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Inflammation, the stress response system and the kynurenine pathway in the pathogenesis of depression and response to ECT

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Thesis submitted for the degree of Doctor of Philosophy

at the University of Dublin, Trinity College

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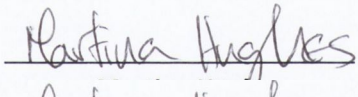

Martina Hughes

II. Summary

Major depression is a serious psychiatric disorder and a leading source of disability worldwide. However, the precise biological basis of depression remains elusive. Evidence suggests that activation of the inflammatory response system may have a pivotal role to play in the pathogenesis of certain subtypes of depression. Patients with severe treatment-resistant depression or with a history of childhood trauma are often characterised by a pro-inflammatory phenotype. In accordance with this, in this thesis, assessment of the circulating inflammatory profile in a cross-sectional cohort of severely depressed patients and additionally, in an independent cohort of severely depressed patients referred for ECT, revealed that both cohorts of depressed patients were characterised by increased circulating concentrations of the inflammatory cytokine IL-6 and the acute phase protein, C-reactive protein (CRP). While elevated concentrations of inflammatory mediators are often observed in depressed cohorts, a more targeted symptom-wise approach was employed to study the association between inflammatory and neuroendocrine markers and depressive symptomology in the cross-sectional cohort of depressed patients. Interestingly, positive correlations were revealed between specific PAXgene whole blood monocytic and inflammatory expression markers and the affective depressive symptoms, including core depression and anxiety. By contrast no association was observed between inflammatory markers and somatic symptom clusters. Therefore, while the onset of affective depressive symptoms may be associated with an activated inflammatory response system, alternative mechanism(s) appear to underlie the manifestation of somatic symptomology. In addition, the severity of childhood trauma in the depressed cohort was negatively associated with circulating brain derived neurotrophic factor (BDNF) concentrations and the whole blood mRNA expression of the glucocorticoid receptor (GR) and its co-chaperone FK506 binding protein 5 (FKBP5), which were also found to be decreased specifically in patients who were neglected as children. Increased mRNA expression of IL-1β and IL-6 were observed in depressed patients with a history of abuse. Taken together, these results indicate alterations in the inflammatory and neuroendocrine systems, which may play a role in the biological basis of major depression.

Activation of the kynurenine pathway and depletion of tryptophan for serotonin synthesis has been proposed as a mechanism by which inflammation can precipitate depression. Assessment of the inflammatory signature, tryptophan depletion and kynurenine pathway activation in the two independent cohorts of severely depressed patients, revealed that despite the presence of a mild circulating inflammatory phenotype and depleted tryptophan concentration, there was no evidence of peripheral kynurenine pathway activation. Despite this, further assessment in

severely depressed patients referred for ECT, revealed that isolated peripheral blood mononuclear cells (PBMCs) from these patients were capable of producing inflammatory cytokines and inducing kynurenine pathway activity and tryptophan depletion, in a similar manner to the controls, in response to immune stimulation with anti-CD3 or LPS. In addition, adaptive immune cells (T cells) had a greater propensity to deplete tryptophan and induce kynurenine pathway activation than innate immune monocytic cells. Interestingly, a successful course of ECT resulted in the restoration of circulating tryptophan concentrations and an increase in unstimulated PBMC tryptophan concentrations, which even when subjected to secondary immune stimulation remained unaltered and elevated with respect to pre ECT concentrations. In addition, the effect of ECT or its therapeutic efficacy reduced the ability of adaptive immune cells to produce inflammatory cytokines and activate the kynurenine pathway. However, the increased circulating inflammatory profile was unaffected by ECT.

The relative contribution of the adaptive immune system was also investigated through the assessment of T-cell subset gene expression and cytokine production. The increased unstimulated expression of CD25 and FoxP3 in the depressed cohort prior to ECT, relative to healthy controls, indicated the presence of T-reg cells. In addition, PBMC stimulation with the T-cell mitogen anti-CD3 revealed a decreased IL-2 mRNA expression in the depressed cohort relative to controls, indicative of an impaired immunoproliferative response in the face of an immune challenge. Interestingly, successful treatment with ECT normalised the expression of the T-reg cell markers in addition to restoring IL-2 mRNA expression levels.

This thesis supports a role for a dysregulated inflammatory and stress response system in the aetiology of severe depression. It highlights a potential role for immunosuppressive T-reg cells in depression and the value of a more targeted symptom-wise approach in the search for biomarkers for depression, with an ultimate goal to develop personalised treatment strategies for patients with major depressive disorders. In addition, the findings in this thesis indicate that in the absence of kynurenine pathway activation, alternative mechanism(s) may be mediating the depleted tryptophan concentrations in idiopathic depression. Finally, this thesis presents novel findings which demonstrate the positive effects of ECT treatment on the expression of immunoproliferative and T-reg cell markers, along with increasing tryptophan availability, both at a cellular level and in circulation. These findings highlight the need for a more in-depth assessment of the contribution of T-cell subsets in the pathogenesis of depression and suggest that the effect of ECT may be mediated, in part, through its impact on inflammatory processes.

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Chapter 1

Introduction

1.1 Major Depressive Disorder

Major depression is a serious and disabling psychiatric disorder with a high relapse rate and a lifetime prevalence of 16%. Predominantly affecting women more than men in a ratio of 2:1, approximately 70% of depressed patients suffer from recurring episodes of which the severity and duration intensifies overtime (Frank and Thase, 1999). A strong genetic component also predisposes individuals to the onset of a depressive episode which affects 15% of women and 8% of men (Kessler et al., 1994). Consequently, this lifelong condition is a leading cause of illness worldwide and is predicted by the World Health Organisation (WHO) to be the second main contributor to the global burden of disease by 2020 (Kessler et al., 2003). In Ireland alone approximately 300,000 people suffer from depression with an average of 500 completed suicides a year (AWARE). Without appropriate treatment, a depressive episode can persist for an average of 9-12 months while recurrent and chronic depressive episodes can prevail for an average of 5-8 years (Frank and Thase, 1999). The debilitating symptoms of major depression include cognitive and affective symptoms such as depressed mood, anhedonia, feelings of helplessness, hopelessness and worthlessness, guilt, anxiety and suicidal thoughts and somatic or vegetative symptoms such as sleep disturbance, fatigue or lack of energy, significant weight change, psychomotor retardation and diminished ability to concentrate. Diagnostic criteria for major depression outlined in the Diagnostic and Statistical Manual for Mental Disorders IV (American Psychiatric Association, 2000), include feelings of depressed mood and impaired functional ability in the presence of at least five of the above mentioned symptoms to occur consecutively over a prolonged period. However, to date the diagnosis of depression is not etiologically or biologically derived given the heterogeneity of major depression and the lack of specificity of the proposed biological markers (Mossner et al., 2007).

Depression severity and the degree of impairment can be categorised as mild, moderate or severe. Additionally, major depression is a highly heterogeneous condition that presents in a variety of sub-types. For example, reactive depression may emerge as a result of a traumatic life event such as bereavement while biological or chemical alterations are thought to underlie the development of endogenous depression. Within this, clinical depression can be categorised into individual sub-types such as melancholic depression or atypical depression.

Melancholia is a very severe form of depression commonly resulting in hospitalisation and predominately affects people ≥40 years of age. Extreme self-reproach, manifest in feeling of helplessness, hopelessness and worthlessness feeds into a dread of future prospects at the thought of their apparent self-deficiency. Other consistent features of melancholia include severe anhedonia and loss of libido, diminished interest and un-reactive mood, insomnia, reduced

appetite and weight loss and diurnal variation in mood with greater depression severity experienced in the morning (Rush and Weissenburger, 1994). In contrast, atypical early onset and often chronic depression, experienced by approximately 15-30% of the depressed population, is characterised by inverse neurovegetative symptomology evidenced by increased appetite and weight gain, hypersomnia and the progression of depressive symptomology severity over the course of the day. In contrast to melancholia, patients with atypical depression have the ability to respond positively to pleasurable experiences (Levitan et al., 1997, Thase, 2007).

1.2 Pharmacological therapies for Major Depression

Currently, treatment with selective serotonin reuptake inhibitors (SSRIs) is the first line treatment choice for moderately severe major depression. SSRIs increase the availability of serotonin in the synaptic cleft and prolong stimulation of post synaptic receptors through inhibition of reuptake via the pre synaptic cells (Berton and Nestler, 2006). Other commonly prescribed antidepressants include serotonin noradrenaline reuptake inhibitors (SNRI), noradrenergic and specific serotonergic antidepressants (NaSSA), tetracyclic antidepressants/serotonin modulator and tricyclic antidepressants (TCA) all which act through monoaminergic mechanisms. The therapeutic efficacy of this range of antidepressants is similar. However, SSRIs as compared with first generation antidepressants such as the TCAs (which non-selectively inhibit the reuptake of multiple neurotransmitters in the brain such as dopamine noradrenaline and serotonin) are more tolerable with fewer, less severe side effects (Ferguson, 2001).

Recent advances in antidepressant therapies have led to the development of the first melatonergic antidepressant treatment which is not dependent on monoaminergic mechanisms (de Bodinat et al., 2010). Rather, aglomelatine acts to normalise the dysregulated melatonin output by stimulating the melantonin M1 and M2 receptors on the pineal gland in synergy with antagonising serotonin $(5\text{-HT})_{2c}$ receptor activation in the brain which functions to decrease adrenergic and dopaminergic neurotransmission (Aloyo et al., 2009). As depression has been associated with disrupted melatonergic output and a subsequent alteration in circadian rhythms, normalisation of these biological rhythms have been shown to have a positive impact on key depressive symptoms; mood, sleep, appetite and libido (Souetre et al., 1989, Srinivasan et al., 2009).

1.3 Responsiveness to pharmacotherapy

While pharmacotherapy targeting monoamine neurotransmission is the first line treatment choice for major depression, rates of response to initial anti-depressant treatment are extremely poor with only 30-45% of patients achieving remission (Thase et al., 2001). Subsequent dosage augmentation, altered treatment duration, treatment with another class of antidepressant or combination treatment is therapeutically efficacious for some patients with remission achieved following multiple trials (Bauer et al., 2002). However, approximately 30% of those affected by chronic or severe depressive episodes do not respond to adequate courses of antidepressant treatments while others are too severely ill or unable to tolerate the adverse side-effects of antidepressant medication and subsequently have been termed medication or treatment-resistant (McCall, 2001). For these patients, electroconvulsive therapy (ECT) is an important treatment option and may be the treatment of choice (UK ECT Review Group, 2003).

1.4 Electroconvulsive therapy

ECT is the most effective antidepressant treatment available; however, despite this, it is rarely a first-line treatment for major depression. Rather, it is most often offered to treatment-resistant patients as a last resort (McCall, 2001). While the therapeutic efficacy of ECT is reduced in treatment-resistant patients relative to those without recognised treatment-resistance, studies have shown that approximately 60% of treatment-resistant patients do actually remit, while therapeutic efficacy for non-resistant patients is very high, with remission rates approaching 90% (Prudic et al., 1996, McCall, 2001, Husain et al., 2004, Eranti et al., 2007). However, ECT is also associated with adverse effects on memory and executive function. Although these effects largely abate within weeks of completing a course of ECT (Semkovska and McLoughlin, 2010, Semkovska et al., 2011), these short-term cognitive alterations, coupled with the need for anaesthesia, have a negative impact on the perception and uptake of this treatment with pharmacotherapy thought to be a safer treatment choice.

ECT can be administered bilaterally (bitemporal position) or unilaterally on the right [Figure 1.1]. While bitemporal ECT had been found to be therapeutically more effective, right unilateral ECT (RUL) is associated with fewer cognitive side-effects (Sackeim et al., 1993). By increasing the electrical stimulus dose at least 6 times above the seizure threshold the effectiveness of RUL ECT can be greatly enhanced and is comparable with the effectiveness of suprathreshold bitemporal ECT (McCall, 2001). However, increasing the electrical stimulus is also associated with a dose dependent increase in cognitive side effects (Kellner et al., 2010). Whether or not these cognitive

side effects are of a smaller magnitude than those induced by bitemporal ECT remains to be definitively elucidated. While the mechanism(s) by which ECT exerts its therapeutic efficacy are largely unknown certain hypotheses have been put forward (Wahlund and von Rosen, 2003).

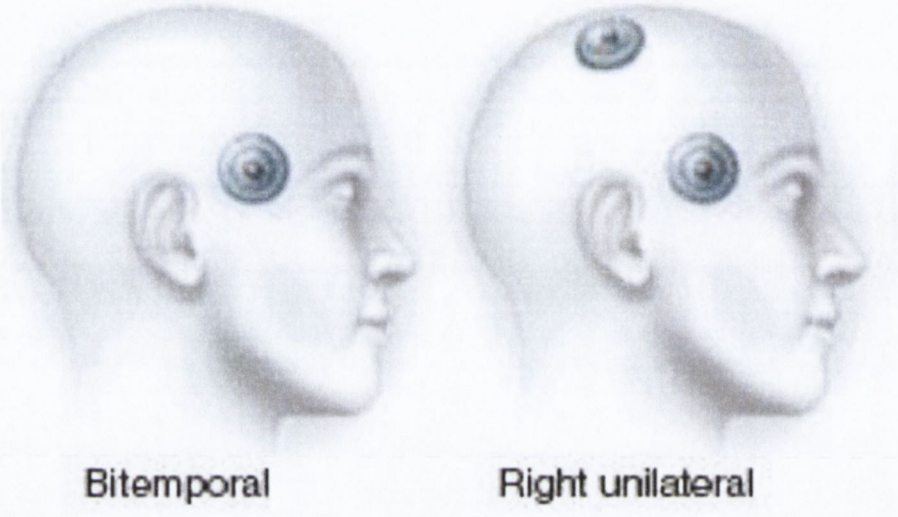

Figure 1.1 Illustration showing the most frequently employed electrode configurations for administering ECT (Reprinted with permission from the Massachusetts Medical Society; Copyright Massachusetts Medical Society 2011).

1.4.1 The effect of ECT on monoamines

In addition to the therapeutic effects of pharmacological agents targeting monoaminergic neurotransmission, ECT is also thought to enhance the transmission of dopamine, noradrenaline and serotonin in particular (Yoshida et al., 1998). Specifically, preclinical evidence suggests that electrical stimulation of the medial prefrontal cortex induces a current-dependent increase in serotonin output which is not evident upon stimulation of other regions in the limbic system (Juckel et al., 1999). This is interesting given the clinical findings implicating alterations in prefrontal cortex activity in depressed patients (Soares and Mann, 1997) and may represent a mechanism by which ECT induces its therapeutic effect.

1.4.2 The effect of ECT on BDNF

Alterations in neurotrophin expression and function, particularly brain derived neurotrophic factor (BDNF), have been widely implicated in the pathogenesis of depression and it is thought that ECT may have a positive effect on the neurotrophin system and specifically BDNF. BDNF has a central role in neuronal proliferation, regeneration and survival, neurogenesis and synaptic plasticity (Tapia-Arancibia et al., 2004). However, both stress and inflammation can disrupt the expression and function of BDNF (Schaaf et al., 1998, Barrientos et al., 2003). In accordance with this, Karege et al. (2005) have shown decreased BDNF expression in suicide victim post mortem hippocampal and prefrontal cortex tissue, while others have repeatedly reported reductions in the circulating concentration of BDNF in depressed patients (Karege et al., 2002, Cattaneo et al., 2010). Consequently, reduced availability of BDNF and the subsequent loss of neuronal protection and neurogenesis, which may have a role to play in the pathogenesis of depression, has led to the neurotrophin hypothesis of depression (Martinowich et al., 2007).

Furthermore, studies have demonstrated increased expression of BDNF following successful antidepressant treatment (Cattaneo et al., 2010), while others have demonstrated a role for BDNF in the metabolism and synthesis of serotonin (Siuciak et al., 1996). In accordance with this, preclinical evidence suggests that ECT may also have a positive impact on neurogenesis and increased synaptic connectivity. Specifically repeated electroconvulsive shock (ECS) has been shown to increase neurogenesis and synaptic connectivity in the rodent brain and particularly in the dentate gyrus of the hippocampus in a dose-dependent manner (Stewart and Reid, 1993, Scott et al., 2000). Further to this, numerous clinical studies have shown an increase in the circulating concentrations of BDNF in patients who respond therapeutically to ECT (Okamoto et al., 2008, Piccinni et al., 2009). Collectively, these findings suggest that the therapeutic efficacy of ECT may be in part attributable to its positive effects on neurotrophins and BDNF in particular.

1.5 The monoamine hypothesis of depression

The monoamine hypothesis of depression is the oldest and most widely accepted hypothesis of depression to date. Evidence in support of the monoamine hypothesis of depression arises from the observed mood altering effects elicited by pharmacological treatments that specifically target serotonergic and noradrenergic neurotransmission (Maes and Meltzer, 1995). The ability of TCAs and later SSRIs to increase synaptic concentrations of monoamines by inhibiting reuptake and metabolism in association with alleviating depressed mood was further supported by the discovery that serotonin depleting agents or decreased tryptophan availability for serotonin

synthesis induced the onset of depressive symptomology (Freis, 1954, Delgado et al., 1990, Delgado et al., 1999). Consequently, altered or dysfunctional monoamine neurotransmission was proposed as a biological risk factor for the onset of a depressive disorder. However, the mechanism(s) which drive alterations in monoamine activity, receptor sensitivity and transporter function remain to be elucidated. Additionally, pharmacotherapy is only therapeutically efficacious in a portion of individuals, with up to 30% of depressed patients classed as non-responders to adequate antidepressant treatment (Thase et al., 2001). Consequently, a number of other theories have been postulated in an effort to decipher the underlying biological basis of major depressive disorder (MDD).

1.6 The monocyte-T-lymphocyte theory of depression

The monocyte-T-lymphocyte theory of depression, devised by Smith and Maes in the early 1990's, proposes that increased pro-inflammatory cytokine secretion in the form of interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) is responsible for the initiation and maintenance of a depressive episode (Smith, 1991, Maes et al., 1995c). In support of this, there is a large body of evidence implicating a dysregulated inflammatory system in the pathogenesis of major depression. However, before describing the evidence in support of inflammation associated with depression, a brief introduction to the immune system is provided and the mechanisms by which cytokines signal and influence brain functions are outlined.

1.7 The innate immune response

In response to stress, injury or invading pathogens the body's first line of defence is the activation of the non-specific innate immune response. The primary task for the hosts innate immune cells is to detect pathogen and mount an immediate defensive response resulting in the activation of an inflammatory response followed by the initiation of a highly diverse, antigen-specific adaptive immune response and subsequent immunological memory (Hoffmann et al., 1999, Medzhitov, 2001).

Cells of the myeloid lineage including neutrophils, basophils, eosinophils, macrophages and dendritic cells and many epithelial cells bear germ-line encoded pattern recognition receptors (PRR) such as the Toll-like receptors (TLRs), which are stimulated upon recognition of microbial pattern associated molecular pathogens (PAMPs) expressed by foreign bodies. PAMPs are highly

conserved exogenous signals that alert the host to the presence of invading pathogen. However, in response to stress, trauma, tissue damage or unplanned cell death, host cells release endogenous danger signals or damage associated molecular pattern molecules (DAMPs) which activate the inflammatory and complement cascade in an effort to restore homeostasis (Bianchi, 2007).

The bacterial endotoxin lipopolysaccharide (LPS) found on the outer cell wall of gram negative bacteria is an example of an exogenous danger signal and is a potent stimulus of the innate immune response [Figure 1.2]. Recognition of LPS by TLR-4 is facilitated by the association of lipid binding protein (LBP) and cluster of differentiation 14 (CD14). The complete complex of LPS, TLR-4 and MD-2 (also known as Lymphocyte antigen 96) which assists LPS binding, results in the recruitment of adaptor proteins and the initiation of signalling cascades (Fitzgerald et al., 2004). TLR-4 signals via two distinct pathways (Lu et al., 2008). The myeloid differentiation primary response gene (MyD88) dependent pathway involves the recruitment of a series of adaptor proteins, including TNF receptor associated factor 6 (TRAF-6), resulting in the downstream activation of I kappa B (IκB) kinase and mitogen activated protein kinase (MAPK) pathways. Subsequent phosphorylation of the inhibitory molecule IκB by the IκB kinase (IKK) complex results in protein degradation and the release of the transcription factor nuclear factor kappa B (NFκB) which translocates from the cytosol to the nucleus where it initiates the transcriptional expression of inflammatory cytokines such as IL-β, TNF-α and IL-6. Alternatively, in response to viral or bacterial pathogens, TLR-4 may signal via the MyD88 independent pathway otherwise known as the toll interleukin 1 domain containing adaptor protein inducing interferon beta (TRIF) dependent pathway. Activation of interferon regulatory factor 3 (IRF3) and NFκB results in the production of type 1 interferons and interferon-inducible genes such as interferon alpha and beta (IFNα/β) (Lu et al., 2008) [Figure 1.2].

