 and culture filtrate protein-10, CFP-10) not found in *M. bovis* BCG due to deletion of region of difference 1 (RD-1). They have proved more useful in diagnosing latent than active disease and are not confounded by BCG vaccination [34].

## ***Treatment***

Latent disease can be treated with one or two drugs for 3-6 months. Treatment of symptomatic disease is more complicated with anti-mycobacterial antibiotics needed for at least six months. Combinations of three or four drugs, such as

isoniazid, rifampicin, pyrazinamide, streptomycin and ethambutol are used for two months with a further four months of isoniazid and rifampicin. Extensive tissue destruction (such as pulmonary cavitation or cranial nerve palsies) develop over the long periods of time that disease is present before treatment is commenced. Furthermore exposure of antigen to the immune system at the onset of treatment can lead to worsening of symptoms before cure. This situation is complicated by drug-drug interactions, poor absorption and resistance of *M.tb* isolates to these drugs. Resistance to isoniazid and rifampicin is defined as multidrug resistant TB (MDR-TB). If isolates are resistant to all first line drugs, fluoroquinolones and second line injectables (e.g. kanamycin) it is defined as extensively drug resistant TB (XDR-TB). MDR-TB and XDR-TB carry a poorer chance of cure but individualised treatment can be efficacious [35]. However, with HIV co-infection and no anti-retroviral therapy XDR-TB is almost universally fatal [36].

Resistance can be present in the infecting organism or develop during treatment due to poor compliance. Drug resistance is currently only a consequence of TB resurgence and poor treatment, not yet a cause of rising rates. In today's global world, like in many areas of microbiology, the rise of organisms resistant to drugs considered the backbone of TB treatment since the late 1940's threatens to return us to the pre-antibiotic era [37]. Although new drug classes are being developed and older drugs, such as the fluoroquinolones, being tested in TB novel approaches to treatment are urgently needed. It is in this context that the study of TB immunology and in particular the host response to infection by *M.tb* has an important role to play.

## ***CNS TB – specific aspects***

### **Epidemiology**

Accurate estimates of the burden of tuberculous disease due to involvement of the CNS are difficult to obtain because the greatest number of cases of TB are in the developing world where access to diagnostic tools and central record keeping are limited. Therefore the best estimates are from the USA where 1.2% of all TB reported to the CDC involved the meninges, making up 5.9% of extra-pulmonary TB [38]. Worldwide, using this rate to extrapolate, this equates to approximately 20,000 cases a year. However, this is likely to be an underestimate as the US has a lower burden of HIV positive TB cases, where CNS TB is more likely to occur. Thus the UK data presented above broadly reflects US figures. Other risks reported for CNS TB include excess ethanol use, immunosuppression and carcinomas.

### **Pathogenesis**

TB affects the central nervous system as meningitis (TBM), CNS tuberculomas and rarely as a brain abscess. Classic experiments, using intra-carotid injection of bacilli in guinea pigs and rabbits by Rich and McCordock in 1933 demonstrated that the local rupture of a granuloma into the subarachnoid space was the most likely explanation for spread of disease into the CNS, rather than blood-borne bacilli crossing the blood brain barrier (BBB) directly [39].

Nevertheless *M.tb* bacilli are able to penetrate and cross brain endothelial cells *in vitro*, but these endothelial models do not have complete BBB structure [40].

Rich and McCordock confirmed their animal model work with an extensive review of TBM patients' post-mortems, showing that in almost all cases they

could identify a specific point where a granuloma was juxtaposed to subarachnoid tissue [39].

Once *M.tb* has reached the CNS leucocytosis of the CSF, a prominent feature of TBM, occurs. This is mediated by pro-inflammatory cytokines, chemokines and matrix metalloproteinase (MMP) release [41-43]. TNF- $\alpha$  plays a central role in BBB breakdown and immunopathology in TBM. Rabbits inoculated intracisternally with a virulent strain of *M. bovis* had increased survival rates when thalidomide, a drug with anti-TNF- $\alpha$  secretion activity, was co-administered with anti-tuberculosis drugs [44, 45]. In the same rabbit model, using an *M. bovis* BCG strain overexpressing murine TNF-  $\alpha$ , higher CSF concentrations of TNF- $\alpha$  were correlated with higher CSF white cell counts and increased tissue destruction [46]. However, modulation of TNF- $\alpha$  in CNS TB patients has proved disappointing [47, 48].

CSF IL-6 concentrations were independently associated with severe TBM in a study of patients with TBM from Vietnam but there is no published mechanism to explain this effect [49]. In the same study HIV positive patients with lower CSF IFN- $\gamma$  concentrations had poorer clinical outcomes, with IFN- $\gamma$  levels mirroring lower peripheral and CSF CD4 counts [49]. Subsequently CD3+CD4+ lymphocytes and monocytes predominate in HIV negative TBM but CSF neutrophils are an early feature of TBM, suggesting these cells may play an important role in the pathogenesis of TBM [50, 51]. Although treatment is effective in many patients with CNS TB it is clear that inflammation takes much longer to abate. Many months after treatment has commenced patients still

have evidence of BBB dysfunction and elevated CSF concentrations of many inflammatory molecules [50, 52].

Toll like receptors (TLRs), pattern recognition molecules used by the innate immune system, are known to sense mycobacteria. TLR2, 4 and 9 have all been implicated in the immune response to TB [53-56]. TLR2 genotype 597CC was associated with susceptibility to TBM, especially when patients had miliary disease, suggesting it controls the pathogen's ability to disseminate [57]. Patients with TLR2 597TC allele were more likely to be infected with *M.tb* of East Asian/Beijing genotype, with the Euro-American lineage of *M.tb* appearing to cause pulmonary rather than meningeal disease. Host and pathogen genotypes may depend upon the geographical regions where they co-evolved as often these associations are not repeated in other parts of the world [58].

Not all CNS tissue destruction is host mediated though. *M.tb* strain background affects phenotype. East Asian/Beijing genotype was associated with shorter duration of illness and fewer CSF leucocytes [59]. Different strains of *M.tb* caused varying amounts of CNS damage in the rabbit TBM model, in part mediated by pathogen polyketide synthase-derived phenolic glycolipid (PGL) [60]. The W-Beijing family is said to be the only group of *M.tb* strains able to synthesise PGL, perhaps explaining the more aggressive clinical phenotype seen with this strain [61-63]. Silencing the *pks1-15* gene, required for the synthesis of PGL, in H37Rv *M.tb* does not give a pathogen with the same phenotype as that originally described for the HN878 isolate [62]. Perhaps this is because pathogen strain may also affect host type I interferon induction and

have negative effects on pro-inflammatory signalling pathways via production of negative regulators of cytokine pathways such as suppressors of cytokine signalling (SOCS) [64]. What becomes clear from these data is that pathogen stimulated, host driven tissue destruction is central to many of the clinical problems observed in CNS TB.

### **Clinical features**

TBM presents as the classic triad of fever, neck stiffness and headache in up to 90% of patients unless aged over 60 years [65-67]. Additional factors that aid a diagnosis of TBM over the main differential of bacterial meningitis (BM), especially where prior antibiotics have been administered, are being younger and HIV positive. Duration of illness is usually greater than 10 days in TBM. In contrast it is usually only 2-3 days in BM [68, 69]. Cranial nerve palsies are statistically more common in TBM (~25% patients) but occur in BM frequently too. HIV infection does not appear to affect clinical presentation, although co-existent extra-pulmonary TB disease may be clinically apparent [66, 70, 71].

### **Diagnosis**

CSF opening pressure is raised during lumbar puncture (LP) and total white cell count is usually less than 900 cells/ $\mu$ L. It can be over 10,000 cells/ $\mu$ L in BM, which is usually accompanied by a systemic leucocytosis unlike TBM. Although neutrophils predominate early in both diseases, it is rare for an LP to be done at this time in TBM so a mononuclear cell infiltrate is usually apparent. When clinicians are uncertain of the diagnosis a repeat lumbar puncture 48 hours after the first LP is usually unchanged in TBM where anti-tuberculosis treatment has not been administered. In BM this second LP would usually demonstrate a fall

in opening pressure, CSF white cell count, lactate and protein but a rise in the CSF: blood glucose ratio and the proportion of CSF lymphocytes [65].

TST and IGRA appear to be less useful in the diagnosis of TBM than latent TB, in keeping with findings for active pulmonary disease [49, 72, 73]. It is known that malnutrition (leading to micronutrient deficiencies) and HIV affect the TST, both risk factors for TBM [74-76]. Rates of TST positivity in TBM vary between 20-50% in most series, despite high levels of BCG vaccination [77, 78]. CSF has been used with the IGRA kits to diagnose TBM [79]. Sensitivity in this small series was 84% and specificity 92% but culture positivity was only 16%, meaning a large number of diagnoses were based on clinical features. However, it does suggest that the CNS TB immune response is tissue specific, analogous to findings in pulmonary TB where peripheral blood, but not pulmonary, responses to mycobacterial antigens are often negative [80, 81] .

CSF smear, performed on fresh CSF, stained for acid fast bacilli by the Ziehl-Neelsen method is the initial diagnostic test performed in most laboratories. Centrifuging more than 6 mls of CSF for 15 minutes at 3000g, then viewing areas of high cellularity for at least 30 minutes yielded a smear sensitivity of 58% in a study of over 100 patients, confirming the published evidence from 50 years previously [68, 82]. Even so this is not always possible where only a small volume of CSF has been taken from the patient, e.g. in cases of suspected paediatric TBM. Smear alone will also give no information about *M.tb* drug susceptibility.

The gold standard of TBM diagnosis is therefore culture of *M.tb* from the CSF, which is very dependent upon the laboratory's experience of CNS TB cases. Therefore published rates of sensitivity vary between 20-90% [82, 83]. CSF is more likely to be smear and culture positive in HIV positive patients and when CSF neutrophils predominate [51, 66, 70]. *M.tb* has a doubling time of about one day and so grows in 28-42 days on standard solid media, like Löwenstein-Jensen; too slowly to influence the early management of patients with suspected TBM. Using liquid culture systems reduces time to positive culture, has an increased sensitivity and can be used to perform direct sensitivity testing by introducing anti-tuberculosis drugs into culture medium. However, these automated systems are prohibitively expensive for most laboratories in low income settings. In addition the increased sensitivity is at the cost of increased contamination from bacterial overgrowth and cross-contamination from other positive specimens being handled in the laboratory concurrently. More recently microscopic observation drug sensitivity assay (MODS), known to work well with respiratory specimens from suspected TB patients, has been validated in TBM [84-89]. MODS is a liquid culture system, developed with low income settings in mind, that utilises the characteristic beaded appearance of *M.tb* observed down a light microscope to enhance the speed of diagnosis. Anti-tuberculosis drugs are included in Middlebrook liquid culture medium in some culture wells allowing direct sensitivities to be read by observing a lack of growth. MODS out-performed standard liquid culture giving a positive result in a median of only 6 days compared to 16 days using the best liquid system [84]. The main drawback is that it is heavily reliant upon personnel as plates need

viewing by a trained technician every few days, but in low income settings this is currently more economical than computer assisted systems.

PCR techniques are no more sensitive than a well performed CSF smear but require addition expertise, finance and time. Laboratory contamination has been a particular problem using such a highly sensitive system too. The role of PCR appears to be in confirming a diagnosis of TBM and suggesting possible drug resistance as specificity and positive predictive values are high [90]. Many other laboratory tests for TBM have been assessed: detection of anti-mycobacterial specific antibodies, secreted/released mycobacterial antigen, tuberculostearic acid and detection of lymphocyte inflammatory molecules (e.g. adenosine deaminase) with variable success dependent upon whose hands the test is in [91]. Unlike the MODS technique test success appears to be difficult to transfer between laboratories and may be also vary dependent upon the population being tested.

Both computerised tomography (CT) and magnetic resonance imaging (MRI) are useful tests in the diagnosis of TBM. In unselected patients with CNS symptoms of meningitis it is not possible to diagnose TBM confidently on neuroimaging alone as the differential diagnosis is wide, including non-infectious causes such as sarcoidosis, vasculitis and tumours. Where clinical suspicion and other tests are suggestive a combination of basal enhancement, hydrocephalus and evidence of infarction appears to be the best indicator of TBM in children and adults [92-96]. In older adults and HIV positive patients

imaging is less good, perhaps due to a poorer inflammatory response in the subarachnoid space [97, 98].

The diagnosis of tuberculomas or TB brain abscesses is more difficult as clinical features usually include evidence of a space occupying lesion and so LP is rarely undertaken. CNS imaging series confirm that any number of lesions may be apparent. Only 30% have solitary lesions and lesions can be of any size [99-101]. Confusing the diagnosis with other infectious causes of multiple CNS mass lesions such as cysticercosis, a common cause of epilepsy in South Americans and Indians, is well described [102]. TB can still be a surprising finding for surgeons biopsying a mass lesion thought to be a tumour [103]. Histopathology of excised tissue reveals typical granuloma formation and acid fast bacilli in some cases [104]. Concurrent TB discovered elsewhere occurs in about 25% cases of CNS TB so is worth investigating for.

### **Prognosis and outcome**

Absolute numbers of cases with CNS involvement are much lower than other forms of TB. Nevertheless these manifestations account for a large percentage of the attributable morbidity and mortality of TB disease with overall global mortality from CNS TB estimated at 60-70% although rates in the UK presented above are lower [66, 69, 105]. In a paper reporting on 292 post-mortems performed in patients dying from TB performed between 1972 and 1976 in India TBM was the main cause of death in both children (70% deaths) and adults (55%). Isolated TBM was more common in adults than children (50% versus 22% respectively). TBM affected all age groups equally, unlike miliary disease which was almost twice as common in the under 15 year old group (39.5%

versus 23.9% respectively). BCG vaccination appeared to protect children from TBM as is generally thought to be the case [106]. Like the UK data above tuberculomas seem to have a more benign course in this series as only 3% of autopsied patients had them as the main finding. Although this paper gives no indication to the denominator value of how many other deaths due to TB were not autopsied during the same period it gives an insight into how over represented CNS TB is in TB mortality figures.

Once diagnosed with TBM or CNS tuberculomas patients have a risk of mortality of 20-40% if HIV negative, rising to 80% if co-infected despite the use of dexamethasone [71, 107]. Neurological disability is seen in up to 50% of all survivors of CNS TB followed-up long-term [108, 109]. This is partly because they have a broad differential diagnosis and treatment is often delayed. Repeatedly clinical stage, extent of coma and focal neurological signs on admission to hospital are predictive of poor outcome from TBM [110-112].

Laboratory parameters can also predict outcome: high initial CSF concentrations of lactate and protein, low numbers of CSF white blood cells (in particular neutrophils) and low CSF glucose levels are associated with poorer outcome [52, 67, 73, 113]. Single drug resistance in *M.tb* isolates does not affect outcome but MDR-TB can [113-115]. There are no data currently on XDR-TB.

## **Treatment**

Therapy, when started, is prolonged and frequently complicated by worsening of symptoms before recovery [83, 95, 116]. This is likely to be due to an

increase in tissue destruction driven by an enhanced inflammatory reaction to revealed mycobacterial antigens [117]. Such a reaction is especially poorly tolerated in the CNS where there is less room for expansion of lesions and development of hydrocephalus may be a pre-morbid event in parts of the world where access to neurosurgery is sparse [118].

As tissue destruction is widely apparent in CNS TB concomitant steroid therapy might be beneficial. Clarification that adjunctive steroid therapy reduces mortality is only available in TBM [119]. In a randomised, placebo controlled trial of 545 patients receiving intravenous dexamethasone at a starting dose of 0.4mg/kg/day receipt of dexamethasone led to a 30% reduction in death rate over placebo but no effect on the numbers of severely disabled patients was observed [107]. Additionally the same authors found less clear benefit of dexamethasone in HIV positive patients. Similar evidence for a beneficial effect of dexamethasone in children has been reported [120, 121]. The mechanism for this effect has not been described despite extensive analysis of CSF inflammatory mediators [50]. There are no randomised, controlled trials of the effect of steroids in treating CNS tuberculomas but anecdotal evidence has persuaded physicians to use them in this setting, supported by the benefits in TBM and the high likelihood that concurrent meningeal disease may be present with tuberculomas. Clearly a trial is long overdue in this setting.

Oral anti-tuberculosis therapy is generally administered for 9-12 months, although there is no evidence for the efficacy of prolonging treatment for more than six months. As in pulmonary TB four drugs, tapered to two after two

months seems to be the consensus view. Usually therefore isoniazid, rifampicin, ethambutol and pyrazinamide are commenced but regimens vary [91]. When injectables are needed streptomycin, isoniazid, rifampicin, fluoroquinolones and second line agents such as capreomycin and aminoglycosides are available. Intrathecal administration of therapy is only possible with good neurosurgical input and seems to be reserved for intractable MDR TBM [122]. All four first line drugs cross an inflamed BBB but rifampicin and ethambutol are less good at crossing the BBB as treatment continues but steroids do not seem to have an influence on CSF penetration of anti-tuberculosis drugs [123-125].

### ***Neuroinflammation***

In order to understand the context of the pathogenesis of CNS TB presented above more fully a more general introduction to neuroinflammation is helpful. Inflammation is a complex set of interactions between soluble molecules and cells arising in all tissues in response to trauma, infection, ischaemia, toxins or autoimmune injury. This process has evolved to lead to elimination of the invading pathogen and to healing [126]. Neuroinflammation is no different in many respects but has certain unique features that set it apart from systemic inflammatory processes.

### **The Blood Brain Barrier**

The BBB is the highly specialised interface between the CNS and the rest of the body, allowing a microenvironment for efficient neural function (Figure 1). It is composed of endothelial cells joined by high resistance tight junctions, astrocyte foot processes interfacing with brain capillaries and pericytes (macrophage lineage cells), which are embedded in the basal lamina [127, 128]. This is a

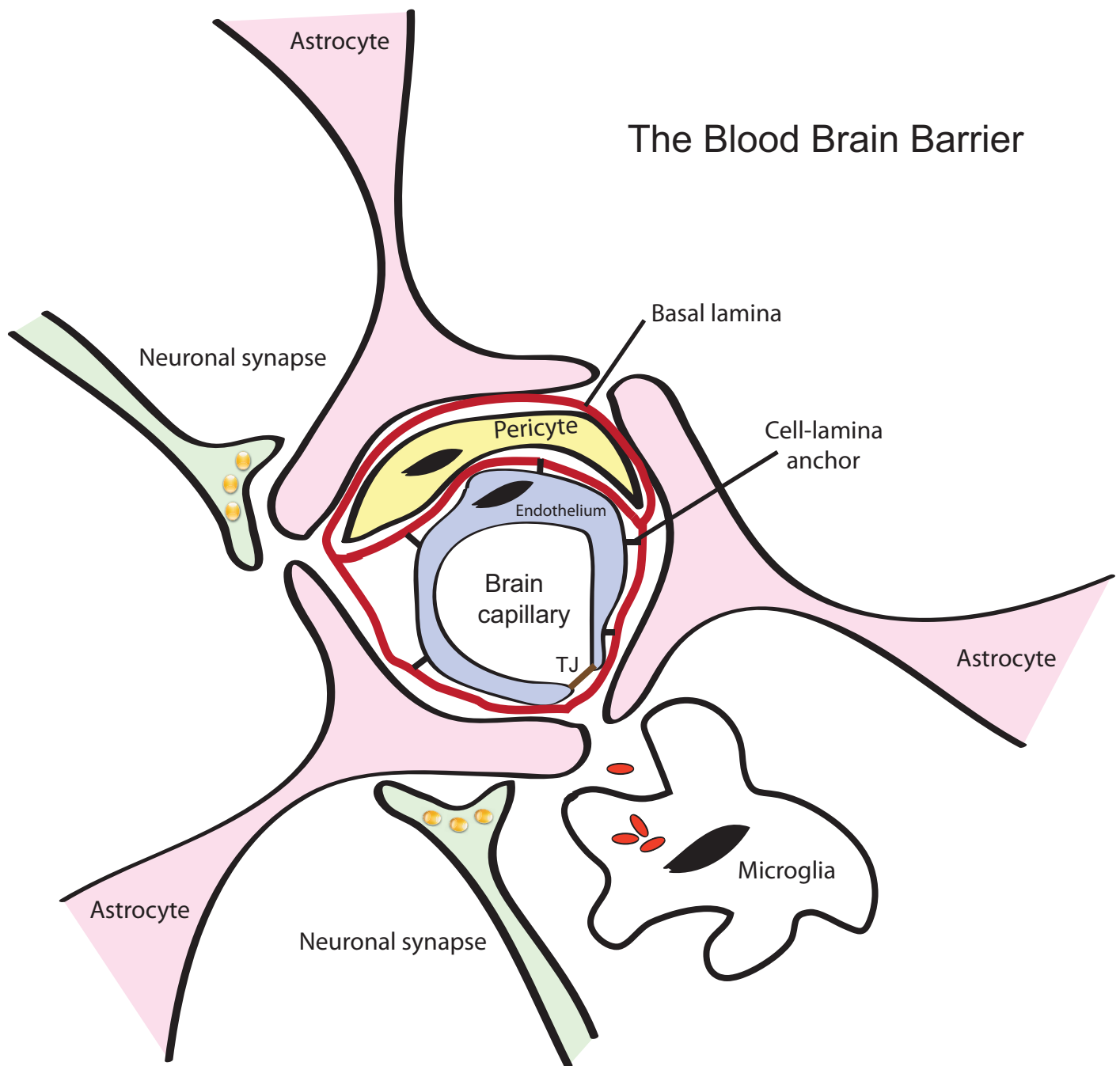


Figure 1. Schematic representation of the blood brain barrier (BBB). Astrocyte foot processes make intimate contact with the basal lamina, which divides them from pericytes and endothelial cells lining brain capillaries. Basal lamina is made up of type IV collagen, laminin, aggrecan and fibronectin. Microglial cells, seen engulfing *Mycobacterium tuberculosis*, do not make up the BBB but are found in adjacent brain parenchyma. Endothelial cells are tightly opposed by tight junctions (occludens, cadherins and claudin molecules link the cell membrane to the intracellular actin cytoskeleton) and cell-lamina interactions with aggrecan.

complex mix of extracellular matrix, such as type IV collagen, laminin, fibronectin and agrin [129]. Tight junctions in endothelial cells are made up of transmembrane claudin and occludin molecules, linking to intracellular zonula occludens (ZO-1 to 3) molecules that in turn bind the actin cytoskeleton. Junctional adhesion molecules (JAM-A to C) help maintain the integrity of the BBB. The tight junction system is so efficient even  $\text{Na}^+$  and  $\text{K}^+$  ions have to cross intracellularly as resistance across the cells is  $>1000 \text{ ohm cm}^2$  (usually 2-20  $\text{ohm cm}^2$ ) [128].

### **“Immune-privilege” in the CNS**

The BBB functions to keep the CNS an “immuno-privileged site” by acting as a physical barrier to infiltrating leucocytes and immune mediators. This is reflected by the lack of resident lymphocytes within the CNS, although immuno-surveillance does occur in the resting state [130]. In addition it serves to emphasise the importance of microglia in protecting the CNS during the initial stages of any inflammatory insult such as infection because they are the only professional immune cells present. Nevertheless a diverse spectrum of organisms can invade the CNS, causing meningitis or encephalitis which typically is accompanied by an intense inflammatory response. This suggests that this “immune-privilege” is only sustainable in the resting state.

### **Leucocyte migration across the BBB**

Leukocyte-endothelial interactions at the BBB are essential for trafficking of leucocytes into the CNS, which occurs by a process of transmigration [43]. Endothelial anchored chemokines initiate leukocyte integrin aggregation and conformational change, leading to leukocyte arrest within the brain capillary

system at inter-endothelial junctions. Leukocytes then extend protrusions through the inter-endothelial junctions, in search of chemokine gradients that drive transmigration. Extravasating cells accumulate in the perivascular space by the basal lamina (sometimes called the glia limitans). It is here that MMPs may be involved as leukocytes traverse the glia limitans into the brain parenchyma. Some *Mmp* null mice have significantly attenuated BBB disruption in response to inflammatory stimuli, also seen with broad spectrum MMP inhibitors [131, 132].

## ***Microglia***

### **Introduction**

Better insight into immune responses in the subarachnoid space and brain parenchyma might help develop more efficacious adjuvant therapeutic approaches to a variety of CNS inflammatory disorders [133]. Relatively little is known about effectors of tissue destruction in CNS disease but innate immune responses are likely to be key [52]. Microglia are the resident macrophages of the CNS, most likely of mesodermal monocyte-lineage in origin and are therefore the principle CNS innate immune cell [134-137]. Although more predominant in white matter, microglia are found throughout the CNS and in some areas are as numerous as neurons [138]. Although far outnumbered by astrocytes and oligodendrocytes they can make up to 17% of CNS cells in the resting situation [138]. The microglial cell has a scavenger role to maintain homeostasis when quiescent, so-called ramified cells, but like all sentinel cells it is pre-stationed to sense inflammation. Microglia are activated by many stimuli, including LPS, cytokines and amyloid-related proteins, to secrete an array of

inflammatory mediators including IL-1 $\beta$ , TNF- $\alpha$ , chemokines, growth factors and MMPs, although probably not interferons [139, 140]. Microglia express a wide range of cell surface molecules including MHC I and II, integrins, complement, cytokine/chemokine, CD68, CD14 and mannose receptors [141]. All nine Toll-like receptors (TLR) are expressed at high levels, whilst astrocytes and oligodendrocytes, the other CNS glial cells, primarily express only TLR2 and TLR3 [142, 143]. In addition microglia can take on the functional characteristics of dendritic cells in order to present antigen to Th1 lymphocytes during inflammation [144].

### **Microglia as effectors of CNS inflammatory disease**

Microglia are implicated in the pathogenesis of a number of non-infectious CNS diseases, the most notable being multiple sclerosis (MS) and Alzheimer's disease. MS is an adaptive immune response driven inflammatory disorder leading to demyelination of neuronal sheaths. However, the damage to neurons is effected by T-lymphocyte activated infiltrating macrophages and microglia engulfing and degrading myelin [145]. In addition these cells secrete neuronal pro-apoptotic mediators and proteases, including MMPs, that not only degrade myelin basic protein but cause BBB breakdown [131]. Alzheimer's disease appears to be more dependent upon a microglial innate immune response because  $\beta$ -amyloid reactive T-cells are scarce in the early stages of disease [133, 146-148].

Thus microglia have the ability to become the effectors of CNS tissue destruction [149]. Breakdown of microglial control mechanisms play a central part in development of disorders such as HIV-associated encephalitis and

Alzheimer's disease [149-151]. There is gathering evidence that regulation of CNS inflammation may have clinically beneficial effects in these diseases and that this may be modulated via disruption of microglial function [152, 153]. The exact mechanisms of neuronal death during CNS inflammation are complex but frequently involve microglia. Many insults such as infection, hypoxia, acidosis and toxic molecule secretion occur simultaneously, derived from different cell types. For example HIV infected microglial/macrophage secreted proMMP-2, activated by neuronal MMP-14, cleaves the astrocyte derived chemokine CXCL12 (SDF-1), which is itself neurotoxic [151]. Additionally microglial secreted molecules such as TNF- $\alpha$  are known to be pro-apoptotic to neuronal cells. Control of inflammatory responses in microglia is known to be via IL-10 and TGF- $\beta$  as well as exogenous neuroregulators such as neurotrophins [139, 154].

Although direct microglial responses to pathogens and inflammatory molecules occur monocyte-microglial networks are likely to be important in CNS TB. Peripheral monocytes traffic into the CNS due to BBB breakdown, following chemokine gradients and secreting a number of pro-inflammatory cytokines that can affect microglia as outlined already. Also once matured into macrophages it is hypothesised that peripheral monocyte-derived macrophages have a more inflammatory phenotype than their CNS counterpart microglia [155]. However, as deciphering the origin of mature macrophages once in the CNS is not possible this remains speculation. In summary microglia have a complex role during CNS inflammatory immune responses acting as effectors, controllers and the controlled.

## Microglia and CNS TB

*M.tb* causes a meningo-encephalitis clinically and histopathologically. 70% of patients who initially present with features of meningitis will have radiological evidence of parenchymal involvement at some stage of their illness [95].

Microglia are infected by *M.tb* more readily than the most abundant glial cells astrocytes. When infected there are also more intracellular bacilli per cell than in astrocytes suggesting an active phagocytic process in microglia is occurring [156]. *In vitro* infection of microglia and astrocytes resulted in the secretion of a wide array of pro-inflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , IL-6, CCL2, CCL5 and CXCL10 in microglia, whereas astrocytes only secreted CXCL10 [156, 157]. TNF- $\alpha$  expression has been shown to be biphasic in microglia suggesting a down-regulatory interaction between host and pathogen, supported by the observation that non-pathogenic mycobacteria did not up-regulated TNF- $\alpha$  secretion at all [158]. Nevertheless the CNS TB granuloma would appear to be little different from those in other organs. Primary porcine microglia, infected with *M. bovis*, rapidly formed multinucleated giant cells in a TNF- $\alpha$ , CD14 and CD18 ( $\beta_2$  integrin) dependent fashion [159]. Consistent with the immune-privilege hypothesis are data that suggest non-opsonised *M.tb* is readily phagocytosed by microglia in a CD14 dependent manner, obviating the need for an active humoral immune response in early CNS TB [160]. Of note dexamethasone has a potent down-regulatory effect on cytokine secretion in *M.tb* infected microglia but not on phagocytic ability [156].

## Animal models of CNS TB

The study of CNS TB has been somewhat hampered by differences between human and murine CNS responses to *M.tb* challenge. In addition it has proven

difficult to establish CNS disease models from latent infection. Mice need to have an intra-cisternal injection of live bacilli in most models. Human microglia do not produce nitric oxide (NO) in response to *M.tb* infection, a distinct difference from mouse models of TBM, which are poor mimics of human disease [161]. Therefore the recent report that *iNOS* null mice infected with *M.tb* developed serious clinical manifestations and granulomatous lesions containing tubercle bacilli throughout the meninges, much more in keeping with human form of the disease than previous mouse models, may allow further in depth studies [162]. Currently therefore the rabbit remains the best model of CNS TB as very little research has been performed in non-human primates despite some evidence that macaques may develop TBM in response to low doses of intra-tracheally administered *M.tb* [163-165].

## ***Matrix metalloproteinases***

### **Introduction**

Events leading to CNS inflammation, from any stimulus, involve multiple steps, cells and mediators. One such important group of mediators, acting as effectors of inflammation, are the proteases. Proteases are classified into four groups by the biochemistry of their active site: aspartic, cysteine, serine and metallo-proteases. Aspartic and cysteine proteases are lysosomal and have an optimal pH of 2-5 and 3-6 respectively. Although Cathepsin S maintains some activity at neutral pH these proteases are most important in extracellular tissue destruction only in highly acidic pericellular compartments. Serine proteases are active at neutral pH and are secreted by inflammatory cells but are rapidly depleted within 24 hours [166]. Only the MMPs are sustainably released and

active at neutral pH. MMPs are a family of 23 zinc dependent, calcium requiring, proteases which degrade extracellular matrix and can cause tissue destruction. MMPs perform multiple roles in development, reproduction and the normal immune response such as facilitating leucocyte recruitment, cytokine and chemokine processing, defensin activation and matrix remodelling [167]. MMP activity is controlled at the transcriptional level and by four tissue inhibitors of metalloproteinase (TIMP-1 to -4), as well as by compartmentalisation and secretion of pro-forms (zymogens). MMPs have already been implicated in the aetiology of a broad range of destructive inflammatory diseases such as rheumatoid arthritis [168], sarcoidosis [169] and atherosclerosis [170] as well as CNS disorders like multiple sclerosis [171] and HIV encephalitis [151]. Generally increased MMP activity is felt to be detrimental in pathological conditions but higher MMP concentrations can inhibit tumour progression and be beneficial for host response to infection [172, 173].

The origin of MMPs lies in the 1962 discovery by Gross and Lapiere of a collagenolytic enzyme responsible for tail involution in tadpoles whilst working on mechanisms controlling metamorphosis in amphibians [174]. Subsequently 22 more MMPs have been described in humans, all involved in the turnover of ECM. Nine are encoded on chromosome 11 at 11q14.3–23.2 suggesting evolution of some MMPs has occurred by gene duplication [175].

## **Structure**

Structure of the MMPs follows a similar pattern and is summarised in figure 2 [167, 176-181]. The pro-domain is ~80 amino acids long and contains the consensus sequence PRCXXPD (with X denoting any amino acid), where the

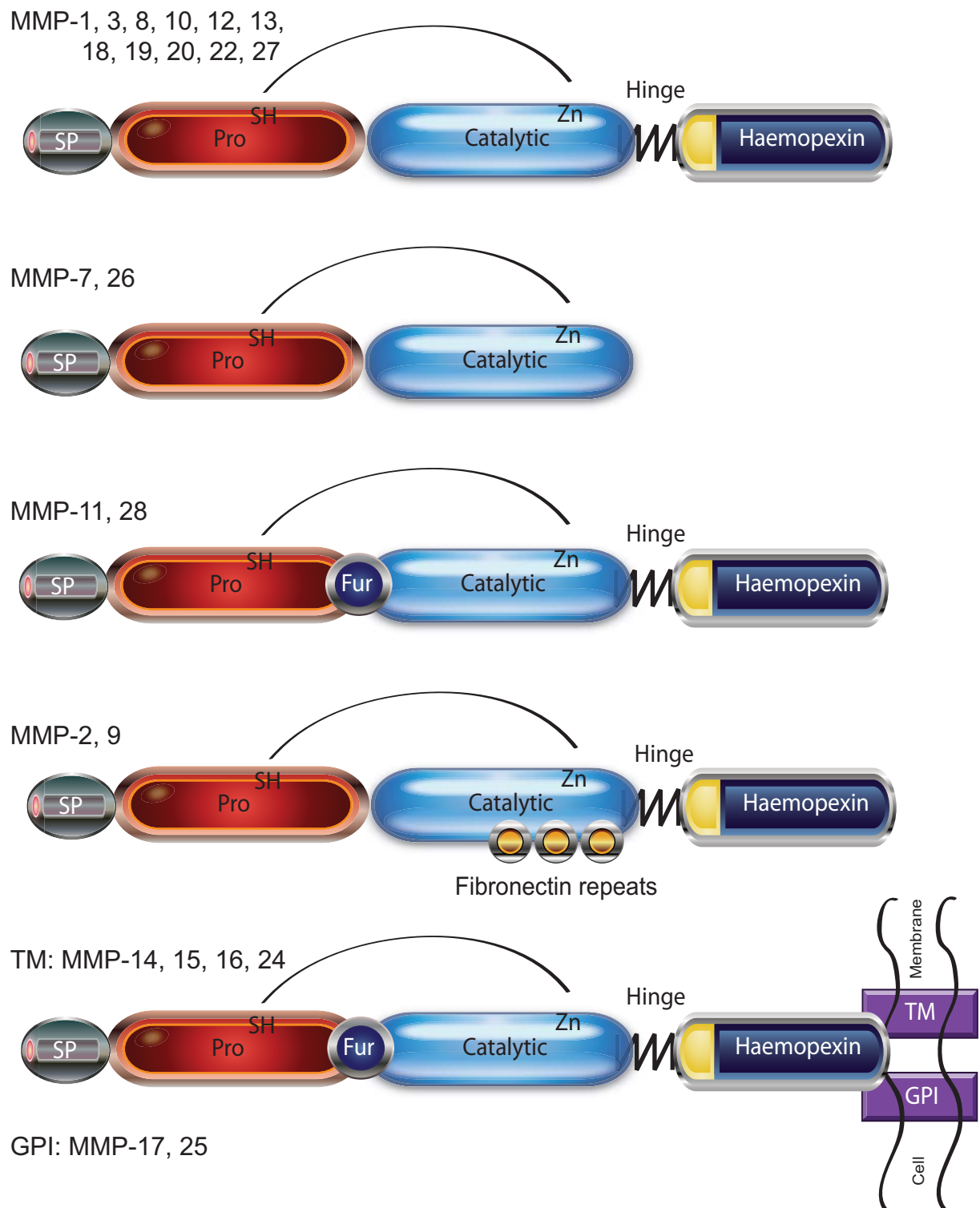


Figure 2: Schematic structure of major MMP groups. MMP groups are shown divided by differences in domain structure, which do not predict substrate affinity (see table 3). Thiol group and zinc ion interaction folds pro-domain into catalytic site: SP, signalling peptide; Pro, prodomain; SH, thiol group; Fur, furin cleavage site; Catalytic, catalytic domain; Zn, zinc 2+ ion; Hinge, hinge region; Haemopexin, haemopexin domain; TM, transmembrane domain; GPI, glycosylphosphatidylinositol phosphate; Fibronectin repeats, fibronectin domains for gelatin binding.

cysteine residue (C) interacts with the zinc ion in the catalytic domain to maintain the zymogen form. The catalytic domain (160-170 residues) contains that zinc ion ( $\text{Zn}^{2+}$ ) in the active site, ligated to three conserved histidine residues in the sequence HEXGHXXGXXHS/T. The glutamic acid residue (E) in this catalytic motif provides the nucleophile that severs peptide bonds by promoting the attack of a water molecule on the carbonyl carbon of the substrate. The structure of the hydrophobic pocket gives substrate specificity. The backbone structures of the MMP catalytic domain are similar to those of the astacin-, adamolysin- and serralsin-family metalloproteinases, as well as certain snake venom MMPs [179]. The adamolysins are so called because they consist of *A* Dysintegrin and *Metalloproteinase* (ADAMs, 13 members) and *A* *Dysintegrin and Metalloproteinase with Thrombospondin repeats* (ADAMTS, 19 members).

MMPs have a proline-rich hinge region, important in activation events. This is up to 75 residues in length and has some homology to type V collagen. All but MMP-7 possess a C-terminal haemopexin-like domain (200 residues) which functions in substrate recognition and delivery to the active site [182]. Terminal cysteine residues are joined in the haemopexin domain leading to a final folded structure resembling four propellers. Signal peptides, about 17-20 amino acids in length, function to allow export from the endoplasmic reticulum. Only MMP-17 does not have a signal peptide.

MMPs can be divided into a number of subgroups dependent upon substrate specificity that is reflected in different additional domains. For example the

gelatinases (MMP-2 and 9) have a gelatin binding domain made of three fibronectin repeats. Other major groups are the interstitial collagenases (MMP-1, 8 and 13), stromelysins (MMP-3, 10 and 11), matrilysins/elastases (MMP-7, 12 and 26) and membrane-type MMPs (MT1-6 MMP). The latter of which posses a furin cleavage site necessary for intracellular activation, needed to deliver active enzyme to the membrane. Interactions with other membrane molecules such as integrins means that secreted MMPs can also be membrane associated thus keeping high concentrations of protease locally. For example MMP-9 binds membrane expressed CD44. CD44 is the principle cell surface receptor for hyaluronate thus juxtaposing substrate and enzyme more efficiently [183]. Not all enzymes are categorisable in this manner, mainly because their *in vivo* substrates are yet to be determined (see table 3 for further details of MMP substrates and groupings).

### **Control of MMP secretion and activity.**

MMPs are potent proteases thus the body needs to retain tight control over them. As already stated control is at four major points: gene regulation, secretion of pro-forms requiring activation, compartmentalisation (where and how MMPs are released e.g. membrane association versus secretion) and physiological inhibitors. Signalling events up-stream of gene expression are important points of control for secretion of MMPs, which if identified might better enable targeted modulation of MMPs in disease. Cytokines, TLRs, mitogen activated protein kinases (MAP kinases) and nuclear transcription factors are all possible targets.

**Table 3. Summary of major MMP groups and published substrates.**

| Name                 | Alternative name     | ECM substrate                                                      | Other substrates                                                                                    | CNS substrate/role                                                                          |
|----------------------|----------------------|--------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|
| <b>Collagenases</b>  |                      |                                                                    |                                                                                                     |                                                                                             |
| <b>MMP-1</b>         | <b>Collagenase-1</b> | Type I, II, III collagen, aggrecan                                 |                                                                                                     | Neuronal apoptosis, brevican, tenascin                                                      |
| <b>MMP-8</b>         | <b>Collagenase-2</b> | Type I, II, III collagen, aggrecan                                 |                                                                                                     | Brevican, tenascin                                                                          |
| <b>MMP-13</b>        | <b>Collagenase-3</b> | Type I, II, III collagen, aggrecan                                 |                                                                                                     | Brevican                                                                                    |
| <b>Gelatinases</b>   |                      |                                                                    |                                                                                                     |                                                                                             |
| <b>MMP-2</b>         | <b>Gelatinase A</b>  | BM, Type IV, V & XI collagen, gelatin, aggrecan, laminin           | CCL-7, CXCL12, $\beta$ -amyloid                                                                     | Neuronal apoptosis, dystroglycan, brevican                                                  |
| <b>MMP-9</b>         | <b>Gelatinase B</b>  | BM, Type IV, V & XI collagen, gelatin, fibronectin, laminin        | Latent TGF- $\beta$ & VEGF, $\alpha$ 1-proteinase inhibitor, CXCL8, CXCL1, 4 & 11, $\beta$ -amyloid | BBB breakdown, zona occludens-1, myelin formation, cerebellar maturation, dystroglycan, NG2 |
| <b>Stromelysins</b>  |                      |                                                                    |                                                                                                     |                                                                                             |
| <b>MMP-3</b>         | <b>Stromelysin-1</b> | Fibronectin, laminin, type IV collagen, aggrecan                   | Latent TGF- $\beta$ , E-cadherin, IL-1 $\beta$ , $\beta$ -amyloid                                   | Neuronal growth & apoptosis, NTs, myelin, occludins, claudins, brevican, tenascin           |
| <b>MMP-10</b>        | <b>Stromelysin-2</b> | Laminin                                                            | ProMMP-1, 7, 8 & 9                                                                                  | Brevican                                                                                    |
| <b>MMP-11</b>        | <b>Stromelysin-3</b> | Type VI collagen, aggrecan, laminin                                | Apoptosis                                                                                           |                                                                                             |
| <b>Matrilysins</b>   |                      |                                                                    |                                                                                                     |                                                                                             |
| <b>MMP-7</b>         | <b>Matrilysin</b>    | Elastin, gelatin, fibronectin, type IV collagen, aggrecan, laminin | E-cadherin, syndecan-1, pro-defensins, FasL (CD95L), latent TNF- $\alpha$ , CXCL1                   | Synaptic plasticity, NTs, brevican, tenascin                                                |
| <b>MMP-26</b>        | <b>Endometase</b>    | Fibrin, fibronectin, vibronectin, gelatin                          | Serpins                                                                                             |                                                                                             |
| <b>Membrane-type</b> |                      |                                                                    |                                                                                                     |                                                                                             |
| <b>MMP-14</b>        | <b>MT1-MMP</b>       | Type I, II, III collagen, laminin, aggrecan                        | ProMMP-2, syndecan-1, SLPI, DR-6, CCL7                                                              | Tenascin                                                                                    |
| <b>MMP-15</b>        | <b>MT2-MMP</b>       | Fibrin, aggrecan, laminin, perlecan                                | ProMMP-2, anti-apoptotic                                                                            | Tenascin                                                                                    |
| <b>MMP-16</b>        | <b>MT3-MMP</b>       | Type III collagen, fibronectin, gelatin, laminin, vibronectin      | ProMMP-2                                                                                            |                                                                                             |
| <b>MMP-24</b>        | <b>MT5-MMP</b>       | Proteoglycans                                                      | ProMMP-2, cadherins                                                                                 | Axon pathfinding, synapse plasticity & remodelling                                          |

... table 3 continues

Table 3 (continued)

| <b>GPI anchored</b> |                        |                                                           |                                                                                                                 |                                        |
|---------------------|------------------------|-----------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|----------------------------------------|
| <b>MMP-17</b>       | <b>MT4-MMP</b>         | Gelatin                                                   | ProMMP-2, ADAMTS4 activation                                                                                    | Expressed in neurons                   |
| <b>MMP-25</b>       | <b>MT6-MMP</b>         | Type-IV collagen, gelatin, fibronectin, fibrin, laminin   | $\alpha$ 1-proteinase inhibitor, ProMMP-2                                                                       |                                        |
| <b>Others</b>       |                        |                                                           |                                                                                                                 |                                        |
| <b>MMP-12</b>       | <b>Metalloelastase</b> | Type V collagen, laminin, elastin, fibronectin            | Latent TNF- $\alpha$ , CXCL11, $\alpha$ 1 proteinase inhibitor, EGFR, OMP A, CXCL1-3, 5, 6 & 8, CCL2, 7, 8 & 13 | Macrophage migration, myelin formation |
| <b>MMP-20</b>       | <b>Enamelysin</b>      | Type V collagen, aggrecan, amelogen, ameloblastin         |                                                                                                                 |                                        |
| <b>MMP-19</b>       | <b>RASI1</b>           | Type IV collagen, aggrecan, laminin, fibronectin, gelatin | T-cell development, IGFBP-3                                                                                     | Expressed in MS lesions, tenascin      |
| <b>MMP-21</b>       |                        | Gelatin                                                   |                                                                                                                 |                                        |
| <b>MMP-28</b>       | <b>Epilysin</b>        | Casein                                                    |                                                                                                                 | Myelin degradation                     |

Notes: MMP-4-6 do not exist. MMP-18 only found in *Xenopus* sp. MMP-22, 23 and 27 no published information. IGFBP-3, Insulin-like growth factor binding protein-3; DR6, Death receptor-6; SLPI, Secretory leucocyte protease inhibitor; EGFR, Epidermal growth factor receptor; OMP A, Outer membrane protein A ( of *E. coli*); NTs, neurotrophins.

### *Cytokines and chemokines*

TNF- $\alpha$  and IL-1 $\beta$  are key pro-inflammatory stimuli leading to MMP secretion.

Most MMPs can be induced by these molecules but the range of MMPs secreted is often cell-type dependent. Local autocrine and paracrine networks involving TNF- $\alpha$  have already been described in *M.tb* induced MMP secretion [184]. Many MMP promoters have IL-1 $\beta$  transcription factor binding sites, such as activator protein-1 (AP-1) [185]. IL-1 $\beta$  and TNF- $\alpha$  synergise with other cytokines such as oncostatin M, a member of the IL-6 superfamily, that signal via Janus kinase (JAK) / signal transducers and activators of transcription (STAT) and the MAP kinase pathways, to up-regulate MMPs [186].