1.7.1 TREM-1

LPS stimulation also induces an increased expression of triggering receptor expressed on myeloid cells 1 (TREM-1) [Figure 1.2]. This innate immune activating receptor is expressed by cells of the myeloid lineage and is thought to synergise with TLR4, thereby amplifying the inflammatory response and the production of monocytic inflammatory cytokine production (Bouchon et al., 2001). The functional ability of TREM-1 is dependent on the association of the cytoplasmic signalling domain and adaptor molecule DNAX activation protein of 12 kDa (DAP-12). Phosphorylation of the cytoplasmic tail and the recruitment of tyrosine kinases instigate the

activation of signalling cascades culminating in transcriptional activity which potentially interacts with NFκB to produce a greater pro-inflammatory response (Colonna, 2003). Additionally, studies have shown that activation of NFκB following TLR4 stimulation may up-regulate the expression of TREM-1 further promoting an inflammatory state (Zeng et al., 2007). Increased expression of Purine-rich Box 1 (PU.1) has been shown to negatively regulate TREM-1 activity and consequently dampen the inflammatory response (Zeng et al., 2007).

1.7.2 The acute phase response

The acute phase response, which is a non-specific biological reaction and an integral part of the innate immune system, is activated in the presence of inflammation, pathogen invasion and tissue injury. Activation of this early defence mechanism results in the up-regulated secretion of a range of acute phase proteins (Pepys and Hirschfield, 2003). Of particular interest to depression is C reactive protein (CRP) which is secreted from hepatocytes in response to IL-6 stimulation. Consequently, circulating CRP concentrations are a useful general marker of inflammation. With regard to depression, numerous studies have reported increased circulating concentrations of CRP, indicating the presence of an activated inflammatory response system (Howren et al., 2009, Dowlati et al., 2010).

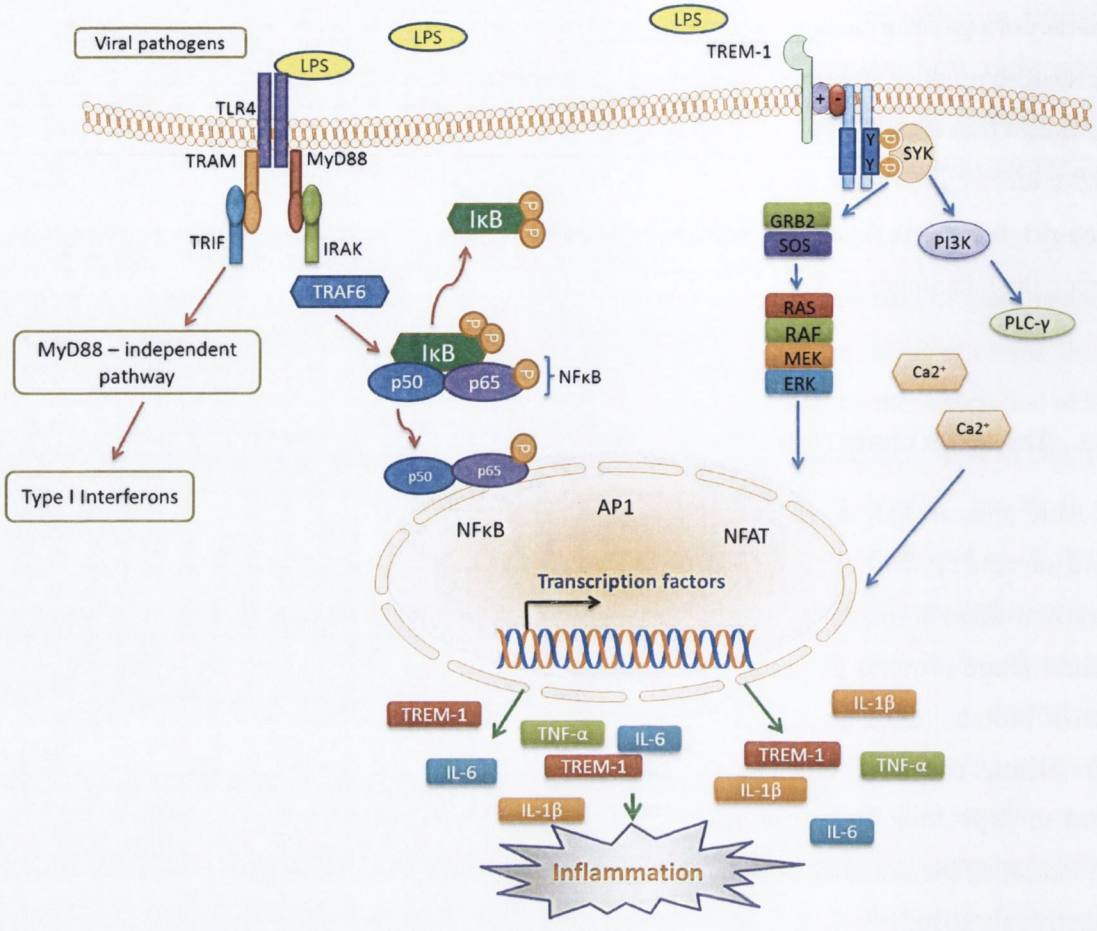

Figure 1.2 Diagrammatic representation of TLR4 and TREM-1 signalling pathways during an inflammatory response. LPS binds to TLR4 and signals via the MyD88-dependent pathway which results in the activation of the adaptor protein TRAF6 and the subsequent phosphorylation of the inhibitory IκB complex. This results in the release of NFκB, which translocates to the nucleus and promotes the expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α and inflammatory mediators such as TREM-1. Upon ligand binding, TREM-1 complexes with its co-chaperone DAP-12. Phosphorylation of DAP-12 provides docking sites for SYK family protein tyrosine kinases. The subsequent recruitment of GRB2 and activation of PI3K and ERK signalling pathways results in calcium ($Ca2^{+}$) mobilisation and the activation of the transcription factors NFκB, AP-1 and NFAT which in turn promote the expression of pro-inflammatory cytokines. It is thought that TLR4 and TREM-1 work in synergy to amplify the pro-inflammatory response. Alternatively, viral pathogens can bind TLR4, resulting in the initiation of the MyD88-independent pathway (TRIF) which mediates the activation of Type I interferon genes (based on Colonna (2003) and Lu et al. (2008)). AP1: activator protein 1; ERK: extracellular-signal-regulated kinase; GRB2: growth factor receptor binding protein 2; IκB: I kappa B; NFAT: nuclear factor of activated T- cells; PI3K: Phosphatidylinositol 3-kinase; PLC-γ: Phospholipase C-gamma.

1.7.3 Cytokines

Cytokines are a heterogeneous group of small cell signalling molecules with a key role in growth, differentiation, activation of the inflammatory response and the induction and maintenance of the adaptive immune response (Connor and Leonard, 1998). Released from numerous cell types in the brain and periphery, cytokines act in synergy or antagonistically to direct specific immune responses through the orchestration of immune cell trafficking and the cytokine induced differentiation of immune cells with roles in innate, cytotoxic, cell-mediated, humoral and auto-immunity in association with immune suppression (Borish and Steinke, 2003, Commins et al., 2010). Broadly speaking, cytokines can be classed as pro-inflammatory, anti-inflammatory or immunosuppressive/regulatory. However, the pleiotropic nature of these molecules enables them to exert a range of effects, depending on the circumstance, the target cells involved and the stage of the inflammatory response (Borish and Steinke, 2003, Commins et al., 2010).

A large variety of cytokines are produced by the monocytic and dendritic cells of the innate inflammatory response. Upon encountering PAMPs, monocytic cells respond by producing a variety of interleukins such as IL-1β, IL-6 and IL-12 along with TNF-α. Additionally, the process of dendritic cell maturation and the presentation of antigen to T-cells results in the production of many inflammatory cytokines and chemokines (mediate for immune cell trafficking), which dictate the direction of the inflammatory response (Borish and Steinke, 2003). As IL-1β, IL-6 and TNF-α have all been implicated in the biological basis of depression a brief description of each is provided.

1.7.4 Innate immune cytokines

1.7.4.1 IL-1β

IL-1β is produced by many immune cells which include mononuclear phagocytes, neutrophils, endothelial cells and microglia, the resident immune cells in the brain, in response to bacterial endotoxins and other pathogenic agents recognised by PRR (Dinarello, 1988). IL-1 signals via the type 1 IL-1 receptor (IL-1R1), which in turn induces the signal dependent activation of NFκB and MAPK and transcription of IL-1β inducible genes (Cao et al., 1996, Malinin et al., 1997). The production of IL-1β has a key role to play in the induction and maintenance of the adaptive immune response, promoting increased expression of IL-2 receptors and IL-2 secretion, thereby impacting on T-cell differentiation and B cell activation (Gillis and Mizel, 1981, Kaye et al., 1984). IL-1β up-regulates the expression of endothelial cell adhesion molecules, facilitating leukocyte adherence to vascular endothelium and cell signalling (Pober et al., 1986). Additionally, IL-1β also

has a critical role to play in the manifestation of sickness behaviour, a strategic physiological response induced by the immune system in an effort to conserve energy in order to fight infection (Hart, 1988, Dantzer, 2006). Symptoms of sickness behaviour include fever, malaise, loss of appetite, and fatigue in association with an activated stress response system (Konsman et al., 2002). Preclinical evidence suggests that the actions of IL-1β in the brain have a pivotal role to play in the sickness response. This is supported by the manifestation of symptomology and sickness behaviour following an immune challenge with IL-β or LPS, which abates in the presence of the IL-1 receptor antagonist (IL-1Ra) (see for review Dantzer (2006)). IL-1Ra, expressed in the presence of numerous cytokines such as anti-inflammatory IL-4, IL-13 and transforming growth factor-beta (TGF-β), antagonises the actions of IL-1β, limiting the detrimental effects of chronic IL-1β production (Morita et al., 2001).

Prolonged activation of IL-1β in association with IL-6 and TNF-α may result in a maladaptive sickness response and the subsequent manifestation of depressive symptoms (Dantzer et al., 2008). Additionally, others have shown that IL-1β and to a lesser extent IL-6, TNF-α and IL-2 have a role to play in the modulation of neurotransmitters (Dunn, 1992, Palazzolo and Quadri, 1992, Song et al., 1999, Zhu et al., 2006). Under stress, dysregulation of this system and particularly serononergic neurotransmission may be in part responsible for the alterations in mood, emotion and cognitive processing that are major characteristics of a depressive episode. Additionally, Reichenberg et al. (2001) have shown that healthy male volunteers treated with low dose endotoxin display increased concentrations of IL-1Ra, IL-6 and TNF-α which are positively correlated with emotional and cognitive disturbances evidenced by depressed mood, memory impairments and anxiety.

1.7.4.2 IL-6

IL-6 is produced by many cells types such as T and B cells, endothelial cells and hepatocytes amongst others; however, the major producers of IL-6 are innate immune mononuclear phagocytes (Commins et al., 2010). IL-6 is a pleiotropic cytokine with both pro and anti-inflammatory effects. It is induced by bacterial endotoxin and the inflammatory cytokines IL-1β and TNF-α. Similar to IL-1β, IL-6 is important for immune cell growth and differentiation, specifically in relation to B and T cell differentiation and plasma cell maturation (Horn et al., 2000). Accordingly, IL-6 in combination with TGF-β polarise Th-17 cell differentiation. IL-6 is the strongest mediator of the acute phase response, stimulating the release of proteins such as CRP and albumin from the liver along with playing a role in immune-mediated HPA axis activation,

perhaps via direct interaction with IL-6 receptors in the paraventricular nucleus of the hypothalamus thereby inducing the stress response and hyperthermia (Lenczowski et al., 1999, Horn et al., 2000). In accordance with this, while numerous studies implicate elevated HPA axis activation in depressed patients, other reports also suggest that patients suffering from depression have an increased core body temperature (Szuba et al., 1997, Rausch et al., 2003).

IL-6 exerts its effects through interaction with the IL-6 receptor and the subsequent dimerization of the cytoplasmic glycoprotein 130 subunit, stimulating the activation of the Janus-Kinase – signal transducer and activator of transcription (JAK-STAT) signalling pathway. Specifically, upon IL-6 receptor dimerization, the IL-6 receptor-associated JAKs (JAK1 and JAK2) with protein kinase activity cross phosphorylate and activate each other. Subsequent tyrosine phosphorylation of the cytoplasmic tail facilitates STAT-3 binding and phosphorylation by JAKS. Phosphorylated STATs dimerise and translocate to the nucleus where they activate the transcription of IL-6 inducible genes (Aaronson and Horvath, 2002). IL-6 signalling is negatively regulated by members of the suppressor of cytokine signalling 3 (SOCS3), family which negatively regulates JAK-STAT signalling. However, chronic production of IL-6 has been widely implicated in many inflammatory, neurodegenerative and psychiatric disorders such as depression. With regard to the potential role of IL-6 in the pathogenesis of depressive symptomology, Wright et al. (2005) have shown that negative changes in mood following inoculation with a typhoid vaccine are correlated with elevated IL-6 concentrations.

1.7.4.3 TNF-α

TNF-α is a multi-functional cytokine that plays a central role in mediating host defence against intracellular pathogens and bacterial endotoxin (Pfeffer, 2003). Similar to IL-6 and IL-1β, TNF is produced by many cell types including neutrophils, lymphocytes and endothelial cells; however, the major producers of TNF-α are mononuclear phagocytes (Beutler and Cerami, 1989). The TNF-α receptor is expressed on almost all cells in the body. It is also found in soluble form due to the cleaving actions of the metalloproteinase integrin, TNF-α converting enzyme (TACE) (Black et al., 1997). There are two distinct forms of the TNF receptor; TNF-receptor I (p75) and TNF-receptor II (p55) both of which induce similar effects. Upon TNF receptor binding, complex signalling pathways are initiated resulting in the recruitment of TRAF6 and the subsequent signal dependent activation of NFκB and MAPK (Chen and Goeddel, 2002). TNF-α plays a critical role in the inflammatory response, promoting inflammation via the stimulated expression of IL-1β and IL-6 in association with promoting lymphocyte proliferation, however, TNF-α also maintains normal

immune function through the initiation of programmed cell death and the induction of cytotoxic immunity in response to tumor cells (Yokota et al., 1988, Fong et al., 1989, Grell et al., 1999). Additionally, under acute inflammatory conditions, TNF-α increases the expression of endothelial cell adhesion molecules, thereby assisting cell trafficking to sites of inflammation (Pober et al., 1986) along with playing a role in the induction of fever and sickness behaviour as discussed in relation to IL-1β (See section 1.7.4.1) (Rodriguez-Angulo et al., 2013). However, chronic low grade TNF-α activity has detrimental consequences for the host, with numerous inflammatory conditions, such as multiple sclerosis and Crohn's disease, implicating the involvement of TNF-α in the pathogenic process (Hofman et al., 1989, Murch et al., 1991). Additionally, Cope and colleagues have reported that chronic TNF-α exposure results in decreased T-cell receptor responses by attenuating T-cell receptor signalling, which consequently impairs normal cell-mediated immune responses (Cope et al., 1994, Cope et al., 1997).

1.7.5 Adaptive immune cytokines

The adaptive immune cytokines IFN-γ and IL-2 are the most widely implicated in the pathogenesis of depression.

1.7.5.1 IL-2

IL-2 has a central role to play in the generation of the immune response. Produced predominately by $CD4^+$ T-cells, IL-2 can also be produced, although to a lesser extent, by $CD8^+$ T-cells and natural killer (NK) cells. Stimulated from naive T-cells in response to the process of antigen presentation, IL-2 production and the up-regulation of the IL-2 receptor are key mediators of immune cell activation and T-cell proliferation. Additionally, along with activating macrophages and playing a central role in orchestrating cell-mediated immune activation, IL-2 is required for the growth and differentiation of NK cells and B-cells (Caruso et al., 1993).

The IL-2 receptor complex is composed of IL-2 receptor alpha chain (CD25), beta chain (CD122) and gamma chain (CD132). CD25, otherwise referred to as 'T-activated', is induced and expressed only after T-cells, B-cells and monocytic cells are activated and consequently is considered a unique marker of immune cell activation (Waldmann, 1986, Caruso et al., 1993). CD25 has the highest binding affinity while CD122 is responsible for IL-2 signal transduction (Waldmann, 1991). A soluble form of the alpha chain of the IL-2 receptor can also be found in circulation and is often used as an indicator of T-cell activation with numerous reports providing evidence in support of

increased T-cell activation in the pathogenesis of many infectious and immune related conditions (Caruso et al., 1993).

1.7.5.2 IFN-γ

IFN-γ is the solitary member of the type II interferon class and is predominantly produced by T-cells and to a lesser extent by natural killer cells and macrophages with a functional role in host resistance against disease (Young and Hardy, 1995). Specifically, activated cytotoxic and $CD4^+$ T-helper 1 (Th-1) cells are the most potent producers of IFN-γ, the expression of which is amplified greatly in the presence of the innate immune cytokine IL-12 (Wu et al., 1993). Upon receptor binding, IFN-γ transmits its signals via the JAK-STAT signalling pathway activated upon cross-phosphorylation of specific JAK1 and JAK2 proteins and the subsequent phosphorylation and dimerization of STAT1, which in turn translocates to the nucleus and promotes the transcription of IFN-inducible genes (Aaronson and Horvath, 2002). The magnitude and duration of IFN-γ signalling is negatively regulated by the increased expression of the interferon-inducible SOCS1 protein which inhibits the recruitment of STAT through the inhibition of JAK phosphorylation (Davey et al., 2006).

In addition to its anti-viral properties as an interferon, IFN-γ has a diverse set of roles orchestrating specific immunological responses and consequently is generally considered more of an interleukin than an interferon. IFN-γ has a central role in the development and differentiation of the immune response. It is a critical stimulus for the induction of acquired macrophage activation and differentiation and promoting a pro-inflammatory response via the stimulated activation of macrophage cytotoxic activity, increased intracellular pathogen killing and antigen presentation in association with the elevated expression of the macrophage cytokine IL-12, thereby maintaining IFN-γ production and the inflammatory response (Pace et al., 1983, Torrico et al., 1991). IFN-γ also acts alone and in synergy with LPS to induce increased nitric oxide production with cytotoxic properties. IFN-γ plays a part in the negative regulation of B-cell differentiation (Reynolds et al., 1987, Ding et al., 1988). The pro-inflammatory effects of IFN-γ are counter balanced by anti-inflammatory cytokines IL-4, TGF-β and IL-10. However, despite this, IFN-γ is implicated in numerous inflammatory conditions, autoimmune diseases and psychiatric disorders (Skurkovich and Skurkovich, 2007).

1.7.6 The adaptive immune response

The innate and adaptive immune responses collaborate in a bi-directional manner to maintain a homeostatic balance (Reiner, 2007). However, initial activation of lymphocytes, the key effector cells of the adaptive immune response, is highly dependent on antigen presentation, cytokine production and co-stimulatory molecules from the innate response system (Hoebe et al., 2004). Engulfed and phagocytised microbial molecules by immature dendritic cells in the first 4 hours of an immune response stimulates the migration of these cells to peripheral lymphoid tissues where they mature into specialised antigen presenting cells (APC) and express the processed pathogenic particles as antigens on the cell surface (Banchereau and Steinman, 1998). In the lymph nodes, interaction between the antigen presenting major histocompatibility complexes (MHC) and the T-cell receptor CD3 complex initiates the activation of naïve T-cells (Banchereau and Steinman, 1998). Following this, the interaction between CD40 and its ligand expressed on naïve T-cells promote the expression of the B7 molecules CD80 and CD86 on the APC and in turn complex with the CD28 on T-cells, thereby providing the co-stimulatory interaction necessary for T-cell activation (Lenschow et al., 1996). The production of inflammatory cytokines also has a key role to play in T-cell activation and polarisation (Medzhitov and Janeway, 1997, Vyas et al., 2008). The increased IL-2 receptor expression and secretion upon T-cell activation promotes T-cell proliferation and differentiation (Medzhitov and Janeway, 1997, Maes, 2011). The complex adaptive cellular immune network and T-cell differentiation results in direct and specific immune responses to various pathogens as well as offering protection against potentially harmful effector responses and autoimmunity. The cells of the adaptive immune response include,

(1) B cells which are essential in orchestrating humoral immunity and immunological memory and transform into plasma cells that produce antibodies, which neutralise foreign bodies,

(2) $CD8^+$ cytotoxic T-cells which are activated upon MHC class I antigen presentation. MHC class I molecules present intracellular antigenic fragments from viral pathogens, intracellular bacteria or cellular transformations to naïve $CD8^+$ cytotoxic T-cells (Vyas et al., 2008). Upon activation, $CD8^+$ cytotoxic T-cells exert their defensive effects via the direct killing of viral or pathogen infected cells,

(3) $CD4^+$ T-helper cells which are activated upon extracellular antigenic particle presentation on MHC class II molecules (Vyas et al., 2008). Activation and differentiation of the various $CD4^+$ helper T-cells results in a variety of effector responses which work in association with innate immune cells to clear pathogen and regain homeostasis (Reiner, 2007).