Molecules that principally target TNF- $\alpha$  and IL-1 $\beta$  are already in use in the clinic in chronic inflammatory conditions such as rheumatoid arthritis, psoriasis, Crohn's disease and ankylosing spondylitis. Currently based on monoclonal

antibody technology these drugs have proved extremely efficacious. MMPs are implicated in the pathogenesis of all these diseases and anti-TNF- $\alpha$  is known to reduce MMP secretion in patient samples and *in vitro*, but a causal link is yet to be proven [187, 188]. Anti-TNF- $\alpha$  therapy has been used as an adjuvant for the treatment of TBM but with little success [47, 48, 189]. Indeed one of the adverse events of drugs such as infliximab is TB reactivation. This is more frequently seen than opportunistic infection, demonstrating the importance of this molecule in TB host defence [18]. Whether the disorganised granuloma structure seen in biopsies from patients with anti-TNF- $\alpha$  associated TB is related to that seen in *Mmp9* null mice is not clear [190].

IFN- $\gamma$ , a major activator of macrophages, suppresses MMP secretion in a number of models, opposing TNF- $\alpha$  driven gene expression. This can be via a direct effect on TNF- $\alpha$  driven Ets-1 and NF $\kappa$ B binding to the MMP-3 promoter or via IFN specific JAK/STAT pathways involving the sequestration of the co-activator CBP/p300 by IFN- $\gamma$  induced STAT1 [191]. TGF- $\beta$ , IL-4 and 10 all down regulated MMP secretion in the same way they do for other molecules. TGF- $\beta$  signals via smads, which can sequester the nuclear-located CBP/p300 histone acetyltransferase, leading to less IL-1 $\beta$ -induced MMP-1 transcription [192]. It is also worth remembering that cytokine-MMP interactions can be bi-directional as MMP processing of cytokines affects the inflammatory milieu (described in detail later). In certain circumstances pro-inflammatory signals such as TNF- $\alpha$  can down-regulate MMP secretion acting at the gene expression level, so once again cell and disease specific control exists [193].

IL-17 is an important cytokine in autoimmune disease. IL-17 can drive MMP secretion from a number of different cell types. IL-17 is also known to drive TNF- $\alpha$  and IL-1 $\beta$  from macrophages offering an indirect mechanism of MMP up-regulation [194]. Although levels of IL-17 producing regulatory  $\gamma\delta$  T-cells are higher in TB patients than controls but currently there are published links between IL-17 and MMPs in TB [195].

CCL2 (MCP-1), CCL4 (MIP1 $\beta$ ), CCL5 (RANTES), CXCL8 (IL-8), and CX3CL1 (Fractalkine) can all cause MMP secretion from microglial cells [196]. HIV infected leucocytes require additional CCL2 to up-regulate MMP-2 and -9 secretion in order to cross the BBB, an important step in the development of HIV related CNS dysfunction [197]. Chemokine processing by MMPs is an important feature of many inflammatory processes and is discussed in detail later.

### *TLRs*

TLRs are a group of nine pattern recognition molecules crucial for the subsequent release of many cytokines in response to infectious challenge. The most well-defined is the interaction between TLR4 and lipopolysaccharide (LPS), a key component in the cell wall of gram negative bacteria, leading to MyD88/NF $\kappa$ B dependent TNF- $\alpha$  release. Stimulation of any TLR can lead to MMP secretion. TLR2, 4 and 9 have already been implicated as important in the host response to *M.tb*. Microglia are known to express a wide variety of TLRs, whereas astrocytes and oligodendrocytes express mainly TLR2 and 3, meaning that microglia are better placed to respond in initial innate responses to invading pathogens [142]. In neuroinflammation MMPs can be up-regulated before pro-

inflammatory cytokines such as TNF- $\alpha$  suggesting innate mechanism are functioning early in disease [198].

#### *Growth factors and hormones*

A large number of growth factors stimulate MMP secretion e.g. epidermal growth factor (EGF), nerve growth factor (NGF) and platelet derived growth factor (PDGF). Vascular endothelial growth factor (VEGF) is an important growth factor in stimulating vascular growth and vascular permeability in normal physiology and in the development of tumour blood supply. Mammary tumour secreted VEGF can induce MMP-9 secretion from T-cells and MMPs in turn can affect VEGF function [199]. MMPs have important roles in uterine remodelling during normal menstrual cycling, pregnancy and delivery suggesting oestrogen and progesterone levels affect MMP function.

Cleavage of other cell surface molecules such as peroxisome proliferator-activated receptors (PPAR) and integrins leads to intracellular signalling and can result in MMP secretion. PPAR $\gamma$  agonist (-glitasones) already have a clinical role in the treatment of insulin resistance in diabetes. In a murine model of cerebral ischaemia pioglitazone reduced delayed neuronal damage through inhibition of active MMP-9 secretion [200].

#### *Cell-cell and cell-matrix interactions*

Cell-cell and cell-matrix interactions are mediated by integrins and cadherins. For example fibroblasts grown in a 3-D collagen matrix will often produce a different spectrum of MMPs than those grown on tissue culture plastic as functional interplay exists specifically between matrix collagen and fibroblast

expressed integrins. This interaction is responsible for subsequent MMP-14 secretion as neither 2-D gels nor gelatin (degraded collagen) were able to substitute for intact collagen in this process [201].  $\alpha_3\beta_1$  integrin signalling has been described to affect mRNA stability of MMP transcripts in keratinocytes using actinomycin-D chase experiments so signalling crosstalk mechanisms are important too [202]. Pericytes appear to be important modulators of endothelial and astrocyte MMP secretion in the context of BBB function. Removal of pericytes from astrocyte co-cultures reduced subsequent MMP expression and activity [203].

#### *Direct control of gene expression*

Although there are many stimuli to MMP/TIMP secretion the final common pathway is often via alterations in gene transcription and stability. Gene transcription is modulated by a variety of processes such as MAP kinases, JAK/STATs, transcription factor binding to *cis*-acting elements, histone (de)acetylation, DNA methylation and mRNA stability. A number of these factors are important in the control of MMP/TIMP gene expression, dependent on the cell examined.

The MAP kinase pathway involves a phosphorylation cascade of a series of protein kinases, with signalling leading to 10,000-fold amplifications. Three main pathways exist:

- i. Stress activated protein kinases (SAPK) also known as extracellular signal-regulated MAP kinases (ERK) play a role in cell turnover and phosphorylation of a number of transcription factors and microtubule associated proteins.

- ii. The p38 MAP kinase subfamily regulates cell differentiation, apoptosis and is important in cellular response to inflammation. Many cytokines signal via p38 and this pathway can be activated via TLRs in response to bacterial pathogen stimulation. p38 is also known to affect gene expression kinetics by post transcriptional regulation of mRNA stability. It has four subunits  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ .
- iii. c-Jun N-terminal kinase (JNK) acts ultimately via the phosphorylation of the c-Jun subunit of the transcription factor AP-1.

All MAP kinase pathways can ultimately phosphorylate transcription factors increasing their ability to drive gene transcription. p38 has been described as an important point of divergence between MMP and TIMP secretion in tuberculosis. Pulmonary epithelial cells secreted MMP-1 in response to *M.tb* infected monocyte derived cytokines. Blockade of p38 led to abrogation of MMP-1 but an increase in TIMP-1 secretion [204]. Astrocyte MMP-9 secretion has been confirmed in brain biopsies from CNS TB patients and *in vitro* MMP-9 secretion was dependent upon an IL-1 $\beta$ /MAP kinase/AP-1 pathway. p38 MAP kinases also increase the cellular amount of the AP-1 proteins indirectly by increasing transcription from the c-jun and c-fos promoters. p38 inhibitors have reached phase II clinical trials in cardiology so exploring these pathways in TB seems appropriate.

### *Transcription factors*

Specific sequences in gene promoters, *cis*-acting elements, can bind transcription factors leading to up-regulation of RNA polymerase activity. Three distinct structures of the promoter region are found in MMP genes. Most (e.g.

MMP-1, -3, -7, -9, -12, -13, -19 and -26) have a TATA box (-30 bp from the transcriptional start site) and a proximal AP-1 site (-70 bp). Some (e.g. MMP-8, -11 and -21) have a TATA box but no AP-1 site. MMP-2, -14 and -28 lack both, often leading to high levels of constitutive expression, controlled by the ubiquitous Sp family of transcription factors, which bind to a proximal GC box [205]. The TATA box contains a conserved A-T rich sequence (usually TATAAAA) which plays an important role in the transcription of eukaryotic genes by RNA polymerases.

MMP secretion is often dependent upon NFκB and AP-1 but PEA3, Sp-1, β-catenin/Tcf-4 are also important in [206]. NFκB is a transcription factor held in the cytoplasm complexed with its inhibitor IκB. Upon phosphorylation of the IκBα and IκBβ subunits by IκKinase1 (IKK1) and IKK2 the p65 (RelA) subunit of NFκB is released and IκB proteins are ubiquitinated and degraded. p65 dimerises with itself or another NFκB subunit, usually p50, and translocates to the nucleus. Here it binds to the specific up-stream promoter regions of genes increasing mRNA transcription. NFκB has a number of subunits (p50, p65, RelB, cRel and p52) and it is the exact make-up of these subunits at the promoter that differentially affect gene transcription. p65 is known to be up-regulated in microglia infected with *M.tb* and many MMP genes have NFκB consensus sites in their promoter, although there is little literature on *M.tb*/NFκB/MMP pathways [156]. Transcription factors rarely act in isolation, for example NFκB and Sp-1 are both required for efficient MMP-9 transcription and AP-1 and PEA3 are commonly required for other MMP gene transcription [207].

### *MMP promoter polymorphisms and TB*

Single nucleotide polymorphisms (SNP) in MMP promoters are responsible for different levels of protein expression. The guanosine insertion at -1607 in the MMP-1 promoter creates an Ets-1 binding site (5'-GGA-3') and leads to increased MMP-1 secretion, which subsequently has been linked to increased risk of a number of cancers [208]. In tuberculosis patients with cavity formation, an increasing trend of the 1G allele frequency was observed but no increased risk of TB disease *per se* over controls was seen [209]. A more recent publication studied patients with endobronchial TB lesions and found that those with the 1G genotype had a 9-fold greater risk of developing subsequent tracheobronchial stenosis [210]. In contrast to the original study monocytes stimulated by IL-1 $\beta$  *ex vivo* in 1G patients demonstrated higher basal and higher up-regulation of MMP-1 secretion than 2G patients. So although risk of TB disease is not affected by MMP-1 promoter polymorphisms disease phenotype is. Data for other MMP promoters in TB are not published.

### *Epigenetic regulation of MMP gene transcription*

Chromatin remodelling by histone acetylation and DNA methylation is an important control point in gene transcription. Deacetylation of histones allows chromatin to coil more tightly leading to a less permissive state for transcription factor access to the basal transcriptional polymerase machinery and thereby effects reductions in gene transcription. Histone modifications occur mainly via histone H3 or 4 acetylation, methylation, or phosphorylation. Chromatin remodelling can be investigated using inhibitors of histone deacetylases (HDAC) such as trichostatin and sodium butyrate. MMP-1 and 13 expression can be blocked in this way but MMP-10 expression is promoted. The final

common pathway for the action of the PPAR agonists mentioned above is felt to be at this point, involving an interaction between HDACs and AP-1 binding to the SW1/SNF chromatin remodelling complex. Increased promoter MMP-3 and 9 methylation caused suppression of secretion in lymphoma and colonic carcinoma cells so the generalisability of the findings is unclear since many MMP promoters contain few CpG islands available for methylation [206].

#### *Post-translational modification of MMP mRNA*

There is evolving evidence for post-translational modification of MMP mRNA as an important mechanism of control. Sequence specific elements at the AU-rich (ARE) 3' or 5'-untranslated regions (UTR) of numerous genes control the decay of their mRNAs by deadenylation. This is strongly attenuated by activating the p38 MAP kinase pathway, which allows Hu family proteins to bind the ARE. HuR 3'-UTR binding is responsible for increased stability of MMP-9 transcript in response to ATP/IL-1 $\beta$  in renal mesangial cells [211]. The 3'-UTR is also responsible for ribosomal engagement and subsequent efficiency of protein synthesis.

#### *MicroRNAs*

Micro-RNAs (miRNAs) are short (21-23 bp) RNA molecules that are involved with control of mRNA translation and degradation [212]. Incomplete base pair hybridisation between miRNAs and mRNA 3'-UTR leads to an as yet unknown mechanism of translational arrest. Complete sequence homology and binding at the 3'-UTR is a characteristic of small interfering RNAs (siRNAs) that lead to degradation of message. MicroRNA-21 (MiR-21) is elevated in glioblastoma cells, known to use MMPs to expand. Silencing of MiR-21 down-regulates

TIMP-3 expression, thereby affecting net-proteolytic ability of the tumour [213].

No data exist for host cells, miRNA and *M.tb*.

### *Dexamethasone*

Corticosteroids are known to affect MMP gene transcription directly, both via nuclear localisation and binding to glucocorticoid response elements, as well as by direct inhibition of NFκB DNA binding, called transrepression.

Dexamethasone also increases the expression of MAPK phosphatase-1, an enzyme that opposes TNF-α induced p38 and JNK MAP kinase activity, without affecting nuclear levels of NFκB [214, 215].

### *MMP activation*

MMPs are usually secreted as pro-forms or zymogens, which is appropriate for potent proteases. They can be activated in a number of ways. Intracellular activation by furin pro-protein convertases allows delivery of fully active membrane bound MMPs (MT-MMPs) to the cell surface [216]. MMP-2/-14/TIMP-2 interact in a mechanism involving TIMP-2/MMP-14 delivery of pro-MMP-2 to another MMP-14 molecule for pro-domain cleavage. Neutrophils release oxidants that activate MMPs by a non-proteolytic mechanism. Cleavage at residues 35-40 of the pro-domain lend the MMP the ability to cleave itself intra-molecularly via the “cysteine switch” mechanism. Once the Cys-Zn<sup>2+</sup> bond in the active site is disrupted proteolysis can occur. Many MMPs can activate one another, particularly MMP-3 [217]. There are reports that the MMP-3 activated product of MMP-1 is a more potent collagenase than the autolytic process produces [178]. All of these mechanisms and those involving

organomercurials have only been described *in vitro* and await confirmation *in vivo*.

## **TIMPs**

There are four members of the tissue inhibitor of metalloproteinase (TIMP) class of molecule. TIMP-1 to -4 are all between 184 and 194 amino acids long [218]. TIMP-1 can inhibit all MMPs by specific 1:1 stoichiometric inhibition. *TIMP1* null mice have impaired learning and memory, as well as increased resistance to pseudomonal infection. *Timp2* null mice have motor defects. *TIMP3* null mice develop alveolar cell damage and an emphysema-like disease, associated with elevated ADAM17 activity, which leads to increased free TNF- $\alpha$ . *Timp4* null mice have no currently described phenotype suggesting some redundancy in substrate inhibition. Less is known about the role of TIMP-4 although levels are suppressed in some forms of meningitis. Astrocytes and microglia do not express TIMP-4 in culture [219, 220].

Control of TIMP secretion is similar to MMPs although basal secretion is often much higher, presumably acting as a mechanism of mopping up excess protease before it can do damage. TIMP-1 levels were suppressed in *M.tb*-infected monocyte-epithelial cell networks and TIMP-1 secretion did not increase in line with MMP-9 in similar monocyte-astrocyte network experiments [204, 221]. Independent of their inhibition of MMPs TIMPs have mitogenic action and regulate survival and apoptosis [185].

Physiological inhibition of MMPs can be performed by non-specific anti-proteases such as  $\alpha$ 2-macroglobulin, a large molecule consisting of four

identical 180 kDa sub-units. Thrombospondins (TSP1 and 2), secreted by astrocytes, increase the quantity of synapses *in vitro* and *in vivo*. TSP1 and TSP2 bind the fibronectin repeats in the catalytic domains of pro-MMP-2 and -9 and inhibit their activation [222].

### **Substrate specificity of MMPs**

Defining the substrates of a particular MMP to a degree of certainty is difficult therefore data in table 3 is from all sources available in the literature. In the strictest sense only evidence of substrate cleavage *in vivo* and loss/gain of function with transgenic mice is acceptable. *In vitro* MMPs cleave all known components of the extracellular matrix (ECM) at neutral pH (an important factor in collagen breakdown). In these model systems overlapping substrate affinity can occur as a laboratory phenomenon because often only the MMP catalytic site will have been expressed. Specificity in secretion, or compartmentalisation, leads to tissue restricted expression *in vivo* (e.g. MMP-12 is mainly expressed by macrophages). Variable substrate affinity means that *in vivo* substrate availability may dictate MMP substrate choice, but there will be some redundancy in the system.

#### *i) Knockout mice*

To date 14 single and some double MMP murine knock outs have been bred, all surviving to birth and most having mild phenotypes until exposed to an inflammatory challenge [223]. Only *Mmp14* null mice die early (3-12 weeks), due to defects in type I-III collagen degradation. *Mmp2* null mice have subtle defects in bone morphology but more extensive mammary gland defects, mirrored in *Mmp3* null mice. The *Mmp20* null mouse displays multiple dental

defects, including enamel hypoplasia [224]. *Mmp9* null mice have vascular remodelling and growth-endplate defects due to inappropriately high galectin-3 levels, a pro-apoptotic molecule [225]. In various models of CNS inflammation these knockouts show less immunopathology, BBB breakdown and cellular infiltration [41, 131, 226]. However, this is at the cost of impaired response to invading pathogens [172]. In the only published paper concerning *M.tb* and *Mmp* null mice defective granuloma formation with poor macrophage recruitment to the lungs was reported in *Mmp9* null mice [190]. There are MMP homologues in lower order organisms, for example drosophila have only two MMP genes. Like their murine counterparts the knockouts survive to the larval stage, when defects in epithelial cell detachment from ECM become apparent [227].

Although *Timp* null mice have been discussed above one with a CNS phenotype warrants further discussion. *Timp3* null mice have reduced cerebral infarct size compared to wild type controls with TIMP-3 expression up-regulated on apoptotic neurons in wild type controls. As TIMP-3 can inhibit MMP-3 driven FasL cleavage on neurones this offers a putative mechanism for the null phenotype protection [228]. However, the authors did not investigate whether unopposed ADAM17 TNF- $\alpha$  release, also pro-apoptotic to neurons, was also partly responsible for this mechanism.

#### *ii) Human genetic defects in MMP genes*

Defects in human MMP genes have been described leading to rare skeletal diseases. Torg, Winchester and Nodulosis-Arthropathy-Osteolysis syndromes are a heterogeneous group of conditions now felt to be due to defects in the

MMP-2 gene. A homozygous recessive nonsense mutation in the MMP-2 gene has been described in consanguineous families leading to stunting, arthritis and osteolysis [229]. An autosomal dominant missense mutation in the MMP-13 gene is responsible for causing a condition called the Missouri variant of spondyloepimetaphyseal dysplasia (SEMD<sub>MO</sub>) [230]. These patients have defective growth and modelling of vertebrae and long bones caused by defects in type II collagen processing. A phenylalanine substitution for a serine residue in the pro-region of MMP-13 means it is auto-activated and degrades not only itself but full length MMP-13 produced from the normal allele intracellularly. Only inactive enzyme fragments are secreted. A mutation in the intron 6 splice acceptor (AG to TG) of the MMP-20 gene leads to a dental disorder called amelogenesis imperfecta [224]. Patients have thin enamel leading to multiple chipped teeth like the null mice. No defects in host immune response have been described in any of these MMP defective patients but they have not specifically been examined.

### *iii) Chemokine & cytokine processing by MMPs*

Chemokine and cytokine stimulation is known to lead to MMP secretion but MMPs have an important role in processing these molecules to modulate their effects [231]. Epithelial injury promotes MMP-7 synthesis and MMP-7 releases CXCL1 (GRO $\alpha$ ) from syndecan-1 sequestration leading to neutrophil recruitment at the site of injury [232]. MMP-9 activates CXCL8 (IL-8) by amino-terminal processing but inactivates both CXCL1 and 4 (PF4) [233]. MMP-9 cleaved CXCL8 is 10 times more potent a stimulus to neutrophil recruitment than the full length peptide suggesting a positive feedback loop exists as the secretion of both molecules will occur in areas of inflammation. The monocyte

chemoattractant CCL7 (MCP-3) is a substrate of MMP-2 and -14 with the cleaved product acting as a competitive antagonist to the parent molecule [234].

proTNF- $\alpha$  is usually cleaved by ADAM17 to its 17 kDa active form. *In vitro* this can be performed by MMP-1, -2, -3 and -9 but it is questionable whether it happens *in vivo*. However, MMP-7 or -12 release of macrophage bound TNF- $\alpha$  may be an important homeostatic or inflammatory mechanism [235]. *M.tb* and LPS specifically up-regulate macrophage MMP-7 but not ADAM17 secretion *in vitro* and TNF- $\alpha$  autocrine loops are important in monocytic cells for MMP secretion [184, 236].

An increasing area of research is now centred on the role of MMPs in the resolution of inflammation. Those same MMPs that were responsible for tissue destruction during the inflammatory process can also aid in its down-regulation. MMP-9 is a known TGF- $\beta$  activator, which can lead to detrimental effects in oncology [237]. However, this same mechanism probably evolved to enable a negative feedback mechanism whereby TGF- $\beta$  down-regulates MMP-9 secretion [238]. Similarly IL-1 $\beta$  is a potent stimulus of MMP-3 secretion, which in turn activates other IL-1 $\beta$  molecules in a caspase-1 independent manner. However, the biologically active IL-1 $\beta$  is itself degraded by MMP-3 [239]. *Mmp8* null mice are at increased risk of chronic skin inflammation in poorly closing experimental wounds [240]. These mice had reduced neutrophil influx suggesting that MMP-8 mediates early acute inflammation, essential for rapid resolution of inflammation.

*iv) Cell surface molecule cleavage*

During normal wound repair MMP-7 enables leucocyte trafficking between loosened epithelial cells by cleavage of E-cadherin [241]. Cadherins are transmembrane proteins that mediate  $\text{Ca}^{2+}$  dependent cell–cell adhesion. N- and E-cadherin are found concentrated around CNS synapses and function in synapse plasticity. MMP-24 (MT5-MMP) can cleave N-cadherin leading to intracellular signalling thereby affecting synaptic adhesion [242]. MMP-7 cleavage of membrane bound FAS ligand (CD95L) is also an important step in the pro-apoptotic pathway of epithelial cells [243]. Neutrophil recruitment is an important step in pulmonary inflammatory processes. Pulmonary epithelial cell epidermal growth factor receptor (EGFR) cleavage by MMP-12 (macrophage elastase) led to CXCL8 secretion [244].

*v) Role of MMPs in host defence to pathogens*

Innate immune responses to invading pathogens are a key initial defence mechanism. Evidence of a direct anti-microbial effect of MMPs is rare but in a murine model of *E.coli* sepsis and bone marrow transplantation MMP-12 appeared to have an anti-microbial effect [245, 246]. MMP-12 was found to kill *E. coli* by degrading outer membrane protein A, localized on the bacterial surface, mediated by the haemopexin domain. Indirect mechanisms are more common. MMP-7 activates gut epithelial secreted pro $\alpha$ -defensins, important anti-microbial peptides involved in gut protection from enteric bacteria [247]. Minocycline, an MMP inhibitor, impeded African trypanosome invasion of the brain by reducing parasite migration across the BBB [248]. MMP-9, MMP-3, and MMP-7 but not MMP-2 or MMP-12 are needed for murine resistance to *Pseudomonas aeruginosa* corneal infections [249]. Other mechanisms are

unexplained such as *Mmp9* null mice being protected against endotoxic shock [250].

## **MMPs in TB**

TB is an inflammatory disease and a number of MMPs have been described in its pathogenesis, although the majority of evidence surrounds MMP-9.

### *i) Murine studies of TB*

There are relatively few published murine studies of MMPs and TB. Infection of BALB/c or severe combined immunodeficiency (SCID) mice with *M.tb* induced substantial increases in MMP-9 activity in infected tissues. *In vitro* this was mediated via TNF- $\alpha$  and IL-18, but down-regulated by IFN- $\gamma$  [251]. This latter effect has been reported in monocytes, where it is mediated by caspase-8 in a non-apoptotic manner [252]. Dissemination of *M.tb* occurred earlier in C57BL/6 TB resistant mice compared to the TB susceptible CBA/J strain. Nonspecific blocking of MMPs in C57BL/6 mice reduced early haematogenous spread of the bacilli, although this was not MMP-9 dependent. *Mmp9* null mice instead showed poor macrophage recruitment to the granuloma, which had a disorganised appearance [190].

### *ii) MMP-9 & TB*

MMP-9 is quantitatively the most significant MMP released by macrophages and concentrations are increased in lungs and pleural fluid of TB patients [253, 254]. Serum MMP-9 concentrations may be raised and associated with disease severity [255]. *In vitro*, MMP-9 is secreted from monocyte cell lines in response to stimulation by a number of TB components such as lipoarabinomannan (LAM), a process dependent upon the transcription factor AP-1 [253, 256].

MMP-9 activity was also dependent upon mannose receptors but not  $\beta$ -glucan receptors. Multinucleate giant cells in tuberculous lymph-node biopsy samples expressed high MMP-9 levels adjacent to areas of necrosis, confirmed *in vitro* [257]. Regulation of MMP-9 secretion by live *M.tb* in monocytic cells is dependent upon autocrine and paracrine networks involving TNF- $\alpha$  [184]. MMP-9 is secreted from pulmonary epithelial cells in networks involving monocyte derived cytokines synergising with *M.tb* antigen, although in this case not LAM dependent [258]. In a mouse mycobacterial model, cytokine-dependent MMP networking effects also appear key in control of MMP gene expression and secretion [251].

### *iii) MMP-1, -3 and -7*

Investigation of gene expression of all known MMPs, ADAMs and TIMPs using real-time PCR focusing on the role of primary macrophage-derived MMPs showed that MMP-1, -7 and -9 mRNA were up-regulated. There was also limited up-regulation of MMP-3 and -10 mRNA. In the same study *M. bovis* BCG did not up-regulate MMP-1 secretion as potently as *M.tb* but both strains increased MMP-7 secretion. MMP-1 is a potent collagenase, an important structural component of lung tissue. This divergence may explain why *M.tb* causes lung cavitation but other non-tuberculous mycobacteria do not.

In the same study investigation of down-stream signalling pathways showed that only secretion of MMP-1 was prostaglandin dependent. MMP-1 was also secreted by monocytes but at lower concentrations and delayed compared to MMP-9 [259]. In monocytes protein kinase C, protein kinase A and tyrosine kinases controlled secretion. Work with respiratory epithelial cells showed that

MMP-1 up-regulation and TIMP-1 suppression in response to *M.tb* may be a more ubiquitous response. In the lung epithelium, this is driven by a monocyte-dependent network involving IL-1 and TNF- $\alpha$ , key cytokines in the immune response to *M.tb*, as well as GPCR [204].

The granuloma is surrounded by a cap of stromal cells, mainly of fibroblast origin. In response to networks involving monocyte derived oncostatin M and TNF- $\alpha$  MMP-1 and -3 are secreted without a compensatory rise in TIMP-1 [186]. There is very little other data on MMPs in TB, except in the context of CNS TB which is specifically discussed below.

### **MMPs in CNS disease**

MMPs can degrade brain extracellular matrix and components of the BBB basement membrane such as type IV collagen, laminin and fibronectin (Table 3). There are many cellular sources of MMPs in the CNS as all CNS cells have been described to secrete MMPs or TIMPs. MMPs are important in neurogenesis and axonal development as neural stem cells express MMP-2 and all four TIMPs, although the exact mechanism is undefined [185]. *Mmp* null mice do not appear to have prominent neurological phenotypes at birth but the literature is currently slender. As in other areas of embryogenesis MMPs may be more important postnatally, as MMP-9 is implicated in postnatal cerebellar maturation [260]. Axonal elongation and guidance involves MMPs. MMP-3 can be localised to the neural growth cone and inhibition is detrimental to this process. Other factors important in neural development such as neurotrophins (NTs) can induce MMP secretion. Neuronal guidance during embryogenesis is an MMP dependent process. Netrin-1, an axon outgrowth-promoting factor

originally isolated from chick brain, binds deleted in colon cancer (DCC) receptors. MMP shedding of DCC receptors has been proposed as a mechanism of axonal guidance [261].

*i) Microglial MMP secretion*

The MMP milieu of any specific condition and its temporal phase will define which molecules are active at any one time as the product of one set of cells may activate those of another. During neuroinflammation infiltrating leucocytes will be an external source of MMPs but their relative contribution over resident CNS cells is unclear. Activated monocytes and neutrophils tend to produce quantitatively and qualitatively more MMPs than T- or B-cells. In a study of MMP secretion from healthy blood donors leucocytes were stimulated with a variety of inflammatory ligands appropriate for the cell stimulated. *Ex-vivo* MMP-2, -11, -17, -26 and -27 were enriched in B cells, while MMP-15, -16, -24 and -28 were prominent in T-lymphocytes. Monocytes and neutrophils produced a wider array of MMPs: MMP-1, -2, -3, -9, -10, -14, -17, -19 and -25 [262]. In a separate study LPS stimulation of adult human brain microglia showed increased mRNA of MMP-1, -3, -8, -10, and -12, qualitatively similar to other leucocytes (B- and T-lymphocytes, monocytes and neutrophils) tested in the same way, but produced transcript in much greater quantities. In addition MMP-7, -13, -14, and -19 mRNA rose to a lesser extent and MMP-15, -16 and -21 transcript levels fell [263].

*ii) Mechanisms of CNS damage by MMPs*

Association of high MMP expression during neuroinflammation is not evidence of causation. More compelling evidence is that injection of MMP-7, -8 and -9

directly into the brain of laboratory animals led to inflammation and that MMP-7 and -9 can cause myelin breakdown and axonal injury [264, 265]. Myelin basic protein fragments, produced from MMP-3 cleavage, are known to be potent T-cell stimuli [145]. Integrin cleavage can lead to apoptosis in the CNS driven by cells sensing they are no longer attached to basement membrane (“anoikis”) [266]. Inhibitors of MMP-9 reverse the neuronal apoptotic effects of MMP-9 [267]. MMP-1 was toxic to human neurons grown in dissociated cultures and IL-1 $\beta$  could stimulate the secretion of MMP-1 from astrocytes [268]. An indirect interaction involving apoptotic neuronal release of MMP-3, which in turn activates microglia by an unknown mechanism to produce pro-inflammatory cytokines, has also been described [269].

### *iii) Multiple sclerosis*

Stimulated peripheral leucocytes from multiple sclerosis (MS) patients secrete more MMP-2 and -14 compared to controls [262]. MS is the prototypical CNS MMP-mediated disease and MMP inhibition in preliminary safety trials have reported promising results [171, 270]. Treatment of MS patients with the immunomodulatory drug IFN- $\beta$  reduced CSF MMP concentrations, restored BBB integrity and increased peripheral TIMP-1 levels [271, 272]. *In vitro* this is mirrored by reduced MMP-9 secretion from IFN- $\beta$  stimulated leucocytes, although MMP-9 itself can cleave IFN- $\beta$  [273]. CSF MMP-9 concentrations correlate with the frequency of inflammatory MS lesions on brain scans and MMP-9 concentrations are reduced by high dose steroid therapy in relapsing and remitting MS [274]. *Mmp9* null mice have a better outcome from experimental autoimmune encephalitis (EAE), the best animal model for MS [275]. EAE animals also show reduced levels of MMP-15 to -17 but the

significance of this is unclear. MMP-7 has also been described in MS lesions from postmortem studies [276].

*iv) HIV dementia*

HIV dementia was a common condition prior to anti-retroviral therapy. HIV itself can cause encephalitis at all stages of disease, including at seroconversion. Chronic disease is characterised by microglial activation, BBB breakdown and neuronal loss that leads to dementia [42]. The description of MMP-2/MMP-14/CXCL12 (SDF-1) mediated neurotoxicity has already been discussed briefly but the mechanism warrants discussion as neuronal loss is key to deterioration in HIV dementia [151]. The cleaved product of CXCL12 only lacked 4 N-terminal amino acid residues. However, it failed to bind to its cognate receptor (CXCR4) but bound CXCR3 instead. Stimulation of this receptor then led to neuronal apoptosis [277].

*v) Alzheimer's disease*

As has been alluded to already Alzheimer's disease is now felt to be an inflammatory disorder [150]. Evidence in clinical studies suggests protection from onset in aspirin takers is mediated by a reduction in neuroinflammation [152].  $\beta$ -amyloid protein can activate microglia to secrete pro-inflammatory cytokines known to influence MMP secretion from CNS cells. In addition it directly stimulates MMP-3, MMP-12 and MMP-13 secretion from the same cells [148]. However, some MMPs, notably MMP-2, -3 and -9 can also degrade  $\beta$ -amyloid so the exact role of MMPs in this disease is unclear.

*vi) Cerebral ischaemia*

During cerebral ischaemia a number of MMPs are known to increase. Neuronal nitric oxide synthase co-localised and activated MMP-9 that then induced apoptosis of neurons in a murine model of ischaemia [278]. MMP-9 seems to be activated by MMP-3, that is itself neurotoxic in other models [217, 279]. Again the temporal aspect of which MMP is expressed when is important. Treatment with MMP inhibitors 7 days after inducing stroke in mice suppressed neurovascular remodelling, perhaps by effects on VEGF, leading to increased brain injury and impaired recovery [280].

*vii) Meningitis*

In the UK an estimated 10,000 cases of meningitis occur every year. However, most will be of viral aetiology, caused by enteroviruses such as echovirus and Coxsackie viruses [281]. Bacterial meningitis, usually cause by *Neisseria meningitidis* or *Streptococcus pneumoniae* has a much poorer outcome and is accompanied by more severe tissue damage. Fungal meningitis is rare outside of the context of immunosuppression, particularly HIV disease. TBM is discussed below. Steroids are currently recommended as adjuvant therapy for pyogenic meningitis suggesting that interrupting the inflammatory process is beneficial [282, 283].

MMP concentrations are raised in the CSF of meningitis patients. Children with viral disease have high MMP-9 but also TIMP-1 concentrations [284]. High concentrations of MMP-8 and -9 were associated with increased BBB permeability and poorer neurological outcome [285]. In this study CSF white cell counts did not correlate with MMP concentrations and so the authors concluded

that CNS cells were a significant source of MMPs but other studies contradict this result [286]. MMP-8, -9 and -13 were up-regulated in a study of viral and bacterial meningitis in children [287]. There is less evidence in other forms of meningitis but MMP-9 seems implicated in fungal and parasitic (eosinophilic meningitis) disease [219, 288]. One of the problems with the published data is that very few of the 23 MMPs known are measured in these studies.

Animal models of pyogenic meningitis demonstrate that MMPs are important in tissue destruction. Murine models of meningococcal and pneumococcal disease have shown MMPs to be important and inhibition by non-specific mechanisms such as doxycycline (a broad spectrum MMP inhibitor) or steroids was beneficial [289-292]. However, one report of reduced host defence to pneumococci in *Mmp9* null mice urges caution [172]. Fungal meningitis in a rabbit model and eosinophilic meningitis in a mouse both implicate MMP-9 in driving tissue destruction [293, 294].

### **MMPs and leucocyte trafficking into the CNS**

As described above the BBB is a complex mix of cellular and matrix components (Figure 1). Leucocytes traffic into the CNS during periods of neuroinflammation in response to chemokine gradients. The influence of MMPs on these molecules has been discussed, but the cleavage of CXCL8 by MMP-9 into a more potent form will influence neutrophil trafficking more than T-cells. Further examples of chemokine/MMP processing, although not CNS specific, reveal the complexity of these interactions with one set of leucocytes often influencing another. Macrophage derived MMP-12 inactivated CXCL1-3, 5, 6 and 8 and the monocyte chemotactic proteins CCL2, 7, 8, and 13 generating a

host of CCR antagonists. This offers a down-regulatory role for macrophages in inflammation, as *Mmp12* null mice had increased levels of neutrophils and macrophages in lungs [295]. T-cell recruitment to the lung is affected in the same way. CXCL11 (I-TAC) was processed by the PMN-specific MMP-8, macrophage-specific MMP-12 and the more ubiquitously secreted MMP-9 [296].

MMP influence on the BBB via loosening of cell-cell contacts by cadherin cleavage has been discussed but other mechanisms are known. Dystroglycan, a transmembrane receptor that anchors astrocyte endfeet to the basement membrane via laminin binding, is cleaved by MMP-2 and -9. *Mmp2Mmp9* null mice were protected from neuroinflammation in an EAE model [226]. The balance between MMPs and TIMPs has also been shown to be important for BBB breakdown. TNF- $\alpha$  stimulation of porcine choroid plexus epithelial cells led to a dramatic decrease in electrical impedance, mediated by fluxes in MMP-3 and -9. Although TIMP-1 levels were high there was no change in its secretion in response to TNF- $\alpha$  but TIMP-3 levels were suppressed leading to a net increase in MMP activity [297].

The biggest role for MMPs described in the literature regards their degradation of the BBB basal lamina, the specialised basement membrane that acts as both a physical barrier and cellular structural support. It is composed of a number of known MMP substrates, in particular type IV collagen (a known substrate of MMP-2, -3, -7, -9 and -19) [291], fibronectin (MMP-3, -7, -9, -12, -19, -25 and -26) [298], aggrecan (MMP-1, -2, -3, -7, -8, -9, -11, -13, -14, -19, -20, MT1-MMP and MT2-MMP) [299] and laminin (MMP-2, -3, -7, -9, -11, -12, -19 and MT6-

MMP) [222]. The MMP cellular source is likely to depend upon the stage of inflammation but CNS parenchymal cells are known to contribute and their products interact with leucocyte derived molecules [217, 228, 300]. *Mmp3* null mice were protected from the effects of intracerebral LPS by reductions in tight junction protein cleavage, specifically occludin (a 60 kDa membrane protein), claudin-5 and laminin degradation [301]. This was associated with less CNS neutrophil trafficking, perhaps mediated by MMP-3 controlled reduction in MMP-9 activity. MMP-9 is implicated in other studies showing decreased BBB permeability with less occludens-1 cleavage, a murine tight junction protein. However, in this model with *Mmp9* null mice occludin was not affected [131]. Indirect effects of MMPs on the BBB have been demonstrated in MS via IL-1 $\beta$  activation of the hypoxia inducible factor (HIF)-VEGF axis [302]. MMPs can influence this indirectly via VEGF processing [303].

MMP substrates in the brain parenchymal will be universally expressed proteins such as collagens as well CNS specific molecules. Pyramidal neurons, important in the control of motor function, are embedded in perineuronal nets composed of brevican (a member of the lectican family of chondroitin sulphate proteoglycans) and the glycoprotein tenascin-R. *In vitro* brevican is cleaved by MMP-1, -2, -3, -7, -8, -10, and -13 but not MMP-9 [304]. Tenascin is also cleaved by MMP-1, -3, -7, -8, -13, -19, MT1-MMP and MT2-MMP [299].

### **MMPs as effectors of repair in CNS disease**

MMPs have accepted roles in the regenerative processes involved after CNS injury. Most of the published evidence regards MS and spinal cord trauma and many of the mechanisms have already been covered: cell survival can be

dependent upon MMP driven apoptosis (FasL is expressed on CNS cells), MMP driven integrin signalling allows cell trafficking, growth factor release from ECM stimulates recovery and MMP/NTs direct axonal regeneration.

MMPs are known to be involved in myelin formation. Myelin is the insulation laid down around axons by oligodendrocytes that allows saltatory conduction to occur. Oligodendrocytes express MMP-9 as they put out cellular processes during remyelination so they can recontact cells. *Mmp9* and *Mmp12* null mice have poor process formation. MMP-9 clears a proteoglycan, NG2, that acts as a barrier to oligodendrocyte maturation. In addition MMP-9 cleavage of CXCL1 (GRO $\alpha$ ) is a controlling step in this maturation process.

#### *Demyelination in CNS TB*

Although not common white matter changes are described in CNS TB.

Perivascular demyelination is felt to be due to a hypersensitivity reaction to TB antigen, perhaps in the same way reversal reactions occur in leprosy [305]. This “allergic tuberculous encephalopathy” is characterised by cerebral oedema, myelin pallor (because of loss of myelin sheaths) and perivascular macrophage infiltration [306]. The demyelination is proposed to be as a consequence of delayed hypersensitivity and may also manifest clinically as radiculomyelopathy.

The evidence for this was published in 1975 [307]. Guinea pigs were sensitized to tuberculin and then either live or killed sonicated tubercle bacilli, tuberculin, or tuberculin purified protein derivative (PPD) was injected into the central or peripheral nervous system. Local inflammatory reactions were produced and demyelination was consistently observed. As there is similarity in this

neuropathology to leprosy it is of some interest that MMPs are now the focus of research in this disease. It has been reported that myelinated axons, infiltrating macrophages, Schwann cells, endothelial cells, and perineural cells stain positive for MMP-2 and -9 in neuritic leprosy biopsies [308].

### **MMPs as therapeutic targets**

Given the amount of evidence that MMPs are involved in the pathological processes in a number of important diseases it may be surprising that MMP inhibition is not included in treatment regimens for any of them currently [309]. Only one compound is licensed in the US, Periostat for treatment of periodontal disease. A number of non-specific MMP inhibitors reached phase III trials in oncology in the late 1990s. Unfortunately there were a number of important safety issues surrounding unexpected musculoskeletal side effects that led to the early termination of many trials [173]. Based mainly on being hydroxamic acid analogues these peptidomimetics chelate zinc ions in the MMP active site. A compound called BMS-275291 has been tried in early breast cancer, Kaposi's sarcoma (KS), late stage prostate and non-small cell lung cancer all to no avail. Marimastat has been used in gliomas with no observed efficacy too.

It is now thought that being so non-specific other proteases, principally the ADAM and ADAMTS families, were also affected leading to the observed side effects. In addition most patients had late stage cancers that were unresectable, unlike the murine models used to test the drugs where early stage disease was usually studied. Some of the early trials compared MMP inhibition to placebo and so measurement of hard clinical endpoints were difficult because these drugs do not cause shrinkage of cancer, being only designed to prevent

metastasis and local expansion. Subgroup analyses did show promise, especially for early disease. More specific drugs have been developed, such as tanomastat which targets MMP-2 and -9 and Metastat (COL-3) has shown promise in a Phase I trial treating KS.

Outside of oncology there is more optimism. Two non-controlled efficacy finding trials in MS using minocycline have reported promising evidence of reduced relapses [270, 310, 311]. Other CNS conditions such as stroke and trauma where murine studies have shown efficacy with early treatment are good candidates for trials of this nature. What is clear from these trials is that the timing of delivery of inhibition is crucial and can only be decided after a full understanding of pathology. MMPs have both detrimental and beneficial effects dependent upon where they are and at what stage the disease is at. More specific drugs will become available. In addition targeting the stimulus to MMP secretion or MMP substrate may represent a better pharmaceutical option.

### **MMPs and CNS TB**

Much less is known about MMPs in CNS TB. CSF levels of MMP-2 and -9 are raised in TBM with no concomitant rise in TIMP-1 or -2, suggesting that in excess MMPs lead to a matrix-degrading phenotype with breakdown of the BBB [288, 312, 313]. Elevated CSF MMP-9 concentrations were associated with local tissue damage and patient death. Initial high concentrations of MMP-9 fall during TBM treatment, although not to baseline levels months later, suggesting ongoing BBB permeability [52, 312]. This permeability required host-cell actin cytoskeletal rearrangements in an *in vitro* model of TBM and was associated with pathogen as well as host transcriptional changes [40]. Whether *M.tb* is truly

neurotropic is a matter of ongoing research but RFLP analysis of isolates from a small number of CNS TB patients revealed 13 distinct restriction patterns compared to pulmonary patients [314]. The strain differences in the rabbit model of TBM and the Beijing strain association with shorter duration of illness have already been discussed but there are no data to support or refute MMP involvement in this area of the literature.

*M.tb*-infected monocytes drive a network involving IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$  and NF $\kappa$ B resulting in increased astrocyte MMP-9 secretion without altered TIMP-1/2 secretion indicating a role for glial cells in the development of the matrix degrading phenotype present in CNS TB [221, 315]. This is mediated via transcriptional control involving AP-1.

Therefore, in summary, microglia and MMPs are central to the pathogenesis of neuroinflammation. Microglial derived MMPs have been implicated as effectors of this process but there are no publications specifically discussing the role of microglial derived MMPs in CNS TB.

## ***Hypothesis and aims***

This project aimed to investigate the hypothesis that interactions between cells of mononuclear and glial lineage are critical for the up-regulation of MMPs during the development of an inflammatory response to *M.tb* in the CNS.

I hypothesised that microglia might control the CNS immune response to *M.tb* infection via the production of a different spectrum of MMPs compared to monocyte/macrophages and other glial components. In addition, by investigating intracellular signalling mechanisms, targets subsequently amenable to specific manipulation via small molecules might be identified.

The specific aims were to:

1. Develop an *in vitro* model of CNS TB that could be used to investigate monocyte-microglial interactions leading to MMP/TIMP secretion.
2. Investigate signalling pathways for the MMPs identified as key, specifically to look at the MAP kinase and NFκB pathways.
3. Confirm these findings in patients by using immunohistochemical analysis of archived brain biopsies and quantification of CSF MMP/TIMP concentrations.

## Chapter 2 – Materials & Methods

Standard tissue culture chemicals were purchased from Sigma-Aldrich (Poole, UK), tissue culture plastic from TPP (Trasadingen, Switzerland) and tissue culture medium from Invitrogen (Paisley, UK) unless otherwise stated.

### ***M.tb* culture and colony counting**

The virulent *M.tb* strain H37Rv Pasteur and a green fluorescent protein (GFP) expressing variant (a gift of Dr Brian Roberson, Imperial College) were cultured from frozen stocks in Middlebrook 7H9 broth (BD Biosciences, Oxford, UK) with 10% OADC enrichment medium (BD Biosciences), 0.2% glycerol, and 0.02% Tween 80 in a rocking incubator. An optical densitometer (Biowave Cell Density Meter, WPA, Cambridge, UK) was used to assess growth of *M.tb*. *M.tb* was passaged regularly by sonicating to reduce clumping and pipetting 100-400  $\mu\text{L}$  of stock with an optical density (OD) of 0.4 – 0.8 into 2 mls complete 7H9 broth. Stocks of *M.tb* were replaced every two months. Experiments were performed with cultures having an OD of 0.6 sonicated for 30 seconds. This has previously been shown to correspond to  $10^8$  colony forming units/ml (cfu/ml) and the mid log-growth phase. The endotoxin level was  $<0.03$  ng/ml LPS as previously measured by amoebocyte lysate assay (Associates of Cape Cod, East Falmouth, MA, US). Multiplicity of infection (MOI) used in each experiment was checked by performing colony counts: 100 $\mu\text{L}$  of experimental broth was serially diluted eight times in 900 $\mu\text{L}$  7H9 and 10 $\mu\text{L}$  was plated from each dilution, in triplicate, on Middlebrook 7H11 agar (BD Biosciences) containing ADC enrichment medium (BD Biosciences) and amphotericin 2.5  $\mu\text{g/ml}$ . After two and four weeks triplicates of the dilution with individual colonies were counted,

averaged and the appropriate calculation performed to give a final cfu/ml and hence MOI for the experiment.

### ***Monocyte isolation and *M.tb* infection***

Peripheral blood mononuclear cells (PBMCs) were isolated from single healthy blood donor residual buffy coats (UK Blood Transfusion Service) using Ficoll-Paque (GE Healthcare, Little Chalfont, UK) density gradient centrifugation. Cells were diluted 50:50 with room temperature HBSS and layered onto 15mls Ficoll-Paque. After a 30 minute spin at 480g the buffy coat layer was pipetted off and washed with medium a total of 5 times, with a 5 minute 308g spin cycle between washes. Between washes 3 and 4 monocytes were counted after a 5 minute incubation at 37°C using a Neubauer chamber slide. Monocytes adhere and appear granular and larger than non-adherent cells such as lymphocytes. Once resuspended in RPMI 1640 containing 2mM glutamine and 10 µg/ml ampicillin (non-mycobacteriocidal at this concentration) monocytes were plated at a density of 250,000 cells/cm<sup>2</sup>. After one hour of adherence at 37°C and 5% CO<sub>2</sub> cells were washed three times with warmed medium. This protocol has previously been demonstrated to give cell purity >95% by anti-CD14 flow cytometry [204].

Monocytes were infected with *M.tb* or 7H9 medium alone at an MOI of 1. At 24 hours the cell culture medium was collected and filtered via a 0.2-µm pore size sterile filter (Anopore, Whatman, Brentford, UK or Nalgene, Hereford, UK) inserted into a 50 ml centrifuge tube and spun in a sealed bucket benchtop centrifuge for 1 minute at 2000 rpm to remove infectious particles. This conditioned medium from *M.tb* infected monocytes was termed CoMTb and

conditioned *medium* from uninfected, **control** monocytes was termed CoMCont. Several batches of CoMCont and CoMTb were produced. To increase homogeneity between experiments where possible different CoM batches were pooled, aliquoted and stored at -20°C.

### ***Microglial cell culture and M.tb infection***

SV40 large antigen transformed human CHME3 microglial cells (a gift of Professor Marc Tardieu, Paris, France and Dr Nicola Woodroffe, Sheffield Hallam University, UK) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (FCS, Biosera, UK) and 3mM glutamine in a humidified incubator with 5% CO<sub>2</sub> at 37°C [196, 316]. For experiments cells were seeded the day previously at 35-50,000 cells/cm<sup>2</sup>. On the day of experiment, which were performed in macrophage serum free medium (MSFM), cells would be 80-100% confluent (equating to 65-85,000 cells/cm<sup>2</sup>). Cell culture medium was harvested at the specified time points and centrifuged for 5 minutes at 12,000g to remove cellular debris and samples were frozen at -20°C for later analysis. In direct infection experiments, *M.tb* was removed by filtration of all samples through a 0.2-µm pore size Durapore sterile filter (Millipore, Watford, UK) to remove infectious particles but retain MMPs [317]. Preparation of conditioned medium from infected microglia was performed as for CoMTb above, except cells were infected at an MOI of 10 in MSFM. It was termed CoMicTb and control medium CoMicCont.

### ***Primary astrocyte cell culture and M.tb infection***

Pure cultures of primary human foetal astrocytes (a gift of Professor V. Wee Yong, University of Calgary, Canada) were obtained as described previously

and maintained in Minimum Essential Medium (MEM) containing 0.1% dextrose, 1mM sodium pyruvate, 0.1mM non-essential amino acids, 0.2mM glutamine and 10% FCS (Biosera) [318]. Flasks were coated with 10 µg/ml polyornithine to aid adhesion. Cultures had been shown to be >99% pure by our collaborators by immunostaining with antibody to the astrocyte specific cell surface expressed glial fibrillary acidic protein [318]. Preparation of conditioned medium from confluent *M.tb* infected astrocytes, termed CoATb, was as per CoMTb above, except an MOI of 10 and serum free MEM were used. Control medium was termed CoACont.

### ***Preparation of nuclear & cytoplasmic extracts***

Nuclear and cytoplasmic extracts were prepared using a commercial kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions. In brief a confluent 60mm dish of CoM stimulated cells was washed and scraped in 1ml ice-cold phosphate buffered saline with phosphatase inhibitors and centrifuged for 5 minutes at 700 rpm at 4°C. Supernatant was discarded and the pellet was resuspended in 250µl of 1x hypotonic buffer and incubated on ice for 15 minutes. 25µl detergent was added and the mixture vortexed and centrifuged for 30 seconds at 14,000g in a centrifuge cooled to 4°C. The supernatant (cytoplasmic extract) was transferred to a pre-cooled microtube and stored at -80°C. The pellet was resuspended in 50µl complete lysis buffer (10mM DTT, Lysis buffer AM1 and protease inhibitor cocktail) and placed on a rocking platform at 150rpm for 30 minutes incubated on ice. The resultant nuclear extract was obtained by vortexing, centrifuging for 10 minutes at 14,000g and storing the supernatant in the same way.

### ***Bradford total protein concentration assay***

In order to ensure equal total protein loading of samples between wells in subsequent western and ELISA assays of cell lysates a Bradford assay was used to calculate total protein concentration in samples. A standard curve was produced between 0 and 1000 µg/ml using a commercial protein assay standard and 200 µL of a 1:5 dilution of developing reagent (both Bio-Rad, Hemel Hempstead, UK). Unknown samples were assayed in duplicate at dilutions that allowed calculation of their concentration from the linear part of the standard curve. Absorbance was read at 580 nm on a spectrophotometer (Bio-Tek, Winooski, Vt, US).

### ***Zymography***

#### **Introduction**

Zymography is substrate based SDS-PAGE electrophoresis. The zymographic process activates pro-enzyme MMPs by releasing the cysteine switch in a non-proteolytic manner, so that gel impregnated substrate degradation allows the detection of both pro- and active enzymes of each MMP being analyzed [319]. Consequently, zymography provides data on all the potentially active MMP present in the sample analysed. Enzyme activity is seen as white bands on Coomassie blue stained gels [320]. In this case the use of 11-12% polyacrylamide gels allows 50-100 kDa proteins to appear in the middle of the gel after 2-4 hours of running time. The molecular weight of each band will give an indication of which MMP is causing proteolysis in the gel, but the exact identification needs to be confirmed by running a recombinant MMP standard and by a second laboratory method such as western blotting. Gels were

analysed densitometrically by digital image capture (UVP Transilluminator, Cambridge, UK) and by Scion Image Analysis software (NIH Image version 1.61, Bethesda, MD, US).

### **Casein zymography**

5µl of loading buffer (0.25M Tris pH 6.8, 50% glycerol, 5% SDS and bromophenol blue crystals) was mixed with 20µl cell culture medium and loaded into precast 12% casein gels (Invitrogen). Gels were run at 125V for 2.5 hours immersed in zymography running buffer (25mM Tris, 190mM glycine and 0.1% SDS). After one hour in 2.5% Triton-X gels were transferred to collagenase buffer (55mM Tris base, 200mM sodium chloride, 5mM calcium chloride and 0.02% Brij; pH 7.4-7.6) for 40 hours incubation at 37°C. Gels were developed with 0.2% Coomassie blue (Pharmacia Biotech, Sweden) for one hour before destaining in acetic acid/methanol/water (1:3:6). A 5 ng MMP-1 standard (Oncogene, Merck, Germany) was run on each gel to allow for between gel variations after construction of a standard curve between 0 and 12.5 ng (Figure 3A). Although three bands are seen on the zymogram only the top band, which is the full length protein, was used to standardise. The lower two bands are autolytic fragments of MMP-1 retaining caseinolytic activity. The lower level of sensitivity for this assay was 1.56 ng total MMP-1 per well.

### **Gelatin zymography**

5µl of loading buffer was mixed with 20µl cell culture medium and loaded into a 15 well 11% polyacrylamide gel (National Diagnostics, Hull, UK) impregnated with 0.12 mg/ml gelatin. Gelatin gels were cast in the laboratory on the day of use and overlaid with a 4% stacking gel. Gels were run at 180V for 3.5 hours

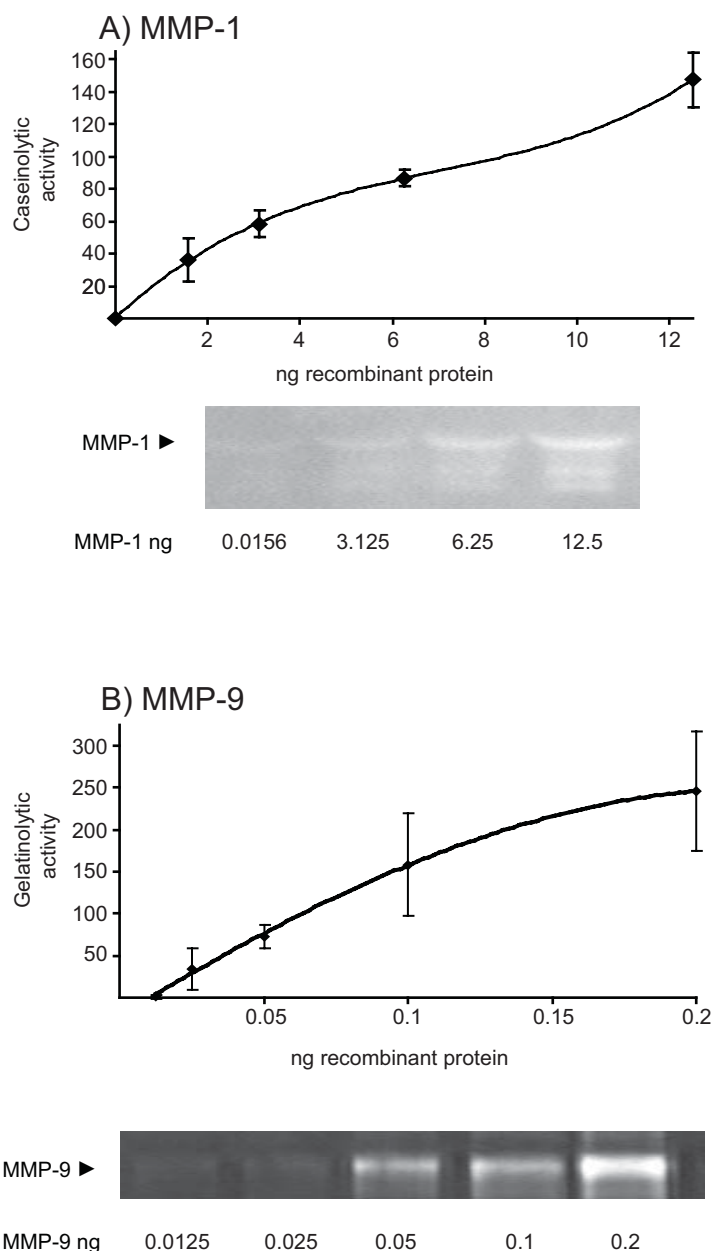


Figure 3. Recombinant human MMP standard curves. A, MMP-1 was serially diluted and analysed by casein zymography. 5ng MMP-1 was used in subsequent experiments. Three bands are seen on the zymogram: the top band is the full length protein and the next two are autolytic fragments retaining catalytic activity. B, MMP-9 was serially diluted and analysed by gelatin zymography. 0.2ng MMP-9 was used in subsequent experiments. Points represent mean values  $\pm$  SD of triplicate samples, representative of at least duplicate experiments performed in triplicate.

immersed in zymography running buffer. After one hour in 2.5% Triton-X gels were transferred to collagenase buffer for 24 hours incubation at 37°C. Gels were developed with 0.2% Coomassie blue with concomitant destaining in acetic acid/methanol/water as previously described. A 0.2ng MMP-9 standard (Oncogene) was run on each gel to allow for gel to gel variability after standard curves were run with 0.0125 to 0.2 ng total recombinant MMP-9 (Figure 3B). The unknown band OD values were then expressed as a percentage of the OD for the standard. The lower level of sensitivity for this assay was 25 pg total MMP-9 per well. Hence weight for weight gelatin zymography is about 60 times more sensitive than casein zymography.

### **Optimisation of collagenase buffer pH**

Biochemical theory suggests MMPs perform better at pH 5.5 yet their optimum working pH for zymography is said to be pH 7.5 [178]. An experiment to optimise the pH of the collagenase buffer used to incubate the gels overnight was performed. Identical casein gels containing two concentrations of recombinant MMP-1 (25 and 2.5 ng) were run as described above. One gel was incubated in collagenase buffer at pH 5.5 and the other at 7.5 (Figure 4). Sample values were normalised to the pH 7.5 25ng MMP-1 OD value. A 93.9% reduction in band intensity was seen with use of pH 5.5 collagenase buffer for the 25ng sample. The 2.5ng sample was below the level of sensitivity of the zymogram at pH 5.5. Therefore pH 7.5 buffer was subsequently used in all experiments.

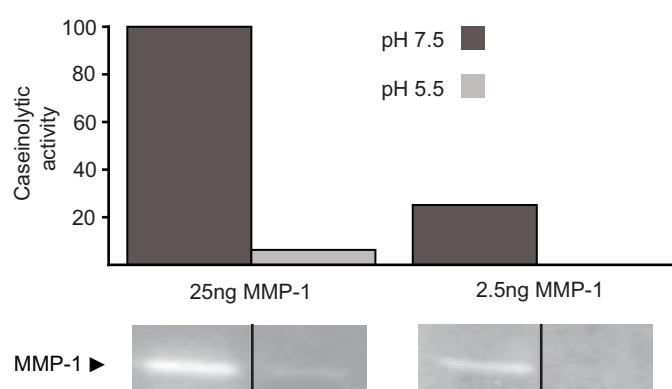


Figure 4. The effect of collagenase buffer pH on MMP-1 zymographic bands. 25 and 2.5 ng of recombinant human MMP-1 was run on two separate casein zymograms. The gels were then incubated for 40 hours in collagenase buffer adjusted to pH 7.5 (dark grey bars) and 5.5 (light grey bars). Collagenase buffer at pH 7.5 was better than 5.5 for supporting enzymatic activity of MMP-1 and was used in subsequent experiments.

## ***Western Blotting***

### ***MMP-1***

Standard western blotting was performed. For MMP-1 40 $\mu$ L of cell culture medium was heat denatured with 1x loading buffer (10% glycerol, 5% 2-ME, 2% SDS, 0.06M Tris pH 6.8 and bromophenol blue) and loaded onto 10% polyacrylamide gels, run at 200V in running buffer (25mM Tris base, 192mM glycine and 0.1% SDS) for 3.5 hours. Gels were electro-transferred to a nitrocellulose membrane (GE Healthcare) and blocked for one hour in 5% milk protein / 0.1% Tween 20. Overnight incubation in 1:1000 sheep anti-MMP-1 primary antibody (The Binding Site, Birmingham, UK) was followed by washing and incubation with 1:2000 donkey anti-sheep peroxidase-conjugate secondary antibody for an hour (The Binding Site). Bands were visualised by chemiluminescence with the ECL plus system (GE Healthcare) according to the manufacturer's instructions.

### ***MMP-3***

MMP-3 western blotting was performed using 1:5000 rabbit anti-MMP-3 primary (Chemicon, Millipore) in 5% bovine serum albumin (BSA) and 1:2000 HRP-linked anti-rabbit secondary (Cell Signalling Technology, Danvers, MA, US) in 5% milk protein.

### ***I $\kappa$ B $\alpha$ and $\beta$ -actin***

For cytoplasmic I $\kappa$ B $\alpha$  Western blots cytoplasmic extracts were prepared and 15 $\mu$ g protein aliquots were processed and probed with 1:1000 rabbit anti-I $\kappa$ B $\alpha$  primary antibody (Chemicon, Millipore, UK) and 1:2000 HRP-linked anti-rabbit

secondary (Cell Signalling Technology). For cytoplasmic  $\beta$ -actin I $\kappa$ B $\alpha$  membranes were striped for 15 minutes with Restore Plus Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL, US), reblocked with 1x TBS, 5% milk protein / 0.1% Tween 20 and probed with 1:1000 mouse anti- $\beta$ -actin primary (Sigma) then 1:5000 anti-mouse secondary (Jackson, West Grove, PA, US).

#### *MAP kinase*

For MAP kinase experiments cells were lysed using 200 $\mu$ l of SDS sample buffer (62.5mM Tris pH 6.8, 2% SDS, 10% glycerol, 50mM DTT and 0.01% bromophenol blue) and immediately frozen at -80°C. 40 $\mu$ l aliquots were processed on western gels as described and probed with 1:1000 rabbit primary antibodies to both phosphorylated and unphosphorylated variants of p38 or ERK MAP kinase. A 1:2000 dilution of anti-rabbit HRP-linked secondary antibody was used (all Cell Signalling Technology).

#### ***Analysis of MMPs by Luminex multianalyte technology***

Casein zymography gives a semi-functional analysis of caseinolytic MMPs, assessing all potentially active enzyme in any one sample. However, total enzyme concentration (pro-, active and degraded) is not assessed by this method. Therefore some samples were analysed for MMP secretion using Fluorokine multianalyte profiling kits (R&D Systems, UK) that is similar in principle to an ELISA but read using flow cytometry laser based technology on a Luminex machine. Luminex relies on the conjugation of fluorescent dye impregnated beads coated with primary antibody to the analyte of interest. As the beads for each analyte have slightly different quantities of two fluorescing red

dyes the Luminex dual laser system detects which bead is passing through the reader using the red 635nm solid state laser and how much phycoerythrin (PE) the bead is coated with (proportional to analyte quantity as secondary antibody is conjugated to it) using the green 532nm Nd-Yag laser.

Standard curve, diluted samples and bead mixtures were loaded onto a permeable membrane bottomed 96-well plate (Millipore) and left for two hours. Excess standard/sample was washed three times with 250  $\mu$ L 1x PBS/1% Tween 20, aspirated through the membranous bottom of the 96-well plate using a vacuum manifold after each wash, thereby retaining the beads/bound analyte but removing excess sample/standard or wash. Biotinylated secondary antibody was added for one hour and washed in the same way. Streptavidin-PE conjugate was then added for 30 minutes, the plate washed and beads finally resuspended in 100  $\mu$ L wash. Sample concentrations were detected using the Luminex platform Bio-Plex 200 system (Bio-Rad) and Bio-Plex manager software (version 5.0) was used to construct standard curves and calculate unknowns. The minimum level of detection for MMP-1 was 10 pg/ml, MMP-2 80 pg/ml and MMP-3 15 pg/ml.

### ***MMP/TIMP & TNF- $\alpha$ ELISAs***

MMP-1, -2, -3, -7, -8, -9, -10, TIMP-1, -2, -4 and TNF- $\alpha$  levels in cell culture medium and CSF were also sometimes measured by standard ELISA technology (R&D Systems, UK) using the manufacturer's instructions to prepare a standard curve. MMP-1, -2, -7 and -10 had primary antibody pre-coated onto the plate but MMP-3, -8, -9, TIMP-1, -2 and -4 were paired antibody kits. The primary antibody concentrate was diluted to 2 $\mu$ g/ml in 1x PBS and 100  $\mu$ L used

to coat the 96 well flat bottomed plate overnight at room temperature. After washing three times in 1x PBS/0.05% Tween 20 the plate was blocked for one hour with 1x PBS/1% BSA and a seven point standard curve produced by serial dilutions. The samples and standards were left for two hours at room temperature, the plate washed and 50ng/ml biotinylated secondary antibody added for two hours. After three further washes 100  $\mu$ L streptavidin-HRP conjugate was added and developed using a stabilised hydrogen peroxide solution (R&D Systems). The reaction was stopped by addition of 50  $\mu$ L 2M sulphuric acid and read at 450nm, using a 540nm reference wavelength to correct for refractive errors. The lower level of sensitivity for the ELISAs were 156 pg/ml for MMP-1 and 7, 78 pg/ml for MMP-2 and 10, and 31 pg/ml for TNF- $\alpha$ , MMP-3, 8, 9, TIMP-1, 2 and 4.

### ***Freeze-thaw cycle stability of MMPs***

As any one experiment may be used for analysis of multiple MMP/TIMPs I was concerned that freeze-thawing samples might affect concentrations of MMPs differentially depending upon analyte and detection methodology. Therefore three samples from CoMTb stimulated microglia were aliquoted and subjected to between 1 and 9 freeze-thaw cycles. Samples were removed from the freezer for two hours then refrozen for 22 hours. Samples were then analysed by casein, gelatin zymography and MMP-3 ELISA. There was no detectable decrease in MMP stability over the 9 cycles (Figure 5). The most any sample was freeze-thawed during the laboratory work towards this thesis was 7 times.

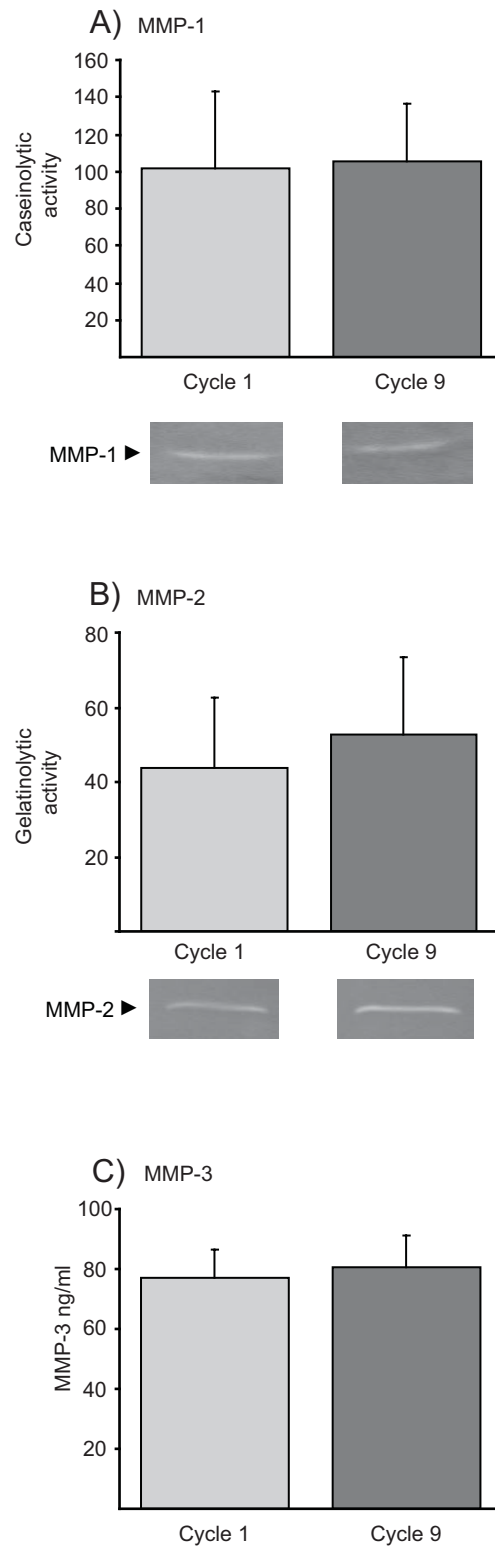


Figure 5. Multiple freeze-thaw cycles do not affect the subsequent activity of secreted MMPs. Nine 80  $\mu$ L aliquots from three separate cell culture supernatants from CoMTb stimulated microglia were left for 2 h at room temperature followed by 22 h freezing up to a total of nine times. Samples were then analysed for MMP activity. A, MMP-1 analysed by casein zymography. B, MMP-2 analysed by gelatin zymography. C, MMP-3 analysed by ELISA. Cycle 1 (light grey bars) and cycle 9 (dark grey bars) were compared by Student's t-test. No sample was used more than 7 times during the thesis.

### ***Size exclusion fractionation of CoMTb***

In order to decipher which pro-inflammatory molecules within CoMTb were responsible for secretion of various MMPs/TIMPs a holistic approach to detection was taken by fractionation of CoMTb using Microcon centrifugal filter units (Amicon, Germany) that specifically allow solutes through a membrane based on molecular weight. 500  $\mu$ L of CoMTb was pipetted into a 10 kDa - Micron YM-10 regenerated cellulose membrane spin column and centrifuged at 14,000g for 20 minutes. The filtered solute (480 $\mu$ L-490 $\mu$ L) contained the <10 kDa fraction and the retentate >10kDa. The columns were then turned upside down and spun at 1,000 rpm for 3 minutes to recover the retentate. Retentate (10-20 $\mu$ L) was reconstituted in RPMI (480 $\mu$ L-490 $\mu$ L) and labelled as the >10kDa fraction. To ensure sterility in cell culture medium both fractions were reconstituted to 500  $\mu$ L using sterile RPMI as used in preparation of CoMTb and sterilised by centrifugation (14,000g, 30 seconds) through a Micron 0.22 $\mu$ m Ultrafree filter column (Amicon).

### ***Modified Kinyoun staining of *M.tb* infected cells***

Initial experiments showed that microglia and especially monocytes adhered better to plastic coated than glass slides. Therefore cells were plated onto 4 well Lab-Tek Permanox chamber slides (Nunc, Roskilde, Denmark) and infected with *M.tb* for two hours as described above. *M.tb* and culture medium were aspirated off and cells were then fixed in 4% paraformaldehyde for 30 minutes and rinsed once in 1x PBS. A modified Kinyoun stain was used to estimate infection rates of microglia and monocytes. Fixed slides were flooded with carbol-fuchsin solution for 3-5 minutes without heating, washed briefly with tap

water, decolourised with 3% acid-alcohol (HCl:C<sub>2</sub>H<sub>5</sub>OH) and counter stained with methylene blue. After air drying the slides were viewed under a 100x oil immersion microscope. Approximately 100 cells were viewed under high power in each of the four different chambers and a mean and standard deviation calculated for the percentage of cells associated with *M.tb*.

### **Confocal microscopy**

Microglial cells and monocytes were plated onto 4 well Lab-Tek Permanox chamber slides (Nunc) and infected with *M.tb* as described above. *M.tb* and culture medium was aspirated off and cells were fixed in 4% paraformaldehyde, rinsed once in 1x PBS and permeabilised for 10 min in 0.2% Triton-X 100 in 1x PBS in the dark to preserve GFP expression. After three washes with 1x PBS cells were incubated for 10 minutes at room temperature in the dark with a TRITC-Phalloidin conjugate (Invitrogen) at 1:500 dilution (final concentration 250 ng/ml) to bind F-actin. After three further washes cells were mounted with 700µl Dakocytomation S3023 mountant (Dako, Glostrup, Denmark), covered with a 22 mm x 50 mm coverslip and left overnight to harden.

Confocal microscopy was performed as previously described, using a Zeiss laser-scanning microscope LSM 510 equipped with a 63x oil immersion objective (Karl Zeiss Inc, Thornwood, NY, US) [321]. Laser excitation at 488 nm and 532 nm was used. Images were collected as horizontal sections taken at 0.1µm intervals through whole cell volumes. Images from representative areas of individual cells (top, middle and bottom) were taken and Y-Z projections of these cell areas were then compiled as composites of the relevant Z-sections using ImageJ software (NIH, Bethesda, MD, US). The composites were

imported into Adobe Photoshop CS3 (Adobe Systems Inc, San Jose, CA, US) and recreated as pseudo colour overlays (phalloidin staining in red and *M.tb*-GFP staining in green).

### ***Gene expression analysis by real time quantitative PCR***

Microglia were lysed with TRI Reagent and total RNA was extracted using a standard chloroform-phenol-isopropanol protocol using phase-lock-gel tubes (Eppendorf, Cambridge, UK) [322]. The RNA was further purified by a DNase step using a commercial kit (Ambio, Austin TX, US). cDNA was prepared from 1µg RNA measured using an Eppendorf Biophotometer, ensuring that the absorbance at 260nm was between 0.1 and 1.0. Reverse transcription of 1µg RNA used a 20 µL reaction volume. Initially 2 µL of random primers (Invitrogen) were annealed to RNA for 10 minutes at 65°C. 200 units of SuperScript III reverse transcriptase (Invitrogen) was added with 1<sup>st</sup> stand buffer, dNTPs, DTT and RNase inhibitor to the annealed RNA/random primers and incubated at 37°C for an hour. After 15 minutes denaturing at 75°C samples were spun and diluted to a final cDNA concentration of 0.5ng/µL for subsequent use.

Quantitative polymerase chain reactions (qPCRs) were done in a 25 µL reaction volume in 96 well plates with a Stratagene Mx3000P machine (Stratagene, La Jolla, US) using 5ng cDNA, Brilliant II qPCR mastermix (Stratagene) and MMP primers and probes as described previously [323]. The cycle threshold ( $C_T$ ) at which amplification entered the exponential phase was determined and this number was used to indicate the amount of target RNA in each sample. The MMP  $C_T$  calculated was also normalised to ribosomal 18s cDNA that was run concurrently.

## ***Immunohistochemistry***

Paraffin embedded brain biopsies from five non-immunosuppressed patients with biopsy-proven CNS *M.tb* infection, two non-infected post-mortem negative controls and two positive controls (patients who had undergone temporal lobectomy epilepsy surgery) were immunostained for the microglial marker Iba-1 (rabbit polyclonal, WAKO Pure Chemical Industries Ltd, Osaka, Japan), MMP-1 (mouse monoclonal, Calbiochem, Merck, Nottingham, UK), cJun (rabbit polyclonal, AbD Serotech, Kidlington, UK), p65 (rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, Ca, US) and phospho-p38 (mouse monoclonal, Sigma). Sections of 5 µm thickness were dewaxed in xylene and rehydrated in decreasing alcohols to water. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in 1x PBS (pH 7.4) for 30 minutes. Antigen retrieval of sections was performed by steaming for 20 minutes in citrate buffer (0.01M citrate pH 6.5) and sections blocked with 10% normal goat serum for 10 minutes (Vector Laboratories, Burlingame, CA, US). The primary antibodies (Iba-1 at 1:400, MMP-1 at 1:150, p65 at 1:100, cJun at 1:200 and p38 at 1:200 dilutions) were applied for 16-20 hours at 4°C after optimisation in placenta (MMP-1) or tonsillar tissue (p65 and cJun). Iba-1 and p38 had already been optimised in the laboratory using glioblastoma sections. On the following day staining was visualised using biotinylated secondary antibodies with an avidin-biotin horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, US) applied onto tissue sections for 45 minutes at 23°C. The reaction product was revealed with 2 µg/mL 3, 3'-Diaminobenzidine (DAB) and 0.0075% hydrogen peroxide in PBS. p65 and cJun were developed using components of the Super Sensitive Polymer HRP

IHC Detection System (BioGenex Laboratories, Dan Ramon, CA, US). Between each step in the immunostaining procedure sections were washed with PBS three times for five minutes. Finally the slides were counterstained with hematoxylin, dehydrated in graded ethanol, cleared in xylene and coverslipped in Di-n-butylPhthalate (DPX). Negative controls included omission of the primary antibody.

### ***Immunofluorescence***

Studies to confirm co-localisation of Iba-1 and MMP-1 were performed using double immunofluorescence. Deparaffinized and rehydrated 5 µm tissue sections were subjected to antigen retrieval utilising a steamer in citrate buffer (pH 6.0). Sections were then treated with 10% normal goat serum (Vector Laboratories) in PBS for 30 minutes to quench the non specific staining. After 16-20 hours incubation with anti-MMP-1 antibody at 4°C the slides were further incubated for one hour with appropriate secondary antibody conjugated to a fluorescent dye (1:200 dilution, Alexa Fluor 488, Invitrogen). After incubation with 10% normal goat serum in PBS, the anti Iba-1 antibody was applied for two hours at 4°C. Slides were incubated with the secondary antibody conjugated to a second fluorescent dye (1:200 dilution, Alexa Fluor 555, Invitrogen) for one hour. Between each step in the staining procedure, sections were washed with PBS three times for five minutes. To reduce autofluorescence caused by lipophilic lipofuscin-like compounds tissue sections were treated with 0.3% Sudan Black B (BDH Chemicals Ltd, Poole, UK) in 70% ethanol. Images were obtained using the Nikon E1000M imaging fluorescence microscope (Nikon, Japan) and a QICAM digital camera (QImaging, Surrey, BC, Canada) and

analysed using Image Pro Plus software (Media Cybernetics, Bethesda, MD, US). For negative controls primary antibodies were omitted.

Ethical consent was obtained from the Hammersmith Hospitals Research Ethics Committee for the use of archived biopsies. Tissue samples were provided by the Human Biomaterials Resource Centre at Imperial College Healthcare NHS Trust.

### ***Detection of nuclear NFκB & AP-1 DNA binding by ELISA***

To investigate the activation of the multiple subunits of NFκB a specific transcription factor assay (TransAM, Active Motif), which is five times more sensitive than EMSA, was performed. Nuclear extracts were added to a 96-well plate containing immobilized oligonucleotides encoding a consensus site (NFκB: 5'-GGGACTTTCC-3'). Active NFκB contained in the nuclear extract specifically bound to this oligonucleotide. The primary Abs used to detect p50, p52, p65, RelB, or cRel recognise an epitope accessible only when the active form of these factors is bound to its target DNA. An HRP-linked secondary antibody was added and the colour change determined by spectrophotometry at 450 nm (Bio-Tek). Competition experiments demonstrated specificity of binding by adding 20 pM/well of either wildtype or mutated NFκB oligonucleotide before assaying with the p65 antibody and demonstrating loss of binding with wildtype but not mutated construct. Similarly the AP-1 subunit kit used the 5'-TGAGTCA-3' AP-1 consensus site. Primary antibodies were to JunB, cJun, JunD, FosB, cFos, Fra-1 and Fra-2.

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### **Promoter Reporter analysis**

Microglial cells were seeded in 12-well plates at 50,000 cells/cm<sup>2</sup> in DMEM 1640 containing 3 mM glutamine, 10% FCS (Biosera), and 10µg/ml ampicillin. For kinetic experiments cells were co-transfected 24 hours later in serum-free medium with 0.8µg of MMP-1 promoter DNA construct (-4372 bp upstream of transcription start site) inserted into the firefly luciferase expression plasmid pGL3 (a gift from Professor Ian Clarke, University of East Anglia, Norwich, UK) and 0.08µg of control reporter plasmid, PRLTK, constitutively expressing low level Renilla luciferase activity, all suspended in 2.4µl of FuGene HD (Roche, Lewes, UK). Cells were stimulated after 12 hours and the luciferase activity of extracts was measured using the Dual-Luciferase Reporter Assay System (Promega, Southampton, UK) analysed with a luminometer (L-Max Lucy 2, Molecular Devices, Sunnyvale, CA, US). Renilla luciferase activity was used to normalise firefly activity to control for transfection efficiency.

### **Deletion and mutation constructs**

A series of deletion constructs decreasing in size from -3830 to -517 bp (Ian Clark) and the full length wild type promoter (-4372 bp) were used to assess regional promoter activity. Two mutation constructs of the full length promoter were used to assess specific *cis*-acting element activities. For the one consensus NFκB site a mutation was made between -2886 to -2878 bp (From 5'-ATGGAA**AAA**-3' to 5'-ATGGAA**CCC**-3') and one of the six AP-1 sites at -1949 to -1955 (From 5'-TGAG**T**TA-3' to 5'-TGAT**T**TA-3) [324].

### ***Phospho-MAP kinase Array***

In order to take a comprehensive approach to the investigation of signalling pathways that might be involved in MMP-1 and 3 secretion a Human Phospho-MAP kinase Array kit (R&D systems) was used to investigate MAP kinase pathways. 60 mm dishes of confluent microglia were stimulated with CoMCont or CoMTb for 30 minutes (without a wash step as initial experiments showed this increased basal p38 phosphorylation). After rinsing in 1ml cold 1x PBS, cells were lysed using Lysis Buffer 6, pipetted up and down and transferred to a pre-cooled microtubule. Samples were rocked on ice for 30 minutes and then frozen at -80°C. On the day of analysis total protein concentration was measured by Bradford assay (Biorad) as described above. The array was used to measure the relative level of phosphorylation of MAP kinases and related serine/threonine kinases. Arrays were blocked for an hour with Array Buffer 1, whilst 250 µg lysate was prepared by adding it to Array Buffer 1 in a 1:5 ratio (lysate:Array Buffer 1) to a final volume of 1.5 mls. After adding this lysate/Array Buffer 1 mix to blocked membranes they were incubated overnight at 2-8°C on a rocking shaker. The following day each membrane was washed three times in 5 mls wash buffer and 1.5 mls 1:1000 diluted biotinylated antibody cocktail added to each membrane for two hours on a rocking shaker. Three further washes were performed and a 30 minute incubation in 1:2000 streptavidin-HRP performed. After three washes the membrane was developed with the ECL system (GE Healthcare) and a digital image taken (UVP). Six positive controls are incorporated onto the membrane. The digital image was then analysed using Scion Image Analysis software (NIH Image version 1.61) and the mean

intensity calculated for CoMCont and CoMTb. Data are presented as the difference ( $\pm$  SD) between mean intensities.

### ***Analysis of CSF MMPs/TIMPS in TBM – Chapter 6***

Please see chapter 6 for further specific methodology for the analysis of CSF specimens from TBM patients.

### ***Statistics***

Data are presented as means ( $\pm$  SD) of three samples and represent experiments performed in triplicate on at least two separate occasions, unless otherwise stated. Paired groups were compared with the Student t-test. Multiple intervention experiments were compared by a one-way analysis of variance and Tukey's correction for multiple pairwise comparisons, with equal variance tested by Levene's test. A  $p < 0.05$  was taken as significant. Error bars in figures represent standard deviations unless stated otherwise. All analysis was done using SPSS software (version 15.0, SPSS corp, Chicago, IL, US).

### ***Ethics***

The research ethics, safety and genetic modification committees of all institutions involved in the research approved the protocols described herein.

## Chapter 3 – MMPs/TIMPs, NFκB and cellular networks

### *Introduction*

#### **Global analysis of microglial MMP/TIMP expression in TB and investigation of the control of MMP-1 expression and secretion**

In order to investigate the hypothesis that microglia control the CNS immune response to *M.tb* and investigate intracellular signalling pathways I had to develop an *in vitro* model of CNS TB. *M.tb* is known to infect CNS cells such as microglia and astrocytes directly, but networks involving infiltrating *M.tb* infected monocytes are also likely to be important for MMP-driven CNS TB immunopathology based on published data [156, 221]. Microglia secrete a wide range of MMPs in response to inflammatory stimuli such as LPS, but a complete analysis of MMP/TIMP expression has never previously been performed in the context of TB.

Therefore I set up the cellular model so that I could test the various interactions between the key cells in CNS TB. Initially it was important to take a broad look at all MMPs/TIMPs to decipher which specific enzymes should be looked at in more detail. Thus I performed gene expression profiling of all 23 MMPs and 4 TIMPs in collaboration with Dylan Edwards and Caroline Pennington at the University of East Anglia, who provided primers, probes and expertise. This suggested that MMP-1 and MMP-3 were the critical microglial MMPs in CNS TB and therefore the control of their expression and secretion was investigated in depth: data are presented in this and the following chapter.

*In vivo* correlation of the cellular model was sought through the use of immunohistochemistry. As monocyte-microglial networks appeared to be central to MMP secretion I checked the specificity of this finding. I have generated data on astrocyte-microglial, as well as microglial-microglial, interactions and show that these networks do not drive MMP secretion in the same way as monocytes networks do.

Gene expression is controlled by a large number of transcriptional activators that bind to the 5' upstream promoter region of genes to enhance transcription by RNA polymerases. NFκB is known to be a central controller of MMP gene transcription and the MMP-1 promoter is known to have only one NFκB consensus site [194, 324]. Transcription factors rarely act in isolation and another key pro-inflammatory transcription factor implicated in control of MMP secretion is AP-1 [325]. Therefore control of gene expression focused on these two molecules.

In this chapter therefore I demonstrate that cytokine networks, specifically between infiltrating monocytes and microglia, acting via NFκB and AP-1, are critical in controlling MMP-1 expression and secretion in CNS TB.

## **Results**

### **MMP gene expression profiling in *M.tb*-infected and CoM stimulated human microglia**

Gene expression of all 24 human MMPs and four TIMPs was analyzed by reverse transcription (RT)–PCR in unstimulated, *M.tb*, CoMCont and CoMTb-stimulated microglia at 24 h. *M.tb* was at an MOI of 10. CoMTb up-regulated MMP-1 3.4-fold over control, MMP-3 3.0-fold over control, MMP-9 2.2 over control (all  $p < 0.05$ , summarised in figure 6). CoMTb also up-regulated MMP-8 6.2-fold over control and MMP-10 5.3-fold over control but these results were not significant. *M.tb* only up-regulated MMP-27 26-fold over control. CoMCont and CoMTb suppressed MMP-24 expression by 40% ( $p < 0.05$ ). A 36% (but non-significant) fall in MMP-2 was also observed between control and CoMTb. MMP-7, -20, -21, -26 and -28 expression was not detected.

The data in figure 6 show changes in MMP mRNA, normalized to 18S rRNA and expressed as fold change over control thus they indicate relative not absolute levels of gene expression in microglia. The  $C_T$  at which PCR amplification enters the logarithmic phase indicates levels of gene expression so  $C_T$  data was used to compare absolute levels of different genes, where a low  $C_T$  value indicates high level gene expression (Figure 7). Of the MMPs up-regulated by CoMTb MMP-1 and -3 were the most highly expressed with mean  $C_T$  thresholds of  $22.9 (\pm 0.6)$  and  $24.8 (\pm 1.4)$  respectively ( $p = 0.003$  and  $p = 0.03$  for t-test between CoMCont and CoMTb), whereas MMP-8, -9, -10 reached only moderate levels with  $C_T$  all over 25 ( $p = 0.05$  for MMP-9 and  $p = 0.01$  for MMP-10

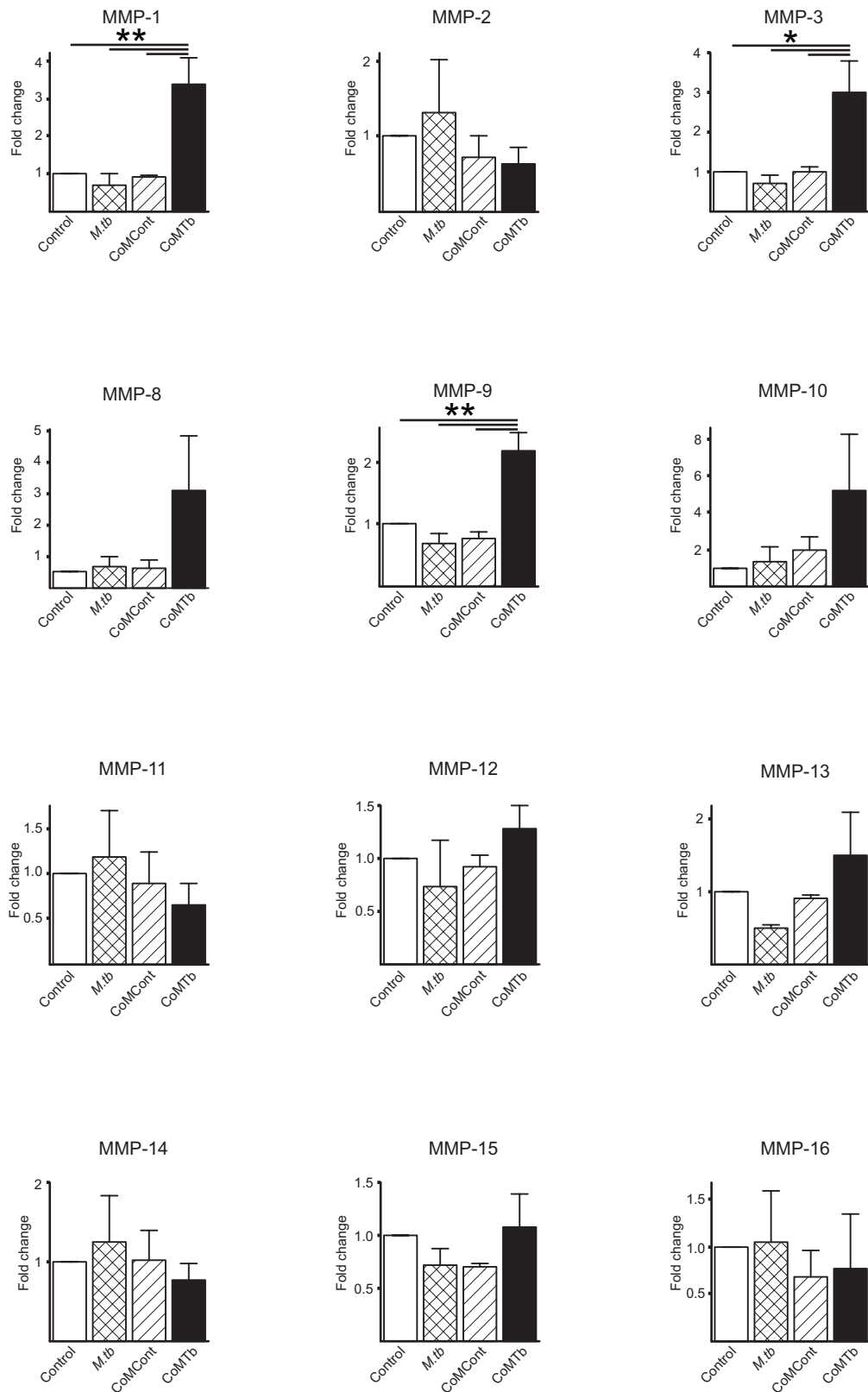
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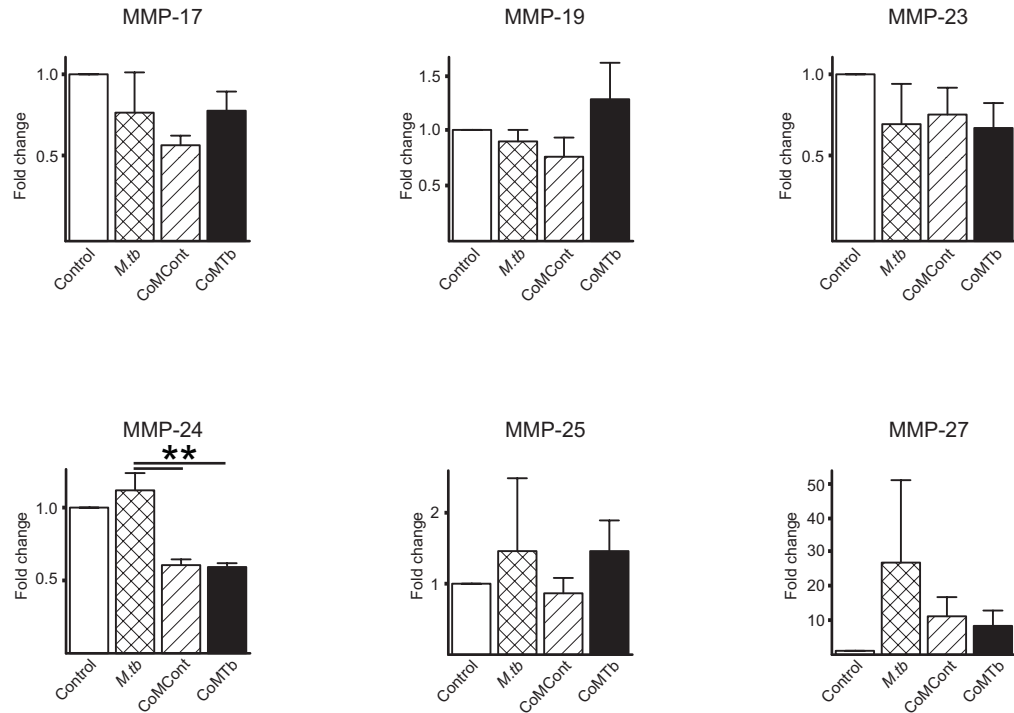


Figure 6. Matrix metalloproteinase (MMP) gene expression in human microglia stimulated by *Mycobacterium tuberculosis* (*M.tb*) and CoMTb. Microglia were stimulated with *M.tb* at a multiplicity of infection (MOI) of 10 or 1:5 CoMCont or CoMTb. Total RNA was extracted at 24 h and mRNA accumulation was analysed by reverse transcriptase–polymerase chain reaction (RT-PCR). For each gene, mRNA levels normalised to 18S RNA are expressed as fold change relative to control mRNA levels at 24 h. All 23 human MMPs were analysed. Open bars depict control, diamond shaded bars depict *M.tb*-stimulated, diagonal shaded bars depict CoMCont and solid bars depict CoMTb-stimulated microglial gene expression. CoMTb stimulated significant increases in MMP-1, -3 and -9 ( $p<0.05$ ). MMP-8 and -10 gene expression by CoMTb and MMP-27 by *M.tb* were increased but did not reach significance. CoMCont and CoMTb suppressed MMP-24 expression. MMP-7, -20, -21, -26 and -28 were undetectable and MMP-4, -5, and -6 do not exist. Mean values  $\pm$  SEM are given for three experiments performed on separate vials of cells. Data were analysed by one-way analysis of variance, followed by Tukey's multiple comparison. Significant effects of a treatment compared with another group are indicated. \* $p<0.05$ .