1.7.6.1 Th-1 cells

Th-1 cells have a key role to play in protecting the host against intracellular bacterial and viral pathogens (Zhu et al., 2010b). While the production of IFN-γ by APC is instrumental in the induction of Th-1 cells, IL-12 also has a critical role to play in the differentiation of Th-1 cells. Released by macrophages and APCs in response to invading pathogens, IL-12 promotes the inflammatory response, inducing IFN-γ production in association with inhibiting Th-2 cell development through the suppression of GATA binding protein 3 (GATA-3), the transcription factor that drives Th-2 cell differentiation (Reiner, 2007). IL-12 receptor binding on activated naïve T-cell results in tyrosine phosphorylation of the cytoplasmaic domain of the IL-12 receptor and the subsequent activation of the JAK-STAT signalling pathway (Romagnani, 2000, Reiner, 2007). This in turn results in the up-regulated expression of the STAT-1 dependent Th-1 specific transcription factor T-bet, responsible for driving the production of T-cell derived pro-inflammatory mediators such as IFN-γ and IL-2 and the up-regulated expression of the IL-12 receptor subunits (Zhu et al., 2010b, Zhou et al., 2009). The formation of IL-12 receptor complexes results in STAT-4 induced IFN-γ production, which collectively results in Th-1-cell differentiation [Figure 1.3]. The production of IFN-γ from Th-1 cells then promotes further activation of monocytes and macrophages resulting in the additional release of monocytic cytokines such as IL-1, macrophage inflammatory mediators such as neopterin, and interferon-inducible genes, thereby sustaining a pro-inflammation phenotype (Zhou et al., 2009, Maes, 2011). The interaction between T-cells and macrophages via the production of a range of inflammatory cytokines is known as cell-mediated immune activation and functions to stimulate and recruit new macrophages, kill infected macrophages and promote and maintain the inflammatory response (Maes, 2011).

1.7.6.2 Th-2 cells

Alternatively, in response to an increased presence of the anti-inflammatory cytokine IL-4 which is produced by mast cells, basophils and eosinophils, naïve T-cells can be differentiated into Th-2 cells. IL-4 receptor binding induces the expression of the STAT-6 dependent Th-2 specific transcription factor and Th-2 cell regulator GATA-3, which drives the production of IL-4, IL-5, IL-10 and IL-13 (Romagnani, 2000). IL-4 is a potent suppressor of IL-12 as it down-regulates the expression of the IL-12p40 subunit, thereby polarising Th-2 cell differentiation and dampening the pro-inflammatory response (Romagnani, 2000). Th-2 cells have a key role in the induction of humoral immunity, B-cell activation and the production of Immunoglobulin E (IgE) antibody and IgG neutralising antibody (Romagnani, 2000).

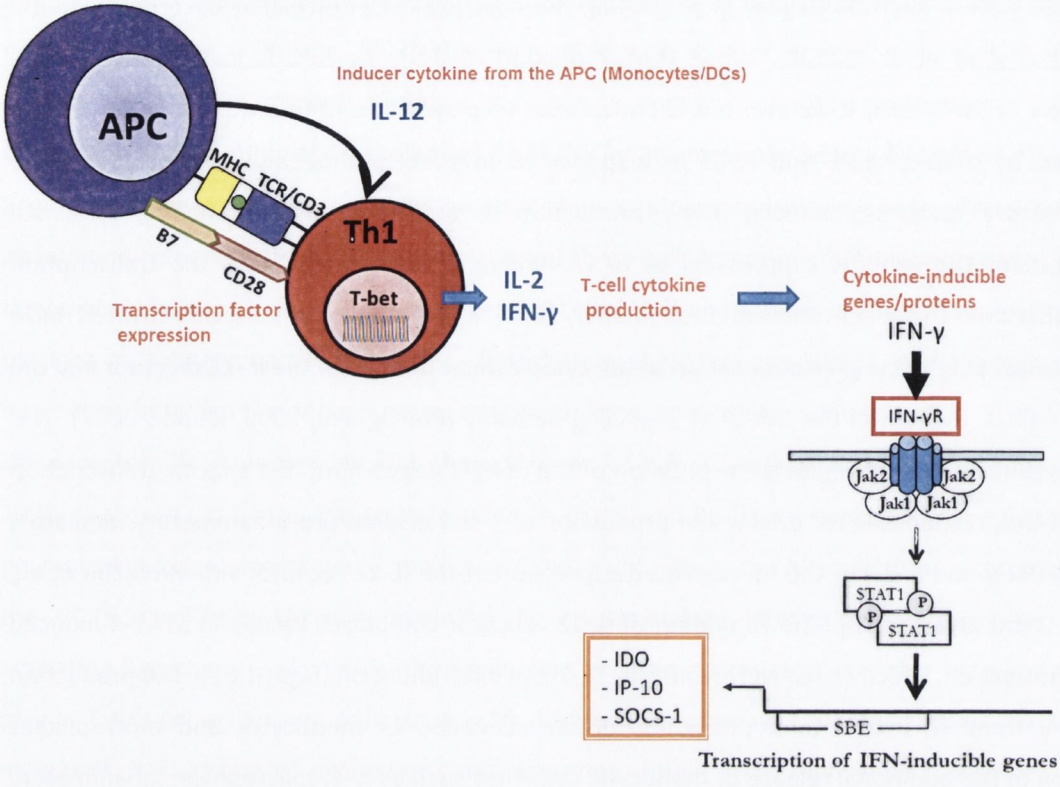

Figure 1.3 Th-1 cell activation. Antigen presentation on MHC class II molecules to the TCR/CD3 complex, in association with effector cytokine release and the essential co-stimulatory interaction between the B7 molecules on APC and CD28 receptor on T-cells, results in T-cell activation. In the case of Th-1 cells, the inducer cytokine IL-12 stimulates the up-regulated expression of the Th-1 specific transcription factor T-bet and the subsequent production of Th-1 cytokines IL-2 and IFN-γ. IFN-γ in turn activates macrophages via cell-mediated immune activation. IFN-γ transmits its signal via the JAK-STAT activation pathway, inducing the transcription of IFN-inducible genes such as IDO. APC: Antigen presenting cell; IDO: indolamine 2,3 dioxygenase; IFN: interferon; IL: interleukin; IP-10: interferon gamma induced protein 10; JAK-STAT: Janus kinase and Signal Transducer and Activator of Transcription; MHC: major histocompatibility complex; TCR: T-cell receptor; SOCS: suppressor of cytokine signalling; T-bet: T-cell-specific T-box transcription factor.

1.7.6.3 Th-17 cells

Th-17 cells, which were discovered only in recent years, have been shown to be induced by IL-23. However, it is now thought that a combination of IL-6 and TGF-β potentially induce the differentiation of Th-17 cells while IL-23 is critical in the maintenance of Th-17 cells (Bettelli et al., 2006). The activation of the STAT-3 dependent Th-17 cell specific transcription factor, RAR-related orphan receptor C-γ (RORC-γ), results in Th-17 cell differentiation and the production of IL-17 and IL-22 (Chen et al., 2007). Th-17 cells are thought to be involved in acute inflammatory responses, orchestrating the recruitment of neutrophils to epithelial cells and promoting growth and integrity of epithelial barriers (Wilson et al., 2007). Differentiation of Th-17 cells is antagonised by the production of IFN-γ and IL-4 from Th-1 and Th-2 cells respectively (Harrington et al., 2005). Th-17 cells have been widely implicated in the pathogenesis of numerous diseases such as Irritable bowel syndrome (IBD) and arthritis. However, their contribution to the biological basis of psychiatric disorders and specifically major depression remains to be elucidated (Tesmer et al., 2008).

1.7.6.4 T-reg cells

The regulatory and suppressive cells of the lymphoid lineage are known as regulatory T-cells (T-regs). Differentiated in the thymus in the presence of IL-2, natural T-regs with specific T-cell receptors for self-antigen are classically defined by the presence of the master regulator and T-reg cell transcription factor Forkhead box P3 (FoxP3) in association with increased expression of the IL-2 receptor alpha chain, CD25 (Sakaguchi et al., 2009). Activation of FoxP3 induces the production of the immunoregulatory mediators IL-10 and TGF-β which function to maintain immunological homeostasis protecting against autoimmunity and down-regulating T-cell activation and inflammatory cytokine production via NFκB inhibition (Bettelli et al., 2005).

IL-10 and TGF-β are also potent inducers of T-reg cell differentiation. TGF-β has been shown to induce T-reg cells that are suppressed in the presence of IL-6, resulting in Th-17 cell polarisation (Bettelli et al., 2006). However, other findings suggest that while natural T-reg cells may be polarised to form Th-17 cells in the presence of IL-6, TGF-β induced T-reg cell expression and functionality are resistant to the IL-6 conversion (Zheng et al., 2008). Additionally, the immunoregulatory cytokine IL-10 is also a key mediator in the induction of T-reg cells in the periphery. However, studies have shown that these cytokines are not essential in all circumstances for normal T-reg cell function (Fontenot and Rudensky, 2005, von Boehmer, 2005). Further to this, in addition to direct cell to cell contact as a means of T-reg cell induction, studies

have shown that T-reg cell mediated immune suppression occurs via the inhibition of IL-2 production from effector T-cells, thereby decreasing the ability of lymphocytes to proliferate further, which, in turn, suppresses immune responses. However, not all reports are consistent with this and others suggest that T-reg cell mediated IL-2 depletion is via the uptake of IL-2 by T-reg cells, which coupled with their inability to actively produce IL-2, culminates in decreased lymphocyte-proliferation and cytokine production and promotes effector cell apoptosis (Thornton and Shevach, 1998, Popmihajlov and Smith, 2008, Cheng et al., 2011). Furthermore, regulatory T-cells can also be induced under circumstances of low tryptophan availability as a consequence of increased IDO activity and are responsible for the maintenance of immune tolerance and protection against autoimmunity and immune pathology (Fallarino et al., 2006). While it is thought that the balance between Th-17 and T-reg cells may be disturbed in depressed patients in favour of increased pro-inflammatory Th-17 cells, no study to date has assessed the relative contribution of Th-17 to the pathogenesis of depression while studies investigating the involvement of T-reg cells are extremely limited (Haroon et al., 2012).

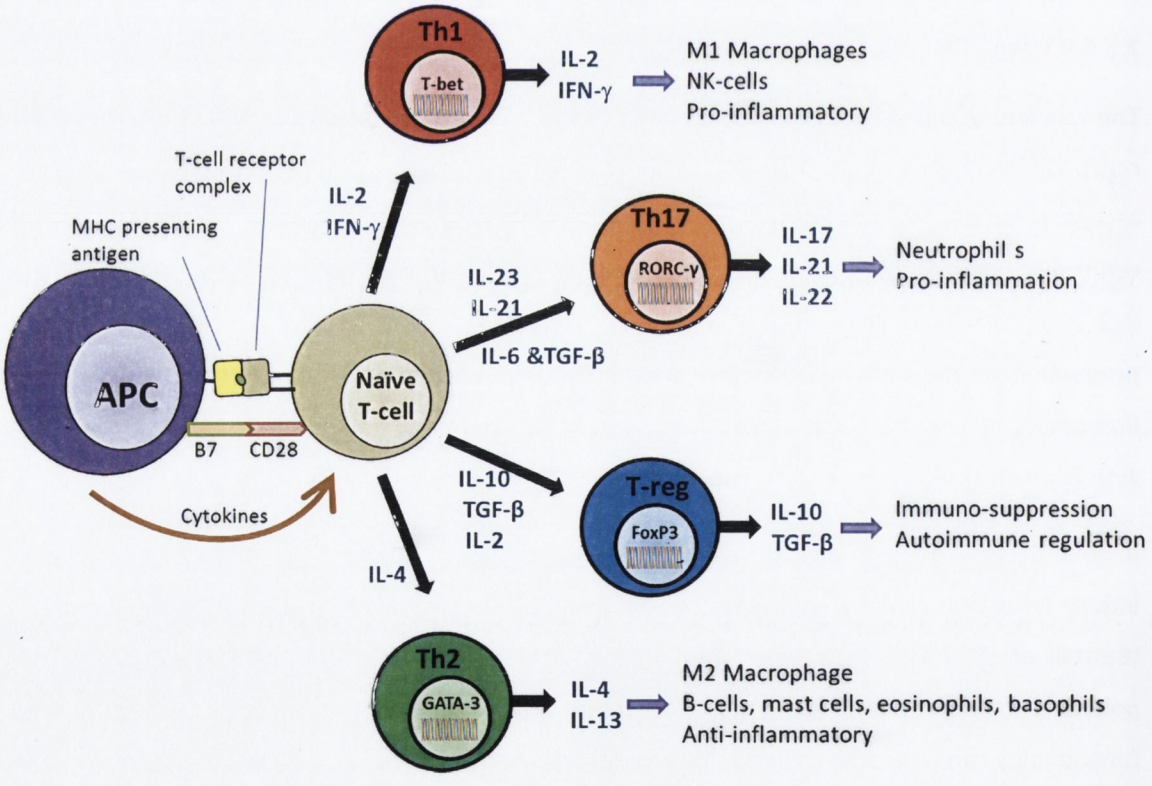

Figure 1.4 CD4$^+$ T-cell subset activation. Simplified overview of the effector cytokines, the transcription factors and T-cell cytokine production from activated Th-1, Th-2, Th-17 and T-reg cells.

1.8 Polarised Macrophage activation

The highly diverse and flexible nature of macrophages enables them to carry out many functions in addition to host defence and inflammation such as tissue repair, humoral immunity and immune suppression (Murray and Wynn, 2011). Depending on the inflammatory and cytokine milieu, macrophage activation is polarised to elicit the appropriate response comparable to that evident in the T-cell subsets. Additionally, T-cells play a key role in the induction and maintenance of distinct macrophage activation states. Similar to Th-1 and Th-2 cells, macrophage polarisation can be defined by two extremes: M1 or classical activation and M2 alternative activation which in recent years has been divided further into various macrophage subsets with diverse functionality (Wolfs et al., 2011).

1.8.1 Classical activation - M1 Macrophages

M1 macrophages are activated in response to immune activation and specifically potent inflammatory cues such as IFN-γ, TNF-α and MyD88-dependent TLR agonists (Mosser and Edwards, 2008). Th-1 cells are potent producers of inflammatory IFN-γ and TNF-α and consequently cell mediated immune responses have a significant role to play in macrophage polarisation (Mosser, 2003). Innate natural killer cell derived IFN-γ and TNF-α cytokine production are also key inducers of the classical macrophage activation state, which functions to present antigen to primed T-cells, sustain cellular immunity and produce inflammatory mediators and noxious agents such as reactive oxygen species and nitric oxide (Mosser and Edwards, 2008). While the acute production of inflammatory mediators from cells of the innate immune system such as NK cells promote classical macrophage activation this is short lived. Therefore, the main M1 macrophage activation stimulus comes from the adaptive immune response which sustains the hosts acquired pro-inflammatory state in response to microbial invasion, stress or tissue damage.

M1 macrophages are defined by a high expression of IL-12 and low IL-10 in association with the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), IDO1 and the production of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, IL-8, and IL-23 (Mosser and Edwards, 2008, Murray and Wynn, 2011). An increased presence of M1 macrophages has been reported in numerous autoimmune and inflammatory disorders such as rheumatoid arthritis, multiple sclerosis and Crohn's disease, which are strongly associated with major depression (Murray and Wynn, 2011).

1.8.2 M2 Macrophages

In contrast to the acquired pro-inflammatory state induced by M1 macrophages, M2 macrophages have diverse roles that encompass immune repair, regulation, suppression and antagonism of M1 responses (Gordon, 2003). In 1992 Stein and colleagues reported that in the presence of IL-4, inflammatory macrophages undergo a phenotypic change resulting in an alternatively activated macrophage capable of reducing the production of M1 pro-inflammatory cytokines and characterised by the up-regulated expression and activity of the phagocytic macrophage mannose receptor, a key marker of alternative immunologic macrophage activation (Stein et al., 1992). Alternatively activated macrophages are also typically characterised by high IL-10 and low IL-12 expression (Gordon, 2003). Since then, researchers have found that there are distinct classes of M2 macrophages which include M2a, b and c amongst others.

1.8.3 M2a – Alternative activation/repair and regeneration

The secretion of IL-4 and IL-13 from activated Th-2 cells in association with the initial and early production of IL-4 from damaged tissue and innate immune basophils and mast cells results in the acquired activation of M2a macrophages (Mosser, 2003, Gordon and Martinez, 2010). The M2a phenotype is characterised by the activation of STAT-6 and subsequent up-regulation of MHC class II (although restricted) and transcription of arginase, which facilitates the production of extracellular matrix precursors and subsequent repair and regeneration of damaged tissue (Stein et al., 1992, Gordon, 2002). IL-4 and IL-13 induce mannose receptor endocytic activity and antagonise IL-1 receptor activity. Additionally, M2a macrophages promote humoral antibody production, alternative inflammation and inducible T-reg cell generation, allergy and anti-parasite responses (Gordon, 2003, Martinez et al., 2009).

1.8.4 M2b - Immunomodulation

Distinct from other M2 macrophages, M2b macrophages retain the ability to produce adequate amounts of pro-inflammatory cytokines such as IL-6, TNF-α and IL-1 in association with high IL-10 and low IL-12 and are activated in responses to immune complexes and TLR ligands such as LPS and IL-1 receptor ligands. The principal function of these cells is to modulate the immune response and promote anti-inflammatory, Th-2 adaptive immune responses (Mosser, 2003).

1.8.5 M2c - immune deactivation

The immunomodulatory cytokines IL-10 and TGFβ and glucocorticoids are instrumental in the activation of M2c macrophages, which are potent suppressors of the immune response (Martinez et al., 2008). In contrast to the effects of IL-4/IL-13, IL-10 down-regulates macrophage MHC-class II expression and the presence of macrophage co-stimulatory molecules, thereby promoting immune cell deactivation and suppression of inflammatory cytokine secretion (Lenschow et al., 1996, Gordon, 2003). Additionally, following LPS stimulation, IL-10 promotes the induction of STAT-3, which acts to inhibit cytokine and nitric oxide production. Furthermore, IL-10 and M2c macrophage activation have key roles to play in tissue repair and remodelling (Perrier et al., 2004).

While the study of macrophage activation states in major depression is limited, studies have reported the presence of a pro-inflammatory macrophage cytokine signature in patients suffering from bipolar depression (Padmos et al., 2008, Drexhage et al., 2011b).

1.9 Acute vs. chronic inflammation

Acute activation of the inflammatory response system (IRS) with an adaptive component is an essential host defence mechanism against stress and infection. Under normal physiological conditions this process is self-limiting with a distinct termination. However, failure of the IRS to resolve, results in the subsequent development of a maladaptive, chronic low grade inflammatory process with detrimental consequences. Evidence of a low grade inflammatory phenotype is apparent in numerous disorders such as cardiovascular diseases, obesity, type 2 diabetes, asthma and psychiatric disorders such as major depression. It is not clear what instigates this maladaptive process, although Medzhitov (2008) suggests that the emergence of chronic inflammation may be a consequence of impaired homeostasis and the dysfunction of physiologic systems not necessarily associated with the host defence or tissue regeneration, which are commonly the principal initiators of an inflammatory response.

Preclinical studies indicate that psychological stress increases gut permeability thereby enabling gut flora to access the systemic system (Bowe and Logan, 2011). In line with this observation, Maes et al. (2008) have reported the presence of antibodies against endotoxin from a number of commensal bacteria in plasma from depressed patients. Consequently, it is possible that LPS from commensal bacteria could stimulate a systemic low grade inflammatory response in depressed patients although this remains to be fully elucidated.

1.10 Mechanisms by which peripherally produced cytokines act on the CNS

Over the last 30 years, great advances have been made with respect to deciphering communication links between the central nervous system and the periphery, and specifically interactions between the immune, endocrine and nervous systems. Prior to this, the general consensus was that the brain was an immune privileged organ, inaccessible and isolated by the blood brain barrier (BBB) from peripheral immune responses (Quan and Banks, 2007). However, advances in establishing communication mechanisms between the brain and the periphery are pivotal to our understanding of psychiatric disorders. With increasing evidence of an inflammatory response in major depression, these mechanisms of communication provide a means by which large peripherally produced cytokine molecules (17-51kD), unable to passively diffuse across the BBB due to their size and structure, can alter neuronal and glial cell function and behaviour via cytokine receptor activation in the CNS thereby potentially impacting on mood and the manifestation of depressive symptomology (Hopkins and Rothwell, 1995, Szelenyi, 2001).

1.10.1 Mechanisms of communication

Fenestrations in the capillary bed, which ordinarily contributes to the formation of the BBB, give rise to permeable, highly vascularised structures known as circumventricular organs (CVOs). Situated around the brain's third and fourth ventricles, these leaky areas of the BBB allow circulating inflammatory mediators to act directly on glial cells, which, in turn, produce inflammatory signals such as cytokines and prostaglandins, thereby relaying the peripheral inflammatory message into the brain parenchyma. In support of this, Saper and Breder (1992) have shown that peripherally produced IL-1 acting on neurons in CVOs induce prostaglandin secretion and diffusion into the hypothalamic regions innervated with IL-1 containing neurons and thereby stimulate the release of centrally produced IL-1 .

Furthermore, there is some evidence to suggest that certain inflammatory cytokines, such as IL-1α and TNF-α, can be actively transported into the brain via saturable transport mechanisms. However, as reviewed by Connor and Leonard (1998), this transport mechanism may only be relevant in instances of high circulating concentrations of inflammatory mediators, as it is thought that the concentrations of cytokines transported into the brain via this mechanism are negligible.