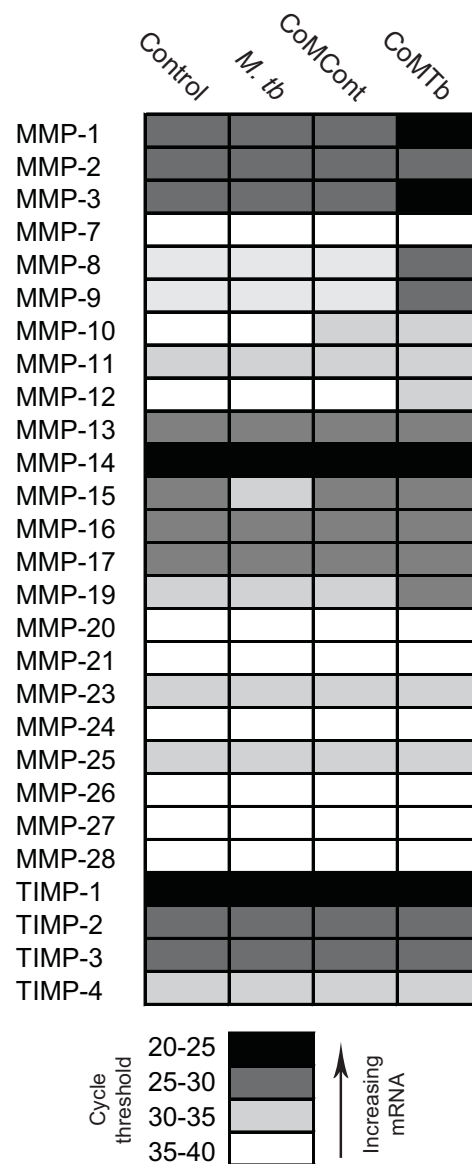


Figure 7. PCR cycle thresholds for matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) gene expression in human microglia stimulated by *M.tb* and CoMTb. The mean cycle threshold (CT) at which PCR amplification enters the logarithmic phase is shown for the RNA samples analysed in Figure 6. A low CT indicates high mRNA levels. Open rectangles represent low expression through shaded to solid rectangles, representing high expression. Of all the MMPs up-regulated CoMTb induced MMP-1 and MMP-3 were the most highly expressed.

for t-test between CoMCont and CoMTb). The  $C_T$  for *M.tb* stimulated MMP-27 expression was very low at 36 suggesting the fold value was biased by a very low control denominator value.  $C_T$  values for all stimuli for MMP-24 were similar (median 30.6, range 30.0 – 30.8). The main changes in MMPs are summarised in table 4.

**Table 4. Summary of main findings from global analysis of MMPs/TIMPs**

|                                 |                                                  |
|---------------------------------|--------------------------------------------------|
| <b>High basal expression</b>    | MMP-14, TIMP-1                                   |
| <b>Increased by CoMTb</b>       | MMP-1, 3 & 9*<br>MMP-8 & 10 <sup>†</sup>         |
| <b>Decreased by CoMTb</b>       | MMP-24 <sup>‡</sup> , TIMP-2, 3 & 4 <sup>†</sup> |
| <b>Increased by <i>M.tb</i></b> | MMP-27 <sup>‡</sup>                              |
| <b>Not detected</b>             | MMP-7, 20, 21, 26 & 28                           |

\* p<0.05; † p=ns; ‡  $C_T$  value very low.

### Conclusions from global analysis of MMPs

Therefore after analysis of the direct and indirect networking effects of *M.tb* driven expression of all 23 known MMPs and 4 TIMPs in human microglia using both 18s corrected and quantitative  $C_T$  data significant up-regulation of MMP-1 and 3 were identified as the key findings and subsequently investigated in depth.

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## **Monocyte-microglial networks drive MMP-1 secretion from human microglia more potently than *M.tb* infection**

Secretion of MMP-1 was then analysed by casein zymography confirmed with western blotting to investigate whether increased gene expression resulted in greater enzyme activity. *M.tb* up-regulated MMP-1 in a dose dependent fashion to a maximum of 4.1 fold over control at an MOI of 10 ( $p < 0.01$ , Figure 8A). However, CoMTb drove MMP-1 secretion more potently with a 20.6 fold increase in secretion over CoMCont ( $p < 0.01$ ) and 3.7 fold greater than *M.tb* ( $p < 0.01$ , Figure 8A). Western blotting confirmed that the bands seen on casein zymography were MMP-1 (Figure 8A). In dose response experiments analysed by Luminex a 1:5 dilution of CoMTb resulted in  $49.9 (\pm 5.3)$  ng/ml MMP-1 secretion (equivalent to  $143.3 \pm 45.2$  units of caseinolytic activity on casein zymogram) at 72 hours and was therefore used in all subsequent experiments ( $p < 0.01$ , Figure 8B). Kinetic studies of MMP-1 expression demonstrated that mRNA had started to rise by 6 hours and had peaked by 48 hours with a  $23.4 \pm 4.7$  fold up regulation of mRNA over time 0 control samples ( $p < 0.01$ , Figure 8C). In kinetic studies MMP-1 secretion was up-regulated by 24 hours but continued to increase until 96 hours (Figure 8D). At 72 hours (the subsequent time point for secretion experiments) MMP-1 was  $67.0 \pm 23.7$  units of caseinolytic activity.

## **Microglial-microglial and astrocyte-microglial networks do not drive MMP-1 secretion**

*M.tb* is known to infect and replicate in macrophages, including microglia the resident macrophages of the CNS, and has been described infecting about 15%

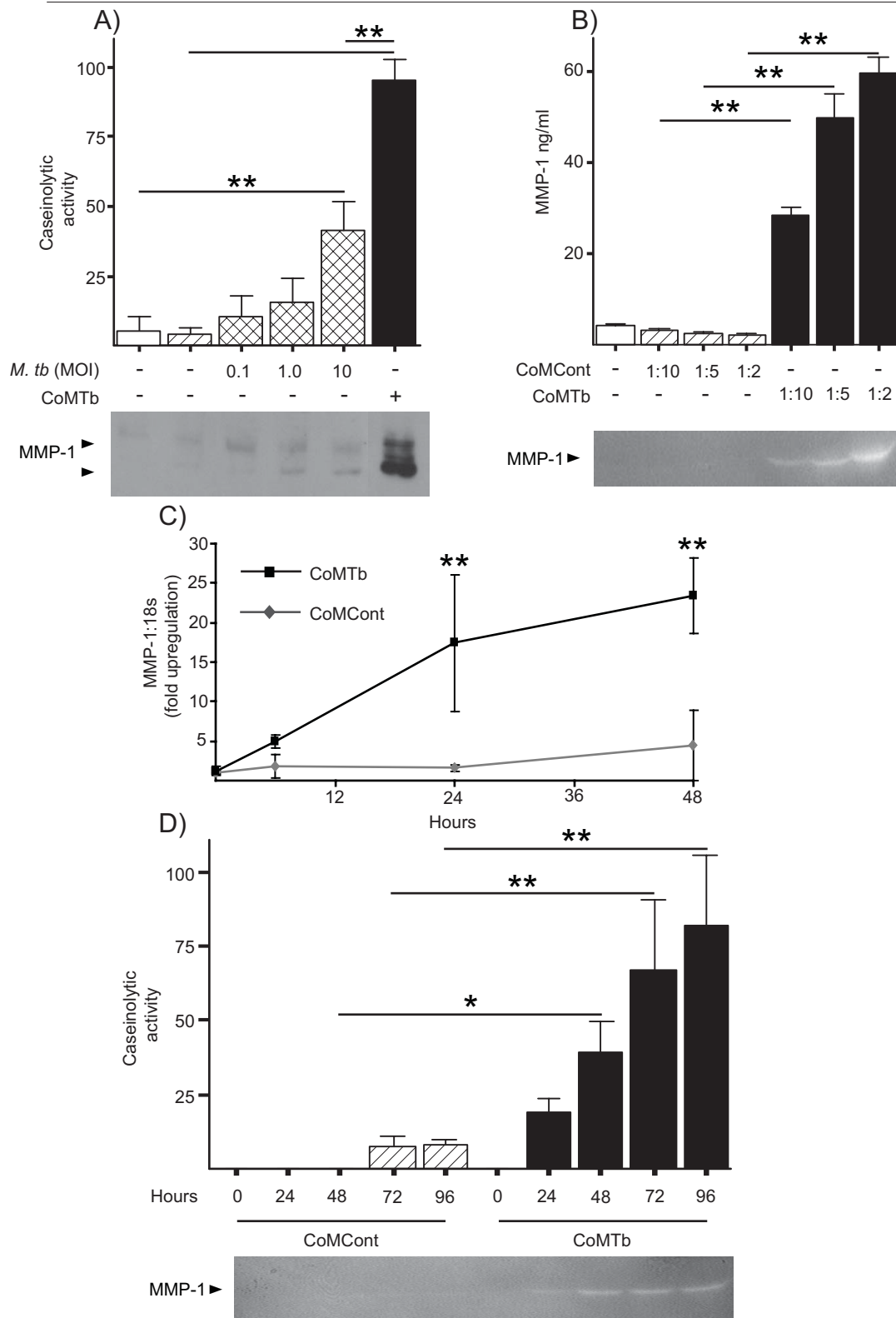


Figure 8. CoMTb drives MMP-1 gene expression and secretion in microglia more than *M.tb*. A, MMP-1 secretion. Microglial MMP secretion at 72 h analysed by casein zymography, confirmed by Western blotting, demonstrated MMP-1 secretion was up-regulated by *M.tb* in a dose dependent fashion but more by 1:5 CoMTb. B, CoMTb dose response. Microglial cells were stimulated with either CoMCont or CoMTb at 1:10, 1:5, and 1:2 dilutions. MMP-1 secretion at 72 h was analysed by Luminex and confirmed by casein zymography. C, Kinetics of MMP-1 gene expression. Microglia were stimulated with CoMCont or CoMTb and total RNA was extracted at 0, 6, 24 and 48 h. mRNA accumulation was analysed by RT-PCR. MMP-1 mRNA levels were normalised to 18S RNA and values are expressed as fold change relative to control mRNA levels at 0 h. Mean values  $\pm$  SD from three experiments are presented. D, Kinetics of MMP-1 secretion. CoMCont and CoMTb stimulated microglia cell culture medium harvested at 0, 24, 48, 72 and 96 h analysed by densitometry of casein zymography. For all experiments, except RNA (C), bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. \* $p < 0.05$ , \*\* $p < 0.01$ .

of astrocytes *in vitro* [156]. Therefore whether *M.tb* acted via networks other than infiltrating monocytes in the model of CNS TB was investigated. CoATb, as well as appropriate control conditioned medium, was prepared from primary human astrocytes from two donors but did not stimulate MMP-1 secretion (Figure 9). Similarly microglial condition medium, CoMicTb, did not up-regulate MMP-1 secretion (data not shown).

### **Chamber slide analysis of *M.tb* and microglial cell association**

I was concerned that the negative findings with CoMicTb and those in the initial global MMP analysis presented in figure 6 and 7 might be as a result of *M.tb* not infecting the microglia as they were a transformed cell line. Therefore I performed chamber slide *M.tb* infections of microglia at an MOI of 10, with modified Kinyoun staining and found 36.1% ( $\pm 13.8$ ) of cells associated with *M.tb*. For comparison I also infected macrophages with *M.tb* and found  $59 \pm 5.7\%$  bacilli associated at an MOI of 1. Confocal microscopy with GFP expressing *M.tb* confirmed that microglia were associated with intracellular bacilli (Figure 10).

### **Immunohistochemistry: MMP-1 is expressed in microglia in patients with CNS TB**

To determine the clinicopathological significance of our cellular findings MMP-1 immunohistochemical analysis of five brain biopsies from non-immunosuppressed patients with histologically proven CNS TB was performed. An antibody to ionised calcium-binding adaptor molecule 1 (Iba-1), a known macrophage/microglial marker, was used to co-localise microglial MMP-1 expression [326]. Two positive control patients were analysed, who had

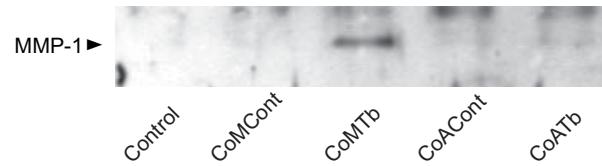


Figure 9. *M.tb*-infected astrocyte-microglial cell signalling does not up-regulate MMP-1 secretion. Astrocytes were infected with Middlebrook culture medium alone (control) or *M.tb* at a multiplicity of infection (MOI) of 10 for 24 h. The cell culture medium was harvested and filtered to remove infectious particles and termed CoACont or CoATb. 1:5 CoACont and CoATb was then used to stimulate microglia and cell culture medium harvested at 72 h and analysed by Western blotting. Blot is representative of triplicate samples performed on at least two occasions.

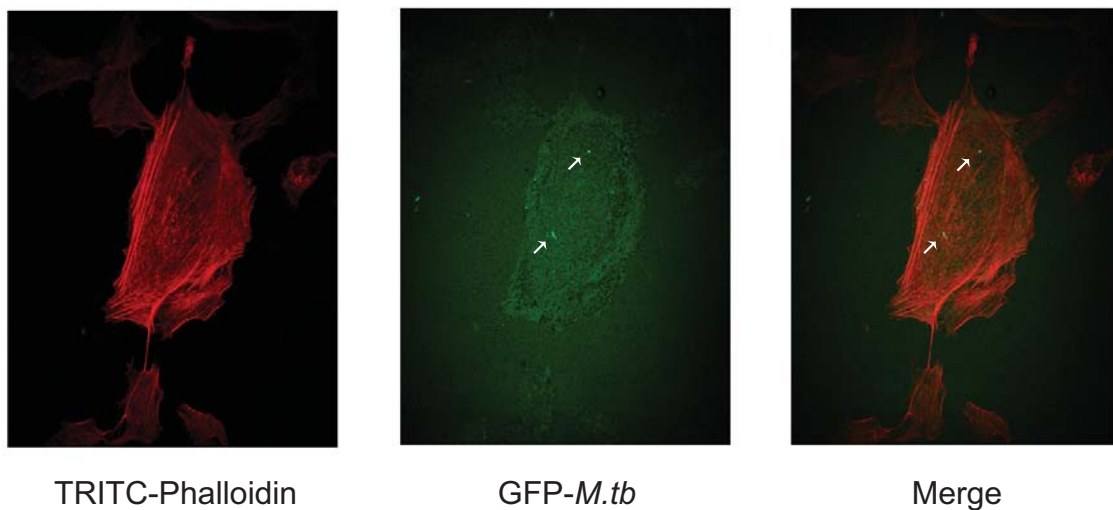


Figure 10. *M.tb* infects microglia. GFP expressing *M.tb* at a multiplicity of infection (MOI) of 10 was used to infect microglia grown in a 4-well chamber slide and confocal microscopy performed. A, Cells were stained with TRITC labelled phalloidin to show F-actin. B, GFP expressing *M.tb* (white arrows). C, Merged image confirms *M.tb* is intracellular. Figure is representative of at least triplicate samples performed on at least two occasions (original magnification x63).

undergone temporal lobectomy for intractable epilepsy, a condition known to induce extensive activation of microglia. Tissue from beyond the epileptic focus at the excision margin was examined. Two negative control tissues examined were from post-mortem, macroscopically normal brains, from subjects with no history of neurological or infectious disease.

Iba-1 immunoreactivity was strong in both parenchymal brain tissue and TB granulomas (Figure 11A). MMP-1 was expressed in resident tissue associated microglia compared to the granular layer neuronal cells (Figure 11B).

Examination of Iba-1 positive granuloma associated microglia/macrophages (Figure 11C) demonstrated that MMP-1 immunoreactivity was strongest in the centre of the granuloma (Figure 11D). Hematoxylin and eosin staining and a representative negative control (omission of primary antibody) are shown for comparison (Figure 11E & F). Astrocytes and neurons did not demonstrate staining for Iba-1 or MMP-1 in any specimens. Positive controls demonstrated that although some microglial and capillary endothelium MMP-1 immunoreactivity was seen this was to a lesser degree than was observed in CNS TB (data not shown). Negative controls showed some background immunoreactivity due to non-specific lipofuscin-antibody binding (a post-mortem artefact) but no specific binding (data not shown). In order to confirm co-localisation of MMP-1 in microglia immunofluorescence was performed for Iba-1 and MMP-1 (Figure 11G-I). Immunofluorescence confirmed that the MMP-1 immunoreactivity observed above was expressed specifically in microglia. In summary immunohistochemistry demonstrated that in CNS TB microglia

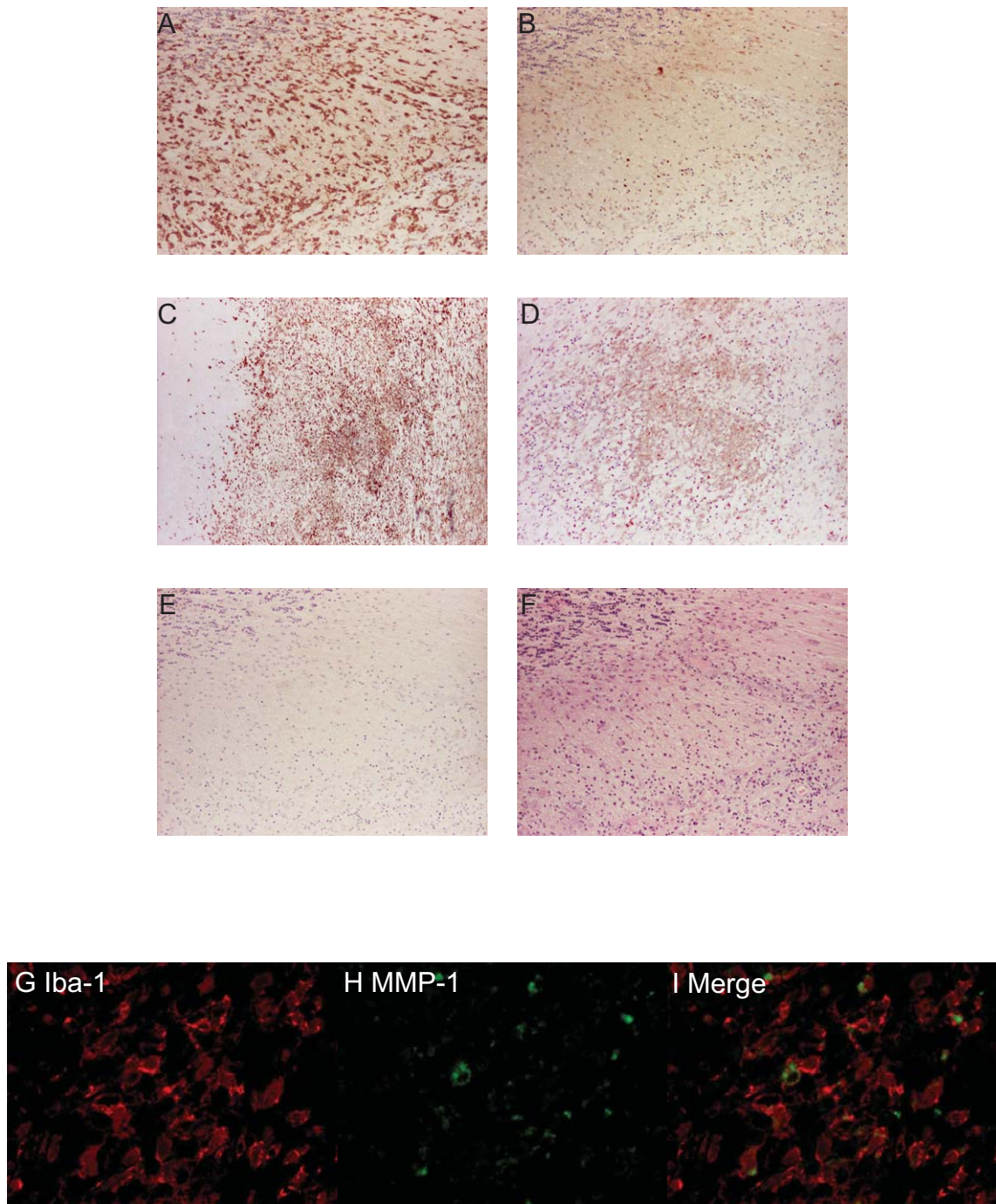


Figure 11. Microglia express MMP-1 in patients with CNS TB. Brain biopsies from five patients with TB and two positive and negative controls were immunostained for the microglial/macrophage marker Iba-1 and MMP-1 as described in materials and methods chapter. Areas of immunoreactivity appear as brown against the blue counterstain. A, In CNS TB, microglial cells are immunoreactive for the monocyte/microglial marker Iba-1 (original magnification 10x). B, Adjacent to TB granulomas microglia are immunoreactive for MMP-1 (original magnification 10x). C, Multiple Iba-1 positive microglia/macrophages are seen within the granuloma (original magnification 10x). D, The centre of the granuloma is strongly immunoreactive for MMP-1 (original magnification 20x). E, Immunostaining with secondary Ab alone demonstrated that binding was specific (original magnification 10x). F, Corresponding hematoxylin and eosin stained section (original magnification 10x). G, Iba-1 immunofluorescence. H, MMP-1 immunofluorescence. I, Merged Iba-1 and MMP-1 image demonstrates co-localisation of staining (G-I, original magnification 40x). Images shown are representative of five biopsies.

express MMP-1 and that this immunoreactivity is more pronounced towards the centre of the TB granuloma.

### **TIMP expression and secretion by *M.tb*-infected and CoM stimulated human microglia**

In contrast to the global analysis of MMPs (Figure 6 and 7) where MMP-1 gene expression was increased, CoMTb did not alter TIMP-1 expression and reduced TIMP-2, -3 and -4 expression by 68%, 64% and 56% respectively, although non-significantly (Figure 12A). The mean  $C_T$  values between stimuli for individual TIMPs were similar but at different levels between TIMPs (Figure 7 bottom): TIMP -1 (median 21.5, range 20.9 – 22.1), TIMP-2 (26.5, 25.8 – 27.5), TIMP-3 (26.3, 26.1 – 27.4) and TIMP-4 (31.8, 31.6 – 32.1). The main changes in TIMPs are summarised in table 4 above.

TIMP-1 secretion was measured by ELISA and was confirmed to be at high concentrations, consistent with gene expression. It was not affected by different stimuli with control secretion of  $31.9 \pm 3.2$  ng/ml representative of the four stimuli values (Figure 12B). In contrast CoMTb suppressed TIMP-2 secretion 29% compared to CoMCont ( $p < 0.01$ , figure 12C) confirming the expression data. TIMP-3 and 4 secretion were not assessed.

### **Fractionation of CoMTb reveals CoMTb-dependent microglial cell MMP-1 secretion is mediated via a >10 kDa protein**

A holistic approach to the identification of the possible mediators of up-regulation of MMP-1 in CoMTb was taken. Fractionation of CoMTb into two aliquots by molecular weight was performed. Aliquots of <10kDa and >10kDa

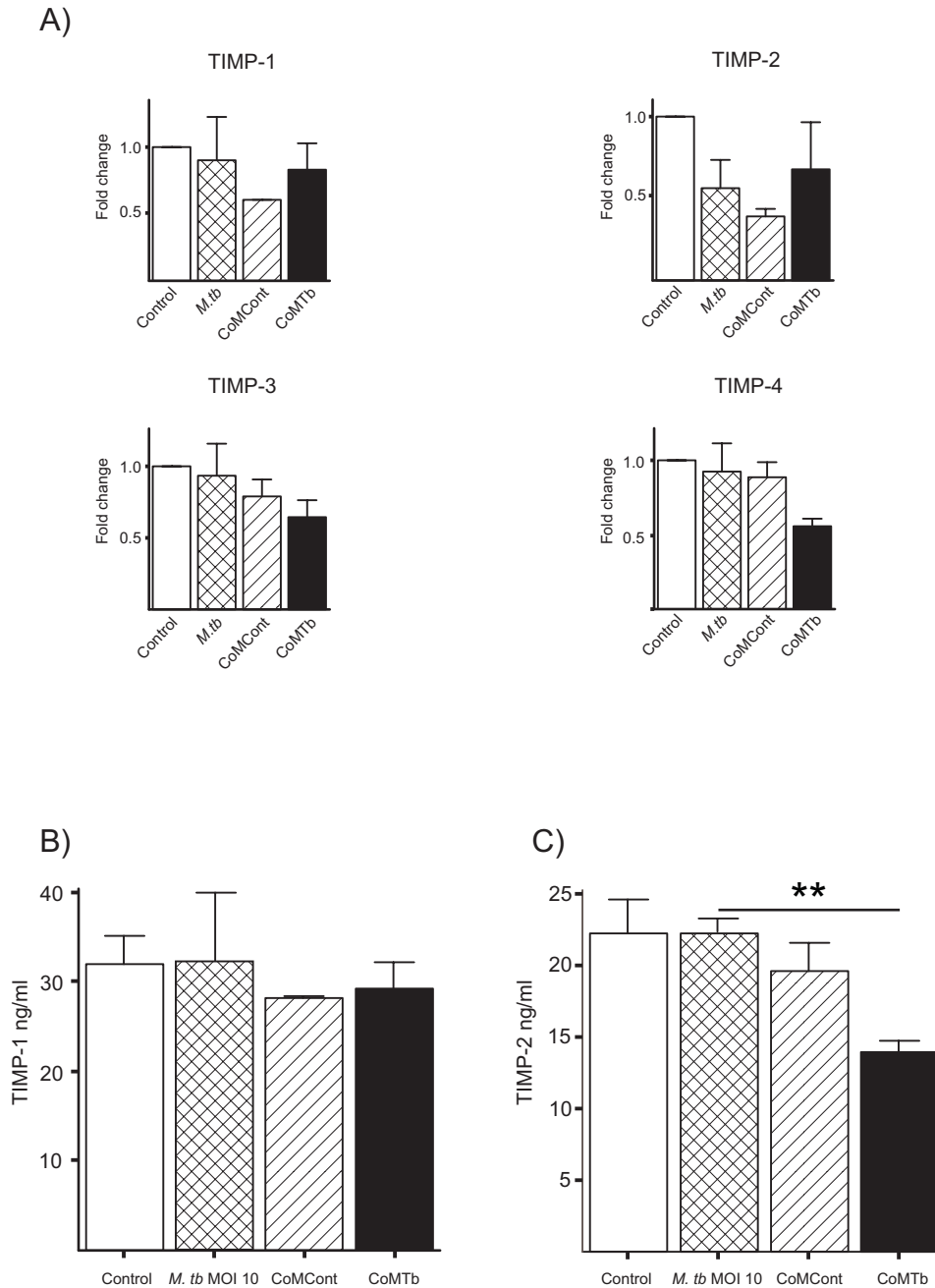


Figure 12. TIMP expression and secretion is not increased by *M.tb* or CoMTb. A, Microglia were stimulated with *M.tb* at a multiplicity of infection (MOI) of 10 or 1:5 CoMCont and CoMTb. mRNA from microglia stimulated for 24 h was analysed by RT-PCR, normalised to 18S RNA and expressed as fold change relative to 24 h control mRNA. All 4 human TIMPs were analysed. Open bars depict control, diamond shaded bars depict *M.tb*-stimulated, diagonal shaded bars depict CoMCont and solid bars depict CoMTb-stimulated microglial gene expression. No significant differences were noted in any of the conditions studied. Mean values  $\pm$  SEM are given for three experiments performed on separate vials of cells. B & C, CoMTb decreased TIMP-2 secretion. Microglial cell culture medium was harvested at 72 h and analysed by ELISA for B, TIMP-1 and C, TIMP-2. Bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. Data were analysed by one-way analysis of variance, followed by Tukey's multiple comparison. \*\* $p < 0.01$ .

fractions were used to stimulate microglia at the same concentration as CoMTb (Figure 13A). The >10kDa fraction up-regulated MMP-1 secretion to 56.8% of the CoMTb value.

### **TNF-α is necessary but not sufficient to reproduce the effect of CoMTb**

As the >10 kDa fraction reproduced some of the effect of CoMTb I hypothesised that the pro-inflammatory cytokine TNF-α (molecular weight ~17 kDa) might be mediating the effect of CoMTb. Therefore CoMTb was pre-incubated with increasing doses of anti-TNF-α antibody (Peprotech, Rocky Hill, NJ, US). Anti-TNF-α abolished the effect of CoMTb in a dose dependent manner (Figure 13B). However, addition of recombinant TNF-α even at 100 ng/ml only restored MMP-1 secretion to 32.9% of CoMTb (Figure 13C).

As synergy between TNF-α and secreted mycobacterial products leading to MMP secretion has previously been shown both TNF and Tb supernatant were used to stimulate cells [258]. Tb supernatant was generated by sterile filtering mid-log growth phase *M.tb* culture through an Anopore 0.2µm membrane (Whatman). The same volume of *M.tb* filtrate present in CoMTb was used to stimulate microglia (Figure 13D). Although there was a moderate increase in MMP-1 secretion (over and above the summated individual values of TNF-α and Tb supernatant) to 54.2% of CoMTb this was not statistically different from TNF-α 100ng/ml alone. In addition the amount of TNF-α in three donor CoMTb batches was measured by ELISA and found to be 22.9 (± 5.0) ng/ml, thus 1:5 diluted CoMTb only contains about 5 ng/ml TNF-α. TNF-α 10 ng/ml and Tb sup only produced 6.5% MMP-1 secretion compared to CoMTb.

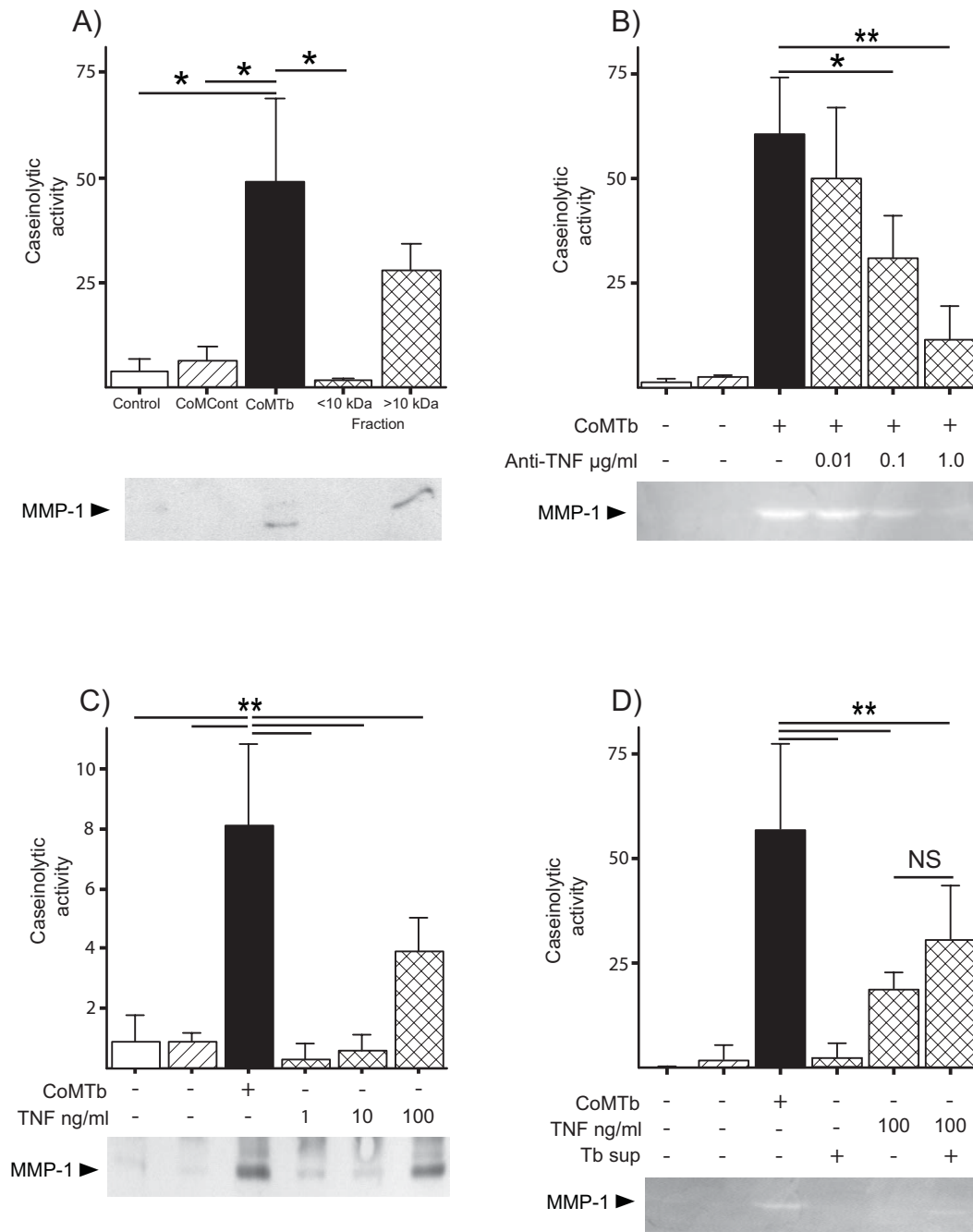


Figure 13. Molecules >10 kDa in CoMTb drive microglial MMP-1 secretion and monocyte-microglial signalling requires TNF- $\alpha$ . A, CoMTb was fractionated by molecular weight exclusion spin columns to produce two aliquots, termed <10 kDa and >10 kDa fractions. Densitometric analysis of MMP-1 secretion by microglia 72 h post stimulation was confirmed by Western blotting. B, CoMTb was preincubated with anti-TNF- $\alpha$  Ab for 2 h. Inhibition of TNF- $\alpha$  activity reduced MMP-1 secretion by CoMTb-stimulated cells. A representative casein zymogram is shown. C, TNF- $\alpha$  alone is a weak stimulus of MMP-1 secretion. Microglial cells were stimulated with TNF- $\alpha$  at concentrations of 1–100 ng/ml. Casein zymography was confirmed by Western blotting. D, *M.tb* derived antigens do not enhance TNF- $\alpha$  driven MMP-1 secretion. Microglial cells were stimulated with TNF- $\alpha$  alone or in combination with Tb sup (*M.tb* filtered culture supernatant, see text). A representative casein zymogram is shown. Bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. Data were analysed by one-way analysis of variance, followed by Tukey's multiple comparison. \* $p < 0.05$ , \*\* $p < 0.01$ .

## **IL-1β is critical for the monocyte-microglial network driving MMP-1 secretion**

IL-1β is also a 17 kDa protein but is at much lower concentrations in CoMTb than TNF-α therefore the role of IL-1β in MMP-1 secretion was investigated using receptor antagonist (IL-1Ra, Peprotech) [327]. IL-1Ra abolished MMP-1 secretion in a dose dependent manner (Figure 14A) but only to  $15.4 \pm 7.4$  units of caseinolytic activity (63% reduction,  $p < 0.01$ ). Add back experiments with IL-1β alone did not increase secretion of MMP-1 over basal levels (Figure 14B).

IL-1β and TNF-α (at concentrations found in CoMTb) were used to stimulate microglia to investigate whether synergy between these two cytokines accounted for all the effect of CoMTb (Figure 14C). TNF-α 10 ng/ml alone stimulated  $28.4 (\pm 8.6)$  units of caseinolytic activity ( $p < 0.05$  *cf* CoMTb). IL-1β and TNF-α together stimulated  $52.4 (\pm 21.1)$  units of caseinolytic activity. In figure 14B IL-1β 1 ng/ml stimulated only  $1.7 (\pm 1.2)$  units of caseinolytic activity. However, as the confidence intervals were wide for these experiments the difference between TNF-α alone and in combination with IL-1β did not reach significance. In addition IL-1β and TNF-α together did not stimulate MMP-1 secretion to the same magnitude as CoMTb, which gave an additional 24.0 units of caseinolytic activity.

## **Analysis of Conditioned Media by Luminex**

In order to try to unpick the final components of CoMTb that were responsible for MMP-1 secretion CoMCont (n=4 donors) and CoMTb (n=6 donors) were analysed using the Luminex platform. By using a low reading 8 point standard curve the lower level of sensitivity for these analytes were all  $< 1$  pg/ml. The

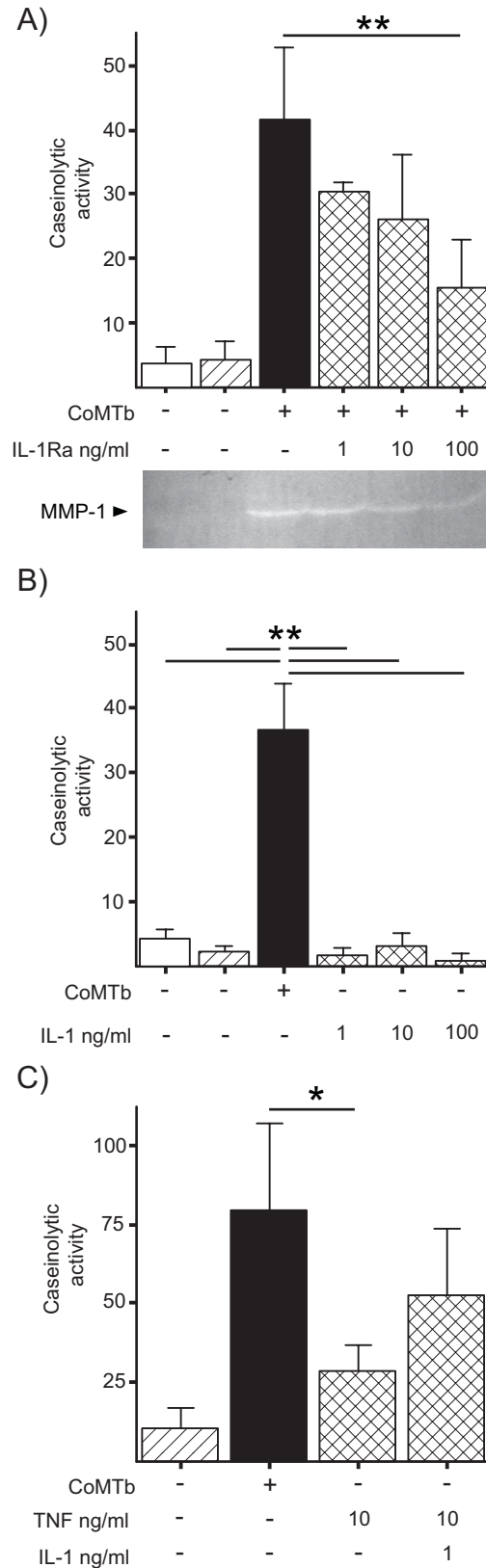


Figure 14. Monocyte-microglial cell signalling requires IL-1 $\beta$ , which does not synergise with TNF- $\alpha$ . A, Cells were preincubated with IL-1Ra for 2 h. Inhibition of IL-1 $\beta$  activity reduced MMP-1 secretion. A representative casein zymogram is shown. B, IL-1 $\beta$  alone is not a stimulus to MMP-1 secretion. Microglial cells were stimulated with IL-1 $\beta$  at concentrations of 1–100 ng/ml. Densitometric analysis of casein zymography is shown. C, Densitometric analysis of TNF- $\alpha$  stimulated microglial cells alone or in combination with IL-1 $\beta$  confirms TNF- $\alpha$  and IL-1 $\beta$  have an additive effect in driving MMP-1 secretion. Bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. Data were analyzed by one-way analysis of variance, followed by Tukey's multiple comparison. \* $p < 0.05$ , \*\* $p < 0.01$ .

results are summarised in figure 15 for analytes in the ng range and in table 5 for those in pg range.

In the nanogram range unstimulated monocytes (i.e. CoMCont) had significant basal secretion of IL-1 Ra 3.0 ( $\pm$ 2.4) ng/ml, CXCL8 (IL-8) 3.7 ( $\pm$ 4.3) ng/ml and CCL4 (MIP-1 $\beta$ ) 1.0 ( $\pm$ 0.8) ng/ml. In the pg range CCL2 (MCP-1) 92.6 ( $\pm$ 82.2) pg/ml, CCL3 (MIP-1 $\alpha$ ) 129.8 ( $\pm$ 232.3) pg/ml and CCL5 (RANTES) 89.7 ( $\pm$ 104.6) pg/ml were secreted. Stimulation of cells with *M.tb* (i.e. CoMTb) led to significant increases in the concentration of IL-1 $\beta$  to 3.9 ( $\pm$ 1.2) ng/ml ( $p$ <0.01), CXCL8 10.5 ( $\pm$ 4.1) ng/ml ( $p$ <0.05), TNF- $\alpha$  8.1 ( $\pm$ 1.8) ng/ml ( $p$ <0.01), CCL4 2.2 ( $\pm$ 0.6) ng/ml ( $p$ <0.05) and CCL2 492.2 ( $\pm$ 263.9) pg/ml ( $p$ <0.05). Although IL-2, -4, -17, IFN- $\gamma$  and CXCL-10 (IP-10) were significantly increased in CoMTb (all  $p$ <0.01) their overall concentrations remained very low. IL-1Ra, IL-10, CCL3 and CCL5 levels did not change significantly in response to *M.tb* stimulation.

### **Pertussis toxin does not affect MMP-1 secretion**

As a number of important chemokines had been identified in the Luminex analysis of CoMTb, cells were pre-incubated with pertussis toxin, a GPCR inhibitor that inhibits chemokine signalling. No change was seen in MMP-1 secretion with pertussis pre-incubation (Figure 16).

In addition recombinant oncostatin M (a member of the IL-6 family), and IL-6 neutralising antibody pre-incubation with CoMTb did not affect MMP-1 secretion (data not shown). IL-12 and 18 were not investigated.

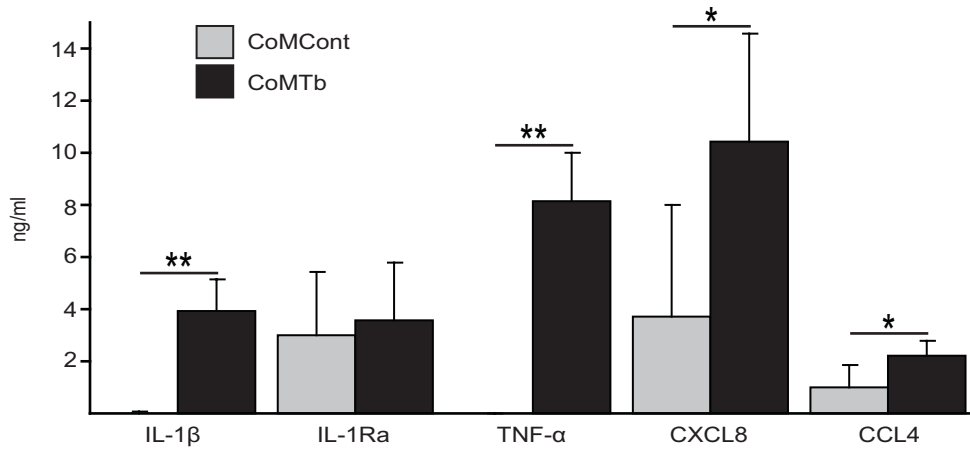


Figure 15. Analysis of cytokine and chemokine concentrations in CoM in ng range. CoMCont (n=4 donors) and CoMTb (n=6) were analysed by Luminex for proinflammatory molecules. Those in ng concentrations are presented here. Bars represent mean values  $\pm$  SD. Data were analysed by Student's t-test. \* $p < 0.05$ , \*\* $p < 0.01$ .