The neural route is another prominent mechanism by which peripherally produced cytokines can impact on neurotransmission in the CNS. Locally produced cytokines secreted from areas such as

the gastrointestinal tract act on peripheral afferent nerve fibres (vagus nerve), which then relay inflammatory signals to their terminus in the nucleus tractus solitarius resulting in the subsequent activation of the hypothalamus and the HPA axis along with specific regions in the limbic system such as the amygdala (Roth and De Souza, 2001).

Additionally, peripheral innate immune mediators such as IL-1 and LPS have been shown to induce the release of inflammatory mediators in the brain through activation and crosstalk between perivascular macrophages and endothelial cells that line the cerebral vasculature. The mechanisms by which these cells of the cerebral vasculature interact to transmit the immune signals into the brain still remain to be fully elucidated. However, it appears that peripheral stimulation and subsequent activation of enzymes COX-2 and prostaglandin E synthase from the cells result in the central production of prostaglandin E2 (PGE2) which is fundamental to the CNS immune response and the induction of fever, sickness and HPA axis activity (Saper, 2010, Serrats et al., 2010). A role for this pathway has been demonstrated in endotoxin induced fever responses (Cao et al., 1997).

1.11 Evidence in support of inflammation associated depression

In the last 25 years, the depression literature has focused on investigating the role of inflammation in the pathophysiology of major depression. While the earliest studies by Kronfol et al. (1983) and Schleifer et al. (1984), investigating alterations in immune function in depressed and stressed individuals, reported a decreased T-cell proliferative response upon mitogen stimulation, much of the research to date has largely focused on the involvement of the innate immune response with numerous reports highlighting an association between major depression and activation of the innate immune response (Dantzer et al., 2008, Miller et al., 2009, Anisman, 2011, Leonard and Maes, 2012). In particular, evidence suggests that depression is associated with increased circulating concentrations of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and IFN-γ in association with elevations in chemokines and the acute phase protein CRP (Maes et al., 1995b, Lanquillon et al., 2000, Cizza et al., 2008, Simon et al., 2008, Diniz et al., 2010, Dowlati et al., 2010, Howren et al., 2009, Liu et al., 2012).

Further to this, it has also been shown that cytokine immunotherapy in the form of IL-2 and IFN-α for the treatment of Hepatitis C (Hep C) and certain types of cancer such as malignant melanoma can induce depression in 30-50% of these patients who are otherwise psychiatrically normal (Capuron et al., 2000, Capuron et al., 2001, Bonaccorso et al., 2001). Additionally, increased concentrations of monocytic cytokines such as IL-6 in association with indicators of cell mediated

immune activation and T-cell subset cytokine production have been highly associated with the onset of depressive symptoms following IFN-α treatment (Bonaccorso et al., 2001, Wichers et al., 2007). Significant symptom overlap between idiopathic and cytokine induced depression has been observed (Capuron et al., 2009). Moreover, the development and progression of the cytokine induced depressive symptoms can be inhibited with antidepressant treatment, suggesting that the therapeutic efficacy of antidepressants may be related to immunomodulatory properties (Musselman et al., 2001, Raison et al., 2005).

In accordance with this, numerous studies have reported on the anti-inflammatory properties of antidepressants (see review by Kenis and Maes (2002)). Specifically, Kubera et al. (2001) suggest that antidepressants may exert their effects via immunoregulatory mechanisms evidenced by elevated IL-10 concentrations and a suppressed IFN-γ/IL-10 production ratio in stimulated whole blood from severely depressed patients treated with antidepressants in vitro. Others have shown reduced whole blood TNF-α concentrations in patients who responded to a 6 week course of amitriptyline (Lanquillon et al., 2000), while Seidel et al. (1995) reported a normalisation of the elevated IFN-γ and soluble IL-2 receptor (sIL-2R) concentrations in the patient cohort following antidepressant treatment. Increased TNF-α plasma concentrations were reduced following a course of ECT (Hestad et al., 2003), while others have shown that SSRI treatment can reduce circulating CRP concentrations in the absence of therapeutic efficacy (O'Brien et al., 2006). Additionally, anti-inflammatory agents have been shown to enhance the efficacy of antidepressant treatment with findings by Muller et al. (2006) showing an enhanced antidepressant efficacy of reboxetine when given in combination with the COX-2 inhibitor, celecoxib, a known inhibitor of PGE-2 and pro-inflammatory cytokines, while Raison et al. (2013) have shown that depressed patients with higher baseline inflammatory markers respond to treatment with the TNF-α antagonist, infliximab.

An increased prevalence of depression has also been observed in association with autoimmune disorders with up to 50% of multiple sclerosis patients developing clinical depression (Feinstein, 2011). Additionally, the chronic inflammatory disorder rheumatoid arthritis is also highly associated with clinical depression symptoms with up to 42% of patients reporting comorbid depression (Bruce, 2008). Therefore, the presence of an autoimmune disease appears to put people at a risk 3 times greater than that posed to the general population.

Elevated T-cell stimulated inflammatory cytokine production prior to experiencing a stressful life event such as military deployment has recently been shown to be a risk factor for the development of depression (van Zuiden et al., 2011). This recent finding represents the emergence of a new body of literature, once again addressing the involvement of the adaptive

immune response in the pathogenesis of depression. Until recently, the depression literature has focused on investigating the innate immune response and with a particular interest in the pro-inflammatory cytokines IL-6 and TNF-α (Dowlati et al., 2010, Miller, 2010). However, of late, there has been a re-emergence of reports in corroboration with early theories by Smith and Maes suggesting the involvement of the adaptive/acquired immune response and cell mediated immune activation in the pathophysiology of major depression (Maes et al., 1995c). Some reports suggest an imbalance in T-cell subset cytokine production with elevated levels of prototypical Th-1 cytokines, such as IFN-γ, while anti-inflammatory IL-4 and TGF-β produced by Th-2 and Th-3 cells, respectively, have been found to be significantly lower in depressed cohorts (Myint et al., 2005, Kim et al., 2007, Sutcigil et al., 2007).

Additionally, in the early 1990's elevated macrophage secreted neopterin levels, which represent increased IFN-γ mediated macrophage activation, in association with increased circulating concentrations of the sIL-2R, were observed in depressed and melancholic patients suggesting an elevated presence of cell-mediated immune activation (Caruso et al., 1993, Maes, 1995). In support of this, recent findings by Celik et al. (2010) also suggest increased cell-mediated immune activity evidenced by elevated neopterin concentrations that are positively correlated with recurrent depression and an increased number of depressive episodes.

1.12 The kynurenine pathway

In recent years theories linking the seronotergic and cytokine hypotheses of depression have emerged (Maes et al., 2011, Leonard and Maes, 2012). The synthesis of serotonin in the CNS is dependent upon the availability of the essential dietary amino acid tryptophan in the blood (Russo et al., 2009). In this regard, the kynurenine pathway is the major metabolic pathway for tryptophan in the body, resulting in the production of kynurenine and many downstream metabolites [Figure 1.5]. Indoleamine 2,3 dioxygenase (IDO) is the rate-limiting, tryptophan degrading enzyme of the kynurenine pathway and is up-regulated in response to immune activation. Specifically, IFN-γ is the most potent inducer of IDO activation. However, IFN-γ independent mechanisms such as PGE2 or IL-6 in combination with TNF-α or IL-1β, are also known inducers of IDO activity (Carlin et al., 1989, Fujigaki et al., 2006, Zunszain et al., 2012). Therefore, IDO induction has been proposed as a mechanism by which inflammation can precipitate depression via tryptophan depletion.

In addition to IDO, activation of the hepatic tryptophan degrading enzyme tryptophan, 2,3 dioxygenase (TDO) in response to psychological stress, glucocorticoids or tryptophan itself, also results in kynurenine pathway activation in the liver (Moffett and Namboodiri, 2003).

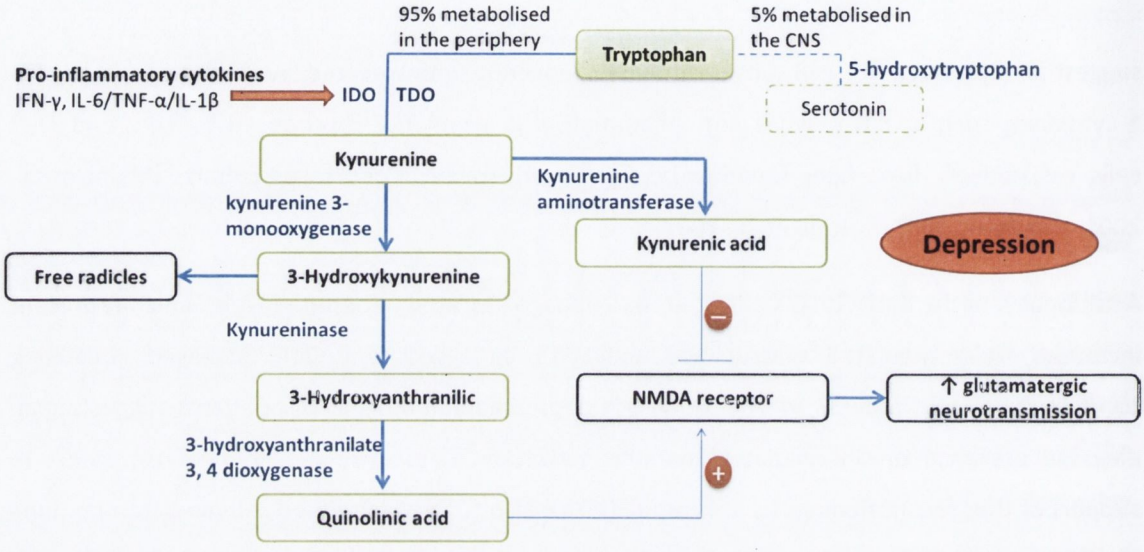

Figure 1.5 Diagrammatic representation of the kynurenine pathway. As a consequence of inflammation, decreased tryptophan availability for serotonin synthesis and an imbalance in the production of downstream kynurenine pathway metabolites, with a shift towards greater production of neurotoxic quinolinic acid, has been proposed as a mechanism by which inflammation may precipitate depression. Metabolites are represented in green boxes, enzymes are represented in blue. NMDA receptor: N-methyl-D-aspartate receptor.

1.12.1 A role for IDO

Under normal circumstances IDO has a key role to play in immune tolerance and suppression. By decreasing the availability of tryptophan during an innate immune response, pathogenic protein synthesis can be inhibited (Taylor and Feng, 1991). IDO activity also acts to regulate host responses and dampen IFN-γ production via negative feedback mechanisms (Moffett and Namboodiri, 2003). The combination of increased kynurenine concentrations in association with reduced tryptophan availability serves to down regulate lymphocyte proliferation, induce apoptosis and promote immune tolerance (Taylor and Feng, 1991). IDO is necessary for maternal tolerance during pregnancy and as a defence mechanism against autoimmunity (Munn et al., 1998, Grohmann et al., 2002). Additionally, high levels of IDO activity and reduced tryptophan

availability have also been found to induce T-reg cells which provides further support for the role of IDO in immune tolerance (Fallarino et al., 2006). However, in the presence of an increased inflammatory drive, as is evident in certain subsets of depressed patients, it has been proposed that the increase in IDO activity may be instrumental in the pathogenesis of depression. Preclinical studies assessing if IDO plays a causative role in the onset of depressive-like behaviour have shown that blockade of IDO activation following peripheral LPS administration prevents the development of depressive-like behaviour (O'Connor et al., 2009b). Additionally, kynurenine administration to mice dose-dependently induces depressive-like behaviour as assessed using the forced swim and tail suspension tests (O'Connor et al., 2009b). Moreover, while bacille Calmette-Guérin (BCG) induces inflammation, IDO activity and subsequent depressive-like behaviour in wild-type mice, IDO-knockout mice inoculated with BCG display an increased inflammatory profile in the absence of IDO activity and depressive like behaviour, thereby suggesting that IDO has a central role to play in the onset of depressive symptomology (O'Connor et al., 2009a).

1.12.2 Tryptophan

Reduced availability of tryptophan for serotonin synthesis or serotonin turnover in the CNS has been shown to be associated with numerous symptoms of depression and have mood altering effects (Christmas et al., 2011, Maes et al., 2011). Studies have shown that experimental depletion of dietary tryptophan, via the administration of tryptophan free diets in combination with increased ingestion of large amino acids which compete with tryptophan to cross the BBB, induces an acute depressive relapse by inhibiting the therapeutic effects of SSRIs which increase serotonin availability in the synaptic cleft (Delgado et al., 1990, Delgado et al., 1999). Moreover, decreased circulating tryptophan concentrations in patients receiving cytokine immunotherapy for the treatment of renal cell carcinoma were positively associated with the severity of depressive symptomology such as severe weight loss, suicidal ideation and an inability to think or concentrate (Capuron et al., 2002b). Despite evidence for IDO activity and subsequent reductions in peripheral tryptophan availability in cytokine-induced depression, Raison et al. (2010) have shown that central tryptophan availability is unaltered following immunotherapy. However, Raison and colleagues have also observed decreased levels of the main serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA) following IFN-α treatment, indicative of alterations in serotonin metabolism that are associated with the onset of depressive symptomology (Raison et al., 2009). Yet, direct evidence in support of tryptophan depletion as a consequence of kynurenine pathway activation in idiopathic major depression is lacking (Hughes et al., 2012).

1.12.3 Kynurenines

In addition to tryptophan depletion as a mechanism by which kynurenine pathway activation may precipitate depression, an imbalance in the production of kynurenine metabolites in favour of the neurotoxic N-methyl-D-aspartate (NMDA) receptor agonists may result in glutamatergic excitotoxicity and neuronal damage and consequently a neurodegeneration hypothesis of depression has emerged (Myint and Kim, 2003). In support of this theory, a number of studies have shown altered glutamatergic neurotransmission in depressed patients and post mortem tissue with elevated concentrations of glutamate and altered glutamate receptor function associated with volumetric changes (Nowak et al., 1995, Sanacora et al., 2004, Bernard et al., 2011). Such alterations in glutamate neurotransmission may have an impact upon normal processing of learning and memory, cognition and emotion (see review by Sanacora et al. (2012)). In addition, based on recent pre-clinical data demonstrating that kynurenine itself produces depressive-like behaviour in mice, a role for downstream metabolism of kynurenine in mediating depressive behaviours has been postulated (O'Connor et al., 2009b).

Kynurenine can cross the blood brain barrier with ease where it is converted by kynurenine 3-monooxygenase (KMO), an enzyme predominately expressed by microglia, into the free radical generator 3-hydroxykynurenine (3-HK). Downstream of this, the increased enzymatic activity of kynureninase results in the production 3-hydroxyanthranilic (3-HAA) and Quinolinic acid (QUIN) (Stone and Darlington, 2002). Given that QUIN is a powerful NMDA receptor agonist, excessive QUIN production may result in excitotoxic glutamate induced neuronal damage and neurodegeneration (Stone, 2001, Nemeth et al., 2007, Muller and Schwarz, 2007). Alternatively, metabolism of kynurenine via the enzymatic activity of kynurenine aminotransferase (KAT I and II), largely localised in astrocytes, results in the production of the neuroprotective agent kynurenic acid (KYNA), which acts to antagonise NMDA receptor activity (Stone and Darlington, 2002). Consequently, an imbalance in the production of downstream kynurenine pathway metabolites and a shift towards greater production of neurotoxic 3-HK and QUIN has been proposed as a mechanism underlying the biological basis of depressive symptomology. Specifically, Wichers et al. (2005) observed a positive correlation between depression severity and the neurotoxic arm of the kynurenine pathway in the absence of decreased tryptophan availability to the brain in cytokine induced depression. Similarly, Raison et al. (2010) have shown a positive association between cytokine induced depressive symptomology and elevated CSF concentrations of kynurenine and QUIN. One study assessing kynurenine pathway activation in idiopathic depression has also highlighted impairment in the neuroprotective arm of the pathway evidenced by decreased concentrations of KYNA (Myint et al., 2007).

1.13 Hypothalamic-pituitary-adrenal (HPA) axis hyperactivity

An individual's capacity to deal with stress is largely controlled by the HPA axis [Figure 1.6]. A dysregulated stress response system, evidenced by hyperactivity of the HPA-axis, represents a vulnerability factor for major depressive disorders and is one of the most consistent findings in patients with major depression, although others suggest that atypical depression may be characterised by HPA axis hypoactivity (Parker et al., 2003, Gold and Chrousos, 2002).

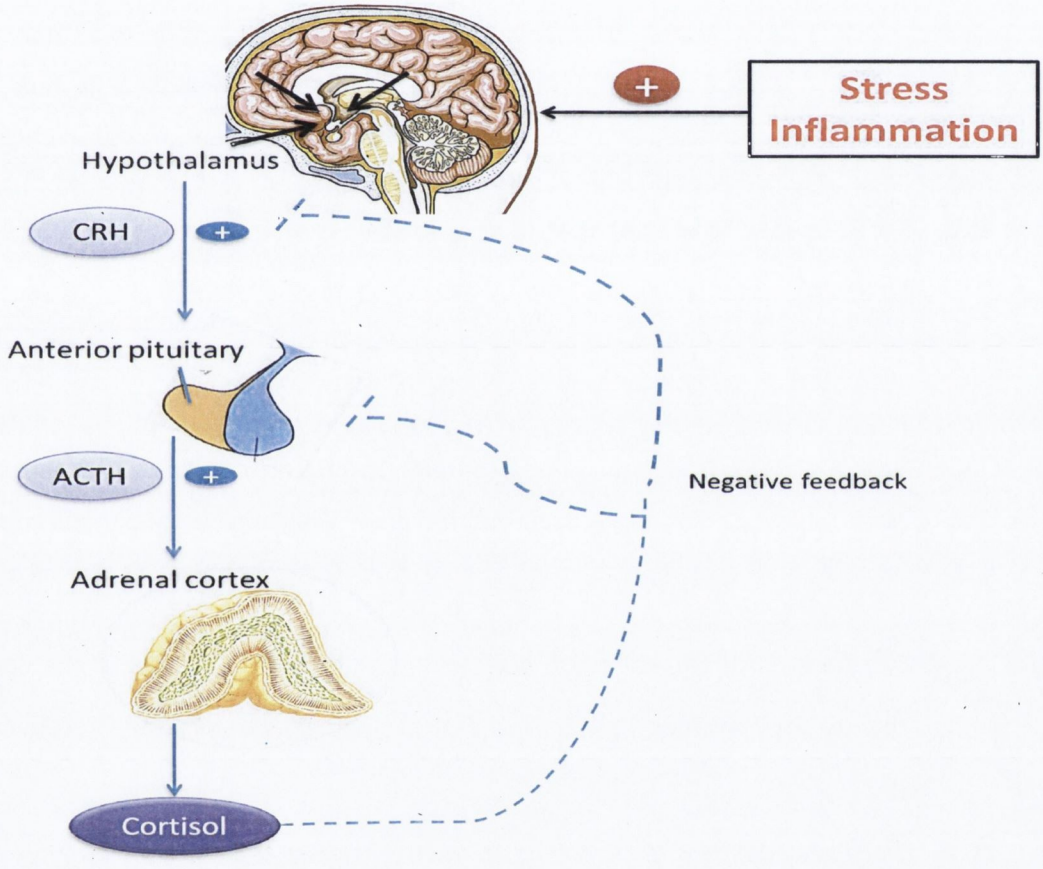

Figure 1.6 Diagrammatic representation of the HPA axis. In response to stress and inflammation the hypothalamus secretes CRH, which stimulates the production of ACTH from the anterior pituitary found at the base of the hypothalamus. Circulating ACTH acts on the adrenal cortex and stimulates the production of the glucocorticoid, cortisol, which acts to restore bodily homeostasis in response to stress. Cortisol also acts to regulate HPA activity through negative feedback at the level of the hypothalamus and pituitary. CRH: corticotropin-releasing hormone; ACTH: adrenocorticotrophic hormone; +: stimulates (based on Hiller-Sturmhofel and Bartke (1998).

In response to psychological or physical stress or a disrupted homeostatic balance with increased concentration of pro-inflammatory cytokines, the paraventricular nucleus of the hypothalamus secretes corticotropin-releasing hormone (CRH) into the hypothalamic-pituitary portal circulation which in collaboration with arginine vasopressin stimulates the production of adrenocorticotrophic hormone (ACTH) from the pituitary. Circulating ACTH acts on the cortex of the adrenal glands stimulating the production of glucocorticoids (cortisol) which have potent anti-inflammatory and inhibitory effects on NFκB inflammatory signalling pathways and function to restore bodily homeostasis (Owens and Nemeroff, 1991). Glucocorticoid receptors (GR) are widely distributed throughout the body and are found on multiple target tissues, including the brain and immune cells. Under normal physiological conditions, the HPA axis is self-regulatory and activity is curtailed via negative feedback inhibition; GR binding in the hypothalamus and the pituitary inhibits the activity of the HPA axis and the subsequent release of glucocorticoids. Additionally, while cytokines such as IL-1β and IL-6 can directly act on the GR in the hypothalamus, activating the HPA axis, glucocorticoids also act to inhibit the synthesis and secretion of inflammatory cytokines, evidenced by the inhibition of endotoxin induced fever in animals treated with exogenous glucocorticoids (Coelho et al., 1992).

However, alterations in many aspects of the HPA axis have been widely reported in depressed patients. Specifically, depressed patients are characterised by hypercortisolemia evidenced by elevated circulating cortisol concentrations in plasma and urine, while increased CRH and cortisol concentrations have been detected in cerebrospinal fluid (CSF) (Carroll et al., 1976a, Carroll et al., 1976b, Nemeroff et al., 1984, Pariante and Lightman, 2008). Moreover, a dysregulation of the diurnal pattern of cortisol secretion is observed in depressed patients with raised cortisol concentrations that do not decrease in the evening as normal, thereby leading to the production of glucocorticoids 24 hours a day (Pariante and Lightman, 2008).

While prolonged elevations in cortisol may be a consequence of chronic stress, alterations in GR function and sensitivity are also commonly reported in depressed individuals. Evidence in support of decreased glucocorticoid sensitivity, commonly referred to as glucocorticoid resistance, in depressed patients arises from studies assessing GR response to the synthetic glucocorticoid dexamethasone and the dexamethasone-CRH (DEX/CRH) stimulation test with studies repeatedly reporting impaired glucocorticoid responsiveness and non-suppression of cortisol secretion, which has been shown to correlate with depression severity and age (Carroll et al., 1981, von Bardeleben and Holsboer, 1991, Holsboer, 2000, Pariante and Miller, 2001, Ising et al., 2007).

Further to this, it is thought that persistent inflammation, observed in subgroups of depressed and stressed individuals such as those exposed to early life adversity, may have a significant role

to play in the onset and maintenance of glucocorticoid resistance, which in turn may fuel the chronic inflammatory phenotype in a feed forward cascade. In support of this theory, assessment of GR expression on HeLaS3 cells, following stimulation with the inflammatory cytokine TNF-α, revealed an increased expression of the inactive beta (β) isoform relative to the active alpha (α) isoform of the GR. The increased protein expression of GRβ, which is unable to bind cortisol and unable to stimulate glucocorticoid inducible genes, was also associated with the development of glucocorticoid resistance (Webster et al., 2001). Additionally, elevated expression of GRβ has been detected in patients with inflammatory conditions such as arthritis and asthma (Chikanza, 2002, Sousa et al., 2000). Further to this, an elevated mitogen stimulated IL-1β and IL-6 production was positively associated with non-suppressed plasma cortisol concentrations following the dexamethasone suppression test in a cohort of depressed patients (Maes et al., 1993a, Maes et al., 1993c). In accordance with this, Pariante and colleagues observed that IFN-α reduced GR translocation and decreased dexamethasone induced GR-mediated gene activity by nearly 50%, further implicating a role for inflammation in the dysregulation and hyperactivity of the HPA axis (Pariante et al., 1999).

The GR is a ligand activated transcription factor. Upon cortisol binding, it disassociates from its co-chaperone heat shock protein (hsp) complex in the cytosol and translocates to the nucleus. There it interrupts NFκB signalling through interactions with the NFκB regulator IκB, thereby exerting anti-inflammatory effects along with promoting the transcription of glucocorticoid inducible genes such as glucocorticoid induced leucine zipper (GILZ) and serum and glucocorticoid regulated kinase 1 (SGK1) (Raison and Miller, 2003) [Figure 1.7]. However, the mechanism of GR activation is tightly regulated by the co-chaperone hsp complex which effectively controls the sensitivity of the receptor and consequently has been implicated in the pathogenesis of major depression. Specifically, the hsp90 co-chaperone FK506 binding protein 51 (FKBP5) under normal conditions acts to negatively regulate the GR. When bound to GR, FKBP5 confers a low cortisol binding affinity on the receptor. However, upon cortisol binding and GR activation, FKBP5 is exchanged for FKBP4 permitting the translocation of the GR complex to the nucleus. This action, along with regulating and inducing gene transcription, also up-regulates the expression of FKBP5, thereby decreasing the sensitivity of the receptor once again (Binder, 2009) [Figure 1.7]. Interestingly, healthy controls carrying certain polymorphisms in the FKBP5 gene display an exaggerated increase in the transcriptional expression of FKBP5 upon GR activation in response to psychosocial stress, thereby decreasing the sensitivity of the receptor as evidenced by insufficient cortisol recovery and the persistent activation of the HPA axis (Ising et al., 2008). Additionally, Zimmermann et al. (2011) have shown that homozygotes of the minor allele of the FKBP5 gene

are more vulnerable to the development of major depression in the wake of severe adverse life events.

Consequently, stressful life events such as childhood trauma, genetics and chronic activation of the inflammatory response system, alone or in combination, may negatively impact on normal functioning and inhibitory control of the stress response system in association with altering the inhibitory feedback mechanisms of glucocorticoids on cytokine secretion, thereby inducing hypercortisolemia (or hypocortisolemia) and subsequently contributing further to the manifestation of a dysregulated inflammatory and neuroendocrine phenotype [Figure 1.8]. These factors potentially culminate in the manifestation of the behavioural and physiological alterations that currently characterise the depressive condition. Additionally, prolonged elevations of circulating cortisol levels, which are known to decrease IL-2 and lymphocyte proliferative abilities (Ross et al., 1990), may be responsible for the blunted T-cell proliferative response to mitogen stimulation frequently observed in depressed patients. Furthermore, as discussed by Frodl and O'Keane (2013) preclinical evidence suggests that elevated cortisol concentrations may have an excitotoxic and neurotoxic impact on brain structures strongly associated with depressive symptomology. However, while hippocampal volumetric changes are evident in depressed patients (Frodl et al., 2012), further clinical assessments are required to decipher if the observed neuronal alterations are a consequence of a dysfunctional stress response and elevated glucocorticoid concentrations.

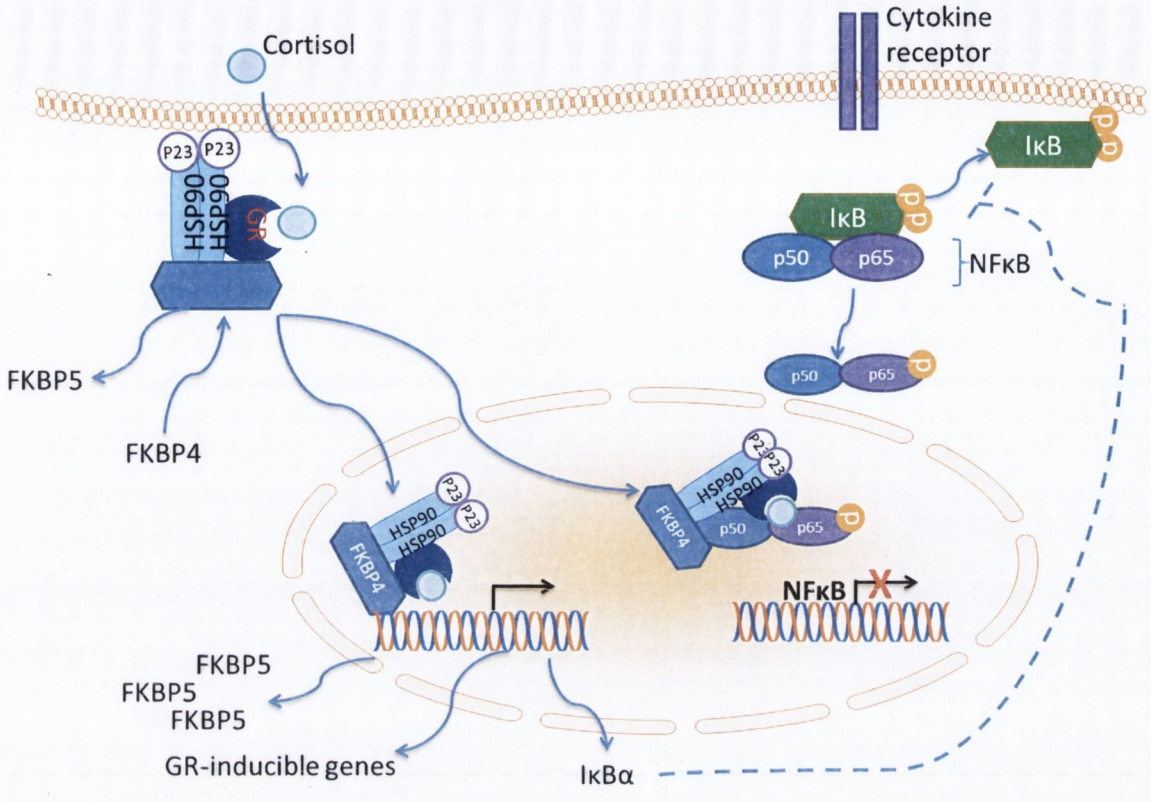

Figure 1.7 Glucocorticoid receptor signalling and suppression of inflammation under normal conditions. GR receptor activation results in the production of anti-inflammatory glucocorticoid inducible genes and the production of FKBP5 which acts as a negative regulator of glucocorticoid receptor activation. Glucocorticoids also act to regulate inflammation via a number of mechanisms, including the production of IκBα which inhibits the translocation of NFκB to the nucleus and the subsequent transcription of inflammatory genes. The glucocorticoid receptor can also interact directly with the p65 subunit, preventing NFκB binding and the transcription of inflammatory cytokines. FKBP: FK506 binding protein; GR: Glucocorticoid receptor; HSP: Heat shock protein; IκBα: I kappa B alpha; NFκB: Nuclear Factor-Kappa B, (based on Smoak and Cidlowski (2004).

1.14 Genetics and the serotonin transporter

Stress is thought to be a main contributing factor for the development of a depressive condition. However, the majority of people who experience stressful life events do not develop depression. Consequently, it is thought that specific genetic risk factors such as the FKBP5 polymorphism described above may predispose individuals to succumb to a depressive episode in the face of adverse life events. Alterations in the serotonin transporter (SERT), a molecule that removes serotonin from the synapse following its release (Benmansour et al., 1999), also appear to pose a risk for the development of a depressive episode following a stressful life event. In accordance with this, a study assessing how variations in the serotonin transporter gene (5-HTT), otherwise known as SERT, may influence the development of depression in response to stress revealed that a functional polymorphism in the promoter region of the SERT gene regulates the impact of adverse life conditions on depression (Caspi et al., 2003). The short (s) allele of the 5-HTT gene linked polymorphic region (5-HTTLPR) confers decreased transcriptional ability of the 5-HTT promoter compared with the long (l) allele (Lesch et al., 1996). In light of this, Caspi et al. (2003) reported that individuals carrying the s allele and exposed to adverse conditions in early adulthood displayed depressive symptomology by the age of 26. Additionally, the presence of the s allele predicted the occurrence of depression and suicidal ideation following stressful life events which was not evident in l/l homozygotes. Furthermore, early adverse life events such as childhood maltreatment also predicted the development of depression in adult s allele carriers (Caspi et al., 2003).

Further to this, SERT may also represent a link between the inflammatory hypothesis and reduced serotonergic function in depression. In this regard, studies have demonstrated that inflammatory cytokines, including IL-1β, TNF-α and IFN-α, increase SERT expression and serotonin reuptake in vitro (Zhu et al., 2006, Tsao et al., 2008). Additionally, a systemic inflammatory challenge with LPS increases SERT expression in the rodent brain (Zhu et al., 2010a). A sustained increase in CNS transcriptional expression of IFN-α and SERT in response to a single systemic challenge with the viral mimetic Poly IC was also found to have functional significance, evidenced by an associated decrease in extracellular serotonin concentrations, quantified by in vivo microdialysis in the pre-frontal cortex of rats (Katafuchi et al., 2005). Furthermore, elevated expression of the inflammatory cytokines IL-1β, TNF-α, IL-6 and IFN-γ on blood leukocytes from depressed patients was associated with an increased transcriptional expression of SERT. However, following chronic SSRI treatment decreased transcriptional expression levels of IFN-γ and SERT were observed in the patient cohort (Tsao et al., 2006).

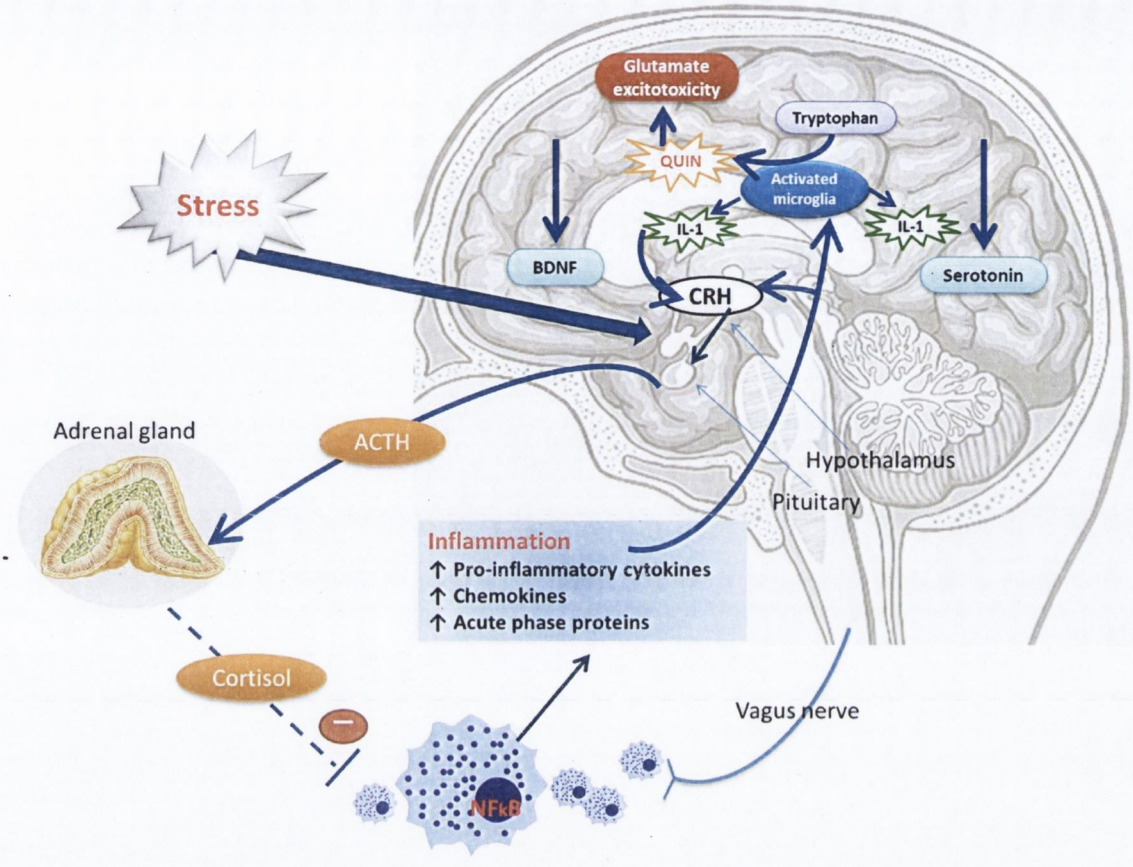

Figure 1.8 Summary of the mechanisms by which inflammation may precipitate depression. Chronic activation of the inflammatory response is thought to impact negatively on many biological systems, potentially culminating in the manifestation of depressive symptoms. Specifically, a chronic inflammatory state may alter serotonergic neurotransmission via the depletion of tryptophan and increased production of neurotoxic and excitotoxic mediators, which in association with inflammation itself, may have a negative impact on the neurotrophin system and BDNF concentrations in the brain. Chronic immune activation is also thought to contribute to the induction of HPA axis hyperactivity and glucocorticoid resistance, thereby, inhibiting the potent anti-inflammatory and immunomodulatory effects of cortisol, which, in turn, contributes further to a dysregulated inflammatory response. Alterations in the inflammatory response system, monoaminergic neurotransmission, the stress response and the neurotrophic system, alone or in combination, may have a detrimental impact on normal brain functioning, potentially culminating in the manifestation of the behavioural and physiological alterations that currently characterise the depressive condition.

1.15 Biomarkers for major depression

As the biological basis of depression still remains elusive, treatment strategies are not always effective and multiple trials often necessary to elicit a response. In the meantime, the patient remains severely depressed and at a possible increased risk of suicide. Consequently, the focus of attention in recent years has been on the identification of biological markers (biomarker) for depression. A biomarker is a measurable biological change associated with a condition which could aid in the diagnostic process, or provide inferences on the severity of the disorder (Mossner et al., 2007). Additionally, changes in specific biological markers may be used to characterise or distinguish depressive subtypes or may predict treatment response and in turn facilitate a more targeted and fast acting approach to treatment (Schmidt et al., 2011). However, while changes in the immune, serotonergic, glucocorticoid and neurotrophic systems are frequently reported in depression and represent potential biomarkers, the alterations are not specific enough to aid in the diagnosis of depression, given the heterogeneity in relation to severity, sub-type and genetic background.

Additionally, while numerous reports implicate endocrine and immune responses and the cross talk between these systems and the central nervous system (CNS) in the pathophysiology of major depression, it must be noted that depression is primarily a disorder of the CNS. However, alterations in the brain are difficult to study. So, many of the consistent findings in relation to immune and endocrine changes in depression emerge largely from the study of peripheral plasma/serum. Only a small proportion of research reports examine more specific CNS related variations in cytokine and metabolite concentrations through the use of CSF and post mortem brain tissue. However, studies on post-mortem brains are limited by many factors, including: analysis at one time-point only, incomplete pre-mortem clinical histories, cause of death and the large range of depressive phenotypes which result in inaccurate associations between biological markers and depressive states (Tochigi et al., 2008). Other concerns include the integrity of the tissue, agonal states of the subjects, sample pH and storage (Lewis, 2002, Shelton et al., 2011). Consequently, many inconsistencies in the literature exist and we have yet to find specific biomarkers which may predict those at greater risk of depression or provide insight into treatment response lending to the development of more efficient treatment strategies.

More recently the focus of attention has turned to PAXgene whole blood mRNA expression which is a novel approach to understanding the biological basis of depression. Emerging evidence in the last 10 years highlights the importance of peripheral blood as a potential diagnostic tool for many diseases including psychiatric disorders (Tsuang et al., 2005) and especially, as it may be used to assess the inflammatory signature in depressed patients. Its importance also arises from studies

by Liew et al. (2006) who show that peripheral blood cells share approximately 80% of the transcriptome with nine non-blood related tissues; specifically they found 81.9% of all genes expressed in the brain to be co-expressed on human blood cells. More specifically, Sullivan et al. (2006) examined the transcriptional profile in 17 individual brain regions and, like Liew et al. (2006), discovered a similar gene expression profile in these brain regions to that evident in whole blood. Given that circulating blood cells respond to the macro and mirco changes occurring around them and come into close contact with brain regions such as the pituitary and hypothalamus, it has been proposed that peripheral blood cells may act as 'surrogates for CNS expression' (Liew et al., 2006, Sullivan et al., 2006). Consequently, the PAXgene system may be thought of as a proxy measure for mRNA expression in brain (Hepgul et al., 2013).

While the use of the PAXgene system enables the analysis of total mRNA expressed by blood cells including erythrocytes (Sunde, 2010), isolated peripheral blood mononuclear cells (PBMCs) provide an insight into the profile of transcriptional expression in white blood cells which are easily obtained from living patients and enable links to be made between depression severity or clinical staging and the biological profile (Le-Niculescu et al., 2007). The use of these molecular biomarkers is of great interest as they may provide an opportunity to identify predisposing risk factors or predict early stage disease progression with greater sub-type specificity that in the context of psychiatric disorders, would eradicate self-report systems and subjectivity (Bell, 2004, Le-Niculescu et al., 2007, Sunde, 2010).

Additionally, PBMCs are very valuable as they provide a means to assess immune cell function following mitogen stimulation. Immunoassays assess the presence or absence of biological mediators or cytokines in circulation; however, these mediators are potentially bound to inhibitory substances and are not necessarily biologically active. Bioassays on the other hand, such as PBMC stimulation, provide an index for the functional activity of the mediators under investigation and consequently their contribution to the inflammatory response (Kronfol and Remick, 2000).

Advances in neuroimaging and positron emission tomography (PET) scanning in living patients also hold great promise for the identification of brain specific biomarkers. Given that depression is primarily a disorder of the CNS, assessment of microglial activation states and brain volumetric changes in depressed patients in association with peripheral immune markers may provide a more targeted and comprehensive approach in the search for biomarkers with an ultimate goal to develop personalised treatment strategies for patients with major depressive disorders (Doorduin et al., 2008, van Berckel et al., 2008, Frodl et al., 2012).

1.16 Aims and Objectives:

The aim of this project was to examine the relationship between activation of the inflammatory and stress response systems and activation of the kynurenine pathway and tryptophan depletion in the pathogenesis of depression and in the therapeutic response to ECT.

Activation of the kynurenine pathway and depletion of tryptophan for serotonin synthesis has been proposed as a mechanism by which inflammation can precipitate depression. In light of this, the aim of the first study was to determine if the inflammatory state observed in major depression was associated with activation of the kynurenine pathway and/or up-regulation of SERT as a mechanism to link inflammation to a serotonergic deficit in depression. Circulating inflammatory markers and PAXgene whole blood mRNA expression from a cross-sectional cohort of depressed patients and healthy control subjects were assessed.