Table 5. Analysis of cytokine and chemokine concentrations in CoMCont and CoMTb in pg range.

| Analyte*          | CoMCont       | CoMTb         |
|-------------------|---------------|---------------|
| IL-2 (pg/ml)**    | 0.5 (0.2)     | 5.6 (1.8)     |
| IL-4 (pg/ml)**    | 0.3 (0.2)     | 4.2 (0.9)     |
| IL-10 (pg/ml)     | 0.4 (0.1)     | 63.7 (99.9)   |
| IL-17 (pg/ml)**   | 3.4 (1.7)     | 30.4 (6.3)    |
| IFN-g (pg/ml)**   | 6.0 (4.8)     | 156.5 (45.6)  |
| CXCL-10 (pg/ml)** | 5.3 (1.6)     | 33.2 (12.5)   |
| CCL2 (pg/ml)**    | 92.6 (82.2)   | 492.2 (263.9) |
| CCL3 (pg/ml)      | 129.8 (232.3) | 363.4 (119.6) |
| CCL5 (pg/ml)      | 89.7 (104.6)  | 206.6 (176.2) |

\* Values presented as mean (sd)

\*\*  $p < 0.01$  for group comparison

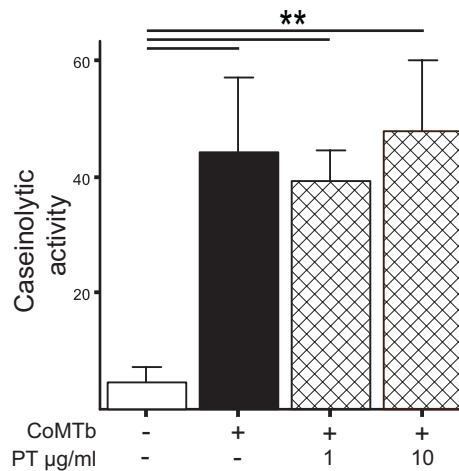


Figure 16. Monocyte-microglial cell signalling does not require GPCR. Pertussis toxin (PT) does not suppress CoMTb-stimulated MMP-1 secretion. Microglial cells were preincubated with PT at 1 or 10  $\mu$ g/ml for 2 h and stimulated with CoMTb. Bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. Data were analysed by one-way analysis of variance, followed by Tukey's multiple comparison. \*\* $p < 0.01$ .

## **NF-κB and AP-1 regulate CoMTb-induced microglial MMP-1 promoter activity and secretion**

MMP-1 but not TIMP-1 or TIMP-2 contains an NF-κB consensus site in its promoter which may account for the differential regulation of gene expression observed [324]. To investigate the role of NF-κB in microglial MMP-1 secretion in response to CoMTb, kinetic analysis of the DNA binding activity of the NF-κB subunits p50, p65, RelB, c-Rel and p52 in nuclear extracts was performed (Figure 17A-E). At 1 hour post stimulation with CoMTb there was a 2.0 fold increase in p50 DNA binding compared to CoMCont stimulated cells, with maximal activity seen at 4 hours. CoMTb induced a 3.1 fold increase in p65 DNA binding at 1 hour. RelB reached a maximal 2.0 fold increase in DNA binding at the 4 hour time point. No change in the activity of c-Rel or p52 was observed. A sustained increase in DNA binding of p50, p65 and RelB subunits was still observed at the 24 hour timepoint, especially for the RelB subunit.

Nuclear p65 kinetics was further investigated in a short timepoint kinetic experiment (Figure 18A). This demonstrated that p65 was detectable in the nucleus by 10 minutes and reached a peak at 30 minutes with sustained activity over 4 hours as previously seen. NF-κB activity is normally regulated by cytoplasmic IκB proteins. Degradation of cytoplasmic IκBα (Figure 18B) occurred by 10 minutes in CoMTb stimulated cells, with resynthesis by 60 minutes. CoMCont did not affect IκBα degradation. Helenalin, an irreversible p65 alkylator, blocked nuclear translocation of p65 and p50 to below basal levels (Figure 18C), suggesting p65-p50 heterodimers are involved in MMP-1 mRNA expression. The importance of the NF-κB pathway was confirmed by

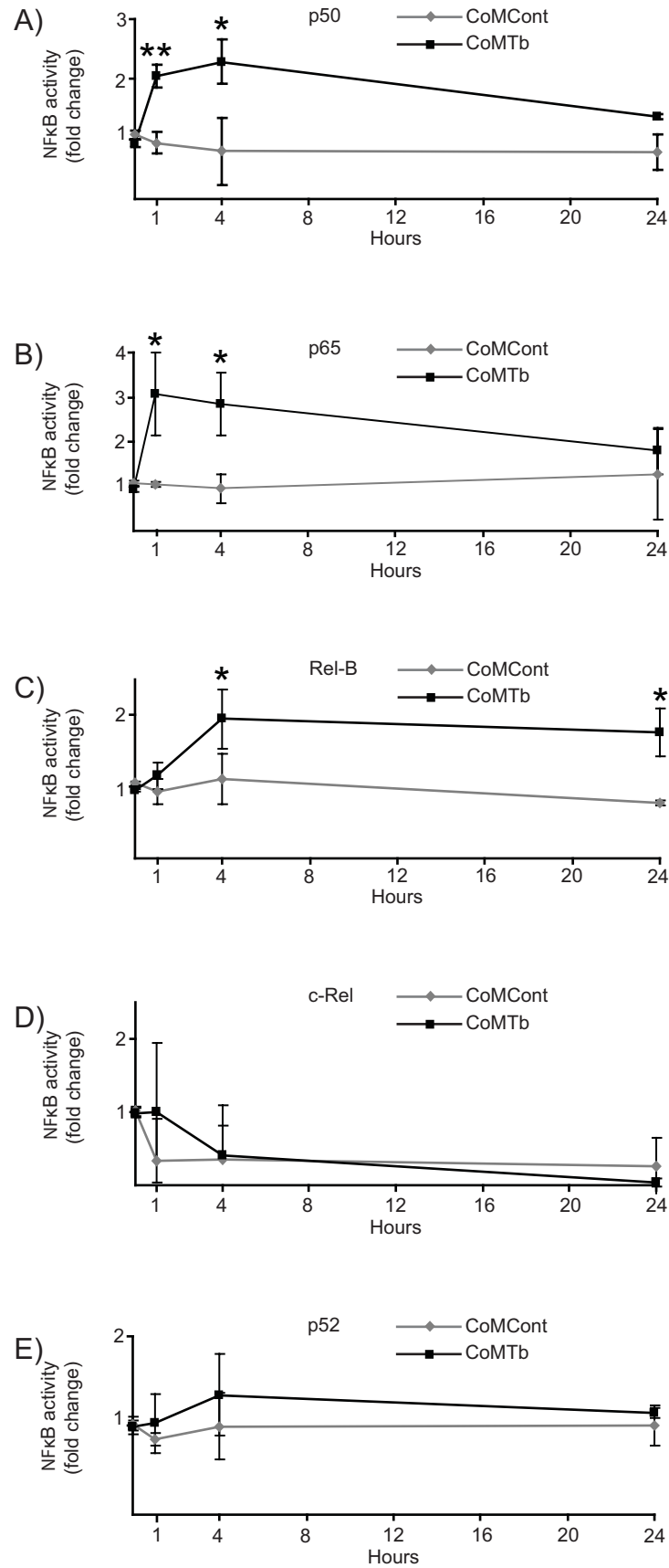


Figure 17. NFkB DNA binding activity in response to CoMTb. Microglial cells were stimulated by CoMCont (grey lines) and CoMTb (black lines). Nuclear extracts were analysed by ELISA for NFkB subunits. A, CoMTb stimulates maximal nuclear p50 binding at 4 hours. B, p65 is maximal at 1 h. C, Nuclear Rel B is sustained beyond 24 hours. D & E, Nuclear cRel and p52 do not change in response to CoMTb stimulation. The y-axis represents DNA-binding activity presented as OD normalised to CoMCont 0 hrs. Each point represents mean  $\pm$  SD of data from three independent experiments. Data at each point were analysed by Student t-test. \*p<0.05, \*\*p<0.01.

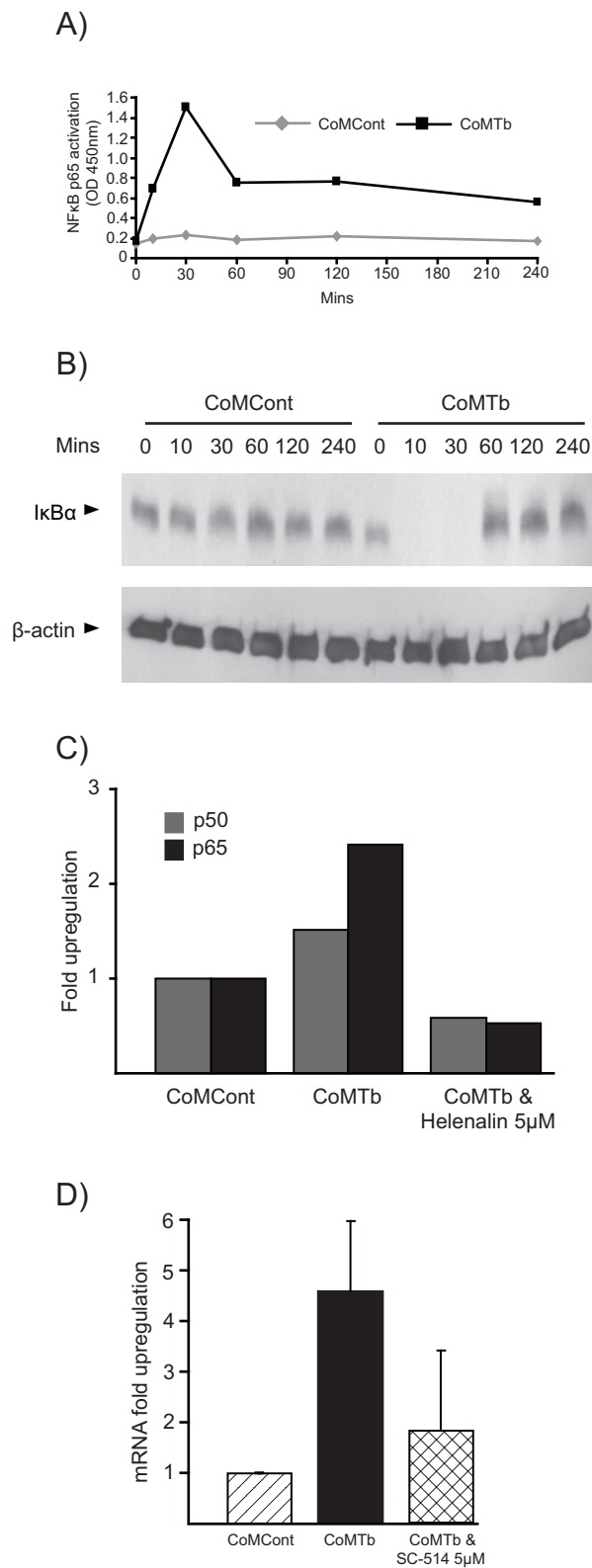


Figure 18. NFkB activation in CoMTb-mediated MMP-1 secretion by microglial cells. A, Nuclear p65 is maximal at 30 mins. Short kinetics of nuclear p65 binding analysed by ELISA. One representative experiment from two similar is shown. B, Representative Western blot of three experiments demonstrating the kinetics of cytoplasmic IκBα degradation after stimulation of microglia with either CoMCont or CoMTb at 10, 30, 60, 120 and 240 mins. β-actin Western blot to show equal loading. C, Analysis of nuclear extracts showing p50 (grey bars) and p65 (black bars) DNA-binding activity after preincubation with 5μM helenalin. D, MMP-1 gene expression is suppressed by NFkB inhibition. Microglia were preincubated with SC-514 5μM for 2 h. mRNA from microglia stimulated for 24 h was analysed by RT-PCR, normalised to 18S RNA and expressed as fold change relative to 24 h CoMCont mRNA. The mean ± SD from three experiments are shown. Data were analysed by one-way analysis of variance, followed by Tukey's multiple comparison.

using SC-514 (Calbiochem) an IKK-2 inhibitor and showing suppression of CoMTb induced MMP-1 mRNA expression (Figure 18D) similar mRNA results for helenalin were observed (data not shown).

To investigate the functional importance of the increase in nuclear p65 cells were pre-incubated with helenalin or SC-514 and MMP-1 secretion was measured (Figure 19A & B). In a dose dependent manner both compounds reduced MMP-1 secretion to basal levels. No change in cell viability was observed in these experiments as measured by trypan blue exclusion.

Since IL-1 $\beta$  was found to be important in CoMTb mediated up-regulation of MMP-1 secretion the role of the IL-1 $\beta$  stimulated transcription factor AP-1 was investigated by performing DNA binding experiments for the JunB, cJun, JunD, FosB, cFos, Fra-1 and Fra-2 subunits. At the 1 hour time point a 111.5% rise in cJun ( $p < 0.05$ ) and 83.4% rise in FosB ( $p < 0.05$ ) DNA binding was seen over CoMCont stimulated cells (Figure 20A). The 6 hour timepoint was also analysed and showed very similar results (Figure 20B) although the differences were not significant because of wide confidence intervals.

The full length wild type MMP-1 promoter (4372 bp up stream of transcription start site) and six 5' deletion constructs were transiently transfected into microglial cells linked to a luciferase reporter to analyse promoter dynamics in response to CoMTb stimulation (Figure 21A for promoter schematics). Kinetic experiments were performed with the full length promoter (Figure 21B). CoMTb increased promoter activity above CoMCont levels by 1.6-fold at 10 h, 2.0-fold

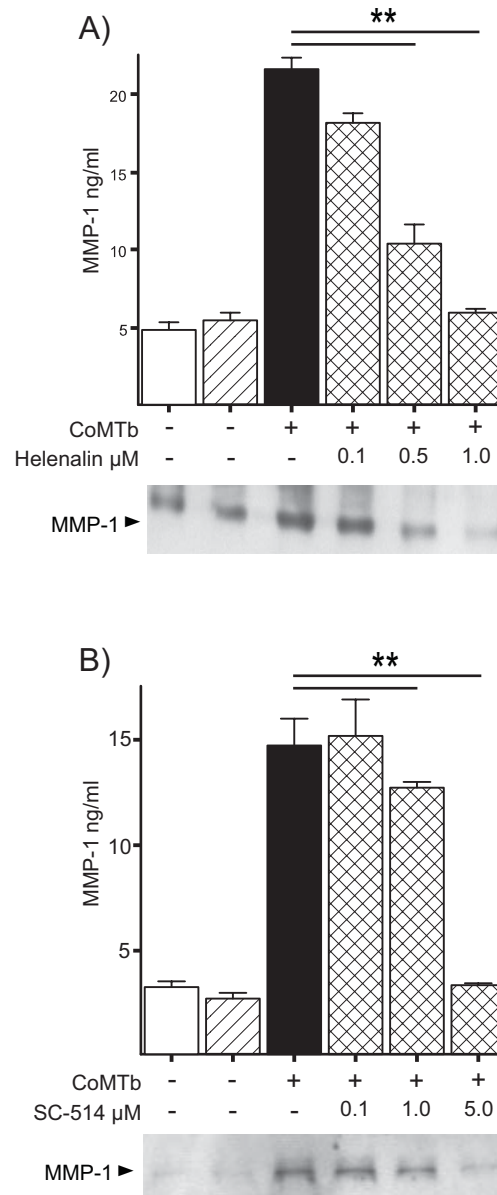


Figure 19. NFkB blockade suppresses MMP-1 secretion. Following a 2 h preincubated with either A, SC-514 or B, helenalin microglia were stimulated with CoMTb. Cell culture medium was analysed by Luminex and confirmed by Western blotting. Bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. Data were analysed by one-way analysis of variance, followed by Tukey's multiple comparison. \*\* $p < 0.01$ .

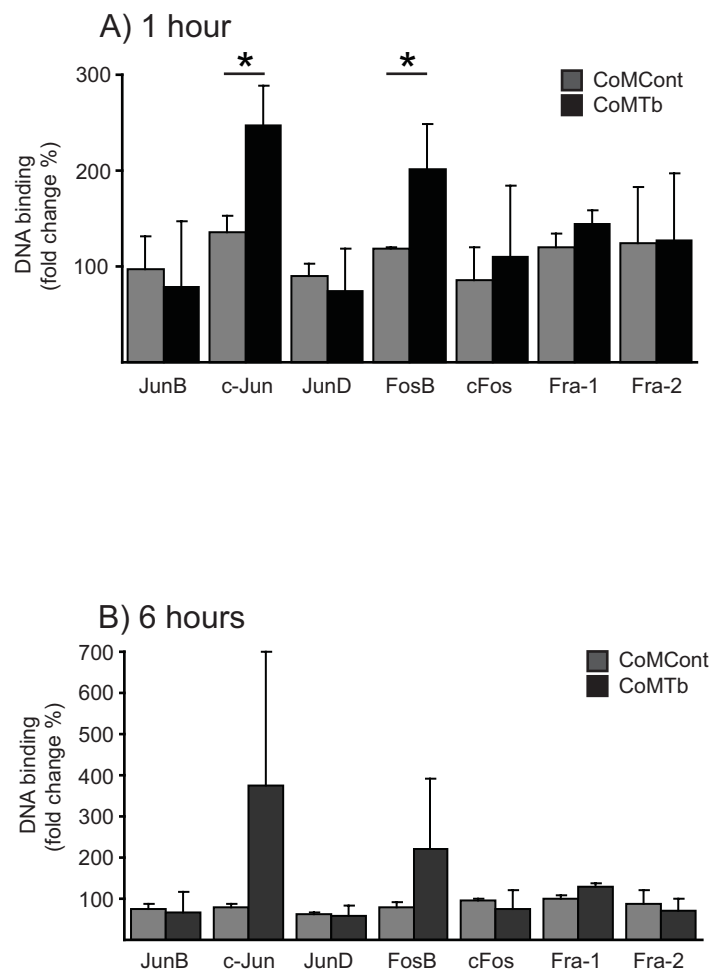


Figure 20. AP-1 DNA-binding activity in response to CoMTb. Microglia were stimulated with CoMCont (grey bars) or CoMTb (black bars) at A, 1 hour and B, 6 hours. Nuclear extracts were assayed for JunB, c-Jun, JunD, FosB, c-Fos, Fra-1 and Fra-2 activity using the TransAM AP-1 family kit. The y-axis represents percentage increase in DNA-binding activity over 0 h value. Each bar is mean  $\pm$  SEM of data from three independent experiments. Data were analysed by Student's t-test. \* $p < 0.05$ .

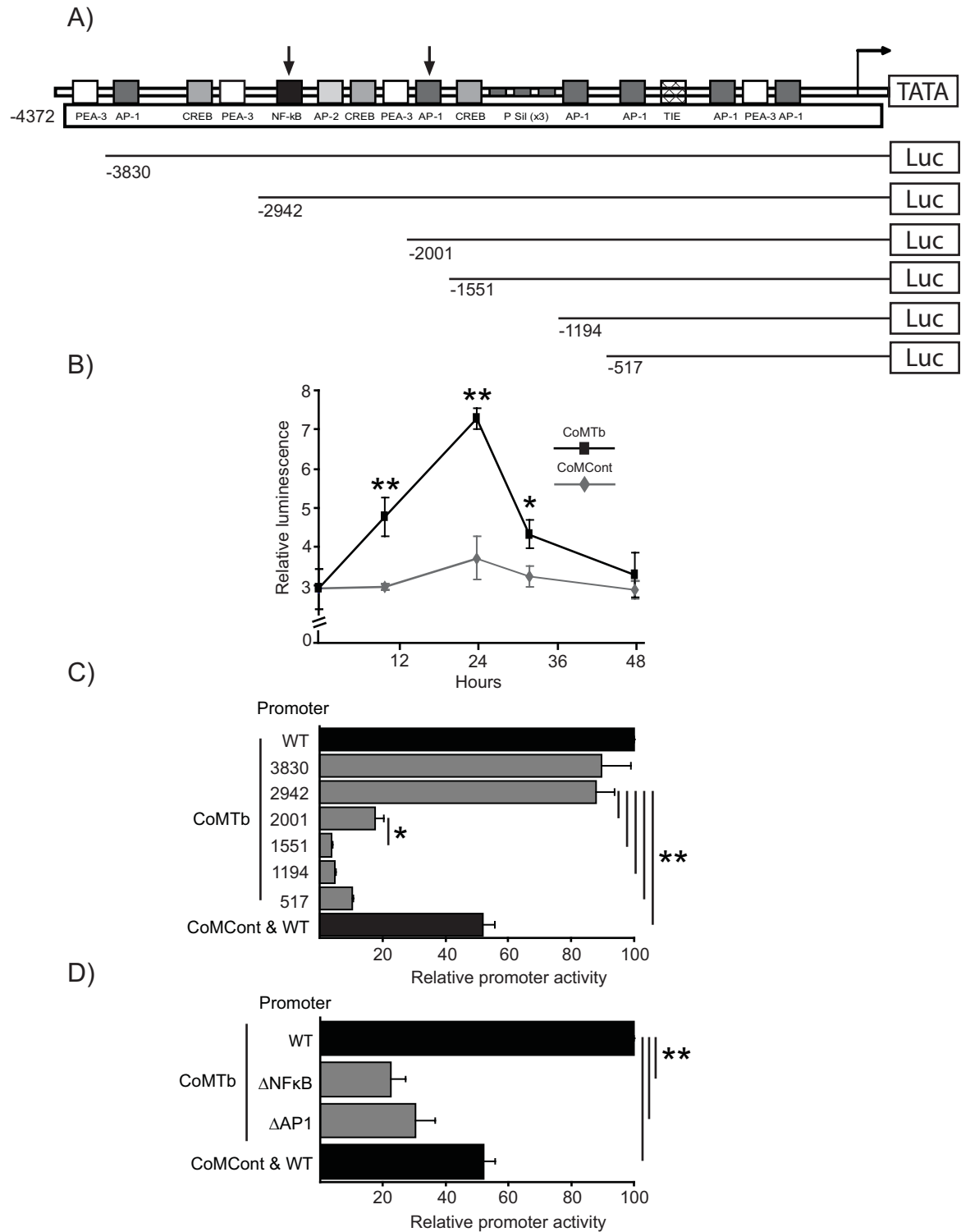


Figure 21. Kinetics of MMP-1 promoter activation and the effect of serial truncations and mutation constructs of the MMP-1 promoter on activity in CoMTb stimulated microglial cells. A, Schematic representation of the full-length (WT) MMP-1 promoter region and MMP-1 promoter constructs truncated by serial deletions (at -3830, -2942, -2001, -1551, -1194 and -517bp). Constructs had been inserted into the firefly luciferase (LUC) expression plasmid pGL3. B, MMP-1 promoter kinetics. Microglial cells were transiently transfected with the WT MMP-1 promoter and stimulated with CoMCont (grey line) or CoMTb (black line). Firefly luciferase activity, normalised to Renilla luciferase activity, was analysed over 48 h. C, MMP-1 promoter deletion constructs. Microglial cells were transfected with WT MMP-1 promoter expression plasmid (black bars) or deletion constructs (grey bars) and stimulated with CoMCont or CoMTb for 24 h and cell extracts analysed for luciferase activity, normalised to Renilla luciferase activity. CoMTb stimulated WT MMP-1 promoter was designated as maximal luciferase activity (100%) and all other results were expressed as a percentage of this maximum. D, MMP-1 promoter mutation constructs. Microglial cells were transiently transfected with full-length WT promoter (black bars) or mutation constructs of an NFkB or AP-1 site (grey bars), stimulated and analysed as per C. Bars represent the mean  $\pm$  SD from three samples and represent at least two separate experiments performed in triplicate. \* $p < 0.05$ , \*\* $p < 0.01$ .

at 24 h, and 1.33-fold at 32 h thus the 24 hour timepoint was used in subsequent experiments. Then the series of 5' deletion constructs showed a drop in promoter activity between -2942 and -2001 bp upstream of the transcription start site (Figure 21C). Using mutation constructs of the known NF-κB and one of the six known AP-1 consensus sites in the wild type promoter (Figure 21A arrows) abolition of CoMTb stimulated promoter activity to 22.5% ±4.7 (p<0.01) and 30.4% ±6.4 (p<0.01) of wild type activity was seen (Figure 21D).

### **Immunohistochemistry for p65 and cJun**

To confirm the *in vivo* relevance of our cellular findings immunohistochemistry was performed on CNS TB and appropriate positive and negative control brain biopsies for p65 and cJun. Tissue associated microglia were immunoreactive for cytoplasmic p65 (Figure 22A), with granuloma associated microglia/macrophages demonstrating strong nuclear p65 immunoreactivity (Figure 22B arrow, enlarged in C). Some lymphocytes and capillary endothelial cells, but not cerebellar granular layer cells, were immunoreactive for p65 in CNS TB (Figure 22A). Neither astrocytes, neurons nor areas of necrosis were immunoreactive for p65. In positive control specimens only capillary endothelial cells were immunoreactive for p65. There were numerous Iba-1 positive activated microglia in these positive control specimens which were not immunoreactive for p65 (data not shown).

Some cJun immunoreactivity was seen in the cytoplasm of tissue associated microglia (Figure 22D & E). However, there was strong nuclear cJun immunoreactivity in microglia/macrophages surrounding the central area of

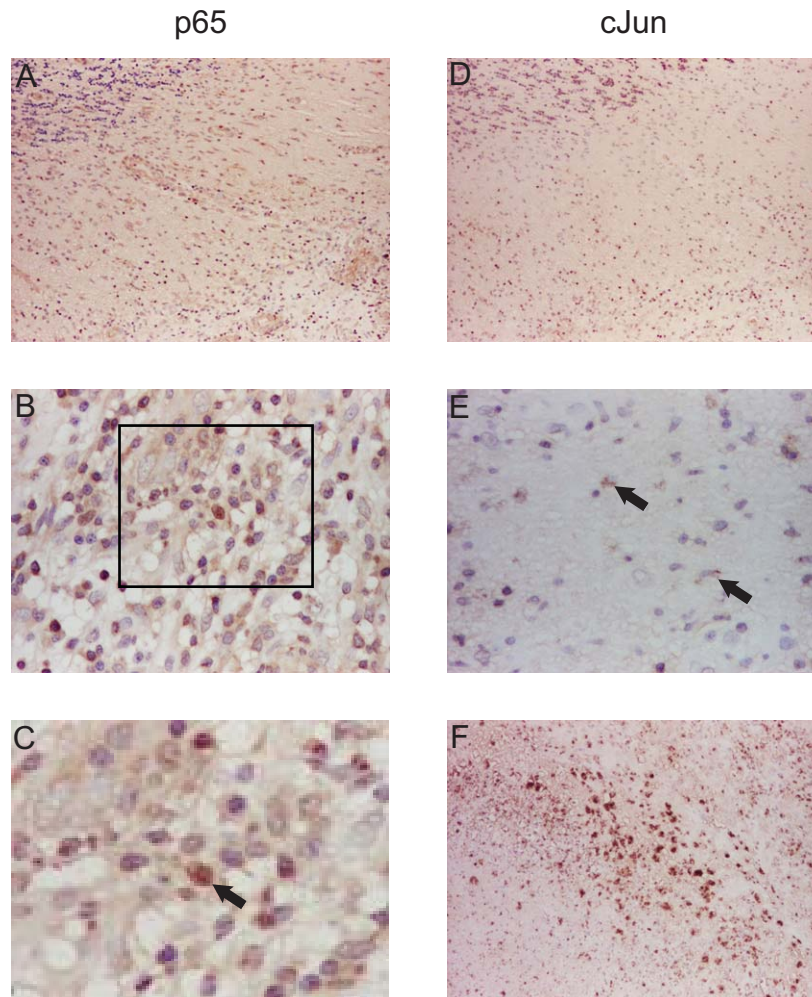


Figure 22. Microglia express p65 and cJun in patients with CNS TB. Brain biopsies from five patients with CNS TB and two positive and negative controls were immunostained for p65 and cJun as described in materials and methods chapter. Areas of immunoreactivity appear as brown against the blue counterstain. Boxed area in B indicates the region magnified in C. *Left: p65. Right: cJun.* A, microglial cells adjacent to TB granulomas are immunoreactive for p65 in cytoplasm (original magnification 10x). B and C (original magnification 40x), Nuclear p65 staining (arrow) is seen in granuloma associated microglia/macrophages. D, cJun immunoreactivity (original magnification 10x). E, Cytoplasmic cJun immunoreactivity (arrow) is seen in microglia adjacent to granulomas (original magnification 40x). F, Granuloma associated microglia/macrophages demonstrate strong nuclear cJun reactivity (original magnification 20x). The negative control is shown in Figure 11E. Representative sections from five CNS TB biopsies are shown.

necrosis in TB granulomas (Figure 22F). In summary increased p65 and cJun immunoreactivity was observed in a gradient from parenchymal brain tissue surrounding granulomas with cytoplasmic staining, increasing towards the centre of the granuloma where staining was nuclear and principally in microglia/macrophage cells.

## **Discussion**

In this comprehensive study of MMP and TIMP gene expression in human microglia I have demonstrated that *M.tb* networks potently increased MMP-1, -3, -9 expression and moderately increased MMP-8 and -10 expression. CoMTb also suppressed MMP-24 expression significantly and MMP-2 moderately. Direct infection with *M.tb* appeared to affect cells very little with a rise in MMP-27 the only significant finding. A number of MMPs were not detected with any of the stimuli suggesting that the spectrum of MMP expression observed is a TB specific finding in microglia. MMP-1 is a potent collagenase and was up-regulated with both the stromelysin MMP-3, capable of activating MMP-1, and the gelatinase MMP-9, able to degrade collagen breakdown products of MMP-1. Thus this suggests that the host CNS response to *M.tb* infection is driven by infiltrating infected monocytes and that the individual microglial MMPs expressed represent a complete spectrum of enzymes necessary for extensive tissue destruction. As noted in table 3 these three MMPs have a number of substrates specific to the CNS: MMP-1 degrades molecules such as brevican and tenascin, MMP-3 and 9 can degrade myelin and tight junction proteins, MMP-1 and 3 are both pro-apoptotic to neurons [268, 328]. In addition TIMP -1 expression remained unchanged and TIMP-2 to -4 were all reduced, suggesting a net shift toward a matrix degrading environment in CNS TB.

Increased gene expression by CoMTb drove high level secretion of MMP-1, confirmed in kinetic and dose response experiments. A moderate dose dependent increase in MMP-1 secretion was also seen with direct *M.tb* infection (4.6 fold increase). Kinetic investigations showed that MMP-1 gene expression was rapidly up-regulated by CoMTb. The fold up-regulation values for these experiments (Figure 8C) are higher than those in the global analysis (Figure 6) because they were normalised to a time 0 control, whereas the global analysis was normalised to 24 hour CoMCont MMP-1 mRNA levels.

There are no published reports to compare these data to directly. However, Nuttal *et al* looked at MMP responses in microglia from human adult brains and described LPS responses in detail [263]. MMP-1, -3, -8, -10 and -12 rose significantly after LPS stimulation. There was an accompanying fall in MMP-2, -15, -16 and -21. No further data on the intracellular mechanism is presented but the authors comment that TNF-α and IL-1β concentration also rise. Studies performed in rat microglia confirm that LPS up-regulated MMP-1, -3 and -8 expression is a cross species response to LPS [329]. Elkington *et al* investigated MMP expression in *M.tb* infected macrophages and found significant elevation of MMP-1, -3, -7, -10 mRNA, as well as a non-significant rise in MMP-8. Although not statistically significant I also found a 6.2 fold rise in MMP-8, suggesting that MMP-1, 3, 8 and 10 up-regulation might be a monocyte lineage specific response to a number of inflammatory stimuli.

TIMP gene expression was either not affected (TIMP-1) or decreased by CoMTb (TIMP-2 to -4). Secretion data was analysed for TIMP-1 and -2 and

reflected the expression data. Thus in CNS TB MMPs are produced in excess of the TIMPs. Loss of neuronal TIMP staining in biopsies from CNS TB patients has been reported and macrophages do not up-regulate TIMPs in response to *M.tb* but there is no data in the literature regarding microglial TIMP secretion in CNS TB [221, 236].

Direct infection did not up-regulate MMPs in microglia to the same extent as CoMTb. Only MMP-27 expression was increased and the qRT-PCR C<sub>T</sub> value for this MMP was very low suggesting up-regulation was moderate and from a very low baseline level. Therefore I confirmed that *M.tb* was associating with microglial cells by Kinyoun staining of chamber slides. Although cell associated *M.tb* was lower in microglia than less heavily infected monocytes the rates were comparable to the literature. Rock *et al* reported that 76% of human foetal microglia were infected by H37Rv *M.tb* [156]. The lower rate reported in this work may be because the microglia were a transformed cell line that have previously been reported to have a lower phagocytic ability than primary cells [316]. Confocal microscopy confirmed that cell associated *M.tb* was intracellular in many cells, but quantitative analysis was not carried out. Whether this means that resident microglia are less good at being professional phagocytes is unclear and warrants further work in primary cells or *in vivo* models. If this were the case it would mean that monocyte infiltration is necessary for an efficient host response in the CNS to *M.tb*. Therefore therapies based on tightening the BBB might be detrimental to patients with CNS TB by excluding monocytes but this remains speculative. Additionally the lack of MMP-1 secretion in response

to *M.tb* driven astrocyte (CoATb) and microglial (CoMicTb) networks supports a central role for the infiltrating monocyte.

The cellular model predicted high level MMP-1 expression in CNS TB patients at the site of tissue destruction and this was observed in clinical biopsy specimens. Microglial markers and MMP-1 co-localised in both brain parenchyma and granulomas suggesting that microglia, as the effector cells of the CNS immune response, may be driving CNS tissue destruction by secretion of MMP-1.

Next I sought to unpick the mediators in CoMTb that were driving MMP-1 secretion by taking a global approach to analysis. Fractionation revealed all the activity to be due to molecules >10 kDa. TNF-α and IL-1-β were proven to be necessary, but not sufficient, to reproduce the effect of CoMTb in driving MMP-1 secretion. Adding back TNF-α and IL-1-β at concentrations in excess of those in CoMTb was necessary to restore MMP-1 secretion to CoMTb levels. TNF-α is known to stimulate MMP-1 secretion from a number of different cell types, but often seems to need co-stimulation with another molecule. Elkington *et al* reported that pulmonary epithelial cells required additional cholera toxin (a GPCR stimulus used as a chemokine surrogate *in vitro*) to up-regulate MMP-1 secretion [204]. Zhou *et al* similarly suggested that monocytes required additional GM-CSF and O'Kane *et al* Oncostatin M in fibroblasts [186, 252]. IL-1-β, however, can stimulate MMP-1 secretion alone in rabbit synovial fibroblasts or gastric epithelial cells [325, 330]. I did not find this to be the case as IL-1-β only stimulated MMP-1 secretion when combined with TNF-α. Both these

cytokines are key inflammatory molecules and have been implicated in the aetiology of other diseases with extensive tissue destruction such as rheumatoid arthritis. However, TNF- $\alpha$  is vital for sustaining the host response to latent *M.tb* infection and therefore therapies reliant upon TNF- $\alpha$  blockade in CNS TB might run the risk of exacerbating infection. Indeed a randomised placebo controlled trial of thalidomide in paediatric TBM showed increased adverse events in the treatment arm and so was terminated early [47].

CoMTb is a complex mixture of host and pathogen derived factors so the small (albeit non-significant) increase in MMP-1 secretion with addition of Tb supernatant to TNF- $\alpha$  (Figure 12D) is of interest. *M.tb* is known to stimulate a number of innate signalling pathways, such as TLRs and mannose receptors. Thus stimulating cells with the combination of TNF- $\alpha$ , IL-1- $\beta$  and Tb sup together may have further increased MMP-1 secretion. Investigating TLR2 pathways involving LAM might be fruitful too as LAM has been shown to up-regulate MMP-9 secretion in monocytic cell lines [256].

The lack of suppression of MMP-1 secretion with pertussis toxin, a 7-membrane span GPCR blocker (e.g. chemokines receptors), suggests that the chemokines up-regulated by *M.tb* in CoMTb, such as CXCL8 and CCL2-5 are unlikely to be important factors in microglial MMP-1 secretion. This is confirmed by the fractionation data (only molecules >10 kDa are important for MMP-1 secretion) as most chemokines are about 8 kDa in size. I also measured a number of cytokines typically produced by T-cells which were at very low (pg) levels in CoMTb, reflecting the purity of the monocyte preparation. Therefore I cannot

comment on the role of other cytokines central to the formation and maintenance of TB granulomas in this model. For example IFN-γ can modulate the secretion of MMPs [252, 315].

As TNF-α and IL-1-β were found to be central in CoMTb stimulated MMP-1 secretion the NFκB pathway was carefully examined. This demonstrated that the canonical pathway involving p65-p50 heterodimer activation of MMP-1 gene transcription is critical for MMP-1 secretion. First nuclear extracts were analysed for all five NFκB subunits but only p50, p65 and Rel-B were enhanced by CoMTb. This was accompanied by rapid IκBα degradation as has been found in other model systems [221]. NFκB has already been described as an important transcription factor for MMP-1 secretion in fibroblasts, where an IκB mutant abrogated secretion [325]. Helenalin, a sesquiterpine lactone that irreversibly alkylates p65 thereby sequestering it in the cytoplasm, blocked both p65 and p50 translocation into the nucleus suggesting p65-p50 heterodimers are central to control of gene transcription. These data were confirmed by using a specific IKK2 inhibitor SC-514 [331]. This molecule slows p65 translocation into the nucleus and increases p65 nuclear egress. In this model it caused a dose dependent reduction in MMP-1 expression and secretion. The alternative NFκB pathway uses p52 subunit dimers derived from proteolytic cleavage of the p100 precursor combined with cRel or RelB. RelB has been shown to be involved in the regulation of antigen presentation function of DCs. Here in microglial cells RelB was found to be up-regulated by CoMTb and is likely to have formed heterodimers with p50, as its other major dimer partner p52 was unchanged by CoMTb. The role of RelB in both TB immunology and MMP secretion is

unknown but *RelB* null mice are known to have defects in innate responses to pathogens such as *Toxoplasma gondii* [332]. There are few chemicals available to block RelB so its role in MMP secretion could not be ascertained further as techniques such as siRNA were not available. Immunohistochemistry demonstrated that p65 activation is a dynamic process *in vivo* too. Cytoplasmic p65 was observed in parenchymal microglia but as the centre of the granuloma was approached this became predominantly a nuclear phenomenon. The antibody used recognises the N-terminus of p65 unbound to IκB proteins so staining observed reflects activation of the NFκB pathway *in vivo*.

IL-1β is known to stimulate AP-1 activity and the MMP-1 promoter has six consensus AP-1 sites [324, 333]. Thus the DNA binding activity of the seven AP-1 subunits were analysed at one and six hours after CoMTb stimulation in nuclear extracts. Both time points demonstrated that c-Jun and FosB were elevated in microglial nuclear extracts. Data from other CoMTb stimulated cells suggests that the AP-1 response is cell specific. In addition to c-Jun and FosB pulmonary epithelial cells up-regulated cFos but in astrocytes the additional subunit is JunB [327, 334]. In microglia cFos was non-significantly raised but JunB fell. Although AP-1 is known to have a central role in LAM induced MMP-9 secretion but there are no published data on *M.tb* induced, AP-1 controlled MMP-1 secretion [335]. Therefore the observation that nuclear cJun is seen *in vivo* particularly in parenchymal microglia and strongly expressed in granuloma associated microglia/macrophages is a key finding from this work.

Promoter reporter analysis confirmed the central role of both NFκB and AP-1. Complete loss of promoter activity was observed with loss or mutation of the NFκB site at -2886 to -2878 bp. A distal AP-1 site at -1955 to -1945 bp was also found to be important, although the NFκB site is likely to be dominant. Similar evidence demonstrating loss of MMP-1 promoter activity with deletion of the NFκB site in primary fibroblasts suggested that it was principally an IL-1β responsive site [324]. TNF-α did not induce promoter activity but was necessary for MMP-1 secretion in synovial fibroblasts despite up-regulating nuclear translocation of NFκB. These data suggest that co-ordinated IL-1β and TNF-α stimulation is required for maximal MMP-1 promoter activity in keeping with the CoMTb data presented here.

In summary I have demonstrated that monocyte-dependent networks are more important than either direct *M.tb* infection or glial-dependent networks in driving MMP expression from human microglial cells. TIMPs were not up-regulated suggesting that *M.tb* drives uncoupled MMP secretion. In addition I have demonstrated that MMP-1 gene expression and secretion is mediated via IL-1β and TNF-α activating through NF-κB and AP-1 *in vitro* and *in vivo*. This suggests that in CNS TB infiltrating monocytes are central in driving MMP-mediated tissue destruction that is the hallmark of this disease. Nevertheless this MMP cannot act alone and MMP-3, a known MMP-1 activator, was up-regulated to the same extent as MMP-1.

## Chapter 4 – MMPs, MAP kinases and dexamethasone

### *Introduction*

In data presented in chapter 3 global analysis of all microglial MMPs/TIMPs in CNS TB suggested that MMP-1 and -3 are key. Data regarding the control of MMP-1 expression and secretion was then presented. In this chapter therefore I focus on the stromelysin MMP-3 as it has a different spectrum of substrates to MMP-1. To compliment the nuclear signalling studies presented in the preceding studies, the role of MAP kinases in differential control of MMP-1 and -3 secretion has also been investigated, as there are data that these kinase cascades, central to cellular stress activated responses, might be amenable to pharmacological manipulation in the future. Dexamethasone is used as an adjunct to anti-tuberculosis therapy in CNS TB, where it has been shown to reduce mortality but the exact mechanism is currently unclear [107]. As corticosteroids are known to affect MMP gene transcription directly the influence of dexamethasone on MMP-1, -3, TIMP-1 and -2 expression and secretion has also been investigated.

The data presented in this chapter demonstrates that monocyte-microglial networks are a greater stimulus than *M.tb* to MMP-3 expression and secretion. In addition p38 MAP kinase appears to be the critical intracellular cytoplasmic regulator of MMP-1 and -3 secretion. Dexamethasone differentially suppresses MMP-1 and -3 over TIMP-1 and -2 suggesting that these molecules may hold the key to the mechanism of dexamethasone action in treatment of CNS TB.

## **Results**

### ***M.tb* infection and monocyte-microglial networks drive MMP-3**

#### **secretion from human microglia**

Secretion of MMP-3 was analysed to investigate whether the increased gene expression (Figures 6 & 7) resulted in greater enzyme activity. *M.tb* up-regulated MMP-3 in a dose and cell-bacillus contact time dependent fashion to a maximum of 2.2 fold over control at an MOI of 10 ( $p<0.01$ , Figure 23A) at 72 hours. However, CoMTb drove MMP-3 secretion more potently (Figure 23B) with a 25.3 fold increase in secretion over CoMCont ( $p<0.01$ ) and 14.1 fold greater than *M.tb* ( $p<0.01$ ). Kinetic studies of MMP-3 expression (Figure 23C) demonstrated that mRNA had started to rise by 6 hours and peaked by 24 hours with a  $34.8 \pm 4.8$  fold up regulation of mRNA over time 0 control samples ( $p<0.01$ ).

### **Astrocyte-microglial and microglial-microglial networks do not drive**

#### **MMP-3 secretion**

As MMP-1 and -3 are often co-regulated I investigated whether *M.tb* acted via networks other than infiltrating monocytes in the model of CNS TB. CoATb, as well as control conditioned medium (CoACont), was prepared from primary human astrocytes from two donors but did not stimulate MMP-3 secretion (Figure 23D). Similarly CoMicTb did not up-regulate MMP-3 secretion above basal levels (data not shown). In addition cells were stimulated with LPS but they did not up-regulate MMP-3 secretion suggesting that MMP-3 secretion is a TB specific response in microglia (Figure 23E).