Depression is a heterogeneous disorder defined by a range of affective and somatic symptoms. Therefore the aim of chapter 4 was to examine the association between inflammatory and glucocorticoid transcriptional markers with refined clusters of the HAM-D scale to include core depression, anxiety, insomnia and somatic symptoms. In addition, given the pertinent role of stress in the onset of depressive disorders, the impact of childhood trauma on the whole blood transcriptional expression of specific glucocorticoid related markers and inflammatory cytokines in a cross-sectional cohort of depressed patients and healthy controls was evaluated. Of particular interest in this study was the role of immune activation markers, T-cell subset gene expression and glucocorticoid inducible genes.

Following on from a cross-sectional design, the aim of chapters 5 and 6 was to assess the circulating inflammatory profile and the functional ability of isolated PBMCs to respond to immune stimuli *in vitro* prior to and following therapeutic intervention with ECT. Specifically, chapter 5 addresses the relative contribution of innate or adaptive immune cells to induce inflammation, kynurenine pathway activation and tryptophan depletion, while chapter 6 focuses on assessing if the various T-cell subsets Th-1, Th-2, Th-17 and T-reg, are altered in depression, and their ability to respond upon mitogen stimulation with anti-CD3.

Chapter 2

Materials and Methods

2.1 Materials

Cell culture

9ml vacuette sodium heparin blood tubes	Cruinn Diagnostics, IRL
Anti-CD3	eBioscience, UK
Dexamethasone hydrochloride	Sigma Aldrich, IRL
Dulbecco's phosphate buffered saline (PBS) (10X)	Sigma Aldrich, IRL
Cryovials (2ml)	Nunc, USA
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, IRL
Fetal Bovine Serum (FBS)	Invitrogen, USA
Ficoll-Histopaque 1077	Sigma Aldrich, IRL
Haemocytometer	VWR International, IRL
Lipopolysaccharide (LPS)	Sigma Aldrich, IRL
Mr. Frosty Freezing Container	VWR international, IRL
Penicillin-Streptomycin	Invitrogen, USA
Plastic Syringe (50 ml)	Becton Dickenson, UK
RPMI 1640 Medium (Gibco®)	Invitrogen, USA
Serological pipette (10 ml)	Sarstedt, IRL
Serological pipette (10 ml)	Sarstedt, IRL
Sterile 48 well Nunc plates	Sarstedt, IRL
Sterile falcon tubes (15 ml)	Sarstedt, IRL
Sterile falcon tubes (50 ml)	Sarstedt, IRL
Sterile microtubes (1.5 ml)	Sarstedt, IRL
Sterile Pasteur pipettes	Sarstedt, IRL
Syringe filter (0.2 µm)	Millipore, USA
Trypan blue	Sigma Aldrich, IRL

General Laboratory Chemicals

β-Mercaptoethanol	Sigma Aldrich, IRL
2-Propanol	Sigma Aldrich, IRL
Bovine Serum Albumin 96% (BSA)	Sigma Aldrich, IRL
Potassium Chloride (KCl)	Sigma Aldrich, IRL
Potassium Phosphate monobasic (KH_2PO_4)	Sigma Aldrich, IRL
Sodium Chloride (NaCL)	VWR International, IRL
Sodium Phosphate Dibasic (Na_2HPO_4)	Sigma Aldrich, IRL
Sodium Phosphate monobasic monohydrate (NaH_2PO_4)	Sigma Aldrich, IRL
Sulphuric Acid 98% (H_2SO_4)	VWR International, IRL
3,3',5,5'-Tetramethylbenzidine (TMB)	Sigma Aldrich, IRL
Tween-20	Sigma Aldrich, IRL

General Laboratory Plastics and Hardware

0.45 µm nylon filter	Nalgene, UK
50ml yellow capped tubs	Sarstedt, IRL
F96 Maxisorp immunoplates for ELISA	Nunc, USA
Glass Inserts	Labquip, IRL
Glass Vials	Labquip, IRL
Mini tubes (0.5ml)	Sarstedt, IRL
Microtubes (1.5ml)	Sarstedt, IRL
Para-film Laboratory Rolls	Sarstedt, IRL
Pasteur Pipettes	Sarstedt, IRL
Pipette tips	Sarstedt, IRL
Plastic Syringe (1 ml)	Becton Dickenson, UK

PCR

Fast 96 well optical reaction plates	Applied Biosystems, UK
Filter tips (1, 200, 1000 µl)	Sarstedt, IRL
High capacity cDNA archive kit	Applied Biosystems, UK
Molecular grade water	Sigma Aldrich, IRL
Molecular grade Absolute Ethanol	Sigma Aldrich, IRL
Optical adhesive covers	Applied Biosystems, UK
PAXgene blood RNA kit	Qiagen, UK
RA1 lysis buffer	Macherney-Nagel, GER
RNA extraction kit	Macherney-Nagel, GER
RNase Zap Wipes	Ambion, UK
RNase-free 1.5 and 2 ml microfuge tubes	Ambion, UK
RNase-free H_2O	Sigma Aldrich, IRL
Taqman® Fast Advanced master mix	Applied Biosystems, UK
Taqman® gene expression assays	Applied Biosystems, UK

ELISA

CRP ELISA Duoset	R&D systems, UK
BDNF ELISA Duoset	R&D systems, UK
Human IL-6 ELISA MAX™ Deluxe Kit	Biolegend, UK
Human TNF-α ELISA MAX™ Deluxe Kit	Biolegend, UK
Human IFN-γ ELISA MAX™ Deluxe Kit	Biolegend, UK
Human IL-1β ELISA MAX™ Deluxe Kit	Biolegend, UK
Human IL-10 ELISA MAX™ Deluxe Kit	Biolegend, UK
Human IL-17A ELISA MAX™ Deluxe Kit	Biolegend, UK
MSD®Multi-spot Human ProInflammatory-1 4-Plex	
Ultra-Sensitive Kit: IFN-γ, IL-1β, IL-6, TNF-α	MesoscaleDiscovery, USA

Kynurenine HPLC

3-Hydroxyanthraniliate	Sigma Aldrich, IRL
3-Hydroxykynurenine	Sigma Aldrich, IRL
Acetonitrile	Sigma Aldrich, IRL
Glacial Acetic Acid	Fisher Chemicals, UK
HPLC Grade Water	Fisher Chemicals, UK
Kynurenic Acid	Sigma Aldrich, IRL
L-Kynurenine	Sigma Aldrich, IRL
L-Tryptophan	Sigma Aldrich, IRL
N-methy-5-HT	Sigma Aldrich, IRL
Perchloric Acid	VWR International, IRL
Quinolinic Acid	Sigma Aldrich, IRL
Zinc Acetate	Sigma Aldrich, IRL

Statement of Contribution

Participant recruitment, blood sampling and clinical diagnostic testing on the cross-sectional cohort, assessed in Chapters 3 and 4, was carried out by Prof. Thomas Frodl's clinical research team in St. James's University Hospital, Dublin 8. Participant recruitment, blood sampling and clinical diagnostic testing on the ECT group, assessed in Chapter 5 and 6, was carried out by Prof. Declan McLoughlin's clinical research team in St. Patrick's University Hospital, Dublin 8. I carried out all experimental work and sample analysis for each study in Trinity College Institute of Neuroscience, Trinity College Dublin.

2.2 In vitro studies

2.2.1 Aseptic technique

All cell culture work, including the preparation of cell culture reagents, was carried out in a laminar flow-hood (Hera safe, category 2) and aseptic technique was utilised throughout. The filtered air in the laminar flow-hood prevents cell culture contamination with air-borne pathogens from the normal atmosphere. Prior to and following use, the interior of the laminar flow-hood was sprayed with 70% ethanol (EtOH) (30% double distilled water (d.d.H_2O) + 70% EtOH v/v) to maintain a sterile environment. All materials taken into the flow-hood were also sprayed with 70% EtOH beforehand preventing the introduction of pathogens. Other aseptic techniques included the use of sterile disposable plastic-ware and any glassware or d.d.H_2O used for culture work was sterilised prior to use by autoclaving at 121°C for 2 hours. Following use, the laminar flow-hood was exposed to ultraviolet (UV) light for 30 minutes. Disposable powder-free latex gloves were worn at all times and they were also sprayed lightly with 70% EtOH before use in the laminar flow-hood. Cells were kept in a Nuaire incubator at 37°C, 95% oxygen (O_2), 5% carbon dioxide (CO_2) and all items placed in the incubator were also sprayed with 70% EtOH to prevent external pathogenic contamination. Both the incubator and laminar flow-hood were cleaned regularly with 70% EtOH to preserve a sterile, pathogen-free environment. It is essential to sustain a sterile working environment for cell culture and to apply aseptic techniques throughout in order to maintain an uncontaminated environment free from bacteria, fungi and viruses which are known to interfere with normal cellular functions or result in cell death.

2.2.2 Preparation of culture media and test compounds

Culture Media: Using a 0.2 micrometre (μm) syringe filter, Fetal Bovine Serum (FBS) and penicillin-streptomycin were filter-sterilised and added to sterile RPMI 1640 1X containing L-Glutamine and 25 mM HEPES to give complete RPMI (cRPMI). Final concentrations of FBS and penicillin-streptomycin in the media were 10% and 1% respectively.

Phosphate Buffered Saline (PBS): 5 ml of Dulbecco's sterile 10X PBS (100 millimolar (mM) NaCl, 80 mM Na_2HPO_4, 20mM NaH_2PO_4) was added to 45 ml of d.d.H2O to yield a 1X working concentration of PBS.

Lipopolysaccharide (LPS): 25 mg LPS [*Escherichia coli* serotype 0111:B4] was dissolved in 1 milliliter (ml) d.d.H2O. The solution was mixed using a vortex and when fully dissolved 500 μl of this 25 mg/ml solution was added to 62 ml of cRPMI (1:125 dilution) to yield a 200 μg/ml stock solution which was then filter-sterilised using a 0.2 μm syringe filter. This stock solution was divided into 1 ml aliquots and stored at -20°C. Prior to use the stock solution was diluted to a working concentration in pre-warmed cRPMI.

Anti-CD3: 0.5 ml of a 1 mg/ml stock solution was stored at 2-8°C. Prior to use the stock was diluted to a working concentration in pre-warmed cRPMI.

2.2.3 Cell culture

2.2.3.1 PBMC collection, isolation and cryopreservation

Fasting whole blood samples were taken from depressed patients and healthy controls in sodium heparin tubes from the antecubital fossa between 07:30am and 09:30am. Participant recruitment and blood sampling was carried out by Prof. Declan McLoughlin's clinical research team in St. Patrick's University Hospital, Dublin. I collected and processed freshly-obtained fasting blood samples in the morning and carried out all the experimental work and analysis on these samples.

Bloods were processed within 1 hour following blood draw. 18 ml of blood was gently layered 1:1 over 18 ml of the density gradient cell separation medium, Ficoll-Histopaque 1077 solution, which had been warmed to room temperature (RT). This was then centrifuged for 30 minutes at 500 x g and 22°C without breaks to allow gradient formation and the mononuclear cells were collected from the interface between the plasma and Ficoll-Histopaque solution. The cells were transferred to a sterile 15 ml falcon tube and centrifuged for 10 minutes at 400 x g and 22°C with breaks to facilitate cell pelleting. The supernatant was discarded and the cells were re-suspended in RPMI

pre-warmed to 37°C before centrifugation for 10 minutes at 400 x g and 22°C. This step was repeated twice more and the cells were counted. The cell pellets were then re-suspended in 5 ml freezing media (90% FBS + 10% DMSO). 5 x 1 ml aliquots were made in 2ml cryovials for freezing. To ensure the cell membrane remained intact, the cells were frozen down slowly in a Mr. Frosty slow freeze container placed in the -80°C freezer for 24 hours. The cells were then transferred into liquid nitrogen and stored long-term until all participants had been recruited.

2.2.3.2 PBMC thawing

To ensure viability and efficient cell recovery the cells were thawed quickly. Following removal from liquid nitrogen storage, frozen cells were placed immediately into a 37°C water bath and held upright for approximately 1 minute. 1 ml of cRPMI pre-warmed to 37°C was then slowly added drop-wise over a 30 second period into the cryovial, the slow addition of media is essential given as cells with DMSO intercalated into their membranes are very fragile. The vials were inverted once to mix before the contents of each vial was added to 5 mls of cRPMI at 37°C. Following centrifugation at 330 x g for 10 minutes at 22°C the supernatant was discarded and cells were re-suspended in 1 ml cRPMI at 37°C.

2.2.3.3 PBMC cell count and integrity

The cells were counted on the Sysmex KX-21N Coulter counter (Kobe, Japan) and cell membrane integrity was evaluated using trypan blue dye exclusion. One part of 0.4% trypan blue was mixed with one part PBMC suspension and allowed to incubate for 1 minute. 10 µl was then placed under a glass microscope coverslip on a haemocytometer. A light microscope (OLYMPUS CKY41) was used to count the unstained (viable) and stained (unviable) cells. Based on these results, the PBMCs were then equalized to 1 x 10^6 cells/ml in cRPMI.

2.2.3.4 PBMC stimulation

For anti-CD3 stimulation, appropriate wells on the 48 well plates were coated with 200 µl of 0.1 µg/ml anti-CD3 and incubated overnight at 4°C. Excess anti-CD3 was aspirated from the wells and 450 µl cell suspension was added. Each sample had two wells which were pooled when harvesting. The plates were incubated for 48 hours at 37°C and 5% CO_2 in a sterile incubator.

For LPS stimulation, 450 µl cell suspension was added to the appropriate wells. The cells were stimulated with 50 µl of 1 µg/ml LPS and incubated for 48 hours at 37°C and 5% CO_2 in a sterile incubator. Each sample had two wells which were pooled when harvesting.

For the PBMC time-course and dose response studies the plates were coated with the appropriate concentrations of anti-CD3 as outlined above and stimulated for 6, 24 and 48 hours as described above.

As all clinical samples were stimulated systematically over a number of weeks, stimulation plates were prepared in advance and frozen, thereby eliminating discrepancies which may arise if treatments were made up on a daily basis. The same batch of culture media and FBS was also used throughout, thereby reducing potential variables in the study.

2.2.3.5 Harvesting supernatant for cytokine ELISAs and kynurenine HPLC and PBMC cells for mRNA analysis

Following stimulation and incubation for the appropriate time, the 48 well plates were removed from the incubator. The culture media were gently pipetted up and down to remove adherent cells from the well before being transferred into 1.5 ml microtubes. Each individual had two wells per treatment and these were pooled into the same microtube before being centrifuged for 10 minutes at 12,000 rpm at 4°C. Keeping the RNA pellet on ice, the supernatant was removed and aliquoted into 3-4 fresh microtubes and stored at -80°C until required for ELISA or HPLC.

The RNA pellet was then harvested for mRNA analysis. RA1 lysis buffer containing 1% β-mercaptoethanol was pipetted onto the cells pellet and vortexed vigorously before freezing at -80°C. When samples were required for RNA extraction they were defrosted slowly on ice.

2.2.4 Real-Time Polymerase Chain Reaction (RT-PCR)

2.2.4.1 Total RNA isolation from cultured cells

NucleoSpin® RNA II kits (Macherey Nagel) were used as per manufacturer's instructions to isolate total RNA from cultured cells. Cultured cells resuspended in lysis buffer (350 µl Buffer RA1 and 3.5 µl β-mercaptoethanol) were defrosted on ice. The lysate was filtered through NucleoSpin® Filter columns placed in 2 ml processing tubes by centrifugation for 1 minute at 11,000 x g. To adjust RNA binding conditions, 350 µl of 70% EtOH was added to the lysate and mixed thoroughly. The lysate was then transferred into a NucleoSpin® RNA II column placed in a 2 ml collection tube.

RNA was bound to the column following centrifugation for 30 seconds at 11,000 x g. The column was placed in a new 2 ml collection tube and 350 µl of membrane desalting buffer was added and centrifuged for 1 minute at 11,000 x g to dry the membrane. To ensure complete DNA elimination from the column-bound RNA, a DNase reaction mixture containing rDNase and DNase reaction buffer (10 µl and 90 µl per sample respectively) was prepared and 95 µl was added directly onto the silica membrane and left to incubate for 15 min at room temperature. The column was subsequently washed with 200 µl buffer RA2 and centrifugation for 30 seconds at 11,000 x g to inactivate the rDNase. The column was placed in a new 2 ml processing tube and 600 µl buffer RA3 was added to the column and centrifuged for 30 seconds at 11,000 x g. The flow-through was discarded. 250 µl of buffer RA3 was added to the column. This was then centrifuged for 2 minutes at 11,000 x g to dry the membrane completely. The column was placed into a 1.5 ml nuclease-free collection tube and the RNA was eluted in 20 µl RNase-free H_2O by centrifugation at 11,000 x g for 1 minute. This elution step was repeated to yield 40 µl total RNA which was subsequently divided into 20 µl aliquots stored at -80°C until required for quantification and equalisation.

2.2.4.2 PAXgene whole blood collection for mRNA analysis

Blood samples were taken from each subject in the early afternoon. 2.5 ml of blood was taken into proprietary PAXgene mRNA sample collection tubes. RNA samples were stored -20°C for 48 hours and then moved to -80°C until RNA extraction was performed. This work was carried out by Prof. Thomas Frodl's clinical research team.

2.2.4.3 Isolation of total RNA from human whole blood

Prior to starting, the RNA preparation area was wiped down using RNase Zap wipes to eliminate contamination and other RNases. A PAXgene Blood RNA kit was used to isolate RNA as per manufacturer's instructions. The PAXgene Blood RNA tubes were removed from the -80°C freezer and left at room temperature for 4 hours to thaw and equilibrate to room temperature before being centrifuged at 3,000 x g for 10 minutes at 22°C. The supernatant was discarded and 4 mls RNase-free water was added to the pellet. The pellet was vortexed until visibly dissolved and centrifuged at 3,000 x g for 10 minutes at 22°C. After discarding the supernatant, 350 µl resuspension buffer (BR1) was added to each pellet and vortexed until dissolved. To ensure complete protein digestion, the sample was transferred into a 1.5ml microcentrifuge tube and 300 µl binding buffer (BR2) and 40 µl Proteinase K was added. This was then vortexed quickly and

incubated for 10 minutes at 55°C in the shaker-incubator at 300 rpm. The lysate was transferred directly into a PAXgene Shredder spin column placed in a 2 ml processing tube and centrifuged for 3 minutes at 19,000 x g to homogenise the cell lysate and remove residual debris. To adjust RNA binding conditions the supernatant was transferred to a new 1.5 ml microcentrifuge tube and 350 µl 96% EtOH was added. 700 µl of each sample was transferred into the PAXgene RNA spin column placed in a 2 ml processing tube and centrifuged for 1 minute at 19,000 x g, enabling RNA binding to the PAXgene silica membrane. The flow-through was discarded and the process was repeated with the remaining sample. To remove any remaining contaminants, 350 µl wash buffer 1 (BR3) was added to the PAXgene spin column and centrifuged for 1 minute at 19,000 x g. The flow-through was discarded and the spin column was placed in a new 2 ml processing tube. To ensure complete DNA elimination from the column-bound RNA, a stock solution containing DNase and DNA digestion buffer (10 µl and 70 µl per sample respectively) was added directly onto the membrane of the PAXgene column and left for 15 minutes. To remove the DNase, the column was washed with 350 µl wash buffer 1 (BR3), centrifuged for 1 minute at 19,000 x g and the flow-through was discarded. 500 µl wash buffer 2 (BR4) was then added to the column, centrifuged for 1 minute at 19,000 x g, the flow-through was discarded. This was then followed by a second 500 µl addition of BR4 which was centrifuged for 3 minutes at 19,000 x g. The PAXgene RNA spin column was then transferred to new 2 ml processing tube and centrifuged for 1 minute at 19,000 x g to completely dry the column. The PAXgene RNA spin column was then placed in a 1.5 ml microcentrifuge tube to which 40 µl elution buffer (BR5) was added and centrifuged for 1 minute at 19,000 x g. This elution step was then repeated with another 40 µl BR5. The RNA was divided into 20 µl aliquots and subsequently stored at -80°C until required for quantification and equalisation.

2.2.4.4 RNA quantification and equalisation

The RNA yield and quality of each sample was quantified based on optical density (OD) using the NanoDrop©ND-1000 UV-Vis spectrophotometer [Thermo Fischer Scientific]. On blanking the machine with 1 µl RNase free water or BR5 for RNA isolated from whole blood, 1 µl of each sample was placed on the spectrophotometer and the RNA concentration was measured at absorbance wavelengths of 260 nm. The quality of the RNA was assessed using the ratio of A260/280 (the range of 1.8-2.1 was an indication of a good quality RNA sample). To prevent contamination, the spectrophotometer was wiped with tissue paper after each individual sample. RNA from whole blood was equalised to 10 ng/µl while RNA isolated from PBMCs was equalised to 2 ng/µl using RNase-free H_2O as diluent.

2.2.4.5 cDNA synthesis

cDNA synthesis was carried out using a High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). A master mix solution was made up (per sample, 2 µl 10X RT buffer, 0.8 µl 25X dNTP mix 100 mM, 2 µl 10X RT random primers, 1 µl Multiscribe™ Reverse Transcriptase, 4.2 µl RNase-free H_2O) and 10 µl per sample was added to an equal volume of RNA. The samples were then vortexed and centrifuged briefly before being placed in the thermocycler [Peltier Thermal Cycler PTC-200] and the program was set according to protocol as follows: 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 seconds. The final step inactivated the reverse transcriptase. Upon completion, the samples were centrifuged for 30 seconds, divided into 10 µl volumes and diluted 1 in 5 with molecular grade water before being stored at -20°C.

2.2.4.6 Multiplex RT-PCR

The StepOnePlus™ Real-Time PCR system (Applied Biosystems) in the 96-well format was used for quantitative (Q)-PCR. To quantify expression of target genes of interest Taqman® gene expression assay's (Applied Biosystems) were used, each of which contained a specific set of forward and reverse primers and a FAM-labelled MGB probe for the target of interest [Table 2.1]. PCR reactions were in a duplex format also containing a Taqman® gene expression assay (primer limited) containing a VIC labelled probe for the endogenous control gene glyceraldehyde-3-phosphatedehyrdrogenase (GAPDH). A 10 µl volume was added to each well, the components of which are as follows: 4 µl of diluted cDNA, 0.5 µl of FAM-labeled Taqman® gene expression assay for the target of interest, 0.5 µl VIC-labeled Taqman® gene expression assay for GAPDH and 5 µl of Taqman®Fast Advanced Master Mix. Samples were assayed over 40-50 cycles using ABI's universal cycling conditions using a fast protocol as follows: 50°C for 2 minutes, 95°C for 20 seconds for polymerase activation (holding stage), 95°C for 1 second for each cycle (denaturation) and 60°C for 20 seconds (annealing). GAPDH was chosen as an endogenous control gene in the amplification system for these analyses as it demonstrated the least variable expression profile among blood samples relative to another endogenous control gene human acid ribosomal protein (HUPO). At the end of the reaction, data analysis was performed with the StepOnePlus™ System Software (Applied Biosystems) and RQ values ($2^{-\Delta\Delta CT}$, where CT is the threshold cycle) of the target genes relative to their own endogenous control were obtained. The RQ values were then used to assess differences between groups or converted into fold change values relative to control group for cross-sectional analysis.

With regard to the cycling conditions, a high temperature of 95°C for 1 second is applied initially to separate the target DNA strands from each other. As the temperature lowers to 60°C the complimentary primers and a fluorgenic probe anneal to the target sequence of interest enabling Taq polymerase to specifically amplify the target sequence. During extension and synthesis of the complimentary DNA, the 5' exonuclease activity of the Taq polymerase cleaves the probe with the subsequent release of the reporter molecule from the quencher, resulting in a fluorescent emission. Consequently, at 60°C the exponential accumulation of PCR product over 40-50 cycles was measured cycle by cycle based on the fluorescent emission.

Table 2.1 List of genes used with the GenBank sequence ref numbers

Target Symbol	Target Name	Taqman Gene Assay ID
Cytokines		
IL-1β	Interleukin-1beta	Hs01555410_m1
TNF-α	Tumor Necrosis Factor alpha	Hs00174128_m1
IL-6	Interleukin-6	Hs00985639_m1
IFN-γ	Interferon gamma	Hs00989291_m1
IFN-inducible genes		
IP10	Interferon gamma induced protein-10	Hs99999049_m1
SOCS1	Suppressor of cytokines signalling-1	Hs00705164_s1
IDO1	Indolamine 2,3 dioxygenase 1	Hs00984148_m1
IDO2	Indolamine 2,3 dioxygenase 2	Hs01589373_m1
KP enzymes		
KMO	Kynurenine-3-monooxygenase	Hs00175738_m1
KAT I	Kynurenine aminotransferase I	Hs00187858_m1
KAT II	Kynurenine aminotransferase II	Hs00212039_m1
Kynureninase	Kynureninase	Hs01114099_m1
SERT	Serotonin transporter	Hs00169010_m1
Immune activation markers		
TREM-1	Triggering receptor expressed on myeloid cells-1	Hs00218624_m1
DAP-12	DNAX activation protein of 12 kDa	Hs00182426_m1
TLR3	Toll-like receptor 3	Hs00152933_m1
TLR4	Toll-like receptor 4	Hs01061963_m1
PTX3	Pentraxin 3	Hs00173615_m1
PDE4B	Phosphodiesterase 4B	Hs00277080_m1
TRAF6	TNF receptor-associated factor 6	Hs00371512_g1

Macrophage markers		
M1		
EGR1	Early growth response protein 1	Hs00152928_m1
IL-12p40	Subunit beta of interleukin 12	Hs01011518_m1
iNOS	Inducible nitric oxide synthase	Hs01075529_m1
IFN-γR1	Interferon gamma receptor-1	Hs00988304_m1
COX-2	Cyclooxygenase-2	Hs00153133_m1
IκBα	I-kappa-B-alpha	Hs00153283_m1
M2		
PU.1	SFFV proviral integration 1 protein	Hs02786711_m1
ARG1	Arginase-1	Hs00968979_m1
IL-4R	Interleukin-4 receptor	Hs00166237_m1
MRC1	Mannose receptor-1	Hs00267207_m1
CD200R	Cluster of differentiation 200 receptor	Hs00793597_m1
T-cell activation		
CD25	Cluster of differentiation 25	Hs00907779_m1
Th-1		
IFN-γ	Interferon gamma	Hs00989291_m1
IL-2	Interleukin-2	Hs00174114_m1
T-bet	T box expressed in T cell	Hs00203436_m1
Th-2		
IL-4	Interleukin-4	Hs00174122_m1
IL-13	Interleukin-13	Hs99999038_m1
GATA3	GATA-binding protein 3	Hs00231122_m1
T-reg		
TGF-β	Transforming growth factor beta	Hs00998133_m1
IL-10	Interleukin-10	Hs00961622_m1
FoxP3	Forkhead box P3	Hs01085834_m1
Th-17		
IL-17A	Interleukin-17A	Hs00174383_m1
IL-21	Interleukin-21	Hs00222327_m1
IL-22	Interleukin-22	Hs01574154_m1
RORC-γ	RAR-related orphan receptor gamma	Hs00172860_m1
Glucocorticoid inducible genes		
GR	Glucocorticoid receptor	Hs00353740_m1
FKBP5	FK506 binding protein 5	Hs01561006_m1
SGK1	Serum/glucocorticoid regulated kinase 1	Hs00178612_m1
GILZ	Glucocorticoid-induced leucine zipper	Hs00608272_m1

2.2.4.7 RT-PCR analysis

The ΔΔCT method (Applied Biosystems RQ software, Applied Biosystems, UK) was used to assess gene expression for all real-time PCR analysis. This method is used to assess relative gene expression by comparing gene expression of treated/ experimental samples to a normal control or untreated control sample, rather than quantifying the exact copy number of the target gene. In this manner, the fold difference (increase or decrease) can be assessed between treated and control samples. The fold difference is assessed using the cycle number (CT) difference between samples. Briefly, a threshold for fluorescence is set, against which the CT is measured. To accurately assess difference between gene expression, the threshold is set when the PCR reaction is in the exponential phase, when the PCR reaction is optimal or 100% efficient. Thus samples with low CT reading demonstrate high fluorescence, indicating greater amplification and hence greater gene expression. When a PCR is 100% efficient a one-cycle difference between samples means a 2-fold difference in copy number (2^2), similarly a five-cycle difference is a 32-fold difference (2^5).

To measure this fold-difference relative to control, the CT of the endogenous control (GAPDH) is subtracted from the CT of the target gene for each sample, thus accounting for any difference in cDNA quantity that may exist. This normalised CT value is called the (ΔCT). The CT difference (ΔCT) of the control is subtracted from itself to give 0, and subtracted from all other samples, this is the ΔΔCT value. The ΔΔCT (cycle difference corrected for GAPDH) is then converted into a fold-difference. As a one-cycle difference corresponds to a two-fold increase or decrease relative to control, 2 to the power of the ΔΔCT (difference in control and sample CT corrected for GAPDH) gives the fold difference in gene expression between the control and treated samples. The control sample always has a ΔΔCT value of 0, thus 0^{-2} gives a $2^{-\Delta\Delta CT}$ of 1, against which all other samples are referenced.

2.2.5 Enzyme-linked immunosorbent assay (ELISA)

2.2.5.1 Blood collection for plasma

A 10ml blood sample was taken from healthy controls and depressed patients in the early afternoon into a heparinised vacutainer and was centrifuged to generate plasma. This was then stored at -80°C until required for cytokine and HPLC analysis. This work was carried out by Prof. Thomas Frodl's clinical research team.

Fasting blood samples were taken from healthy controls and severely depressed patients prior to and following therapeutic intervention with ECT in K2 EDTA tubes between 07:30am and 09.30am. Samples were centrifuged at 2000 rpm for 10 minutes and stored at -80°C within one hour. This work was carried out by Prof. Declan McLoughlin's clinical research team.

2.2.5.2 ELISA for IL-1β, TNF-α, IL-6, IFN-γ, IL-17A and IL-10

Human IL-1β, TNF-α, IL-6, IFN-γ, IL-17A and IL-10 were quantified by sandwich ELISA using BioLegend ELISA MAX™ Deluxe kits as per manufacturer's instructions. The capture antibodies for IL-1β, TNF-α, IL-6, IFN-γ, IL-17A and IL-10 were diluted 1:200 in 1 X Coating buffer and 100 µl of this capture antibody solution was added to all wells on the 96-well maxisorb ELISA plates which were incubated overnight at 4°C. Plates were then washed 4 times with 300 µl/well PBS + 0.05% Tween 20 and blocked with 200 µl/well 1 X assay diluent for 1 hour at RT with shaking at 200 rpm, to eliminate non-specific binding. As before, plates were washed 4 times and 100 µl samples/standards were added to the appropriate wells and incubated for 2 hours at RT with shaking.

Samples were diluted as follows:

Plasma samples for IL-1β, IL-6, TNF-α, IFN-γ all run neat

Unstimulated PBMC supernatants for IL-6, TNF-α, IFN-γ, IL-17A, IL-10 all run neat

Anti-CD3 stimulated PBMC supernatants for IL-6, TNF-α and IFN-γ diluted 1:10

Anti-CD3 stimulated PBMC supernatants for IL-10 diluted 1:2

Anti-CD3 stimulated PBMC supernatants for IL-17A run neat

Standards were prepared as per kit instructions; six two-fold serial dilutions of the 500 pg/ml top standard were carried out with 1X assay diluent for IL-6, TNF-α and IFN-γ. This was repeated for IL-1β with a top standard of 125 pg/ml and for IL-17A with a top standard of 250 pg/ml.

Following 4 washes, 100 µl of detection antibody, diluted 1:200 in 1 X assay diluent, was added to each well and incubated for 1 hour at RT with shaking. This was followed by washes as before and the addition of 100 µl/well Avidin-Horseradish peroxidase (HRP), diluted 1:10,000 in 1X assay diluent, which was incubated for 30 minutes at RT with shaking. 5 washes were completed and 100 µl of freshly mixed TMB substrate solution was added to each well and incubated in the dark until the wells turned blue in colour (approximately 10-20 minutes depending on the ELISA). The addition of 100 µl/well 2N H_2SO_4 stopped the reaction. The absorbance was read at 450 nm using

a microplate reader (Sunrise Tecan, Reading, UK) and was then recalculated as a concentration (pg/ml) using standard curves derived using GraphPad Prism Software Version 5.00 (GraphPad Software, Inc).

2.2.5.3 ELISA for CRP and BDNF

Human BDNF and CRP were quantified by sandwich ELISA (R&D Systems, UK). 100 µl/well of capture antibody diluted 1:180 in 1 X PBS was incubated at room temperature (RT) overnight. Following 3 washes with PBS + 0.05% Tween 20 the plates were blocked for 1 hour at RT with 300 µl/well of 1% bovine serum albumin (BSA). After 3 washes 100 µl of samples/standards were added to each well and incubated for 2 hours at RT. Plasma samples for BDNF were diluted 1:10 while plasma samples for CRP were diluted 1:10,000. Two-fold serial dilutions were prepared from a top standard of 3,000 pg/ml for BDNF and 1,000 pg/ml for CRP. This was followed by washes as before and 100 µl/well of detection antibody diluted 1:180 in 1% BSA was incubated for 2 hours at RT. 3 washes were completed and 100 µl avidin-HRP, diluted 1:200 in 1% BSA, was added to each well and incubated for 20 minutes in the dark. Following washes, the plates were incubated in the dark for 20 minutes with TMB (100 µl/well). The addition of 100 µl/well 2N H_2SO_4 stopped the reaction. The absorbance was read at 450 nm using a microplate reader (Sunrise Tecan, Reading, UK) and was then recalculated as a concentration (pg/ml for BDNF, mg/L for CRP) using standard curves derived using GraphPad Prism Software Version 5.00 (GraphPad Software, Inc).

2.2.6 MSD®MULTI-SPOT Human Pro-inflammatory 1 4-Plex Ultra-Sensitive assay

Human plasma IL-1β, IL-6, TNF-α and IFN-γ concentrations were measured in a sandwich immunoassay format using an MSD® MULTI-SPOT Human Pro-inflammatory 1 4-Plex kit (MesoScale Discovery, USA) as per manufacturer's instructions. Plates pre-coated with capture antibody for IL-1β, IL-6, TNF-α and IFN-γ on spatially distinct spots were blocked with 25 µl/well of Diluent 2 for 30 minutes at RT with shaking at 300 rpm. A top standard of 2,500 pg/ml was prepared and four-fold serial dilutions were carried out, using Diluent 2 as diluting agent, yielding an 8 point standard curve. 25 µl of samples/standard were added to the appropriate wells and incubated for 2 hours at RT with shaking at 300 rpm to accelerate capture. Following this incubation period the plates were washed 3 times with PBS + 0.05% Tween 20. The stock detection antibody blend was diluted 1:50 in Diluent 3 and 25 µl of this solution was added to

each well and incubated for 2 hours at RT with shaking at 300 rpm. 3 washes were completed as before. The 4 X read buffer T was diluted 1:2 in deionised water and 150 µl of this solution was added to each well and the plate was read immediately on the SECTOR Imager. A reverse pipetting technique was employed throughout to provide greater accuracy and prevent bubble formation.

2.2.7 High performance liquid chromatography (HPLC)

Tryptophan and kynurenine pathway metabolites were measured by HPLC. Kynurenine was measured at a wavelength of 230 nm or 250 nm by PDA-UV detection (SPD-M10AVP, Shimadzu). Tryptophan, kynurenic acid (KYNA) and 3-hydroxyantranillic (3-HAA) concentrations were measured fluorometrically (RF-10A XL, Shimadzu) at an excitation wavelength of 254 nm and an emission wavelength of 404 nm.

2.2.7.1 HPLC sample and buffer preparation

Homogenisation buffer was prepared with mobile phase containing 7% perchloric acid to deproteinise the samples and 200ng/20µl N-methyl 5-hydroxy-tryptamine (Sigma Aldrich) as internal standard. The mobile phase consisted of 50 nM glacial acetic acid, 100 mM zinc acetate (Sigma Aldrich) and 3% acetonitrile dissolved in double-distilled NANO-pure HPLC grade water (Sigma Aldrich) at pH 4.9. Plasma samples were centrifuged for 10 minutes at 12,000 rpm and 4°C to clean the samples. 300 µl of plasma or PBMC cell culture supernatant was added to an equal volume of ice cold homogenisation buffer, which was vortexed vigorously before being centrifuged at 12,800 rpm for 20 minutes and 4°C. The supernatants were then passed through 4 mm syringe filters (non-sterile 0.45 µm nylon, Nalgene, UK) and transferred to new 1.5 ml tubes on ice. Samples were analysed immediately.

The standards used for HPLC were as follows: N-methyl 5-HT (internal standard), L-Kynurenine, L-Tryptophan, KYNA and 3-HAA (Sigma Aldrich). Standard stock solutions of 10 mg/10ml were prepared by dissolving the standards in 10 ml of HPLC mobile phase. These solutions were stored at 4°C. Using the stock solutions a 10 ml standard mix containing 200 ng/20µl of each standard was prepared.

2.2.7.2 Measurement of kynurenine pathway metabolites

Samples and standards were then transferred into individual 0.2 ml conical inserts (Labquip, IRL) which were placed in a 2 ml glass vials (Labquip, IRL) and HPLC analysis was conducted with an automated HPLC system (Shimadzu ADVP module). A 20 µl volume of sample was injected onto a reverse phase analytical column (Kinetex Core Shell Technology, Phenomenex, UK) with specific area of 100 mm x 4.6 mm and particle size of 2.6 µm and fitted with a guard column (Lichrosorb RP18, specific surface area 30 x 4 mm, Phenomenex, UK) for separation of metabolites. The flow rate was 0.8 ml/min (LC-10AT pump, Shimadzu) and the acquisition time was 18 minutes. A standard mix was injected every six samples in order to recalibrate the system and curtail divergences in the retention times during sample runs. The auto-sampler was also pre-programmed to self-rinse with methanol between each sample injection. CLASS-VP software (Shimadzu) was used for chromatographic control, data collection and processing. Retention times and peak heights were recorded from the chromatographs generated and together with the data from the standards the concentrations of the metabolites in the samples were quantified and expressed as ng/ml of plasma or PBMC culture supernatant.

2.2.8 Statistical analysis

Data are presented as mean with standard error of the mean (SEM) or standard deviation (SD) where appropriate. Categorical variables were tested using Chi-square (χ^2) test. All data were tested for normality using the Shapiro-Wilk test. Normally distributed data were analysed using the student's *t*-test or two-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test, where appropriate. Non-parametric data were analysed using the Mann-Whitney U test for independent sample comparisons, the Wilcoxon-Signed Rank test for paired comparisons or Kruskal-Wallis one-way ANOVA, where appropriate. Correlational analysis was carried out using Spearman's *rho* correlation statistics. All statistical analyses were considered significant when $p \leq 0.05$. Graphs and statistics were generated using GraphPad Prism Software Version 5.00 (GraphPad software, Inc). All data were analysed using SPSS (Version 16).

Chapter 3

Tryptophan depletion in depressed patients occurs independent of kynurenine pathway activation

The work contained in this chapter is published. (Brain Behav Immun. 2012 Aug;26(6):979-87)

3.1 Introduction

Major Depressive Disorder (MDD) is a serious psychiatric disorder and a leading source of disability worldwide with a lifetime prevalence of up to 16%. Whilst the precise biological basis of depression still remains elusive, evidence suggests that activation of the innate immune system may be involved in the aetiology of depression (Dantzer et al., 2008, Miller et al., 2009, Anisman, 2011, Leonard and Maes, 2012). Specifically, it has been reported that depression is associated with increased circulating concentrations of pro-inflammatory cytokines, soluble cytokine receptors, chemokines and acute phase proteins (Maes et al., 1995b, Lanquillon et al., 2000, Cizza et al., 2008, Simon et al., 2008, Diniz et al., 2010). Moreover, in the case of IL-6, IL-1, TNF-α, sIL-2R and C reactive protein (CRP) these original findings have been supported by recent meta-analyses (Dowlati et al., 2010, Haroon et al., 2012, Liu et al., 2012). It is also known that cytokine immunotherapy can induce depressive symptoms in otherwise psychiatrically normal individuals (Capuron et al., 2000, Bonaccorso et al., 2001, Capuron et al., 2001).

Whilst a functional deficit in the neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) is well-established as a contributor to the pathogenesis of depression (Coppen and Doogan, 1988, Cryan and Leonard, 2000), it is only in recent years that theories have emerged to provide a mechanistic link between inflammation and low serotonin (Maes et al., 2011, Leonard and Maes, 2012). The synthesis of brain serotonin is dependent on the availability of the essential amino acid tryptophan in the blood (Russo et al., 2009). In this regard, the kynurenine pathway is the major metabolic pathway for tryptophan in the body resulting in the production of kynurenine and several downstream metabolites (Stone and Darlington, 2002). Induction of the of the rate limiting enzyme in the kynurenine pathway, indolamine 2,3-dioxygenase (IDO) is driven by the inflammatory cytokines IFN-γ, IL-6, TNF-α and IL-1β (Carlin et al., 1989, Fujigaki et al., 2006, Zunszain et al., 2012). Consequently, IDO induction has been proposed as a mechanism by which inflammation can precipitate depression via tryptophan depletion (Raison et al., 2009, Christmas et al., 2011, Maes et al., 2011). Most particularly, activation of the kynurenine pathway and tryptophan depletion has been observed in depression that occurs secondary to exogenous administration of the cytokines IFN-γ and IL-2 (Capuron and Miller, 2004, Wichers et al., 2005, Raison et al., 2010).