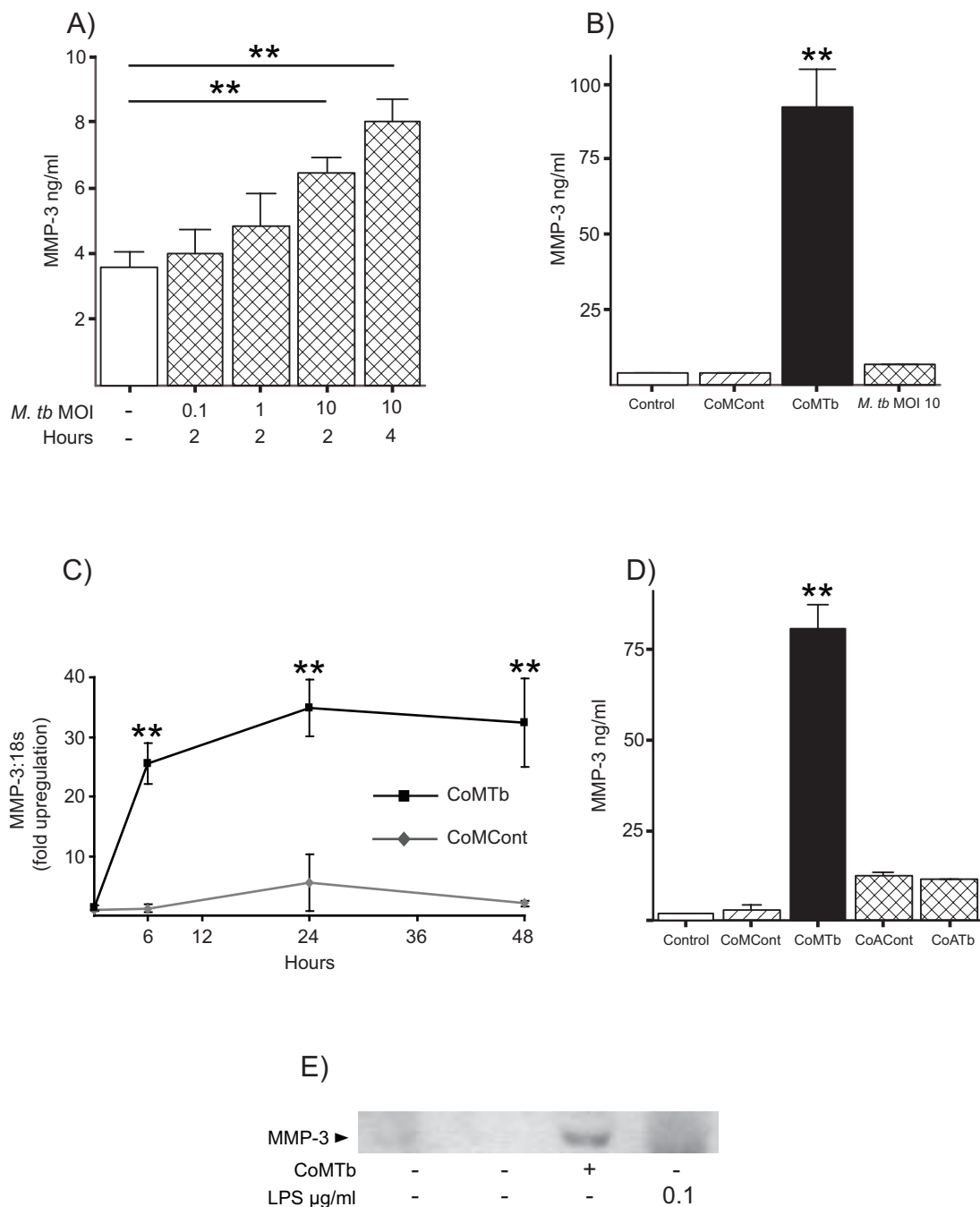


Figure 23. CoMTb drives MMP-3 gene expression and secretion in microglia more than *M.tb* and LPS. A, *M.tb* drives MMP-3 secretion. Microglial were incubated with *M.tb* in mid log growth phase for the multiplicity of infection (MOI) and time (h) indicated. *M.tb* was washed off and culture medium replaced. MMP-3 secretion at 72 h analysed by ELISA demonstrated MMP-3 secretion was up-regulated by *M.tb* in a dose and time dependent fashion. B, Microglial MMP-3 secretion at 72 h analysed by ELISA, demonstrated MMP-3 secretion was up-regulated far more by 1:5 CoMTb than 2 h stimulation with *M.tb* at MOI 10. C, Kinetics of MMP-3 gene expression. Microglia were stimulated with CoMCont and CoMTb and total RNA was extracted at 0, 6, 24 and 48 h. mRNA accumulation was analysed by RT-PCR. MMP-3 mRNA levels were normalised to 18S RNA and values are expressed as fold change relative to control mRNA levels at 0 h. Mean values  $\pm$  SD from three experiments are presented. D, Astrocyte-microglial networks are not important for MMP-3 secretion. *M.tb* infected primary human astrocyte condition medium (CoATb) and control medium (CoACont) were used 1:5 to stimulate microglial cells. MMP-3 secretion was analysed by ELISA. For all experiments, except RNA, bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. \* $p < 0.05$ , \*\* $p < 0.01$ . E, LPS does not drive MMP-3 secretion. Microglia were stimulated with CoMTb or LPS (0.1 µg/ml with 1% human serum) and 72 h cell culture medium was analysed by MMP-3 Western blotting.

**TNF- $\alpha$ /IL-1 $\beta$  synergy is responsible for microglial MMP-3 secretion**

Fractionation of CoMTb resulted in two aliquots <10kDa and >10kDa that was used to stimulate microglia. The >10kDa fraction up-regulated MMP-3 secretion to 37.9% more than the CoMTb effect ( $p < 0.01$ ) but the <10kDa fraction had no effect (Figure 24A). Preincubation of CoMTb and the >10kDa fraction with 1  $\mu\text{g/ml}$  anti-TNF neutralising antibody returned MMP-3 secretion to baseline levels. In complimentary experiments anti-TNF- $\alpha$  abolished the effect of CoMTb in a dose dependent manner (Figure 24B). However, stimulation with 100 ng/ml TNF- $\alpha$  resulted in  $5.2 \pm 0.6$  ng/ml MMP-3 (5% of CoMTb, Figure 24C). A small synergistic effect with the addition of TB supernatant to 100 ng/ml TNF- $\alpha$  alone was observed. Tb supernatant alone gave  $1.6 \pm 0.1$  ng/ml MMP-3, rising to  $9.1 \pm 1.3$  ng/ml with TNF- $\alpha$  (Figure 24C).

Next the role of IL-1 $\beta$  in MMP-3 secretion was investigated. IL-1Ra abolished MMP-3 secretion in a dose dependent manner (Figure 25A) to  $20.6 \pm 2.7$  ng/ml (89.6% reduction compared to CoMTb). Add back experiments with IL-1 $\beta$  alone did not increase secretion of MMP-3 over basal levels (Figure 25B). However, when 1 ng/ml IL-1 $\beta$  was used to stimulate cells with 10 ng/ml TNF- $\alpha$  (similar concentrations of these cytokines found in the analysis of CoMTb in chapter 3) substantial synergy was seen (Figure 25C) with  $37.9 \pm 3.5$  ng/ml MMP-3 secreted (63.1% of CoMTb value,  $p < 0.05$ ). GPCR were blocked with pertussis toxin but there was no influence on MMP-3 secretion (Figure 26).

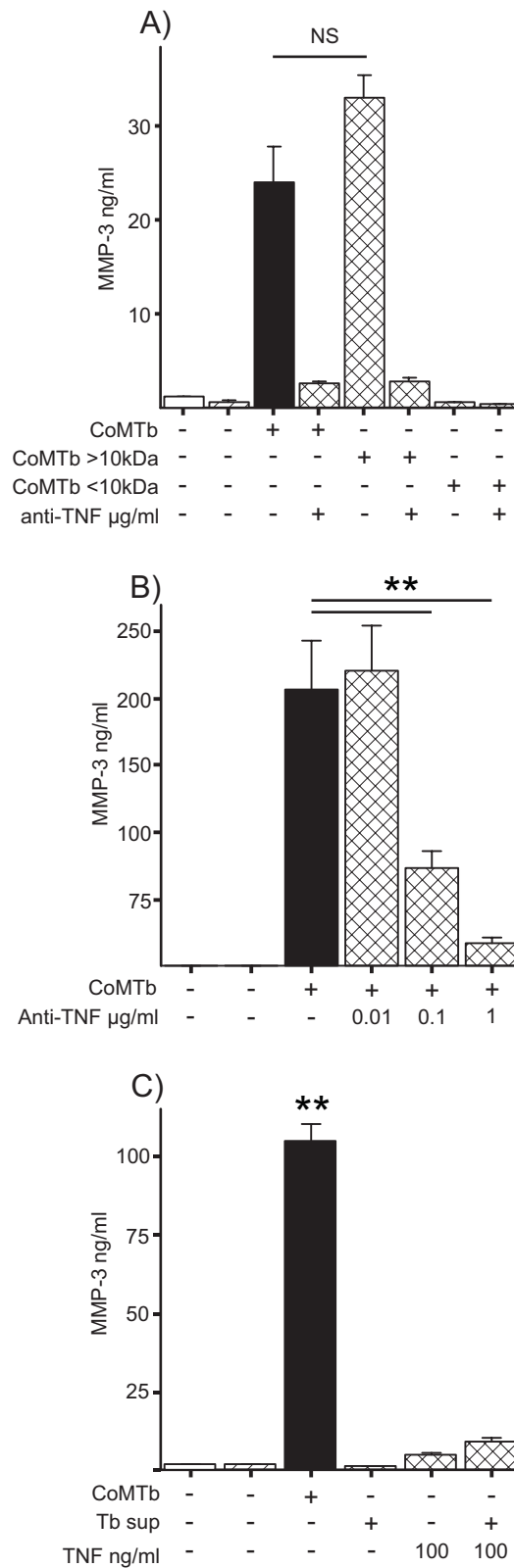


Figure 24. TNF- $\alpha$  is necessary but not sufficient for MMP-3 secretion. A, CoMTb was fractionated to produce aliquots of <10 kDa and >10 kDa fractions. Fractions or CoMTb were preincubated with anti-TNF- $\alpha$  Ab for 2 h where annotated. ELISA analysis of MMP-3 secretion by microglia 72 h post stimulation confirmed inhibition of TNF- $\alpha$  activity, contained in >10 kDa fraction, reduced MMP-3 secretion by CoMTb-stimulated microglia. B, CoMTb was preincubated with increasing doses of anti-TNF- $\alpha$  Ab for 2 h. C, TNF- $\alpha$  alone or in combination with Tb supernatant (Tb sup) is a weak stimulus of MMP-3 secretion. For all experiments bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. \* $p < 0.05$ , \*\* $p < 0.01$ .

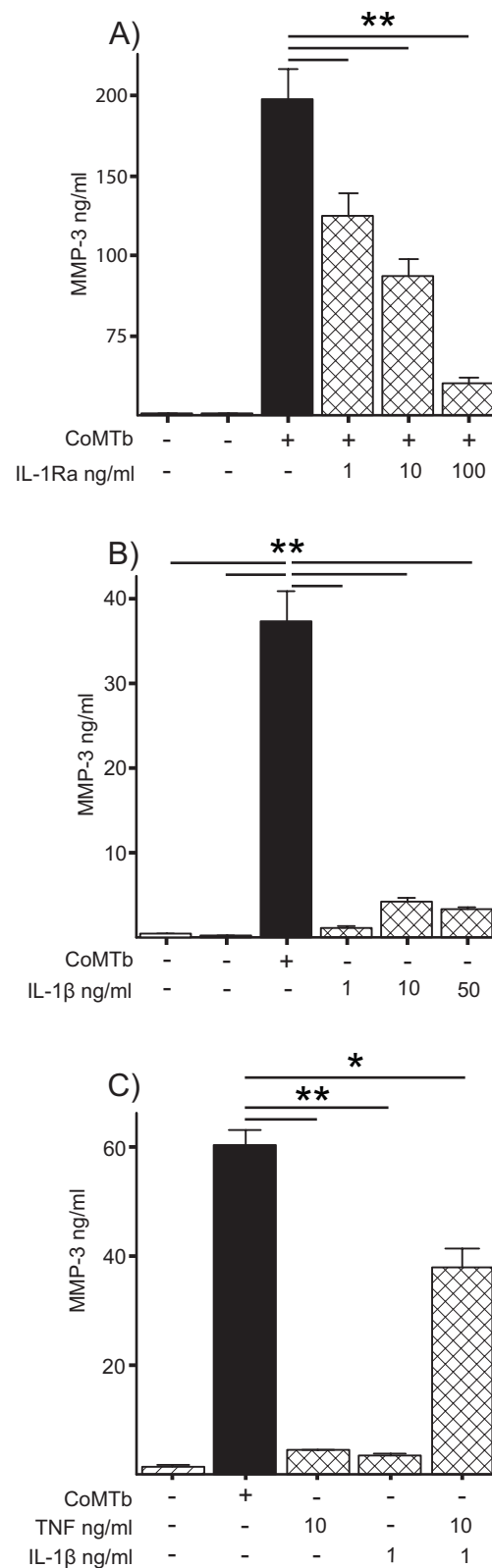


Figure 25. IL-1 $\beta$  synergises with TNF- $\alpha$  to drive MMP-3 secretion. A, Microglial cells were preincubated with IL-1Ra for 2 h. Inhibition of IL-1 $\beta$  activity reduced MMP-3 secretion measured by ELISA. B, IL-1 $\beta$  alone is not a stimulus to MMP-3 secretion. Microglial cells were stimulated with IL-1 $\beta$  at concentrations of 1–50 ng/ml. C, Microglia were incubated with TNF- $\alpha$  and IL-1 $\beta$  alone or in combination. The combined stimulus drove a greater concentration of MMP-3 than the summated values of TNF- $\alpha$  and IL-1 $\beta$  alone. For all experiments bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. Data were analysed by one-way analysis of variance followed by Tukey's multiple comparison. \* $p < 0.05$ , \*\* $p < 0.01$ .

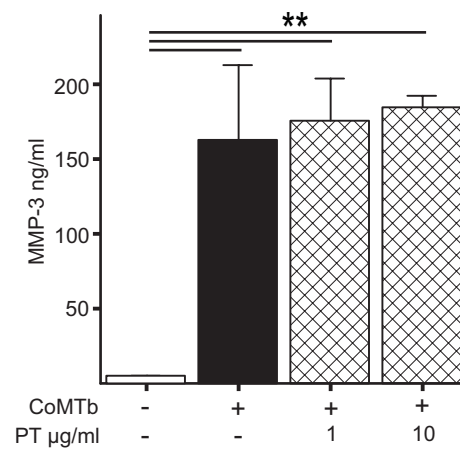


Figure 26. Monocyte-microglial cell signalling does not require GPCR. Pertussis toxin (PT) does not suppress CoMTb-stimulated MMP-3 secretion. Microglial cells were preincubated with PT at 1 or 10  $\mu\text{g/ml}$  for 2 h and then stimulated with CoMTb. Bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. Data were analysed by one-way analysis of variance, followed by Tukey's multiple comparison. \*\* $p < 0.01$ .

### **The NF- $\kappa$ B pathway regulates microglial MMP-3 secretion**

To investigate the role of NF- $\kappa$ B in microglial MMP-3 secretion in response to CoMTb chemical inhibition of IKK2 and p65 nuclear translocation was performed. SC-514 and helenalin blocked MMP-3 secretion in a dose dependent manner to basal levels (Figure 27A & B). At the level of gene expression a similar result was found for SC-514 (Figure 27C, helenalin data not shown but similar). No change in cell viability was observed in these experiments as measured by trypan blue exclusion.

Three 5' MMP-3 promoter deletion constructs linked to a luciferase reporter were available for transient transfection into microglial cells. This demonstrated that CoMTb increased the 2183 bp full length promoter activity above CoMCont levels by 3.4-fold at 24 hours (Figure 27C,  $p < 0.05$ ). Two 5' deletion constructs were also used. A significant rise in promoter activity was seen with the 1612 bp promoter compared to the full length promoter in response to CoMTb stimulation ( $p < 0.01$ ). Promoter activity was abrogated to CoMCont levels when the shorter 642 bp construct was stimulated with CoMTb. Unfortunately no further deletion constructs or point mutation constructs were available to conduct further analysis of these findings.

### **Analysis of serine/threonine kinases in microglia**

To dissect the intracellular signalling pathways that control MMP-1 and -3 secretion an analysis of MAP kinase pathways was performed. A serine/threonine kinase phosphorylation array using a commercial kit that targets a number of important pathways was used. At 30 minutes CoMTb

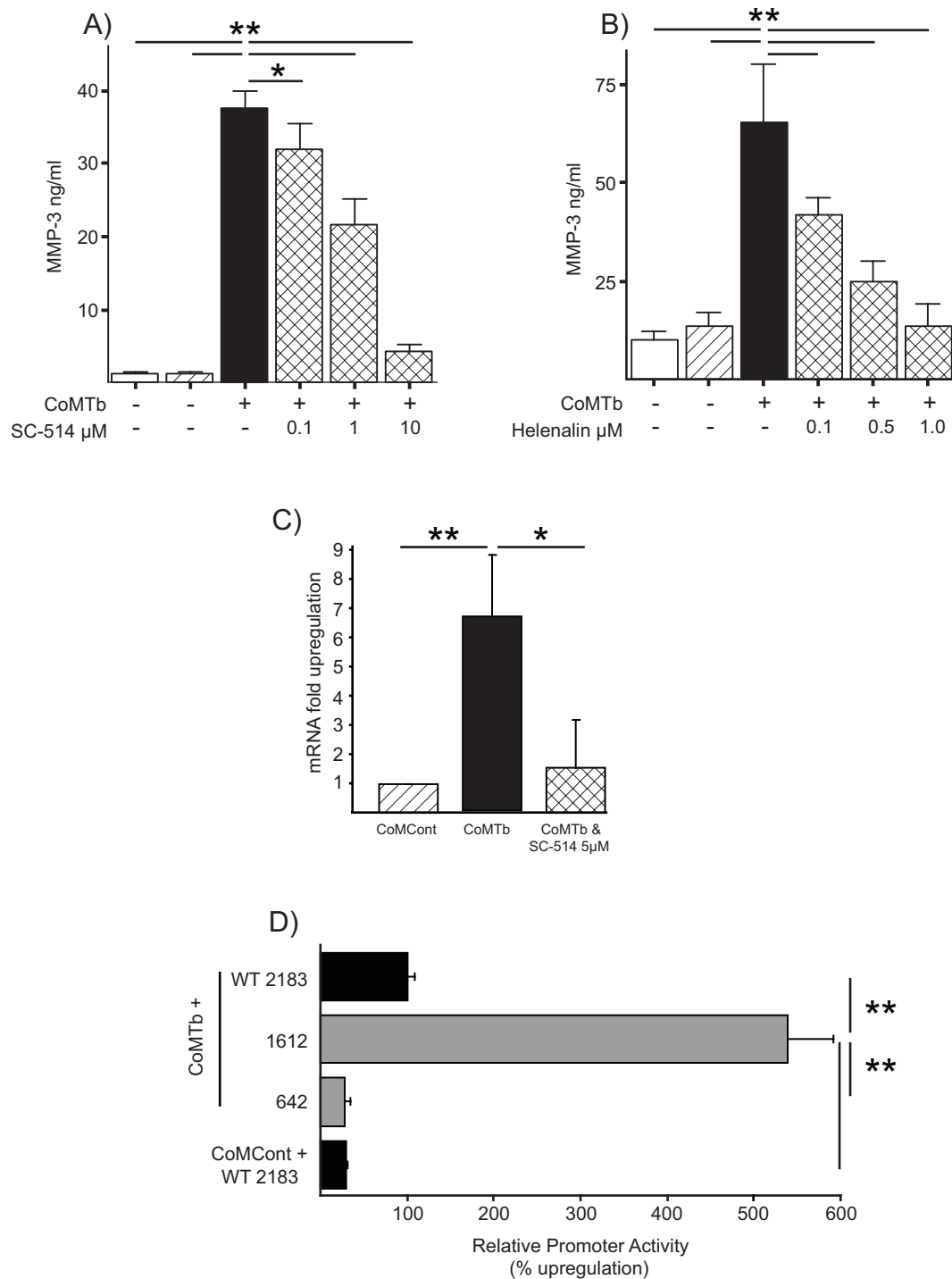


Figure 27. CoMTb driven nuclear signalling controls MMP-3 secretion. Microglial cells were preincubated with A, SC-514 and B, helenalin for 2 h before stimulation with CoMTb. Bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. C, MMP-3 gene expression is suppressed by NF $\kappa$ B inhibition. Microglia were preincubated with SC-514 5 $\mu$ M for 2 h. mRNA from microglia stimulated for 24 h was analysed by RT-PCR, normalised to 18S RNA and expressed as fold change relative to 24 h CoMCont mRNA. The mean  $\pm$  SD from three experiments are shown. D, There is a derepressor between -2183 and -1612 bp in the MMP-3 promoter. Microglial cells were transfected with WT MMP-3 promoter expression plasmid (black bars) or deletion constructs (grey bars) and stimulated with CoMCont or CoMTb for 24 h and cell extracts analysed for luciferase activity, normalised to Renilla luciferase activity. CoMTb stimulated full-length WT MMP-3 promoter was designated as maximal luciferase activity (100%) and all other results were expressed as a percentage of this maximum. Bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. All data were analysed by two way analysis of variance followed by Tukey's multiple comparison. \* $p$ <0.05, \*\* $p$ <0.01.

stimulated multiple signalling pathways including ERK, p38, AKT, GSK, JNK and HSP-27 (Figure 28A). Densitometric analysis demonstrated that high levels of ERK-1/2 and AKT phosphorylation were detected in CoMCont stimulated cells and were increased further by CoMTb (Figure 28B). However, absolute increases in some p38 isoforms were particularly high. CoMTb stimulated p38 $\alpha$  phosphorylation 58.2 units more (311%,  $p<0.01$ ) than CoMCont and p38 $\gamma$  phosphorylation increased by 10.2 units (154%,  $p<0.05$ ). p38 $\beta$  and p38 $\delta$  phosphorylation levels did not change.

#### *p38 phosphorylation kinetics*

To investigate these findings further kinetic Western blot analysis of CoMCont and CoMTb stimulated p38 phosphorylation in microglia was performed over 120 minutes (Figure 28C). Minimal basal phosphorylation was seen. CoMTb stimulated p38 phosphorylation by 15 minutes, which had waned by 60 minutes. However, a second peak of phosphorylation was detected at 120 minutes. ERK phosphorylation was investigated at 30 minutes and confirmed high basal phosphorylation that was further up-regulated by CoMTb (Figure 28D, lanes 1 & 4).

#### *MMP-1 and -3 secretion are p38 dependent but only MMP-1 is ERK dependent*

To study the role of the p38 and ERK pathways in control of MMP-3 secretion specific chemical inhibitors (p38, SB203580 and ERK, PD98059) were pre-incubated with microglia at 1 and 10  $\mu$ M prior to stimulation by CoMTb. For contrast MMP-1 secretion was also analysed. MMP-1 secretion was suppressed 45.6% by 1  $\mu$ M and 92% by 10  $\mu$ M SB 203580 ( $p<0.05$  and  $p<0.01$  respectively, Figure 29A). 10  $\mu$ M PD98059 ERK inhibition similarly decreased

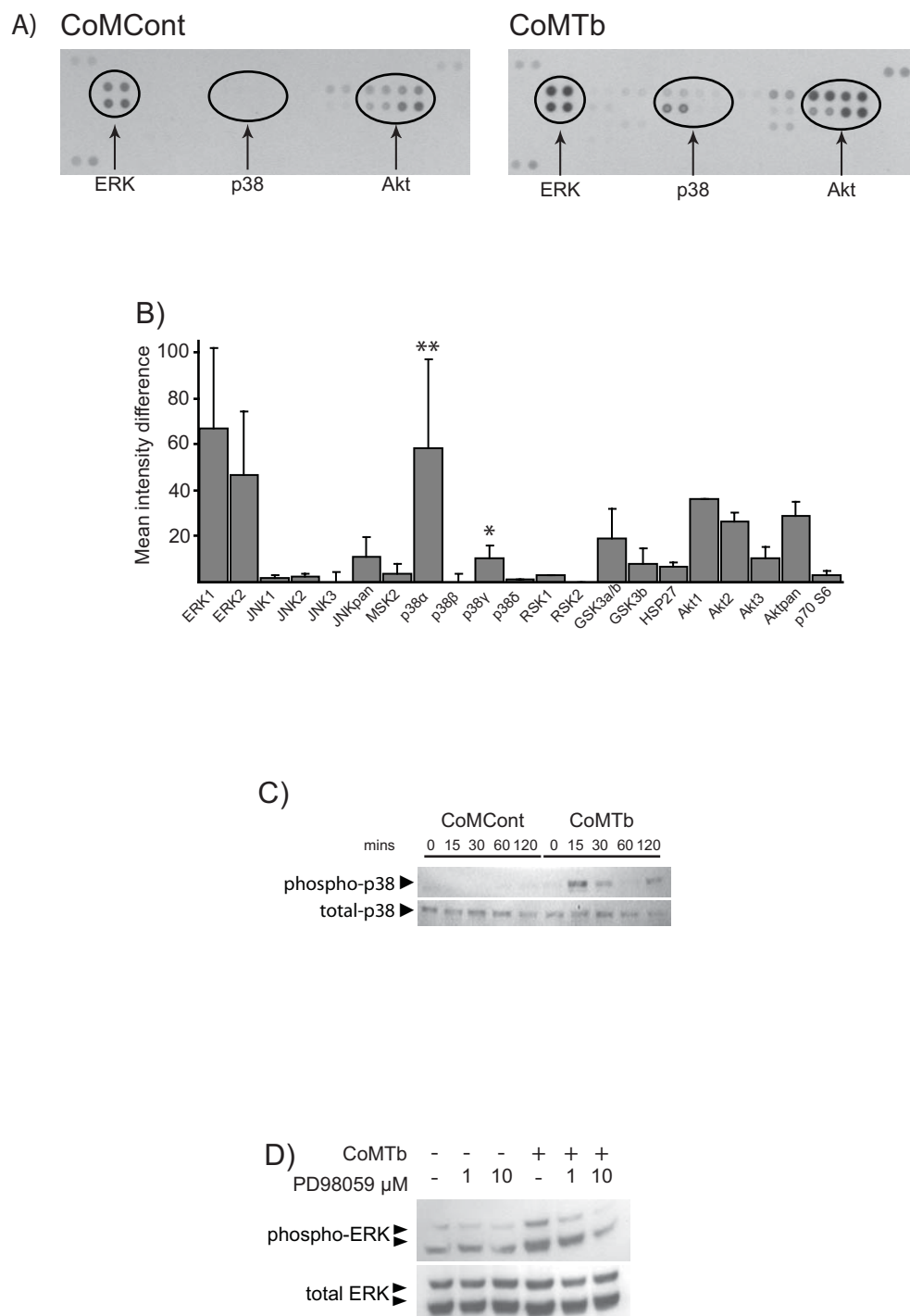


Figure 28. CoMTb drives phosphorylation of signalling kinases in microglia. A, Proteome profiler phosphoarray of CoMCont and CoMTb stimulated microglia at 30 mins. CoMTb drives phosphorylation of multiple signalling pathways compared to CoMCont stimulated cells. B, Densitometric analysis of phosphoarray. Mean intensity difference between CoMTb and CoMCont is presented. High baseline ERK 1/2 and Akt phosphorylation is increased by stimulation and p38 $\alpha$ /p38 $\gamma$  phosphorylation are markedly increased. Bars represent mean  $\pm$  SD from three independent experiments. Data were analysed by Student's t-test. \* $p < 0.05$ , \*\* $p < 0.01$ . C, CoMTb stimulation causes p38 MAP kinase phosphorylation after 15 min. Representative data from an experiment performed on three separate occasions are shown. D, ERK phosphorylation in microglia at 30 mins. Microglia were preincubated with control medium or PD98059 as annotated and stimulated with CoMTb for 30 mins. Representative data from an experiment performed on two separate occasions are shown.

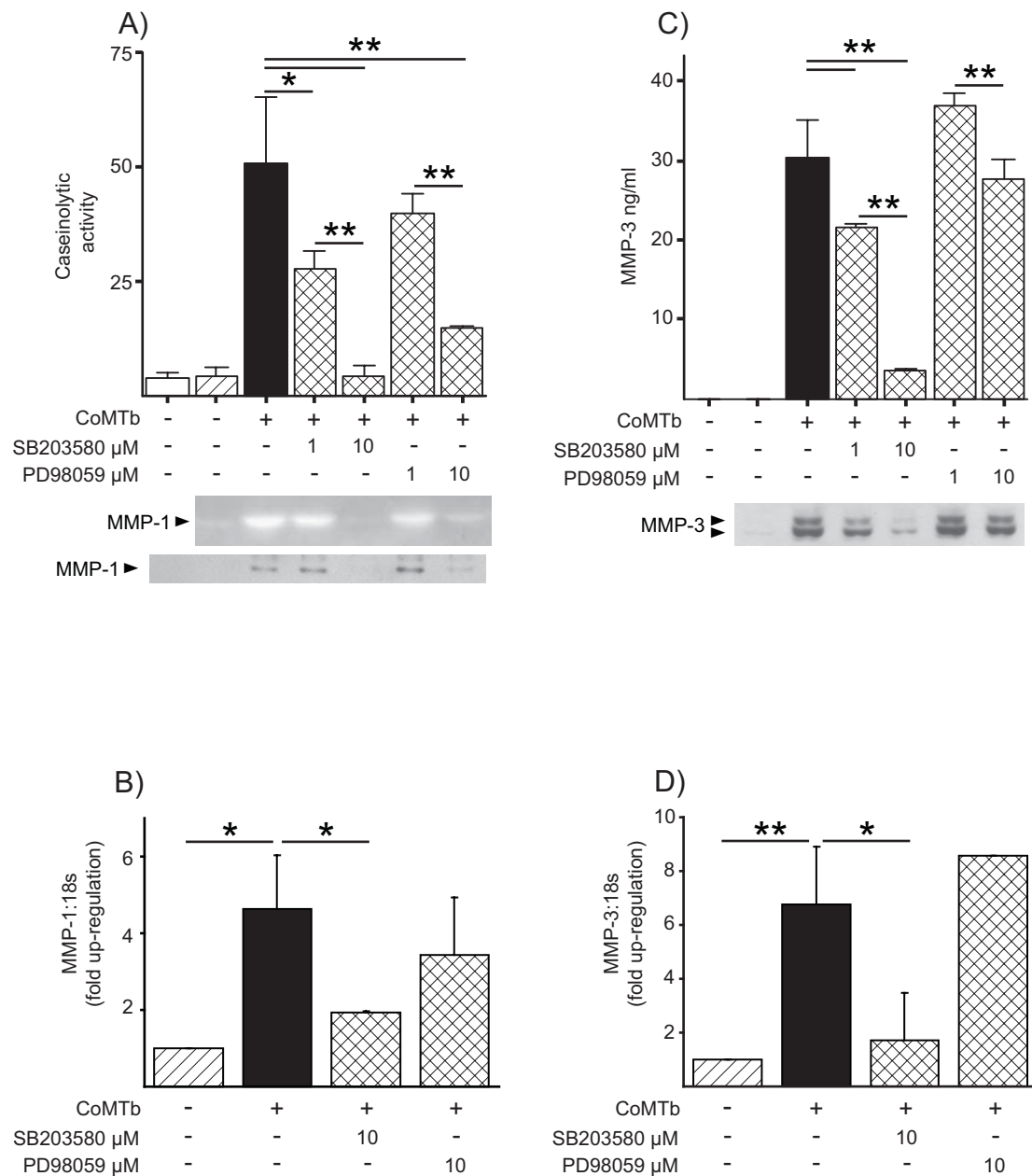


Figure 29. p38 phosphorylation is the key signalling event during MMP-1 and -3 secretion. A, p38 and ERK MAP kinase pathways regulate MMP-1 secretion. Microglial cells were incubated with SB203580 or PD98059 for 2 h and then stimulated with CoMTb. MMP-1 secretion was analysed at 72 h by casein zymography (representative gel shown), confirmed by Western blot and Luminex. B, MAP kinase inhibition suppresses MMP-1 mRNA accumulation. MMP-1 gene expression was analysed by RT-PCR 24 h after stimulation with CoMTb. C, Only p38 MAP kinase regulates MMP-3 secretion. MMP-3 secretion was analysed at 72 h by ELISA. D, p38 MAP kinase inhibition suppresses MMP-3 mRNA accumulation. A & C, Bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. B & D, Mean values  $\pm$  SD from three experiments are presented. Data were analysed by one-way analysis of variance, followed by Tukey's multiple comparison. \* $p < 0.05$ , \*\* $p < 0.01$ .

MMP-1 secretion by 71.3% ( $p<0.01$ ). MMP-3 was found to be p38 but not ERK dependent as SB203580, but not PD98059, suppressed MMP-3 in a dose dependent manner (Figure 29C). 1 and 10  $\mu$ M SB203580 reduced MMP-3 secretion by 28.8% and 83.2% respectively (both  $p<0.01$ ). 10  $\mu$ M PD98059 did not suppress MMP-3 secretion compared to CoMTb ( $p=ns$ ).

Since ERK inhibition did not cause suppression of MMP-3 secretion the inhibitory effect of PD98059 on ERK phosphorylation was checked by phosphorylation Western blot analysis (Figure 28D). At 30 minutes basal ERK phosphorylation was detected (lane 1), which was not significantly affected by PD98059 (lanes 2 & 3). CoMTb up-regulated ERK phosphorylation (lane 4) and this effect was blocked by PD98059 in a dose dependent manner (lanes 5 & 6).

MMP-1 and -3 gene expression were investigated by qRT-PCR (Figure 29B & D). MMP-1 gene expression was up-regulated 4.6 fold by CoMTb over CoMCont ( $p<0.05$ ). This up-regulation was suppressed by inhibition of both the p38 and ERK pathways. SB203580 reduced MMP-1 mRNA expression by 58.7% ( $p<0.05$ ) and PD98059 reduced it by 25.9% although this latter value was not statistically significant. MMP-3 expression was only affected by p38 inhibition, being reduced by 75.1% ( $p<0.01$ ) thereby confirming the secretion data.

### **Effect of dexamethasone on gene expression and secretion of MMP-1 & 3 and TIMP-1 & -2**

Corticosteroids are of proven clinical benefit in TBM therefore the effect of pre-treatment with dexamethasone on expression and secretion of MMP-1, -3,

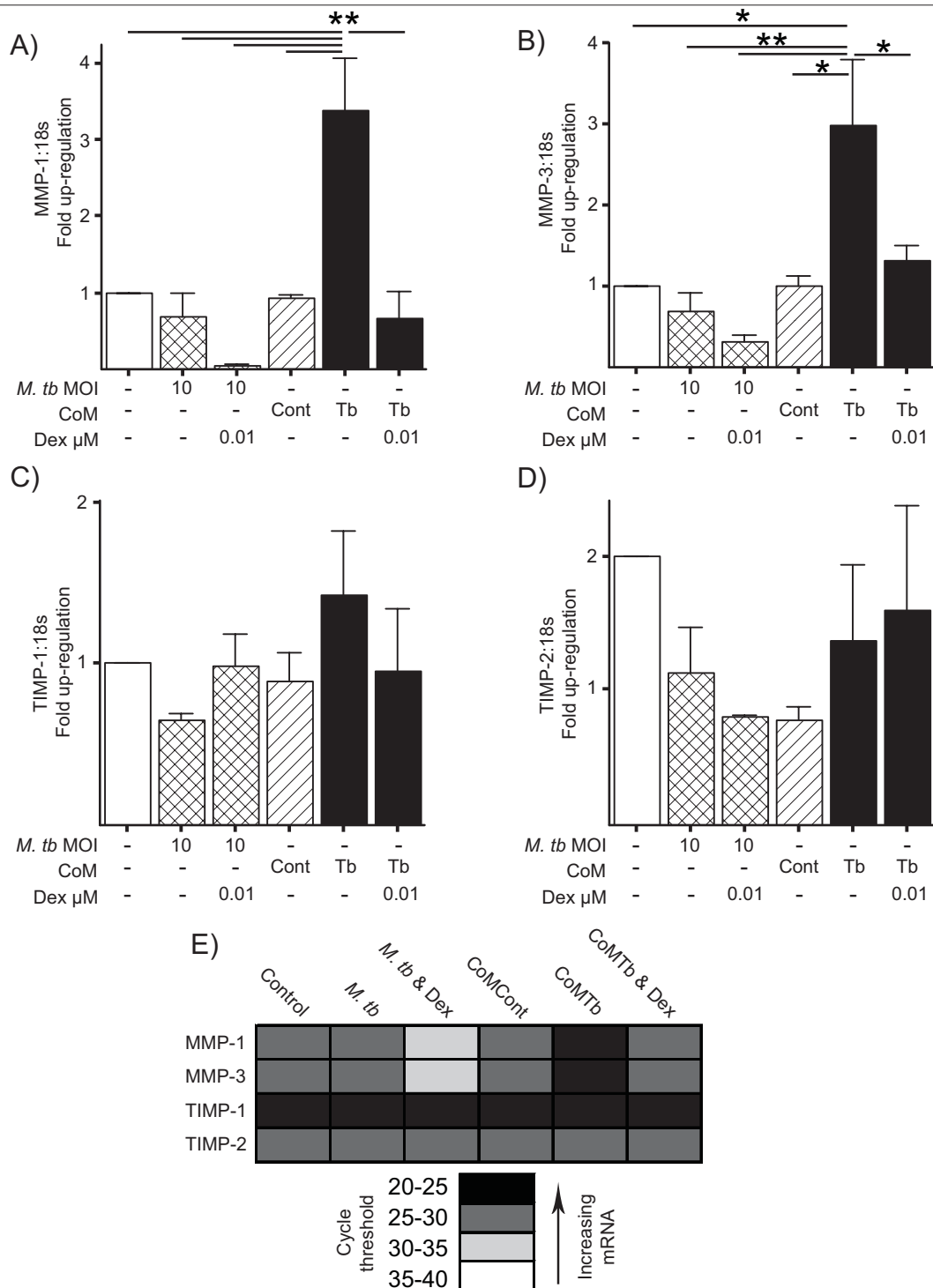


Figure 30. Dexamethasone suppresses CoMTb-induced MMP-1 and -3 gene expression. Microglia were infected with *M.tb* at an MOI of 10 (diamond hatched shading) or stimulated with CoMTb (filled bars) with or without preincubation with 0.01  $\mu$ M dexamethasone for 2 h. Control (open bars) and CoMCont (diagonal shading) are also shown. A, MMP-1 gene expression. Dexamethasone suppressed MMP-1 gene expression at 24 h in infected microglia to below control levels and to basal levels in CoMTb stimulated cells. B, MMP-3 gene expression. Dexamethasone suppressed MMP-3 gene expression to basal levels in CoMTb stimulated cells. C, TIMP-1 gene expression. Dexamethasone partially suppressed TIMP-1 gene expression ( $p$ =ns). D, TIMP-2 gene expression not suppressed by dexamethasone. Mean values  $\pm$  SEM are given for three experiments performed on separate vials of cells. Data were analysed by one-way analysis of variance, followed by Tukey's multiple comparison. \* $p$ <0.05, \*\* $p$ <0.01. E, PCR cycle thresholds for matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) gene expression in human microglia stimulated by *M.tb* and CoMTb preincubated with dexamethasone. The mean cycle threshold (CT) at which PCR amplification enters the logarithmic phase is shown for the RNA samples analysed in A-D. A low CT indicates high mRNA levels. Open rectangles represent low expression through shaded to solid rectangles, representing high expression. Of the MMPs/TIMPs down-regulated by dexamethasone MMP-1 and -3 were the most highly suppressed. Note: in part some of these data were presented in figure 6 & 7.

TIMP-1 and -2 was investigated. Preincubation with dexamethasone at 0.01 $\mu$ M inhibited CoMTb induced MMP-1 mRNA expression to basal levels (Figure 30A,  $p<0.01$ ). In addition dexamethasone reduced *M.tb* induced MMP-1 expression beyond baseline to undetectable levels. Dexamethasone inhibition of CoMTb induced MMP-3 expression to basal levels was also observed (Figure 30 B). Baseline secretion of both MMPs was similarly affected by dexamethasone. In contrast TIMP-1 and -2 mRNA levels were not affected by dexamethasone (Figure 30 C & D). The quantitative  $C_T$  data for this experiment was in accordance with the 18s corrected values (Figure 30E). Secretion data confirmed the expression data for all four analytes (Figure 31). MMP-1 secretion was suppressed further than MMP-3 but both were significantly suppressed by dexamethasone (Figure 31 A & B,  $p<0.01$ ). In contrast TIMP-1 was not affected by dexamethasone preincubation (Figure 31 C). CoMTb suppressed TIMP-2 secretion somewhat but this was not affected by dexamethasone (Figure 31 D).

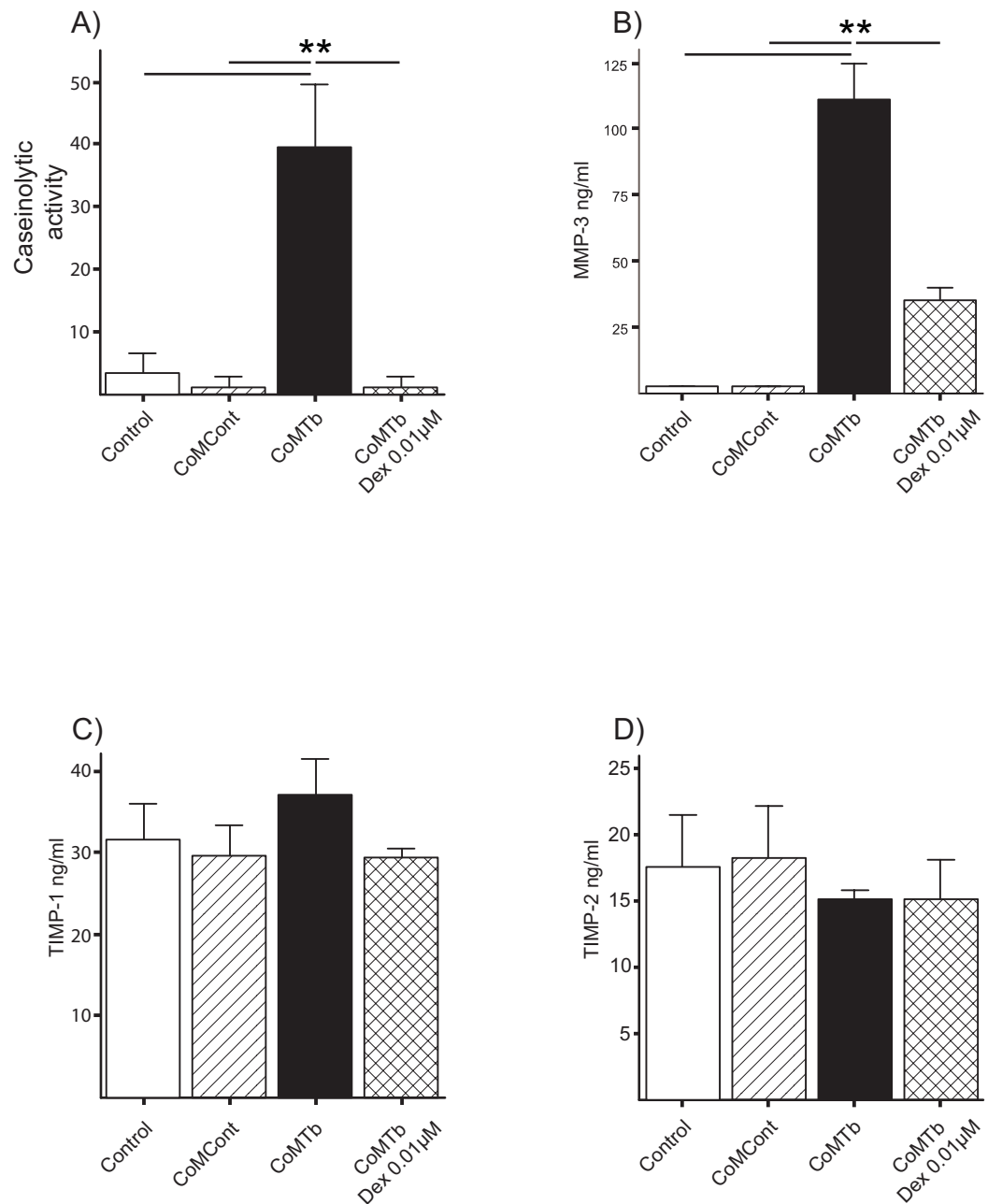


Figure 31. Dexamethasone suppresses CoMTb-induced MMP-1 and MMP-3 secretion. Microglial cells were preincubated with 0.01  $\mu$ M dexamethasone for 2 h and stimulated with CoMTb. A, MMP-1 secretion was analysed by casein zymography. B, MMP-3 secretion was analysed by ELISA. C, TIMP-1 secretion was analysed by ELISA. D, TIMP-2 secretion, analysed by ELISA, was suppressed by CoMTb but not restored by dexamethasone. Bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. Data were analysed by one-way analysis of variance, followed by Tukey's multiple comparison. \*\* $p < 0.01$ .

## **Discussion**

The gene expression profiling discussed in the chapter 3 results section suggested that both MMP-1 and -3 were up-regulated more than any other MMPs. Although MMPs have overlapping substrate affinities, a variety of MMPs need to be secreted into the extracellular environment to degrade both extracellular matrix and other substrates such as chemokines and tight junctions efficiently. Therefore this chapter has focused both on factors in CoMTb that led to increased MMP-3 secretion, as well as intracellular signalling events stimulated by CoMTb leading to MMP-1 and -3 secretion. MMP-3 has a number of CNS specific substrates: it can cause neuronal apoptosis and BBB breakdown in addition to activating MMP-1 [301]. There are little published clinical data on the role of MMP-3 in TB and no data in CNS TB. Extrapolation from *Mmp3* null mice work performed in other models of inflammation would suggest that, in addition to the substrates/activities listed in table 3, MMP-3 is critical for leucocyte influx to areas of inflammation [336]. Modulation of the host inflammatory response is a realistic drug target and therefore the MAP kinase pathways have been explored in some depth to unpick up-stream signalling events leading to MMP-1 and -3 secretion. As dexamethasone is a key adjuvant therapy for TBM its effect on the secretion of MMP-1 and -3, as well as TIMP-1 and -2 was investigated.

The first observation was that *M.tb* infection of microglial cells up-regulated MMP-3 secretion in a time and dose dependent fashion despite the lack of change in gene expression suggesting that *M.tb* infected microglia are not inert. LPS injected directly into the brains of mice led to up-regulation of MMP-3

secretion well before TNF- $\alpha$  suggesting that innate signalling pathways involving TLRs lead to MMP-3 secretion [198]. However, CoMTb drove the secretion of MMP-3 more than 14 fold more than *M.tb* direct infection and this was also confirmed to be occurring at the level of gene transcription in kinetic studies. Increased MMP-3 secretion has been described in response to LPS stimulation in mice, rat and human microglia [220, 263, 329]. However, MMP-3 secretion in human microglia appears to be a TB-specific phenomenon, as LPS did not up-regulate MMP-3 in this model (Figure 23E). I have not analysed TLR4 expression on the microglial cells used in these experiments, but microglia are known to express TLR4 [142, 143]. In the same way as described for MMP-1, networks involving astrocytes and microglia did not influence MMP-3 secretion, once again placing the monocyte as the central player in microglial MMP responses to *M.tb* driven neuroinflammation.

There was substantial synergy between TNF- $\alpha$  and IL-1 $\beta$  leading to MMP-3 secretion. MMP-3 secretion is usually IL-1 $\beta$  dependent, but synergy has been described before in trabecular meshwork cells, orchestrated in part by TNF- $\alpha$  driving increased expression of IL-1 receptors [337]. Fibroblast MMP-3 secretion requires synergy between TNF- $\alpha$  and Oncostatin M [186]. The point of synergy between TNF- $\alpha$  and IL-1 $\beta$  in microglia remains to be elucidated.

The NF $\kappa$ B pathway was found to be important for MMP-3 expression and secretion, as chemical blockade with helenalin and SC-514 abrogated both responses. Unlike MMP-1 the published literature has suggested that there are no true NF $\kappa$ B consensus sites in the MMP-3 promoter [333]. A down-regulatory

effect of p50-p65 NF $\kappa$ B subunits binding at the stromelysin IL-1 responsive element found between -1614 and -1595 bp upstream of the transcription start site has been described [338]. The literature fits with the finding in this work of a derepressor of promoter activity between the -1612 and -642 promoter constructs, but not the NF $\kappa$ B chemical inhibition data. Nevertheless, involvement of NF $\kappa$ B in up-regulation of MMP-3 secretion has been described in human macrophages and rabbit foam cells using NF $\kappa$ B knockdown by over expression of I $\kappa$ B $\alpha$  [339]. Perhaps NF $\kappa$ B is having an indirect effect by up-regulating AP-1 subunits, known to have NF $\kappa$ B sites in their own promoters, as AP-1 is known to be important in regulation of MMP-3 gene expression. Once activated these AP-1 subunits could then secondarily activate MMP-3 transcription [340]. Further work to unpick these mechanisms might include EMSAs with NF $\kappa$ B subunit supershifts, or anti-NF $\kappa$ B subunit chromatin immunoprecipitation with subsequent RT-PCR using MMP-3 promoter specific primers designed for non-consensus NF $\kappa$ B binding sites identified using a blast search of the promoter. Further work on investigating the derepressor in the MMP-3 promoter is also warranted using mutation constructs of potential derepressors, such as PEA3 binding sites.

Next I investigated the role of MAP and serine/threonine kinases in control of MMP-1 and -3 secretion by use of a commercial array. This demonstrated that ERK and the Akt pathways were active in resting cells. These pathways are responsible for cell turnover and therefore high basal expression in cell lines is common. Although there was some up-regulation of phosphorylation of these pathways with CoMTb, the p38 pathway, in particular the p38 $\alpha$  and  $\gamma$  subunits,

stood out as the principle target of CoMTb induced signalling. This was confirmed with kinetic studies, where biphasic phosphorylation of p38 was observed. No specific subunit analysis was performed because the antibody used recognises epitopes common to all four p38 subunits. *M.tb* has also been shown to drive p38 phosphorylation in microglia directly. Yang *et al* demonstrated that sonicated *M.tb* stimulated p38 phosphorylation in a TLR2, dectin-1 independent manner [157]. However, I did not use this profiling kit to investigate which components of CoMTb are required specifically for p38 phosphorylation, but it is reasonable to assume that the TNF- $\alpha$  and IL-1 $\beta$  synergy observed acts at this point, as synergy driving MMP-3 secretion has been reported as acting at this level previously [337]. Those authors reported that the p38 $\gamma$  and p38 $\delta$  subunits were important, not p38 $\alpha$ , as was the principle finding in this study. p38 $\alpha$  appears to be a critical regulator of monocyte lineage inflammatory responses. Conditional knockout of p38 $\alpha$  in murine macrophages differentially affects bacterial phagocytosis and LPS-induced TNF- $\alpha$ , but not IL-6, secretion via the attenuation of the transcription factor cAMP response element-binding protein (CREB), but not the NF $\kappa$ B, pathway [341]. Finally there is now evolving evidence that p38 inhibition may have a therapeutic role in the treatment of cerebral ischaemia, where it reduces both neuronal death and microglial activation [342].

The high ERK basal phosphorylation up-regulated by CoMTb observed in the MAP kinase array was confirmed, as was the efficacy of the inhibitor PD98059, by Western blotting. Secretion of MMP-1 was found to be both ERK and p38 dependent, but MMP-3 was only p38 dependent; thus p38 is the central cellular

control point for MMP-1 and -3 secretion in microglial cells. Gene expression data reflected the secretion data, which is somewhat surprising as p38 is felt to act post-translationally on mRNA stability, as has been shown for MMP-1 in fibroblasts [343]. mRNA stability was not assessed in this work, but would add valuable information to the data presented thus far.

Dexamethasone suppressed MMP-1 and -3 gene expression and secretion without affecting TIMP-1 and -2. The mechanism for the beneficial adjuvant effect of dexamethasone in reducing mortality in TBM is unknown, but total protein was significantly reduced in the CSF by study drug recipients [50]. This was not due to an alteration in BBB permeability, as measured by the albumin index (ratio of CSF to serum albumin), so a reduction in MMP concentration could have contributed to this. In addition, measuring CSF parameters of inflammation does not equate to equivalent parenchymal fluxes of mediators. Microglial pro-inflammatory responses have been shown to be dexamethasone sensitive before. Rock *et al* showed that *M.tb* induced TNF- $\alpha$  and IL-1 $\beta$  concentrations were suppressed by the same dose of dexamethasone used in this study ( $10^{-8}$  M). Harris *et al* and Elkington *et al* showed that astrocyte MMP-9 and macrophage MMP-1 respectively, but not TIMPs, were modulated by dexamethasone pre-incubation. How dexamethasone does this is not clear, as MMP promoters are not as rich in glucocorticoid response elements as other genes [206]. Thus modulation of the p38 pathway, critical to microglial MMP-1 and -3 secretion, by MAPK phosphatase-1 (a corticosteroid induced enzyme that opposes that action of p38 MAP kinase) is a possibility, but I was unable to perform experiments to confirm this mechanism [215, 344].

In summary I have shown that *M.tb* infected monocyte networks and direct infection are both strong stimuli to MMP-3 secretion. This is mediated by synergy between TNF- $\alpha$  and IL-1 $\beta$ , acting principally via p38 MAP kinase. In addition, dexamethasone potently suppresses MMP-1 and -3 gene expression and secretion, reversing the CNS matrix degrading phenotype stimulated by CoMTb. This chapter adds to the literature surrounding the mechanism of tissue destruction in CNS TB and offers credible drug targets for future therapies. However, understanding the effects of targeting pathways such as p38 MAP kinase on other mediators controlled by it will be necessary.

## Chapter 5 – The mechanism of MMP-2 suppression

### *Introduction*

The preceding chapters have focused on up-regulation of MMPs, particularly MMP-1 and -3, in the context of CNS TB. MMP-1 is a potent collagenase, cleaving fibrillary collagen exclusively at the Gly<sub>775</sub>-Leu/Ileu<sub>776</sub> peptide bond and is activated by MMP-3. Both were up-regulated far more by CoMTb than *M.tb*. Nevertheless even together they cannot degrade all components of the ECM. In this respect the gelatinases, MMP-2 and -9, are of particular interest as they can further degrade collagen that has been cleaved by MMP-1. Gelatinases have well described and important roles in ECM turnover as they are quantitatively the most abundant MMPs secreted in most situations. MMP-2 is constitutively expressed at high levels by many cells and pro-peptide activation is felt to be the main mechanism of MMP-2 control. MMP-9 has been implicated in the pathogenesis of many inflammatory diseases including CNS TB [313]. In addition up-regulation of the expression of MMP-9 was noted in the global analysis of MMPs/TIMPs. However, the overall flux of MMP concentrations within an inflammatory environment is likely to be an important factor in determining net changes in proteolytic activity within any compartment.

Therefore this chapter focuses on the observation that microglial MMP-2 secretion was suppressed by monocyte derived TNF- $\alpha$  acting via p38 and NF $\kappa$ B signalling to the level of gene transcription. A possible non-apoptotic role for caspase 8 in this pathway is suggested by preliminary data.

## **Results**

### **MMP-2 secretion suppressed by CoMTb more than *M.tb***

Microglia were stimulated with 1:5 CoMCont, CoMTb or *M.tb* at MOIs of 0.1, 1.0 and 10. MMP-2 secretion was analysed by zymography and confirmed by Luminex (Figure 32A). There was a 12.8 and 31% reduction in MMP-2 secretion due to direct infection of microglia at an MOI of 1 and 10 but CoMTb caused a 72.8% suppression of MMP-2 secretion ( $p<0.01$ ). Kinetic analysis confirmed that this suppression was evident by 24 hours and sustained over 96 hours (Figure 32B). Analysis of mRNA by qRT-PCR demonstrated that CoMTb was acting at the level of gene expression as mRNA levels were suppressed by 50% (Figure 32C). This was a specific effect between monocytes and microglia as conditioned media from *M.tb* infected primary human astrocytes (Figure 32D) and microglia (CoMicTb, data not shown) had no effect on MMP-2 concentrations.

### **Only TNF- $\alpha$ in CoMTb is responsible for suppression of MMP-2 secretion**

TNF- $\alpha$  has been described as a mediator of MMP-2 suppression in astrocytoma cells [193]. Therefore CoMTb was pre-incubated for two hours with increasing doses of TNF- $\alpha$  neutralising antibodies before being used to stimulate microglia (Figure 33A). MMP-2 secretion was restored in a dose dependent manner. Therefore recombinant TNF- $\alpha$  was added to cells at 1, 10 and 100ng/ml. 100 ng/ml was able to suppress MMP-2 secretion by 61.0%, the same extent as CoMTb (Figure 33B,  $p=ns$  for comparison of CoMTb and TNF- $\alpha$ ). However,

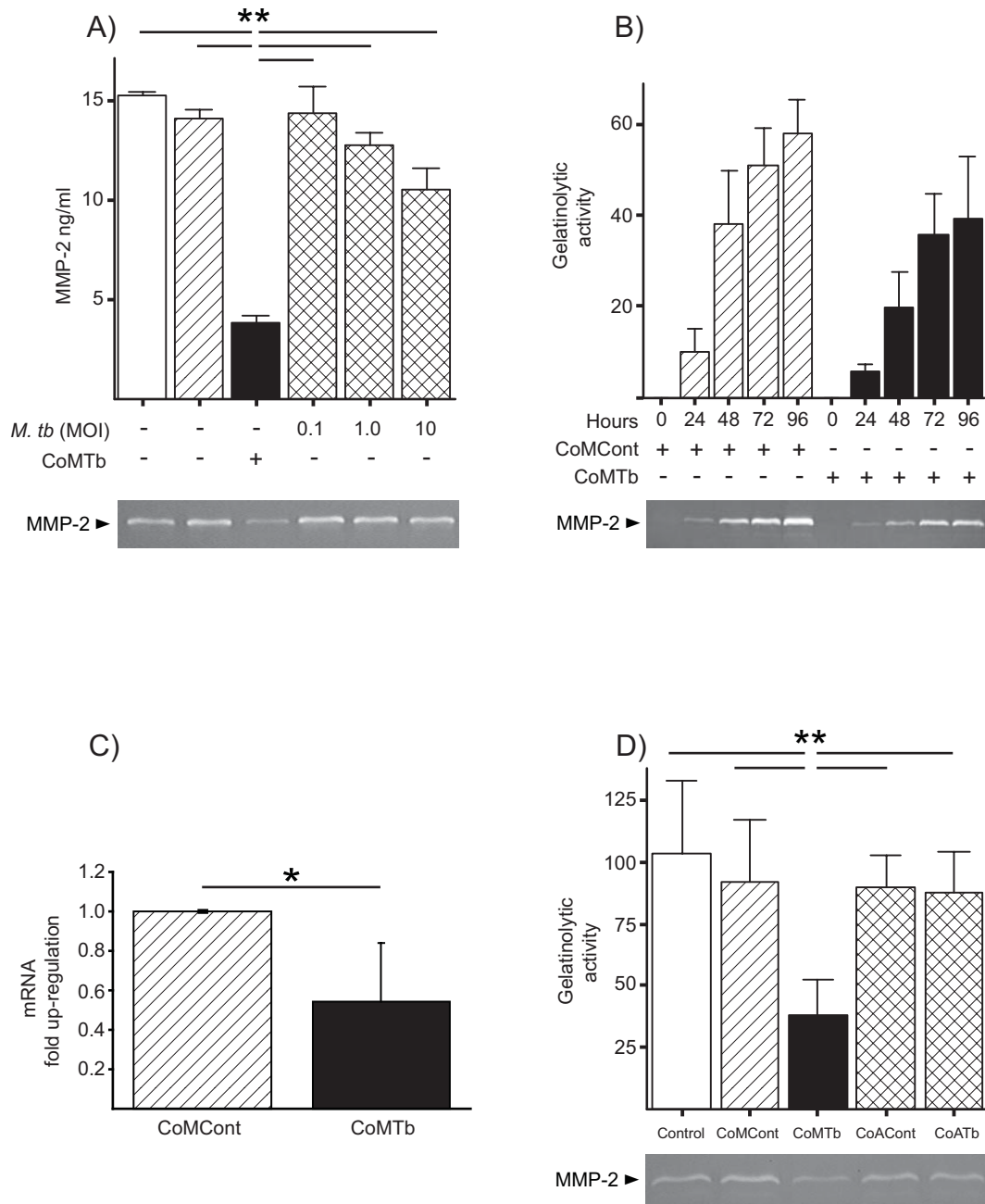


Figure 32. MMP-2 secretion and gene expression is suppressed by CoMTb. A, Microglial cells were stimulated with CoMTb or infected with *M.tb*. 72 h supernatants were analysed by Luminex and gelatin zymography. B, Kinetics of MMP-2 secretion analysed by gelatin zymography (representative zymogram shown). C, MMP-2 gene expression is suppressed by CoMTb. mRNA from microglia stimulated for 24 h was analysed by RT-PCR, normalised to 18S RNA and expressed as fold change relative to 24 h CoMCont mRNA. The mean  $\pm$  SD from three experiments are shown analysed by Student's t-test. D, Astrocyte-microglial signalling networks do not suppress MMP-2 secretion. Conditioned medium from primary human astrocytes was prepared from control (CoACont) and *M.tb* infected cells (CoATb) and used to stimulate microglia 1:5. 72 h cell culture medium was analysed by gelatin zymography. A, B & D, Bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. Data were analysed by one-way analysis of variance, followed by Tukey's multiple comparison. \* $p < 0.05$ , \*\* $p < 0.01$ .

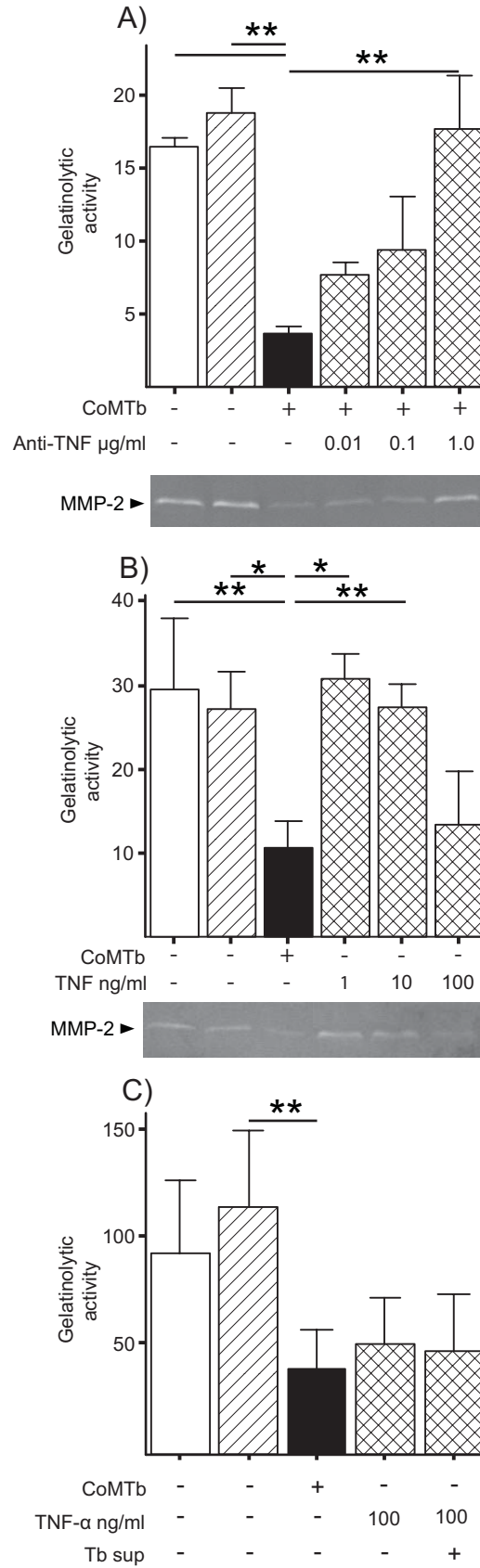


Figure 33. Suppression of MMP-2 by CoMTb is mediated by TNF- $\alpha$ . A, CoMTb was preincubated with increasing doses of anti-TNF- $\alpha$  Ab for 2 h. B, TNF- $\alpha$  suppresses MMP-2 secretion. Cells were stimulated with TNF- $\alpha$  1-100 ng/ml. C, *M.tb* supernatant (Tb sup) does not add to TNF- $\alpha$  mediated MMP-2 suppression. For all experiments bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. Data were analysed by one-way analysis of variance followed by Tukey's multiple comparison. \* $p < 0.05$ , \*\* $p < 0.01$ .

analysis of CoMTb presented in chapter 3 confirmed it contained only ~8 ng/ml TNF- $\alpha$  therefore the role of secreted *M.tb* antigens that are in filtered CoMTb by the addition of Tb supernatant (Tb sup) was investigated. Although Tb sup had been found to be important in the control of secretion of MMP-1 and -3 in chapters 3 and 4 no synergy was found between TNF and Tb sup (Figure 33C). Unlike live *M.tb*, Tb sup alone did not suppress MMP-2 secretion (data not shown). Pre-incubating cells with IL-1Ra in increasing doses or stimulation of cells with recombinant IL-1 $\beta$  did not suppress MMP-2 secretion in the same way as CoMTb (Figure 34A & B). In addition cells were pre-incubated with pertussis toxin for two hours in order to block GPCR before the addition of CoMTb. No effect on CoMTb suppression of MMP-2 secretion was found (data not shown).

### **p38 MAPK controls TNF- $\alpha$ mediated MMP-2 suppression**

As TNF- $\alpha$  is known to signal via MAP kinase signalling pathways cells were pre-incubated for 2 hours with specific chemical inhibitors for the p38 (SB230580) and ERK (PD98059) pathways (Figure 35). Inhibition of the p38 pathway led to a loss of CoMTb mediated MMP-2 suppression. 1  $\mu$ M SB230580 caused MMP-2 secretion to return to the same level as CoMCont and 10  $\mu$ M SB230580 caused a 228% increase in MMP-2 secretion over CoMCont ( $p < 0.01$ ). In contrast ERK inhibition at 1  $\mu$ M PD98059 led to a further 6.1% decrease in MMP-2 secretion over and above the 31.2% due to CoMTb ( $p < 0.05$  for CoMTb to Control group comparison). 10  $\mu$ M PD98059 caused a significant further fall in MMP-2 secretion of 76.6% compared to CoMCont ( $p < 0.01$ ), although statistically this was not lower than CoMTb alone.

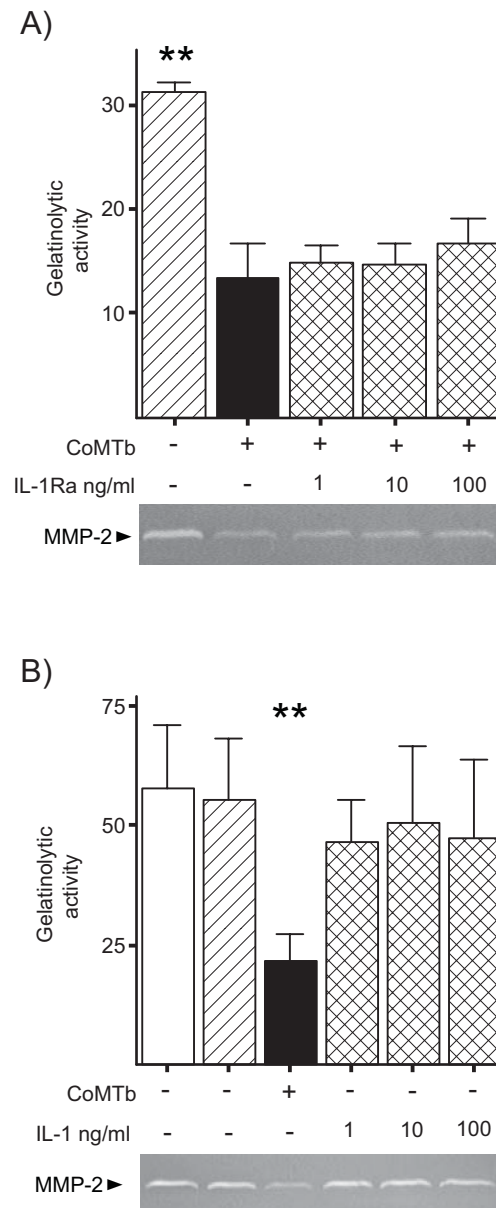


Figure 34. IL-1 $\beta$  does not contribute to CoMTb induced MMP-2 secretion. A, Cells were preincubated with IL-1Ra for 2 h. Inhibition of IL-1 $\beta$  activity did not affect MMP-2 secretion. A representative gelatin zymogram is shown. B, IL-1 $\beta$  alone does not suppress MMP-2 secretion. Microglial cells were stimulated with IL-1 $\beta$  at concentrations of 1–100 ng/ml. Densitometric analysis of gelatin zymography is shown. Bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. Data were analysed by one-way analysis of variance, followed by Tukey's multiple comparison. \* $p < 0.05$ , \*\* $p < 0.01$ .

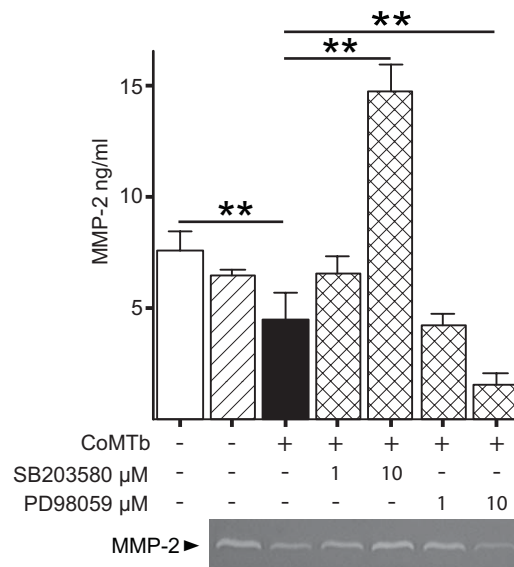


Figure 35. p38 phosphorylation acts as the break for TNF- $\alpha$  suppression of MMP-2 secretion. Microglial cells were incubated with SB203580 or PD98059 for 2 h, and then stimulated with CoMTb. MMP-2 secretion was analysed at 72 h by Luminex and gelatin zymography (representative gel shown). p38 inhibition reverses CoMTb suppression and leads to supra-maximal MMP-2 secretion but ERK inhibition further suppresses secretion. Bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. Data were analysed by one-way analysis of variance, followed by Tukey's multiple comparison. \* $p < 0.05$ , \*\* $p < 0.01$ .

### **NFκB nuclear signalling controls MMP-2 secretion**

As the CoMTb down-regulatory signal was found to be via the TNF-α/p38 pathway (Figure 35), mediated by a fall in mRNA (Figure 32C), the NFκB pathway was studied to unpick further the mechanism of MMP-2 suppression. Helenalin restored MMP-2 secretion to basal levels (Figure 36A). Chemical inhibition of IKK2 with SC-514 led to a dose dependent loss of CoMTb mediated MMP-2 suppression (Figure 36B). This was supra-maximal at 5 μM, 220.7% higher than CoMCont and 423.8% higher than CoMTb (both  $p < 0.01$ ). No supramaximal secretion was seen with helenalin but it was toxic to cells at more than 1 μM if used in secretion experiments as cells were exposed to it for a prolonged period of time.

### **Dexamethasone does not control MMP-2 suppression**

Pre-incubation of cells with 0.01 μM dexamethasone, that had previously suppressed MMP-1 and -3 secretion, had no effect on CoMTb mediated MMP-2 suppression (data not shown).

### **Caspase 8 inhibition restores MMP-2 secretion to control levels**

To investigate the mechanism of CoMTb induced MMP-2 suppression cells were pre-incubated with a caspase 8 inhibitor (z-IETD-fmk, Sigma) and stimulated after two hours. As expected CoMTb suppressed secretion of MMP-2 by 33% ( $p < 0.01$ ). A dose dependent increase in MMP-2 secretion was observed with increasing concentrations of caspase 8 inhibitor, returning secretion to basal levels with 1 μM inhibitor (Figure 37).

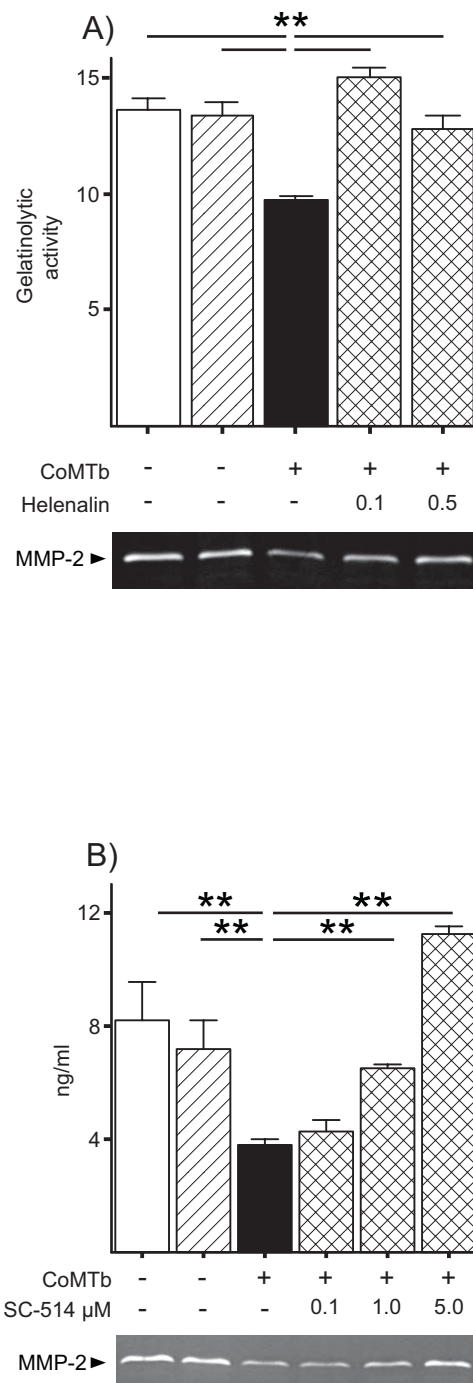


Figure 36. CoMTb suppression of MMP-2 secretion is mediated by the NF $\kappa$ B pathway. A, Cells were preincubated with Helenalin for 2 h and then stimulated with CoMTb. 72 h cell culture supernatants were analysed by Luminex, confirmed by gelatin zymography (representative zymogram shown). B, SC-514 inhibition. Bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. Data were analysed by one-way analysis of variance, followed by Tukey's multiple comparison. \*\* $p < 0.01$ .

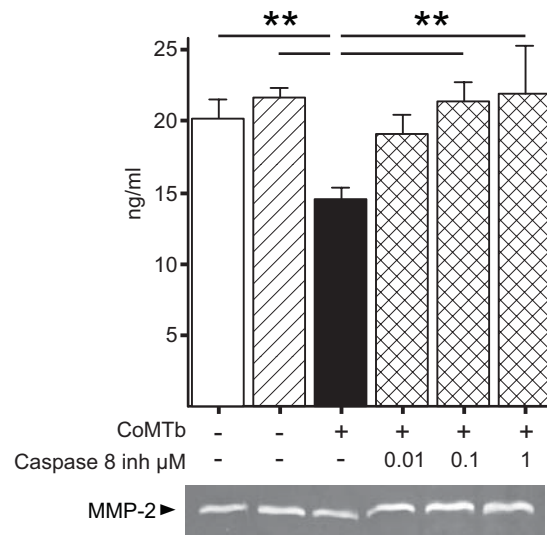


Figure 37. Caspase 8 mediates CoMTb induced MMP-2 suppression. Microglial cells were preincubated for 2 h with 0.01-1  $\mu$ M caspase 8 inhibitor (z-IETD-fmk) and stimulated with CoMTb. 72 h supernatants were analysed by Luminex and confirmed by gelatin zymography. Bars represent mean values  $\pm$  SD of three samples, representative of at least duplicate experiments performed in triplicate. Data were analysed by one-way analysis of variance, followed by Tukey's multiple comparison. \*\*p<0.01.

## **Immunohistochemistry**

To confirm the importance of the *in vitro* p38 findings phospho-p38 immunohistochemistry was performed on the five CNS TB brain biopsies and appropriate controls as described in the preceding chapters. This demonstrated that microglial nuclei were more immunoreactive for phosphorylated p38 than cytoplasm (Figure 38).

## **Technical aspects of quantifying MMP-9 secretion**

As CoMTb induced MMP-9 gene expression was statistically up-regulated in the initial MMP screen (Figure 6 & 7) MMP-9 secretion was investigated. Initial experiments appeared to confirm that CoMTb up-regulated MMP-9 secretion. However, when 1:5 CoMTb itself was analysed it was found to contain high concentrations of MMP-9. This meant that looking for increases in microglial MMP-9 secretion over and above that already in CoMTb (or decreases expected in blocking experiments) was extremely difficult. Interpretation of MMP-9 secretion in microglia using this model system was not possible. Of note no MMP-1 or MMP-3 was detected in CoMTb.

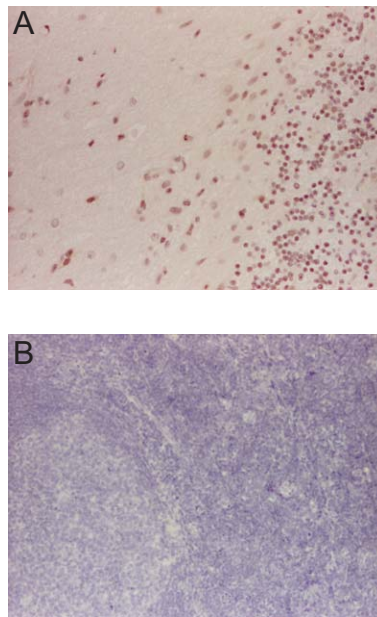


Figure 38. Microglia express phosphorylated p38 in patients with CNS TB. Brain biopsies from five patients with CNS TB and two positive and negative controls were immunostained for p38 as described in materials and methods chapter. Areas of immunoreactivity appear as brown against the blue counterstain. A, Nuclear p38 immunoreactivity is seen in microglia, principally on the left hand side of the figure (original magnification x40). B, negative control (omission of primary Ab) from tonsillar tissue used as positive control for p38 staining (original magnification x20).

## **Discussion**

In this chapter I have explored the mechanism driving the observation that CoMTb down-regulated MMP-2 in microglial cells. I have demonstrated that this occurs via an *M.tb* infected monocyte network involving TNF- $\alpha$ , p38 MAP kinase and NF $\kappa$ B. In addition a role for Caspase 8 is indicated. MMP-2 is frequently constitutively expressed at high levels in many normal physiological states principally because the gene promoter does not have a TATA box [206]. MMP-2 up-regulation is described in the CSF of TBM patients compared with controls or patients with non-infectious neurological conditions in two case series [288, 312]. Although MMP-2 was detected by immunohistochemistry in the meninges of CNS TB patients it was not seen in parenchymal cells suggesting that tissue specific down-regulation may be occurring *in vivo* [288]. It is felt that control of MMP-2 activity is achieved by restricting the activation of the secreted pro-enzyme. This occurs via a mechanism involving a molecule of both MMP-14 and TIMP-2 binding a molecule of proMMP-2 and presenting it to another MMP-14 molecule for proteolysis of the pro-domain [345]. However, in this chapter I have described how MMP-2 down-regulation in microglia is controlled at the level of gene transcription. Outside of the context of carcinomas down-regulation of MMP-2 is rarely reported.

MMP-2 secretion was suppressed by CoMTb more profoundly than by *M.tb* alone. This suppression commenced as early as 24 hours. It was then sustained up to the 96 hour timepoint. Suppression of secretion was shown to be due to suppression of MMP-2 mRNA expression. As in the preceding chapters monocyte networks appear to be key as astrocyte conditioned medium

did not affect MMP-2 secretion. Only TNF- $\alpha$  in CoMTb was responsible for down-regulation of MMP-2 secretion supported by anti-TNF- $\alpha$  neutralising antibodies and add back experiments. This is in keeping with a report from Qin *et al* who observed a similar phenomenon in astroglioma cells mediated by TNF- $\alpha$  and regulated at the level of gene transcription but not by changes in mRNA stability [193]. The negative data from blocking IL-1 $\beta$  and pertussis toxin is consistent with their findings.

Investigation of the ERK and p38 MAP kinase pathways demonstrated p38 acting as a brake to MMP-2 secretion. Addition of 1  $\mu$ M SB203580 restored the MMP-2 concentration to CoMCont levels but 10  $\mu$ M SB203580 led to supra-maximal secretion of MMP-2. The p38 MAP kinase pathway is already known to be a critical regulator of inflammatory processes [346]. p38 acts as a brake in a similar way in pulmonary epithelial cells, where it is the switching point for divergent TNF- $\alpha$  driven MMP-1 and TIMP-1 secretion [204]. The exact mechanism of how p38 acts is not clear either here or in the literature. Microglial mRNA data for SB203580 inhibition of CoMTb stimulation has not been presented but it might be expected to show restoration of MMP-2 mRNA to CoMCont levels. However, as p38 is also known to act post-transcriptionally on mRNA stability, therefore future work would have to include kinetic actinomycin D chase experiments to investigate this proposed mechanism.

Blockade of the NF $\kappa$ B pathway with SC-514 restored MMP-2 secretion to supramaximal levels over controls in the same way that p38 inhibition did. This suggests that both p38 and the NF $\kappa$ B pathway are restricting MMP-2 secretion

from microglia in basal conditions as well when stimulated by CoMTb. Although TNF- $\alpha$  mediates its pro-inflammatory activity via the NF $\kappa$ B pathway down-regulation by the same pathway is well described [347]. TNF- $\alpha$  mediated down-regulation of apolipoprotein E mRNA in adipocytes has been shown to be an NF $\kappa$ B dependent process, with p50 siRNA reversing the inhibition [348]. This suggests that investigation of p50 homodimer binding to the MMP-2 promoter by chromatin immunoprecipitation assays might be the next step although the literature does not support a consensus site for NF $\kappa$ B in the MMP-2 promoter.

However, NF $\kappa$ B has non-DNA binding mechanisms for suppressing transcription. NF $\kappa$ B dependent inhibition of MMP-2 has been described in hepatic stellate cells *in vitro*. Curcumin, a naturally occurring NF $\kappa$ B inhibitor, was shown to increase basal expression of MMP-2, via decreased nuclear p65 but no further data on the mechanism was presented [349]. In p53 (a transcription factor) negative tumour cells, p65 mediated suppression of p21 gene induction. However, in tumour cells expressing p53, competition between p53 and p65 for a limited pool of the coactivator p300/CBP was proposed as the mechanism for the loss of p65 mediated suppression observed [350]. In human monocytes a similar phenomenon was described, this time with CREB and p65 both competing for CBP, rescued by over expression of CBP [351]. Thus I hypothesise that CoMTb driven nuclear p65 competes for p300/CBP coactivator protein complexes with transcription factors known to be necessary for constitutive MMP-2 gene expression such as Sp-1, Sp3 and AP-2 [352].

This may also explain the role of p38 MAP kinase in MMP-2 repression. p38 ( $\alpha$  and  $\beta$  subunits) can feed into the NF $\kappa$ B pathway by forming part of the p65 transcription complex [353]. After p65 release from phosphorylated I $\kappa$ B proteins, in certain circumstances cytoplasmic p38 binds to the p65 nuclear localisation sequence prior to its translocation to the nucleus. Knockdown of p38 then specifically affects NF $\kappa$ B dependent genes such as CXCL8. Additionally p38 has been described as the regulator of p65 phosphorylation. Phosphorylated p65 is a more potent stimulus to gene transcription. p38 phosphorylation of the nuclear located mitogen and stress activated kinase-1 (MSK-1) led to p65 phosphorylation in RAW264.7 murine macrophages [354]. Unfortunately MSK-1 was not on the phosphoarray presented in chapter 4, but there was a non-significant increase in phosphorylation of the closely related MSK-2 (Figure 28).

Finally caspase 8 inhibition also restored MMP-2 secretion in a dose dependent fashion, but not to supramaximal levels as p38 and NF $\kappa$ B inhibition had done. These data suggest that proteolytic cleavage activity of caspase 8 is inhibitory to MMP-2 secretion. Caspase 8 is an aspartate specific cysteine protease that is the most receptor proximal caspase associated with initiation of apoptosis. Subsequently apoptosis is executed by caspase 8 cleaved molecules such as caspase 3. The caspase 8 inhibitor used (z-IETD-fmk) can block the release of cytochrome c as well as the activation of caspases 9 and 3 by its inhibition of caspase 8 [355]. Proteolytic cleavage of kinases and signal transducers by caspases is usually an inactivation step during apoptosis. However, some kinases such as MEK kinase-1 are activated by caspase mediated cleavage [356]. More specifically p38 signalling was essential for caspase 8 activation

linked to *M. avium*-induced death signalling in macrophages. This is cell specific because in some tumour cells treatment with SB203580 and expression of a dominant negative form of p38 did not affect caspase 8 activation [357]. Finally in neutrophils p38 can directly phosphorylate and inhibit the activity of caspase 8 and thereby hinder neutrophil apoptosis [358].

Non-apoptotic roles for caspase 8 in cell proliferation, differentiation, cytokine release and NF $\kappa$ B activation have already been described [359]. Caspase 8 mediates TNF- $\alpha$  activation of NF $\kappa$ B via a p38 independent pathway. In HeLa and HEK293 cells caspase 8 forms a complex with TRAF2, a TNF- $\alpha$  receptor adaptor molecule, and Flice-associated huge protein (FLASH) [360]. The mechanism of how this complex leads to IKK $\alpha$  and  $\beta$  degradation and NF $\kappa$ B nuclear translocation is not known. It is also cell type dependent as other authors have described a role for caspase 8 in T-cell receptor and LPS not TNF- $\alpha$  mediated NF $\kappa$ B activation in T-cells [361].

Inhibition of caspase 8 could have led to suppression of microglial apoptosis thereby increasing the number of cells secreting MMP-2 but this was not analysed. CoMTb mediates significant up-regulation of MMP-1 and -3 secretion and TIMP-1 secretion continues at a high level suggesting that apoptosis is not a significant factor in this model. In addition activation of the canonical NF $\kappa$ B pathway is a recognised anti-apoptotic stimulus. Of interest would be to inhibit Caspase 8 in CoMTb stimulated cells and measure MMP-2 mRNA levels, p38 phosphorylation and nuclear p65 translocation. Caspase 8 activation could be measured by Western analysis of cell lysates too [252].

In summary I have shown that *M.tb* infected monocyte networks involving TNF- $\alpha$ , p38 MAP kinase, NF $\kappa$ B and caspase 8 act to suppress MMP-2 secretion in microglial cells. This serves to remind that it is the overall balance of MMPs and TIMPs within the inflammatory milieu that will affect the absolute level of matrix degradation occurring.

## Chapter 6 – Analysis of CSF MMPs/TIMPs in TBM

### *Introduction*

The preceding chapters have concentrated on analysis of data generated from an *in vitro* model of CNS TB with *in vivo* confirmation from biopsy specimens. Thus findings reflect microglial specific processes to a larger extent. However, TBM is a meningoencephalitis and so analysis of CSF should reflect total net secreted MMP/TIMP load from all CNS cells such as endothelium, astrocytes, microglia, neurons and infiltrating leucocytes. As suggested by data in chapter 5 the flux of MMPs/TIMPs is as important as changes in individual molecules due to potential substrate overlap. In addition, over time the quantity and phenotypic quality of cells involved in any inflammatory circumstance will change leading to alterations in MMP/TIMP concentrations. I now present longitudinal analysis of archived CSF samples from TBM patients that I performed at the Wellcome-Oxford University Clinical Research Unit in Ho Chi Minh City, Vietnam in collaboration with Dr Guy Thwaites (Imperial College).

Previously it has been shown that adjunctive dexamethasone, administered with anti-tuberculosis drugs, improved the outcome of adults with TBM [107], but the mechanism underlying this effect is not understood. Dexamethasone did not have any significant effect on CSF white cell infiltration or cytokine expression in 93 patients recruited to the original clinical trial [50]. A subset of patients had serial brain magnetic resonance imaging which suggested dexamethasone might decrease the incidence of hydrocephalus and infarction [95]. However, no

study has clearly demonstrated the mechanism by which dexamethasone reduced case-fatality from TBM.

In this study, I measured serial CSF concentrations of the MMPs/TIMPs identified as key in TB in a sub-set of adults with TBM recruited to a randomised controlled trial of adjunctive dexamethasone [107]. MMP-9 has already been identified as important in CNS TB [221, 313]. MMP-1, -2, -3, -8, -9 and -10 were identified in the global analysis presented in the preceding chapters.

Macrophages infected with *M.tb* secreted MMP-7, in addition to MMP-1, in a cellular model of TB [236]. As CSF will only contain secreted molecules TIMP-3, which is membrane associated, was not analysed, but TIMP-1, 2 and 4 were.

The aim was to investigate the relationship between dexamethasone treatment, CSF MMP/TIMP expression and clinical outcome.

## **Methods**

### *Study participants and setting*

I studied a subset of patients from a previously reported randomised, double blind, placebo-controlled trial of adjunctive dexamethasone for the treatment of TBM in 545 Vietnamese adults [107]. Disease severity at admission was defined by the Glasgow coma scale (GCS). Assessment of GCS was performed by summation of clinical assessment of eye opening (awarded 1-4 points), best verbal response (1-5 points) and best motor function (1-6 points), where lower values indicate more severe neurological dysfunction [362]. Disease severity was also assessed using the United Kingdom Medical Research Council grade (UK MRC) for TBM [363]: patients in grade I had a GCS of 15 (fully conscious)

and no focal neurological signs; patients in grade II had a GCS of between 11 and 14 and/or focal neurological signs; and patients in grade III had severe coma with a GCS of less than 11.

To be eligible for selection for this study patients had to have been recruited at the Hospital for Tropical Diseases (HTD), Ho Chi Minh City, have a microbiologically confirmed diagnosis of TBM (*Mycobacterium tuberculosis* cultured from the CSF), negative HIV-1 & -2 antibodies, and at least two serial CSF specimens taken within the first 60 days of treatment (when dexamethasone was stopped) available for analysis, unless they had died within this period.

### *Ethics*

The ethical and scientific committees of all institutions approved the study and written, informed consent to participate in the study was obtained from the patients or their relatives.

### *Treatment and specimen collection*

Adults were allocated randomly to start dexamethasone sodium phosphate or placebo as soon as possible after the start of four drug anti-tuberculosis therapy as previously described [107]. Briefly, adults with grade II or III disease received intravenous study drug for 4 weeks (week 1 0.4 mg/kg/day); week 2 0.3 mg/kg/day, week 3 0.2mg/kg/day, week 4 0.1 mg/kg/day), then 4 weeks of tablets starting at 4mg total dose per day, reducing by 1mg per week until zero. Those with grade I disease received 2 weeks of intravenous study drug (week 1

0.3 mg/kg/day, week 2 0.2 mg/kg/day) then 4 weeks of tablets (week 3 0.1 mg/kg/day; then 3mg total per day reducing by 1mg per week until zero).

Serial CSF samples (frozen at -70°C) and routine clinical investigations were collected as part of normal clinical care pre-treatment and on study days 3, 7, 30, 60 and 270.

#### *Assessment of the clinical and CSF inflammatory response to treatment*

The primary outcome of the trial was death or severe disability 9 months after randomisation and was assessed blind to the treatment allocation [107].

Likewise, treatment allocation was unknown to those assessing conventional and experimental CSF inflammatory responses. CSF white cell count, protein, glucose and lactate concentrations were determined by standard methods.

Blood brain barrier integrity was assessed by measurement of paired CSF and plasma albumin concentrations by standard methods. The albumin index was calculated using the formula  $[\text{albumin}_{\text{csf}}]/[\text{albumin}_{\text{plasma}}]$  [52]. The concentrations of CSF cytokines (IFN- $\gamma$ , TNF- $\alpha$ , and the interleukins -1 $\beta$ , -6, -8, -10, and 12p70) were determined by cytometric bead array assay (BD Biosciences) as described [50].

#### *MMP and TIMP concentration measurement*

MMP-1, -2, -3, -7, -8, -9 and -10 and TIMP-1, -2 and -4 were measured by ELISA (R&D Systems) according to the manufacturer's instructions. The lower level of sensitivity for the ELISAs were 156 pg/ml for MMP-1 and 7, 78 pg/ml for MMP-2 and 10, and 31 pg/ml for MMP-3, 8, 9, TIMP-1, 2 and 4. Samples with high MMP/TIMP concentrations were diluted as appropriate. Values below the

level of detection were assigned an arbitrary value halfway between zero and the lower level of sensitivity.

### *Statistical analysis*

Continuous variables were compared by Student's t-test if normally distributed and Mann-Whitney U test if non-normally distributed. Where more than two groups were compared the Kruskal-Wallis test was used for non-parametric data. Categorical data were compared using Fisher's exact test or the  $\chi^2$  test. All p-values were two sided and a value of  $<0.05$  was taken as significant. Baseline MMPs and TIMPs were analysed by univariate analysis to examine relationships between presenting clinical features and outcomes. Continuous variables were modelled using linear regression and binary outcomes using forward and backward likelihood ratio logistic regression. The changes in MMP/TIMP concentration between pre-treatment and follow-up samples were calculated using repeated measures ANOVA with dexamethasone as a covariate. All analyses were performed using SPSS version 15.0 (SPSS Corp).

## **Results**

37 patients, of whom 18 received dexamethasone, satisfied the study entry criteria and their baseline characteristics and laboratory parameters are summarised in table 6. These were similar between the two study groups. Patient median age was 29 (interquartile range [IQR] 21 - 41.5) years and 22/37 (59%) were female. On admission patients were febrile with a median GCS of 13 (IQR 11-15). In addition 14/37 (38%) had at least one cranial nerve palsy, 5/37 (14%) had hemiparesis but only 1/37 (3%) had paraparesis. At the end of treatment a combined poor outcome (death or severe disability) was seen in

8/37 (22%) and 5/37 (14%) of patients died. Baseline CSF laboratory findings, which are summarised in table 7, were similar between the two groups except there was a raised CSF opening pressure in the dexamethasone-treated group ( $p=0.05$ ). In total 141 of 185 (76%) CSF specimens taken were available for analysis of longitudinal changes in MMPs/TIMPs concentrations. 66/94 (70%) were from placebo and 75/91 (82%) from dexamethasone group patients; a mean of 3.8 (+/-1.2) samples per patient. Sample exhaustion accounted for the missing specimens.

**Table 6. Baseline demographics, presenting features and clinical outcome of study patients.**

| Variable**                  | Placebo            | Dexamethasone      |
|-----------------------------|--------------------|--------------------|
| Number (n)                  | 19                 | 18                 |
| Age (yr)                    | 31 (23.0-44.0)     | 25 (19.25-40.5)    |
| Gender (M:F) n              | 7:12               | 8:10               |
| TBM grade n (%) I           | 2 (10.5)           | 6 (33.3)           |
| II                          | 11 (57.9)          | 7 (38.9)           |
| III                         | 6 (31.6)           | 5 (27.8)           |
| Weight mean (sd, kg)        | 49.3 (8.5)         | 48.4 (6.5)         |
| Temperature °C              | 38.7 (38.0 - 39.3) | 39.0 (38.0 - 39.5) |
| GCS at presentation         | 13 (10-14)         | 13.5 (10-15)       |
| Duration of symptoms (days) | 17 (12-21)         | 17 (12-26.25)      |
| HIV negative n (%)          | 19 (100)           | 18 (100)           |
| Cranial Nerve Palsy n (%)   | 8 (42.1)           | 6 (33.3)           |
| Hemiparesis n (%)           | 2 (10.5)           | 3 (16.7)           |
| Paraparesis n (%)           | 0 (0)              | 1 (5.6)            |
| Death n (%)                 | 3 (15.8)           | 2 (11.1)           |
| Combined poor outcome n (%) | 4 (21.1)           | 4 (22.2)           |

\* Values are median (IQR) or number (percentage) unless stated otherwise.

# No significant differences between groups.

**Table 7. Baseline CSF parameters of study patients.**

| Variable*                          | Placebo          | Dexamethasone    |
|------------------------------------|------------------|------------------|
| Number (n)                         | 19               | 18               |
| Opening pressure (cm) <sup>#</sup> | 18.0 (16-32)     | 33 (19.25-39.0)  |
| Total WCC (cells/ $\mu$ l)         | 470 (130-625)    | 250 (147-613)    |
| Neutrophils (%)                    | 30 (18-43)       | 40 (24.5-67.5)   |
| Neutrophils (cells/ $\mu$ l)       | 93 (25-251)      | 101 (38-281)     |
| Lymphocytes (%)                    | 70 (57-82)       | 60 (42-75.5)     |
| Lymphocytes (cells/ $\mu$ l)       | 210 (125-398)    | 164 (86-312)     |
| Protein (g/L)                      | 160 (102-300)    | 200 (150-297)    |
| Lactate (mmol/L)                   | 6.2 (4.6-8.1)    | 6.6 (5.2-8.9)    |
| Glucose ratio – CSF/blood          | 0.17 (0.09-0.30) | 0.25 (0.14-0.34) |
| Albumin ratio – CSF/blood (%)      | 1.12 (0.58-2.84) | 2.03 (0.65-2.73) |

\* Values are median (IQR) unless stated otherwise.

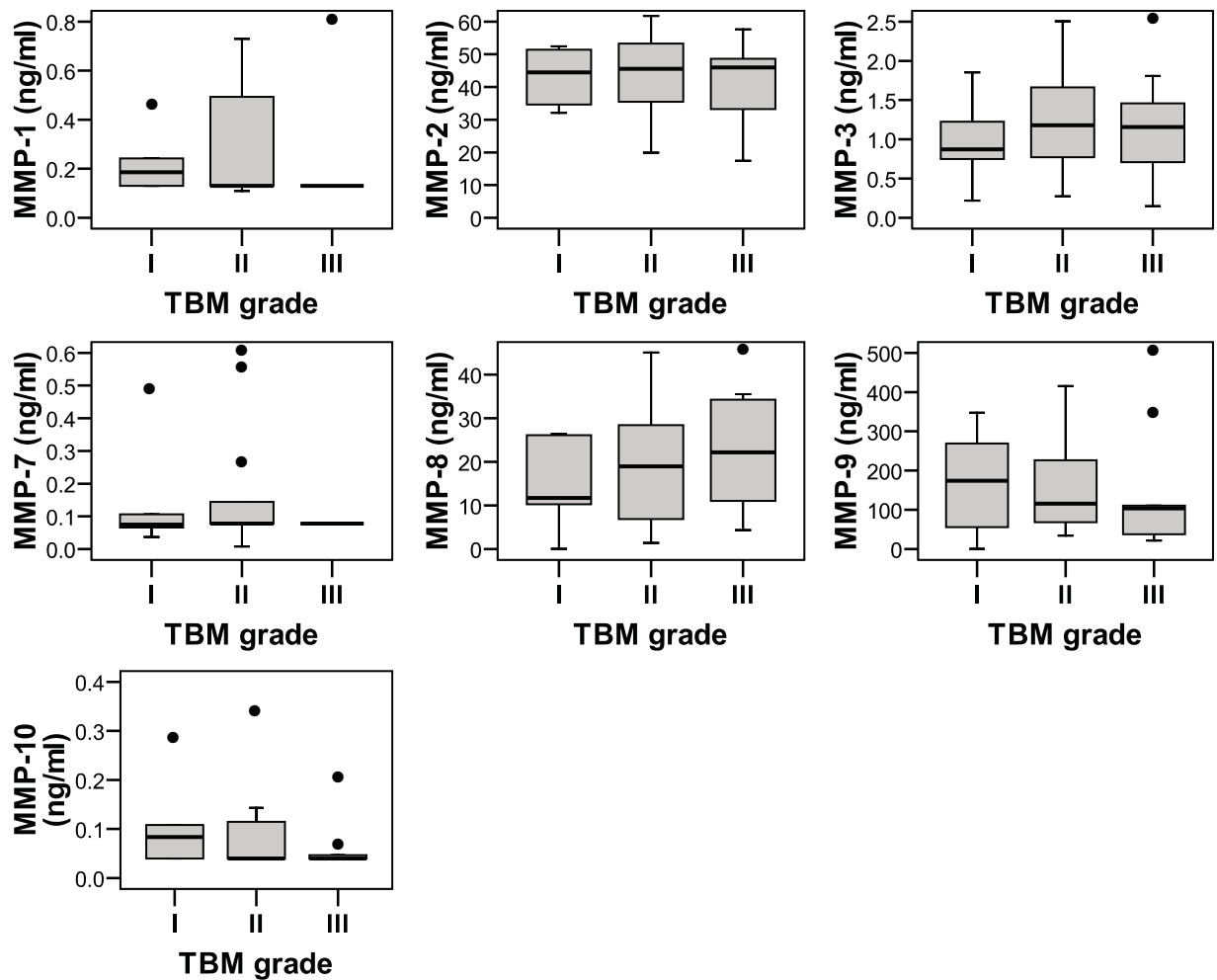
# p=0.05 for group comparison.

*The relationship between baseline CSF MMP and TIMP concentrations, pre-treatment disease features and clinical outcome*

Baseline pre-treatment concentrations of MMPs and TIMPs are summarised in table 8. MMP-2, -3 and -9 concentrations were detectable in all 30 pre-treatment CSF samples and MMP-8 concentrations were detectable in 29 (97%). CSF concentrations of MMP-1 and -7 were only detectable in 9 (30%) patients and MMP-10 in 10 (33%) patients. All 30 patients had detectable TIMP-1 and -2 and 29 (97%) patients had detectable CSF TIMP-4.

Baseline MMP and TIMP concentrations stratified by TBM grade were analysed but no significant relationships were found (Figure 39). Therefore I investigated the relationship between increasing severity of coma (i.e. lower GCS) and CSF MMP/TIMP concentrations (corrected for CSF white cell count) by linear regression. The final model (table 9) showed higher MMP-9 and TIMP-1 concentrations were significantly correlated with higher coma score (lower coma severity, both  $p < 0.05$ ), and MMP-3 showed a strong trend in this direction

## A) MMPs



## B) TIMPs

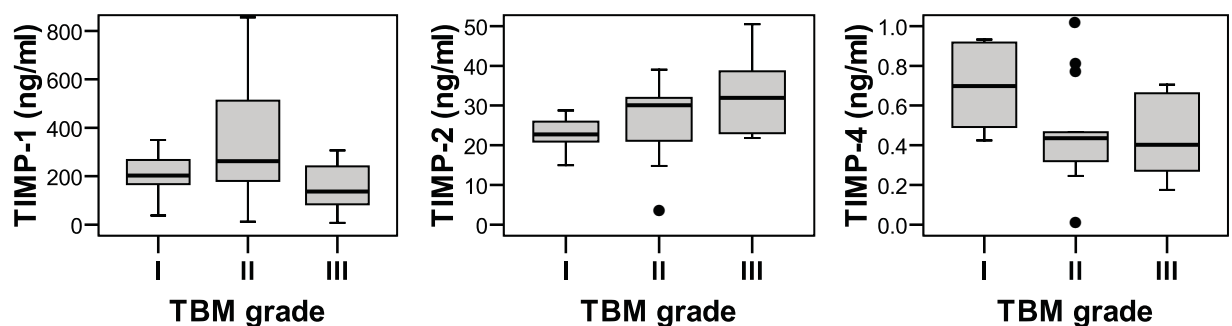


Figure 39. CSF MMP(A)/TIMP(B) concentrations vary by severity of coma at presentation. CSF samples were analysed by ELISA and are presented stratified by MRC TBM coma grade (where I is mild and III severe coma). Outliers are represented by closed circles (●). The black line represents the median value and the box the interquartile range. Whiskers represent 1.5 times the interquartile range away from the box. Data were analysed by the Kruskal-Wallis test.

**Table 8. Baseline CSF MMP/TIMP concentrations\*.**

| Variable                    | Placebo             | Dexamethasone      |
|-----------------------------|---------------------|--------------------|
| Number (n)                  | 15                  | 15                 |
| MMP-1 (ng/ml)               | 0.13 (0.13-0.49)    | 0.13 (0.13-0.24)   |
| MMP-2 (ng/ml)               | 47.0 (37.5-53.2)    | 42.2 (33.3-48.7)   |
| MMP-3 (ng/ml)               | 1.22 (0.80-1.46)    | 0.97 (0.27-1.66)   |
| MMP-7 (ng/ml)               | 0.078 (0.078-0.079) | 0.078 (0.075-0.12) |
| MMP-8 (ng/ml)               | 18.9 (10.9-28.4)    | 12.6 (7.9-28.6)    |
| MMP-9 (ng/ml)               | 108.3 (37.8-226.5)  | 110.8 (93.4-269.2) |
| MMP-10 (ng/ml)              | 0.04 (0.04-0.11)    | 0.04 (0.04-0.11)   |
| TIMP-1 (ng/ml)              | 248.6 (116.2-421.1) | 180.1 (83.9-241.0) |
| TIMP-2 (ng/ml) <sup>#</sup> | 31.6 (28.2-38.6)    | 23.0 (20.9-29.6)   |
| TIMP-4 (ng/ml)              | 0.43 (0.32-0.47)    | 0.49 (0.32-0.87)   |

\* Values are median (IQR) unless stated otherwise.

<sup>#</sup> p=0.01 for group comparison.

(p=0.07). CSF MMP-8 and TIMP-2 were inversely associated with coma score (both p<0.05). Baseline higher MMP-8 and lower MMP-9 concentrations also predicted combined poor outcome in multivariate analysis of all MMPs/TIMPs and CSF WCC (MMP-8: OR=85, p=0.03 [95% CI 2, 4218] and MMP-9: OR=0.02, p=0.04 [95% CI 0.0003, 0.8]).

**Table 9. Summary of multiple linear regression - MMPs/TIMPs that predict more severe coma (lower Glasgow Coma Score) on clinical presentation with TBM.**

| Variable | Unadjusted       |      | Multiple regression analysis |       |
|----------|------------------|------|------------------------------|-------|
|          | Coeff. (95% CI)  | P    | coeff. (95% CI)              | P     |
| MMP-3    | 0.03 (-2.3, 2.4) | 0.98 | 3.2 (-0.3, 6.8)              | 0.07  |
| MMP-8    | -37 (-95, 20)    | 0.19 | -258 (-445, -70)             | 0.009 |
| MMP-9    | -19 (-62, 23)    | 0.37 | 190 (37, 344)                | 0.02  |
| TIMP-1   | 1.0 (-0.8, 2.8)  | 0.27 | 2.7 (0.7, 4.8)               | 0.01  |
| TIMP-2   | -0.9 (-3.3, 1.5) | 0.45 | -7.6 (-12.5, -2.8)           | 0.03  |

Variables entered into model: MMP-1, -2, -3, -7, -8, -9, -10 & TIMP-1, -2, -4 adjusted for CSF WCC (R<sup>2</sup>=0.45, ANOVA p=0.01 for final model).

I found no other significant relationships between pre-treatment MMP/TIMP concentrations and combined poor outcome, mortality, cranial nerve palsy, hemiparesis and paraparesis on admission by multivariate analysis, although

there was a significant relationship between lower pre-treatment MMP-2 concentrations in CSF and hemiparesis ( $p=0.02$ ) on univariate analysis. There was no relationship between duration of symptoms and MMP/TIMP concentrations.

*MMP non-secretion and disease severity at clinical presentation*

21/30 (70%) pre-treatment CSF samples were from patients with undetectable CSF MMP-1. 14 of these 21 patients (67%) also had undetectable MMP-7 and of these 14 patients 8 (57%) had undetectable MMP-10. These 8 patients were categorised as “non-secretors” and compared to the remaining 22 “secretors” by TBM grade. Non-secretors made up 0/6 (0%), 3/15 (25%) and 7/9 (78%) of patients with grade I, II and III disease respectively ( $p=0.003$ ). There was no significant difference in CSF white cell or neutrophil count between groups to explain the discrepancies in MMP secretion. An exploratory univariate analysis revealed a significant relationship between non-secretors and admission GCS ( $OR=1.6$ ,  $p=0.02$  [95% CI 1.1, 2.2]) and a trend with combined poor outcome on admission ( $p=0.08$ ). There was no significant relationship between non-secretors and survival, cranial nerve palsy, hemiparesis or paraparesis on admission. Multivariate analysis with age, sex, GCS, combined poor outcome and CSF white cell count within the model showed GCS as the only independent predictor of failure to secrete detectable CSF MMP-1, -7, and -10 in the model.

*The influence of dexamethasone on CSF MMP/TIMP concentrations*

22 patients had both a pre-treatment and at least one paired ‘early’ follow up sample available for analysis, taken a median 5 days (range 3-8) into anti-

tuberculosis treatment. Where two samples were available from this period the results from the earlier one were used in analysis. CSF MMP-9 concentrations were significantly lower in the early follow-up samples of patients given dexamethasone compared to placebo ( $p=0.01$ , Figure 40), confirmed by repeated measures ANOVA with CSF white cell count as a covariate ( $p=0.002$ ). 3/11 (27%) patients in the placebo group had a fall in their CSF MMP-9 concentration and 9/11 (82%) of the dexamethasone group ( $p=0.03$ ) between pre-treatment and the early follow-up sample. However, there was no significant relationship between those patients whose CSF MMP-9 fell and outcome.

There was a similar but less marked effect of dexamethasone on MMP-8 concentrations. A trend for lower CSF MMP-8 concentrations in the dexamethasone arm ( $p=0.08$ ) was not significant in the repeated measures ANOVA. No other significant relationships were found at this or any later time points for any other MMP/TIMP and treatment group. MMP-9 concentrations at later time points were not statistically different between the two groups. Day 270

**Table 10. Day 270 CSF MMP/TIMP concentrations.**

| Variable       | CSF Concentration [median (IQR)]* | P value <sup>#</sup> |
|----------------|-----------------------------------|----------------------|
| MMP-1 (ng/ml)  | 0.13 (0.13 - 0.13)                | 0.34                 |
| MMP-2 (ng/ml)  | 36.7 (33.0 - 50.7)                | 0.26                 |
| MMP-3 (ng/ml)  | 0.26 (0.12 - 0.45)                | <0.001               |
| MMP-7 (ng/ml)  | 0.08 (0.07 - 0.11)                | 0.63                 |
| MMP-8 (ng/ml)  | 0.03 (0.03 - 0.03)                | <0.001               |
| MMP-9 (ng/ml)  | 4.7 (0.9 - 12.2)                  | <0.001               |
| MMP-10 (ng/ml) | 0.04 (0.04 - 0.05)                | 0.20                 |
| TIMP-1 (ng/ml) | 23.9 (15.2 - 36.0)                | <0.001               |
| TIMP-2 (ng/ml) | 26.4 (23.1 - 37.4)                | 0.82                 |
| TIMP-4 (ng/ml) | 2.1 (1.4 - 3.1)                   | <0.001               |

\* Data are presented unstratified for treatment group, n=19.

<sup>#</sup> Comparison to baseline value in table 8.

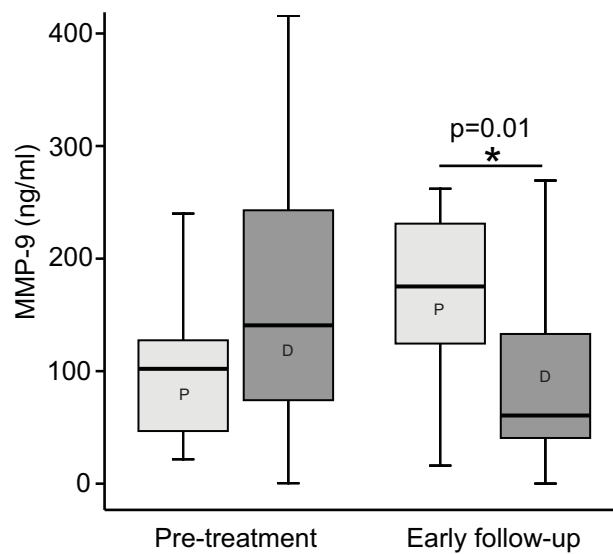


Figure 40. Dexamethasone reduces CSF MMP-9 concentrations in early follow up samples. Paired CSF samples from patients with tuberculous meningitis were taken pre-treatment and at early follow up, a median 5 days (range 3-8) into anti-tuberculosis therapy, and analysed for MMP-9 concentration by ELISA. Patients who received placebo (P, light grey bars, n=11) had significantly higher MMP-9 concentrations in their early follow-up CSF samples than those who received dexamethasone (D, dark grey bars, n=11). The black line represents the median value and the box the interquartile range. Whiskers represent 1.5 times the interquartile range away from the box. Groups were analysed by the Mann-Whitney U test. \*,  $p=0.01$ .

MMP/TIMP concentrations and their statistical change from baseline are summarised in table 10. Of note MMP-3, -8, -9 and TIMP-1 fell and TIMP-4 rose significantly over the course of 9 months anti-tuberculosis treatment.

*The relationship between CSF WCC and MMP-9 concentrations*

Neutrophils play a key but possibly under-recognised role in host defence to mycobacteria and release preformed MMP-9 from granzymes to cross the BBB [364, 365]. CSF MMP-9 concentration and neutrophil count were correlated on analysis of all samples ( $R^2 = 0.19$ ,  $p < 0.001$ ). There was a significant correlation between MMP-9 concentration and CSF WCC ( $R^2 = 0.31$ ,  $P = 0.004$ ) in all early follow-up samples and a stronger correlation with CSF neutrophil count (Figure 41,  $R^2 = 0.52$ ,  $P < 0.001$ ) which was dexamethasone independent.

*The relationship between MMP-9 and other CSF inflammatory parameters*

BBB disruption is potentially related to MMP activity [300], but I could find no relationship between the albumin index and CSF MMP/TIMP concentration. I found significant correlations between CSF total protein (the only CSF analyte to fall significantly due to dexamethasone [50]) and MMP-9 ( $R^2 = 0.18$ ,  $p < 0.001$ ) concentrations. When treatment groups were analysed separately dexamethasone significantly strengthened the relationship between MMP-9 and total protein concentrations (placebo  $R^2 = 0.05$ , dexamethasone  $R^2 = 0.41$ ,  $p = 0.004$ ). I could find no relationship between MMP-9 and TIMP-1, or their ratio and clinical outcome.

I then explored the relationship between CSF concentrations of TNF- $\alpha$ , IFN- $\gamma$ , IL-6, IL-8, IL-10 (data not presented) and MMP-9 in all specimens and found

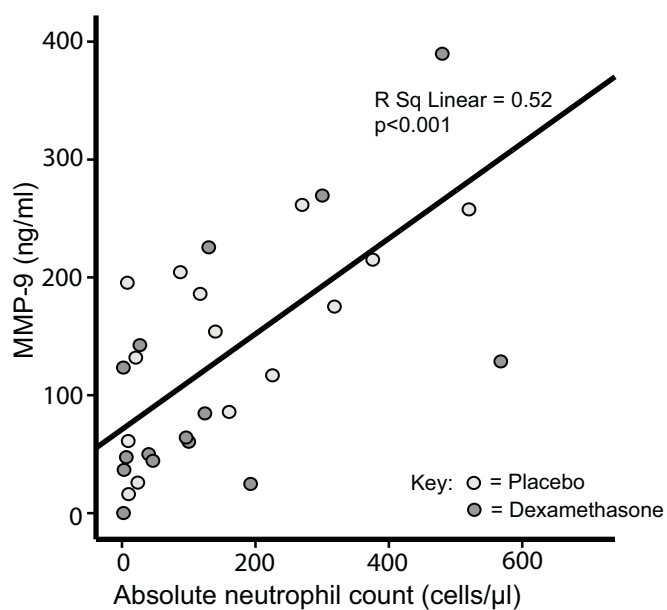


Figure 41. CSF MMP-9 concentration and CSF neutrophil count are significantly correlated in early follow up samples. Early follow-up CSF samples, taken a median of 5 days (range 3-8) into anti-tuberculosis treatment, from patients with tuberculous meningitis were analyzed for correlation between absolute neutrophil count and MMP-9 concentration (measured by ELISA). The  $R^2$  value for Spearman's correlation was 0.52 ( $p<0.001$ ). Patients who received placebo are represented by light grey circles and dexamethasone by dark grey circles.

only IL-10 and MMP-9 to be correlated ( $R^2 = 0.22$ ,  $p < 0.001$ ). Concentrations of IL-1 $\beta$  and IL-12p70 levels were below the level of detection in all but 6 and 8 specimens respectively so were not analysed. I looked specifically at baseline and early follow-up samples but did not find other significant relationships.

## ***Discussion***

In this study I have examined the role of MMPs/TIMPs in the immunopathology of TBM in patients and the influence dexamethasone has on their secretion. I demonstrated a significant relationship between MMP-8, -9, TIMP-1 and -2 and the severity of coma at presentation and MMP-8 and -9 with death or severe neurological disability by 9 months of treatment. There were strong correlations between CSF MMP-9 and the CSF concentrations of neutrophils, and total protein, but not pro-inflammatory cytokines. Dexamethasone decreased CSF MMP-9 concentrations early in the course of treatment and may represent one mechanism by which corticosteroids improve outcome. Higher CSF MMP-9 concentrations before the start of treatment were associated with better outcome. Dexamethasone did not influence the CSF concentrations of other MMPs/TIMPs.

The CSF concentrations of MMP-2, -3, -8 and -9, and TIMP-1, -2 and -4 were elevated in all patients with TBM before the start of treatment. When corrected for CSF white cell count, concentrations of MMP-8 and -9 and TIMP-1 and -2 were significantly associated with severity of coma at presentation. Higher CSF MMP-9 concentrations were associated with less severe coma before anti-tuberculosis treatment and a lower incidence of death or disability by the end of treatment. CSF MMP-8 and -9 concentrations were up-regulated in patients with

pyogenic bacterial meningitis and high MMP-9 concentrations were associated with poor outcome [285]. In a previous retrospective study that compared adults with TBM to those with viral or bacterial meningitis, and controls, increased CSF MMP-9 concentrations were related to increased disease severity and case-fatality, confirmed by others in studies of TBM alone [288, 313]. Taken together, the data from this and previous studies demonstrate that MMP-9 has a key role in the pathogenesis of TBM. MMP-9 may have both protective and detrimental clinical effects. Inhibition of MMPs by doxycycline in mice decreased pneumococcal CNS immunopathology, but MMP-9 knockout mice with experimental bacterial meningitis had reduced host defence mechanisms to the same pathogen [172, 289]. Murine studies suggest MMP-9 is important in host defence to *M. tuberculosis*. Macrophages from mice resistant to *M. tuberculosis* produce more MMP-9 mRNA in response to intracellular infection than susceptible mice [366]. Non-specific MMP inhibition blocked early dissemination of *M. tuberculosis* and MMP-9-knockout mice had both reduced pulmonary recruitment of macrophages and poorer granuloma formation [190].

These data suggest the majority of early CSF MMP-9 is released from neutrophils. Other studies have reported strong correlations between MMP-9 and neutrophils in viral meningitis and with total white cells in TBM [284, 312]. Neutrophils release large amounts of preformed MMP-9 in response to many inflammatory stimuli, which may explain the lack of correlation observed between CSF MMP-9 and the pro-inflammatory cytokines measured [367]. The role of the neutrophil in the pathogenesis of tuberculosis is not well understood. Peripheral neutrophil counts have been inversely related to risk of tuberculosis

in tuberculosis contacts and neutrophil depletion *ex vivo* impaired the ability of whole blood to restrict mycobacterial growth via depletion of secreted anti-microbial peptides [365]. A report that neutrophil derived anti-microbial peptides are released in response to *M. tuberculosis* infection but not expressed in the tuberculous granuloma is consistent with the proposed early role for neutrophils in tuberculosis [368]. In addition, adults with TBM and a high proportion of neutrophils in the CSF have an increased chance of survival [52].

Consistent with the finding of decreased CSF MMP-9 concentrations in patients with more severe coma, non-secretors of MMP-1 (a collagenase), MMP-7 (an elastase) and MMP-10 (a stromelysin) were also associated with more severe coma at presentation. MMP-1 and -7 are present in pulmonary TB granulomas and *M. tuberculosis*-infected macrophages up-regulate expression of MMP-1, -7 and -10 mRNA *in vitro* [236]. However, TBM is an encephalomyelitic process and dissociation between parenchymal and secreted MMPs may account for their lower levels in the CSF [369]. Immunohistochemical analysis of brain biopsies in chapter 3 suggested that the parenchymal TB granuloma is abundant in MMP-1. Alternatively, there is evidence that severe TBM may be associated with a relative immune-paresis, or cellular exhaustion as has been described in measles [370].

MMP-3 concentrations decreased 4-fold during treatment and I found a strong trend towards higher MMP-3 levels predicting more severe coma score on presentation. MMP-3 expression and secretion was up-regulated in microglia (chapter 4) suggesting they are a likely source of CSF MMP-3. MMP-3 mRNA is

known to be up-regulated in TB infected macrophages, but astrocytes have previously been shown not to be a source of MMP-3 in CNS TB [221, 236]. No data exist for oligodendrocytes. An LPS/MMP-3 knock-out mouse model showed less BBB disruption suggesting a role for MMP-3 in neuroinflammation [301]. MMP-3 cleaves myelin basic protein and MMP-3 release from apoptotic neurons activates microglia, perhaps suggesting a mechanism of enhancing further MMP secretion [269, 371]. TIMP-1 levels fell from a high baseline level 10-fold during treatment but were still elevated at the end of treatment. TIMP-1 is constitutively expressed by a number of CNS cells (including microglia and astrocytes) and elevated CSF concentrations have been reported in patients with viral and eosinophilic meningitis [219, 221, 284]. Although neuronal associated TIMP-1 is down-regulated in immunohistochemical studies of parenchymal cerebral TB CSF concentrations in TBM are higher than in viral, but not bacterial, meningitis [221, 313]. TIMP-4 concentrations increased greater than 4-fold during treatment. Similarly suppressed baseline CSF TIMP-4 has been reported in eosinophilic meningitis [219, 372]. TIMP-2 concentration was elevated at baseline and levels did not change during longitudinal analysis consistent with constitutive expression as described [221].

Lower baseline MMP-2 concentration was associated with a significant risk of hemiparesis suggesting that the down-regulation presented in the cellular data (chapter 5) is important *in vivo*. Evidence from a study of experimental olfactory nerve injury in mice suggests that MMP-2 has a role in neuronal regeneration [373]. Peak MMP-2 levels corresponded to the onset of neural repair. Hence lower MMP-2 concentrations maybe detrimental to patients due to defective

healing rather than as a consequence of direct neuronal damage as suggested for MMP-1 and -3.

Dexamethasone, the only known effective adjuvant to anti-tuberculosis chemotherapy, significantly reduced MMP-9 concentrations in early follow-up samples. Dexamethasone may improve outcome by influencing MMP-9 secretion. I was not able to show a direct relationship between those with reduced post-treatment MMP-9 concentrations and improved outcome, but this study was not powered to confirm such an effect. No effect was observed at later time-points or on the other MMPs/TIMPs studied. Limitations in methodology may partly explain this observation. I used ELISAs that detected both active and pro-MMP-9 thus cannot differentiate between biologically active and inactive or degraded MMP-9 [320]. Differences in the activity of MMP-9 (assessed by zymography) have been reported in experimental pyogenic meningitis where treatment with antibiotics alone increased CSF MMP-9 activity, but dexamethasone co-administration suppressed this effect [292]. Additionally differential effects of dexamethasone suppressing TNF- $\alpha$  and IFN- $\gamma$  driven MMP-9 secretion, but leaving TIMP-1 and -2 secretion unchanged in *M. tuberculosis*-stimulated human astrocytic cells have been described [221, 315].

Given the strong correlation between CSF neutrophils and MMP-9 concentrations I investigated the influence of dexamethasone on CSF neutrophil numbers, but did not observe any differences between the treatment groups at any time point. However, dexamethasone may affect function rather than absolute numbers [107]. Corticosteroids can rapidly prevent neutrophil

degranulation independently of effects on protein synthesis [374]. The restricted release of neutrophil derived proteins in dexamethasone treated patients may have contributed to the significant reduction in CSF protein observed in the dexamethasone group in the previous study involving a larger number of patients from the same trial [50].

In summary, this chapter has identified key relationships between baseline CSF MMP-8, -9, TIMP-1 and -2 concentrations, severity of coma at presentation and clinical outcome in TBM patients. I have shown that dexamethasone may influence outcome from TBM by reducing MMP-9 secretion into the CSF as has been shown for MMP-1 and -3, but not MMP-2 *in vitro*. Future research needs to delineate the roles of neutrophils and their MMPs in TBM more clearly.

## Chapter 7 – Summary and conclusions

The experiments presented have investigated the hypothesis that that microglia control the CNS immune response to *M.tb* infection via the production of a different spectrum of MMPs compared to monocytes, macrophages and other glial components. By performing global analysis of all MMPs/TIMPs in human microglia in response to *M.tb* infection and *M.tb* infected monocyte-microglial networking (CoMTb) I found that MMP-1, -3 and -9 gene expression were significantly up-regulated only in CoMTb stimulated cells. MMP-8 and -10 were up-regulated to a lesser degree. In contrast TIMP-1 to -4 expression was either static or suppressed by the same stimuli. Conditioned media from both microglia and primary astrocytes did not up-regulated MMP secretion in the same way as CoMTb suggesting that microglia require monocyte activity to be a source of MMPs. There is published evidence that the astrocyte response to *M.tb* infection is not as robust as that from microglia or monocyte in terms of cytokines and MMPs but investigations of oligodendrocytes are lacking [156, 221]. Thus cells of monocyte lineage, both monocytes and microglia, appear to be the critical cells orchestrating the CNS immune response to TB.

After confirming MMP-1 and -3 expression were accompanied by secretion and observed *in vivo* in patient biopsies or CSF I attempted to unpick the components of CoMTb that were responsible for MMP-1 and -3 secretion. There was a difference between these two key MMPs. Although both were TNF- $\alpha$  and IL- $\beta$  requiring neither cytokine alone could reproduce the effect of

CoMTb at working concentrations found in it. Significant synergy existed between TNF- $\alpha$  and IL- $\beta$  leading to MMP-3, but not MMP-1, secretion. Nevertheless there was a missing factor in CoMTb which I was unable to find. This may have been filtered *M.tb* antigens although the data supporting this were not conclusive. Kinyoun staining and confocal microscopy helped confirm that differences in MMP gene expression seen between direct infection and CoMTb stimulated cells were not simply due to lack of phagocytosis of bacilli, although the cells used did not perform as well as the published microglial literature in this respect.

In depth analysis of nuclear signalling events that led to MMP-1 secretion suggested that p50-p65 heterodimers of NF $\kappa$ B and cJun/FosB AP-1 subunits are rapidly recruited to the promoter, leading to up-regulation of MMP-1 gene expression. Some of this work was also performed in a more limited way for MMP-3. I then focused upon the ERK and p38 MAP kinases, identified as key in a global analysis of serine/threonine kinase pathways activated by CoMTb. The proteome profiler array and subsequent experiments demonstrated that the p38 $\alpha$  pathway is critical for MMP-1 and -3 gene expression and secretion. As dexamethasone is used as an adjuvant to anti-tuberculosis therapy in TBM I investigated its effect on MMP-1 and -3 secretion foreshadowing the clinical study presented in the final results chapter. This demonstrated that both MMP-1 and -3, but not TIMPs, were suppressed by dexamethasone at the level of gene transcription.

As the flux of MMPs is likely to be important *in vivo* due to substrate overlap I next investigated the mechanism of CoMTb mediated MMP-2 suppression. This was driven by a TNF- $\alpha$ /p38/NF $\kappa$ B/caspase 8 pathway, but due to time constraints I was unable to demonstrate convincing links between the individual components of the pathway identified. Additionally technical difficulties (namely the high MMP-9 content of CoMTb) made further analysis of the interesting dynamic whereby MMP-9 is up-regulated at the same time as MMP-2 is down-regulated impossible within the timescale of the thesis.

Analysis of CSF specimens from patients with TBM confirmed that the MMPs identified in the *in vitro* model were present at elevated concentrations in most TBM patients. MMP-3, -8, -9 and TIMP-1 and -2 were predictive of coma severity and outcome from illness, although confidence intervals were wide. A prospective, appropriately powered study ought to be performed to confirm these findings. Additionally MMP-9 was the only MMP studied that was affected by dexamethasone treatment *in vivo*. Although MMP-9 was principally from neutrophils, the cellular source of the other CSF MMPs/TIMPs measured remains to be ascertained, but microglia are likely to contribute. This suggests that MMP manipulation in the clinical context may be a viable pharmaceutical target, although concerns about altered host response to the pathogen warrant further investigation in animal models first.

Therefore, in CNS TB it is likely that once bacilli reach the subarachnoid space or brain parenchyma, the first immune cells they will encounter will be microglia. There will be limited MMP-1 and -3 secretion from these cells once they have

phagocytosed *M.tb*. MMP-3 mediated MMP-1 activation will facilitate monocyte influx into the CNS by their action upon brain ECM (specifically fibrillary collagen, brevican and tenascin) and tight junction proteins like occludens molecules. Once infiltrating monocytes, perhaps following microglial derived chemokine gradients, have engulfed *M.tb* release of proinflammatory cytokines further enhances microglial MMP secretion. In addition to MMP-1 and -3 secretion, MMP-8, -9 and -10 may be released allowing extensive ECM breakdown, chemokine processing and enhanced BBB breakdown. In addition to monocyte influx, T-cell trafficking will mean there is IFN- $\gamma$  secretion in the CNS. I have not investigated how this cytokine effects microglial MMP secretion, but it has been reported to synergise with IL-1 $\beta$  to enhance MMP-9 secretion from astrocytes in CNS TB [315]. Microglia will then efficiently form granulomas with infiltrating monocytes and T-cells, during which MMP-9 function is known to be important [190]. Whilst the action of MMP-1 and -3 are important for these processes, there will be concurrent detrimental effects as these two MMPs are implicated in driving neuronal apoptosis. MMP-2 is the only other MMP reportedly able to effect this process, but I have demonstrated that it is likely to be down-regulated by monocyte derived TNF- $\alpha$ , itself pro-apoptotic to neurons.

If patients are then commenced upon anti-tuberculosis therapy, release of *M.tb* antigens will further activate monocytes and enhance microglial MMP secretion, leading to intensified CNS inflammation and oedema, responsible for hydrocephalus and the deterioration sometimes seen early in therapy. It is tempting to speculate that the adjuvant effect of dexamethasone observed in reducing mortality from TBM is not only due to modulation of CSF MMP-9

concentrations, but also to effects on parenchymal MMP expression. As these MMPs may be membrane associated or simply do not spill over into the CSF this effect is not easily measured in patients by current technology.

In addition, by investigating intracellular signalling mechanisms I have identified the NF $\kappa$ B, AP-1 and p38 MAP kinase pathways as key to the control of MMP gene expression and secretion. p38 inhibitors are in the early stages of clinical development. However, TNF- $\alpha$  secretion is driven by the p38 pathway in monocytic cells, so a cautious optimism for their potential use must be balanced by intense scrutiny of their effects on host-pathogen dynamics before use in patients with CNS TB is attempted. However, if specific p38 subunits are differentially used to drive TNF- $\alpha$  and MMPs, then targeting them for subunit manipulation via small molecules might be possible [375].

In summary, this thesis identifies *M.tb* driven MMP secretion via NF $\kappa$ B, AP-1 and p38 MAP kinase pathways as central to the pathogenesis of CNS TB. Further work is warranted to delineate which MMPs specifically should be targeted to combat the detrimental effect of the excessive inflammation that accompanies the process of pathogen elimination. Perhaps by targeting the signalling pathways leading to MMP secretion described herein better adjuvants to anti-tuberculosis therapy in CNS TB might be developed.

## Chapter 8 – Future directions

This work has concentrated upon developing and testing an *in vitro* model of CNS TB using cell culture and then confirmation *in vivo* in patient samples. Chemical inhibition data ought to be confirmed in primary microglia and with other technology such as siRNA. However, there are significant technological difficulties in transfecting primary monocytic cells to be overcome before this can become routine in the laboratory. Clinical trials of anti-MMP therapy are warranted. The tetracycline derivative doxycycline is known to have both anti-MMP and anti-tuberculous activity, so potentially this drug could be used in patients as a therapeutic adjuvant in CNS TB. Although deciphering the mechanism of any observed beneficial effect would be difficult unless large numbers of patients were recruited to an appropriately powered prospective study.

Before attempting clinical trials of MMP/TIMP modulation further work ought to be performed in an animal model. Unfortunately the mouse model of CNS TB requires direct inoculation of *M.tb* into the brain, and the clinical phenotype of the ensuing disease is not the same as observed in humans. In published work looking at CNS TB in mice *M.tb* was cultured from brain homogenates, meningeal cellular infiltration was observed and inflammatory cytokines released, but mice did not die [161]. Whether this is due to enhanced iNOS production in murine microglia, not seen in human disease, is unclear. A recent report that *iNOS* null mice displayed a more human phenotype, dying with granulomatous lesions containing tubercle bacilli in histological examination of

the meninges may be a viable animal model worth trying. There has been no work using the guinea pig as a model of TBM since chemotherapy trials were published in the 1950s. The rabbit model of TBM is the most successful but suffers from a lack of molecular tools, as well as similar problems with disease phenotype seen in murine studies. Large primate studies, using macaques, would be the most likely to yield relevant results but are expensive and logistically difficult to do in category 3 facilities [165].

Nevertheless *Mmp* null mice, or perhaps *iNOS Mmp* null double knockouts, would compliment a study of MMP inhibition in murine TBM well. Knowing that *Mmp9* null mice have disordered TB granuloma structure, along with the clinical data presented here on dexamethasone and MMP-9, makes this model pertinent to human disease. Unfortunately there are no data using other *Mmp* null mice in murine TB to suggest which avenue of investigation should be tried first. In addition murine cells do not express a functional orthologue of human MMP-1. However, the scavenger receptor A promoter has been linked to the human MMP-1 gene and inserted into the mouse genome, thus activated macrophages can be made to express human MMP-1, a subject of ongoing research in my host laboratory. *p38α* null mice do not survive gestation but there are macrophage conditional knockouts which could be used to test whether loss of TNF- $\alpha$  secretion in response to *M.tb* occurs *in vivo*, as well as whether abrogating this pathway affects pathogen killing [341]. In addition this model, along with cellular studies, could be used to look at the mechanism of p38 control of MMPs by investigating gene stability, transcription factor phosphorylation and p38-p65 complexes.

This study has not addressed the impact that HIV is likely to have on MMP secretion in CNS TB. TB in HIV patients is frequently not accompanied by inflammation until ART is commenced, clinically apparent as immune reconstitution syndrome (IRIS). HIV can infect microglia and adjuvant dexamethasone showed marginal benefit in HIV positive patients with TBM [107]. Thus any data generated from similar studies to those presented here would add valuable information to the current literature.

In summary these studies have identified that microglia secrete MMPs *in vitro* and *in vivo*. CSF examination shows MMPs are key regulators of inflammatory processes in TBM. Further delineation of the control and roles of MMPs, as well as the influence of HIV, in CNS TB pathogenesis by the use of animal models and new technology would aid the planning of large prospective clinical trials of MMP modulation.

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## Appendix 1 – Abbreviations

|          |                                                                       |
|----------|-----------------------------------------------------------------------|
| ADAMA    | dysintegrin and metalloproteinase                                     |
| ADAMTS   | A dysintegrin and metalloproteinase containing thrombospondin repeats |
| AP-1/2   | Activator protein-1/2                                                 |
| ARE      | AU-rich element                                                       |
| BBB      | Blood brain barrier                                                   |
| BCG      | Bacille Calmette-Guerin                                               |
| BM       | Bacterial meningitis                                                  |
| BSA      | Bovine serum albumin                                                  |
| cAMP     | Cyclic adenosine monophosphate                                        |
| CBP/p300 | CREB binding protein/p300                                             |
| CMI      | Cell mediated immunity                                                |
| CNS      | Central nervous system                                                |
| CREB     | cAMP response element-binding protein                                 |
| CSF      | Cerebrospinal fluid                                                   |
| DAB      | 3, 3'-Diaminobenzidine                                                |
| DC       | Dendritic cell                                                        |
| DMEM     | Dulbecco's modified essential medium                                  |
| DNA      | Deoxyribonucleic acid                                                 |
| DPX      | Di-n-butylPhthalate                                                   |
| ECM      | Extracellular matrix                                                  |
| ERK      | Extracellular signal-regulated kinase                                 |
| FCS      | Foetal calf serum                                                     |

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|              |                                                  |
|--------------|--------------------------------------------------|
| GM-CSF       | Granulocyte macrophage colony stimulating factor |
| GPCR         | G-protein coupled receptors                      |
| GRO $\alpha$ | Growth-related oncogene alpha                    |
| HBSS         | Hank's balanced salt solution                    |
| HIV          | Human immunodeficiency virus                     |
| HPA          | Health Protection Agency                         |
| Iba-1        | Ionized calcium-binding adaptor molecule 1       |
| IFN $\gamma$ | Interferon gamma                                 |
| IGRA         | Interferon gamma release assay                   |
| IkappaB      | Inhibitor kappa B                                |
| IL-          | Interleukin                                      |
| JAK          | Janus kinase                                     |
| JNK          | c-jun N-terminal kinase                          |
| LAM          | Lipoarabinomannan                                |
| LP           | Lumbar puncture                                  |
| <i>M.tb</i>  | <i>Mycobacterium tuberculosis</i>                |
| MAP kinase   | Mitogen activated kinase                         |
| MCP-1 to 3   | Monocyte chemoattractant protein-1 to 3          |
| MDR-TB       | Multidrug resistant tuberculosis                 |
| MHC          | Major histocompatibility complex                 |
| miRNA        | Micro RNA                                        |
| MMP          | Matrix metalloproteinase                         |
| MODS         | Microscopic observation drug sensitivity assay   |
| MOI          | Multiplicity of infection                        |
| mRNA         | Messenger RNA                                    |

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|               |                                                              |
|---------------|--------------------------------------------------------------|
| MS            | Multiple sclerosis                                           |
| MSFM          | Macrophage serum free medium                                 |
| NFkappaB      | Nuclear-factor kappa B                                       |
| OD            | Optical density                                              |
| PBMCs         | Peripheral blood mononuclear cells                           |
| PCR           | Polymerase chain reaction                                    |
| PEA3          | Polyomavirus enhancer activator 3                            |
| PF4           | Platelet factor 4                                            |
| PGL           | Polyketide synthase-derived phenolic glycolipid              |
| RNA           | Ribonucleic acid                                             |
| RPMI          | Rosswell Park Memorial Institute                             |
| SAPK          | Stress activated protein kinase                              |
| SDF-1         | Stromal cell-derived factor-1                                |
| SDS-PAGE      | Sodium dodecyl sulphate - polyacrylamide gel electrophoresis |
| SNP           | Single nucleotide polymorphism                               |
| SP-1/3        | Specificity protein-1/3                                      |
| STAT          | Signal transducers and activators of transcription           |
| TB            | Tuberculosis                                                 |
| TBM           | Tuberculous meningitis                                       |
| TGF- $\beta$  | Transforming growth factor beta                              |
| TIMP          | Tissue inhibitor of metalloproteinase                        |
| TLR           | Toll-like receptor                                           |
| TNF- $\alpha$ | Tumour necrosis factor alpha                                 |
| TST           | Tuberculin skin test                                         |
| WHO           | World Health Organisation                                    |

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XDR-TB      Extreme drug resistant tuberculosis

## Appendix 2 – Publications and presentations

### *Papers & published abstracts*

1. **Green JA**, Friedland JS. Astrocyte-leucocyte interactions and the mechanisms regulating matrix degradation in CNS tuberculosis. *Biochem Soc Trans* 2007;35 (4): 686-8.
2. **Green JA**, Elkington PT, Rand L, Fry J, Dholakia S, Graeber MB, Friedland JS. Networks regulate differential MMP and TIMP expression in CNS tuberculosis. *Immunology* 2007;120: S31.
3. **Green JA**, Elkington PTG, Tran HCT, Thwaites GE, Bullen A, Graeber M, Agranoff D, Farrar J, Friedland JS. An investigation into the factors driving tissue destruction in central nervous system tuberculosis by investigating a cellular model and the immunopathology in patients. Oral presentation BIS Spring Meeting 2007 & *J Infect* 2008; 56(4): 301.

### *Presentations*

1. **Green JA**, Dholakia S, Elkington PTG, Moores R, Rand L, Friedland JS. "Matrix Metalloproteinase Driven Tissue Destruction In Central Nervous System Tuberculosis Is Regulated By NFκB." Oral presentation BIS Spring Meeting 2008.
2. **Green JA**, Moores R, Dholakia S, Elkington, PTG, Rand L, Friedland JS NFκB and AP-1 regulate matrix metalloproteinase-1 driven immunopathology in central nervous system tuberculosis. Accepted for poster presentation at the British Society of Immunology annual conference Glasgow November 2008.