However, the role of kynurenine pathway activation in MDD has been less well studied to date, with one study reporting an increase in the IDO activity index (kynurenine/tryptophan (KYN/TRP) ratio) and an decrease in concentrations of the neuroprotective kynurenine pathway metabolite kynurenic acid (KYNA) in depressed patients relative to control subjects (Myint et al., 2007). Based on these results Myint and colleagues suggested that kynurenine was metabolised down the

neurotoxic (3-hydroxykynurenine-quinolinic acid) arm of the kynurenine pathway, although to date neurotoxic kynurenine pathway metabolites or expression of the enzymes that drive their production have not been examined in depressed patients. In addition, to my knowledge no study to date has measured IDO expression in either cytokine-induced depression or MDD. In this regard, all published assessments of IDO activity in this field have relied on measuring tryptophan and kynurenine concentrations.

Following its synthesis by IDO, kynurenine can be further metabolised down one of two pathways. The enzyme kynurenine-3-monooxygenase (KMO) converts kynurenine into the free radical generator 3-hydroxykynurenine (3-HK), which can be metabolised by kynureninase to form 3-hydroxyanthranilic acid (3-HAA) and ultimately 3-HAA can be further metabolised to form the excitotoxin quinolinic acid (QUIN) (Stone and Darlington, 2002). Alternatively, kynurenine can be metabolised into kynurenic acid (KYNA) by kynurenine aminotransferase enzymes (KAT I & II). KYNA is largely regarded as a neuroprotective compound due to its NMDA receptor antagonist properties (Stone and Darlington, 2002). To date, expression of enzymes responsible for downstream metabolism of kynurenine have not been measured in depressed patients. In this regard, a suggestion of an imbalance in production of downstream kynurenine pathway metabolites in depression formed the basis of the "neurodegeneration hypothesis of depression" proposed by Myint and Kim in 2003 (Myint and Kim, 2003).

A second mechanism that has been used to link inflammation to reduced serotonergic function is via the ability of inflammatory cytokines to induce expression of the serotonin transporter (SERT), a molecule that removes serotonin from the synapse following its release (Benmansour et al., 1999). In this regard, studies have demonstrated that inflammatory cytokines including IL-1β, TNF-α and IFN-γ increase SERT expression and serotonin reuptake in vitro (Tsao et al., 2006, Zhu et al., 2006) and that a systemic inflammatory challenge with bacterial lipopolysaccharide (LPS) or the viral mimetic Poly I:C increases SERT expression in rodent brain (Katafuchi et al., 2005, Zhu et al., 2010a).

The aim of this study was to determine if the inflammatory state observed in MDD was associated with activation of the kynurenine pathway and/or upregulation of SERT as mechanisms to link inflammation to a serotonergic deficit in depression. Specifically, circulating concentrations of the inflammatory cytokines IL-1β, IFN-γ, TNF-α and IL-6 and the acute phase protein CRP were measured alongside plasma tryptophan, kynurenine, KYNA and 3-HAA concentrations, and whole blood IDO, KAT, KMO, Kynureninase and SERT mRNA expression in patients with MDD compared with healthy age- and sex-matched control subjects.

3.2 Methods

3.2.1 Participants

The study included 39 adult patients with MDD from the mental health services of the Adelaide and Meath Hospital, incorporating the National Children's Hospital, Dublin or St. James's Hospital, Dublin. The diagnosis of these patients with MDD was a clinical diagnosis based on Diagnostic and Statistical Manual for Mental Disorders IV (DSM-IV, American Psychiatric Association, 2000) criteria and confirmed by an independent psychiatrist using the 21-Hamilton Rating Scale for Depression (HAM-D 21) (Hamilton, 1960). Thirty-nine healthy control subjects were recruited from the local community and the groups were balanced for age and sex. In the depressed group, 14 patients received mono-therapy with an SSRI and 13 with a dual acting antidepressant (mirtazapine or venlafaxine). Twelve were medication-free when they were transferred to the service and were investigated before restarting antidepressant therapy. Antipsychotics or mood stabilisers were exclusion criteria. Other exclusion criteria were age <18 or >65, history of neurological or comorbid psychiatric disorders (Axis I or Axis II), other severe medical illness, head injury or substance abuse. Demographic variables, inclusion and exclusion criteria were documented using a standardised questionnaire and through a structured interview by a psychiatrist.

Written informed consent was obtained from all participants after being given a detailed description of the study which was designed and performed in accordance with the ethical standards laid out by the Declaration of Helsinki and was approved by the ethics committee of St. James and the Adelaide and Meath Hospitals, Dublin.

3.2.2 The Hamilton Rating Scale for Depression

Depression severity was assessed in patients and controls using the HAM-D 21 scale (Hamilton, 1960, Hamilton, 1969). This scale is the most frequently used, validated and standardised assessment for major depression and surveys the range of symptoms most commonly observed in depressed patients. While the HAM-D lists 21 items, the total score is the sum of the first consecutive 17 items and is indicative of global depressive burden. The remainder provide supplementary clinical information. Participants scoring between 0-7 fall within the normal range while scores of 8-13, 14-18, 19-24 and > than 25 are indicative of mild, moderate, severe and very severe depression, respectively (Ruhe et al., 2005).

3.2.3 Measurement of blood inflammatory markers, kynurenine pathway enzymes and SERT expression

A 10 ml blood sample was taken from each subject in the early afternoon into a heparinised vacutainer and was centrifuged to generate plasma. Plasma was stored at -80°C until inflammatory markers were measured. A second blood sample (2.5 ml) was taken into a PAXgene blood RNA tube (Qiagen, UK) and used for whole blood RNA isolation. PAXgene tubes were stored at -20°C for 48 hours and then moved to -80°C until RNA extraction was performed. All blood samples were taken to the freezer within 1 hour after acquisition. All samples were processed in the same batch with respect to the following methods.

3.2.3.1 Plasma IL-6, TNF-α, IL-1β, IFN-γ and CRP measurements

Plasma IL-6, TNF-α IL-1β, and IFN-γ concentrations were measured using ELISA MAX™ Deluxe kits (Biolegend, UK), and plasma CRP concentrations were measured using a CRP ELISA DuoSet (R&D systems, UK) and these immunoassays were performed according to manufacturer's instructions. Absorbance was read at 450 nm using a microplate reader (Sunrise Tecan, Reading, UK) and was then recalculated as a concentration (pg/ml for IL-6, TNF-α, IL-1β and IFN-γ and mg/L for CRP) using standard curves derived using GraphPad Prism Software Version 5.00 (GraphPad software, Inc). Limits of detection for the ELISAs were 4 pg/ml for IL-6 and IFN-γ, 2 pg/ml for TNF-α, 0.5 pg/ml for IL-1β and 5 pg/ml for CRP.

3.2.3.2 Real-time PCR analysis of mRNA expression of IDO1, IDO2, KMO, Kynureninase, KAT I, KAT II and SERT in whole blood samples

RNA isolation was performed using a PAXgene blood RNA kit (Qiagen, UK) and was followed by off column DNase treatment in order to remove contaminating genomic DNA as previously described (see Chapter 2 section – 2.2.4). The PAXgene system has been shown to reproducibly yield high-quality RNA from whole blood that can be used in downstream applications such as real-time PCR or gene chip analysis (Chai et al., 2005). Following RNA quantification and equalisation, cDNA was synthesised using a cDNA archive kit (Applied Biosystems, UK).

Gene expression analysis was conducted using Real-Time PCR employing Taqman® Gene Expression Assays (Applied Biosystems, UK). To quantify expression of target genes of interest Taqman® Gene Expression Assays containing FAM-labelled probes were used (Assay IDs: IDO1: Hs00984148_m1; IDO2:Hs01589373_m1; KMO:Hs00175738_m1; Kynureninase: Hs01114099_m1;

KAT I: Hs00187858_m1; KAT II: Hs00212039_m1; SERT: Hs00169010_m1; Applied Biosystems, UK). PCR reactions were in a duplex format also containing a Taqman® Gene Expression assay (primer-limited) containing a VIC-labelled probe for the endogenous control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Assay ID: 4326317E). Samples were assayed using Applied biosystems universal cycling conditions using a fast protocol on the StepOnePlus™ Real-time PCR system (Applied Biosystems, UK). Fold change in gene expression from the control group was calculated using the ΔΔCt method and GAPDH served as endogenous control in the amplification system. GAPDH was chosen as an endogenous control gene for these analyses as it demonstrated the least variable expression profile among blood samples relative to another endogenous control gene human acid ribosomal protein (HUPO). Data are expressed as fold change in gene expression relative to the control group.

3.2.3.3 Measurement of tryptophan, kynurenine and kynurenine metabolites using HPLC

Plasma tryptophan, kynurenine, KYNA and 3-hydroxyantranillic acid (3-HAA) concentrations were measured by HPLC. Kynurenine was measured at a wavelength of 250 nm by UV detection. Tryptophan, KYNA and 3-HAA concentrations were measured fluorometrically at an excitation wavelength of 254 nm and an emission wavelength of 404 nm.

Homogenisation buffer was prepared with mobile phase containing 6% perchloric acid and 200 ng/20 µl of internal standard (N-methyl-serotonin) (Sigma Chemicals Co., UK). The mobile phase contained 50 nM glacial acetic acid, 100 mM zinc acetate and 3% acetonitrile at pH 4.9. A 1:1 ratio of supernatant and homogenisation buffer was centrifuged at 12,800 rpm for 20 minutes at 4˚C before being passed through a 0.45 µm syringe filter (Phenomenex UK). A 20 µl sample of the resultant filtrate was injected onto a C-18 reverse phase Kinetex core-shell technology column (Phenomenex, UK) fitted with a guard column for separation of metabolites in the sample (flow rate 0.8 ml/min). Retention times and peak heights were recorded from the chromatographs generated and together with the data obtained for the standards (5 ng/20 ul standard mix of tryptophan and kynurenine, KYNA, 3-HAA and N-methyl 5-HT), were used to calculate tryptophan and kynurenine concentrations expressed as ng/ml of plasma.

3.2.4 Statistical analysis

Data are presented as mean with standard deviation (SD) in parentheses. Differences in demographic variables were tested using Student`s *t*-test, Chi-square (χ^2) test for gender distribution and Mann-Whitney U test for differences in non-parametric clinical variables. Normality was tested using the Shapiro-Wilk test of normality. Spearman's *rho* correlation coefficients were used to assess the relationship between plasma inflammatory markers (CRP, IL-6 and IFN-γ) and HAM-D scores, plasma inflammatory markers and plasma tryptophan concentrations, HAM-D scores and plasma tryptophan concentrations and the kynurenine/tryptophan ratio. All statistical analyses were considered to be significant if $p \leq 0.05$. Graphs and statistics were generated using GraphPad Prism Software Version 5.00 (GraphPad software, Inc). All data were analysed using SPSS (Version 16).

3.3 Results

3.3.1 Demographic data for patients with MDD and healthy controls

Consistent with a diagnosis of depression, patients with MDD had a significantly elevated HAM-D 21 score ($p<0.001$) compared to the control group (MDD: 27.7 (5.75) vs. Control: 2.7 (2.74)) (Table 3.1). Analysis revealed that there was no significant difference in age, gender or BMI between the depressed cohort and the control group ($p>0.05$) (Table 3.1).

Table 3.1 Demographic data for patients with MDD and healthy controls. Depicted are demographic variables and clinical depression severity measured with the HAM-D 21 scale

	Patients (n=39)	Controls (n=39)	Statistics (p-value)
Age [years]	41.9 (11.1)	37.1 (12.9)	t=-1.79,df=1,76, (p=0.077)
Gender (female/male)	23/16	22/17	χ^2=0.053, (p=0.819)
BMI	25.3 (3.8)	23.9 (3.5)	t=-1.63,df=1,76, (p=0.098)
Alcohol consumption (units per week)	4.0 (6.1)	4.8 (3.4)	t=-0.60,df=1,76, (p=0.549)
Smoking (Yes/No)	11/28	8/31	χ^2=0.063, (p=0.599)
Hamilton Depression Score (HAM-D 21)	27.7 (5.8)	2.7 (3.0)	t=-23.9,df=1,76, (p<0.001)
Age of onset	25.7 (8.7)		
Cumulative illness duration	8.9 (9.1)		
Medication (free/SSRI/dual acting)	12/14/13		

Data expressed as mean with SD in parentheses and statistical analysis was performed using Student's t-test (Age, BMI, Alcohol consumption) and Chi squared (χ^2) test (Gender and Smoking).

3.3.2 Circulating concentrations of inflammatory cytokines and CRP in depressed patients and healthy controls

3.3.2.1 Circulating inflammatory cytokine concentrations in depressed patients compared with healthy controls

A Mann-Whitney U test revealed a significant increase in the circulating concentrations of IL-6 ($Z=-2.400$, $p=0.016$) and IFN-γ ($Z=-2.001$, $p=0.045$) in the depressed patients relative to the control subjects [Figure 3.1b and d respectively]. There was no significant difference in the circulating concentrations of IL-1β ($Z=-0.991$, $p=0.322$) and TNF-α ($Z=-0.394$, $p=0.694$) between the depressed patients and healthy controls [Figure 3.1a and c respectively].

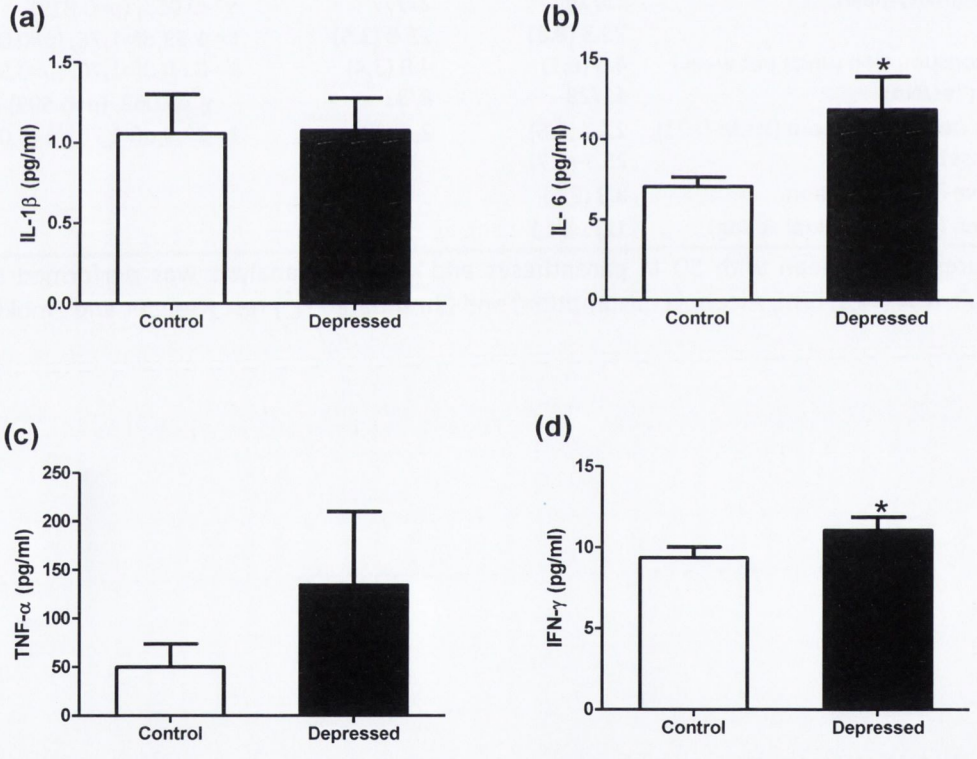

Figure 3.1 Circulating inflammatory cytokines concentrations in depressed patients compared with healthy controls. Plasma concentrations of (a) IL-1β, (b) IL-6, (c) TNF-α and (d) IFN-γ in depressed patients compared with healthy controls. Data expressed as mean with SEM (n=39 per group). *p≤0.05 vs. control (Mann-Whitney U test).

3.3.2.2 Circulating CRP concentrations in depressed patients compared with healthy controls

A Mann-Whitney U test revealed a significant increase in the circulating concentrations of CRP in the depressed cohort relative to control subjects (Z=-2.433, p=0.015) [Figure 3.2].

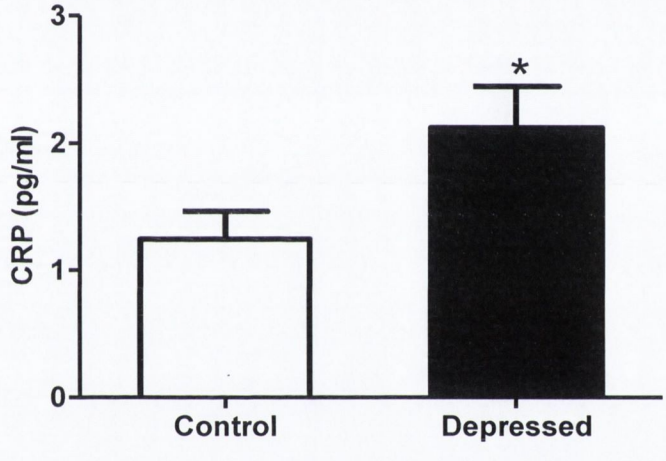

Figure 3.2 Circulating CRP concentrations in depressed patients compared with healthy controls. Plasma CRP concentrations in depressed patients compared with healthy controls. Data expressed as mean with SEM (n=39 per group). *p≤0.05 vs. control (Mann-Whitney U test).

3.3.3 Analysis of kynurenine pathway activation in depressed patients compared with healthy controls

3.3.3.1 IDO mRNA expression in depressed patients compared with healthy controls

Gene expression of IDO1 and IDO2 was measured in whole blood from depressed patients relative to control subjects. Basal expression of IDO1 mRNA was measured in all blood samples studied and the average threshold cycle (Ct) for IDO1 in the control group was 32.4. Basal IDO2 mRNA expression (a Ct of less than 40) was observed in 34 of the 39 control samples and 27 of the 39 samples from depressed patients and the average Ct for IDO2 was 34.9. The average Ct for the housekeeping gene GAPDH was 25, resulting in an average ΔCt of 7.4 for IDO1 and 9.9 for IDO2. Based on these data, basal IDO2 expression in blood is lower than IDO1.

A Mann-Whitney U test revealed no significant difference in the whole blood transcriptional expression of IDO1 (Z=-0.275, p=0.783) and IDO2 (Z=-0.465, p=0.642) between the depressed patients and healthy controls [Figure 3.3a and b respectively].

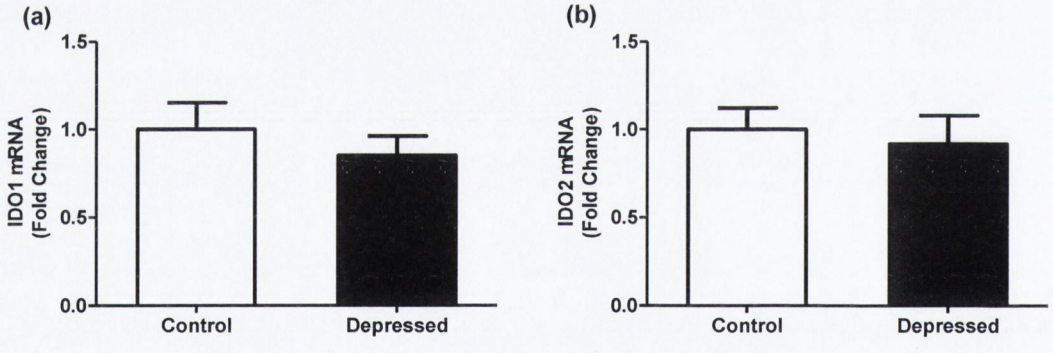

Figure 3.3 Whole blood IDO mRNA expression in depressed patients compared with healthy controls. Relative mRNA expression for (a) IDO1 and (b) IDO2 in depressed patients compared with healthy controls. Data expressed as mean with SEM (n=39 per group), Mann-Whitney U test.

3.3.3.2 Kynurenine pathway metabolite concentrations in depressed patients compared with healthy controls

A Mann-Whitney U test revealed a significant decrease in circulating tryptophan concentrations in the depressed cohort compared with healthy controls ($Z=-2.731$, $p=0.006$), in the absence of a concomitant increase in kynurenine in the patient cohort relative to controls ($Z=-0.215$, $p=0.830$) [Figure 3.4b and a respectively]. A Mann-Whitney U test also revealed a significant increase in the kynurenine/tryptophan ratio in the depressed patients relative to controls ($Z=-2.847$, $p=0.004$) [Figure 3.4c].

3.3.3.3 Kynurenine pathway enzyme expression in depressed patients compared with healthy controls

The transcriptional expression of KAT I, KAT II, KMO and Kynureninase was assessed in whole blood from depressed patients and healthy controls. Basal expression of KAT I mRNA was observed in all blood samples studied and the average Ct for KAT I in the control group was 33. In contrast KAT II was below the limits of detection in most samples. Basal expression of KMO and kynureninase mRNA was observed in all blood samples studied and the average Ct for KMO and kynureninase in the control group was 32.9 and 30.1 respectively. The average Ct for the housekeeping gene GAPDH was 25 resulting in an average ΔCt of 8.0 for KAT I, 7.9 for KMO and 5.1 for kynureninase. Based on these data, the basal expression of kynureninase in blood is lower than KAT I and KMO.

A Mann-Whitney U test revealed no significant difference in the whole blood transcriptional expression of KAT I ($Z=-0.695$, $p=0.487$), KMO ($Z=-0.295$, $p=0.768$) or kynureninase ($Z=-0.235$, $p=0.814$) between depressed patients and controls [Figure 3.5a, b and c respectively].

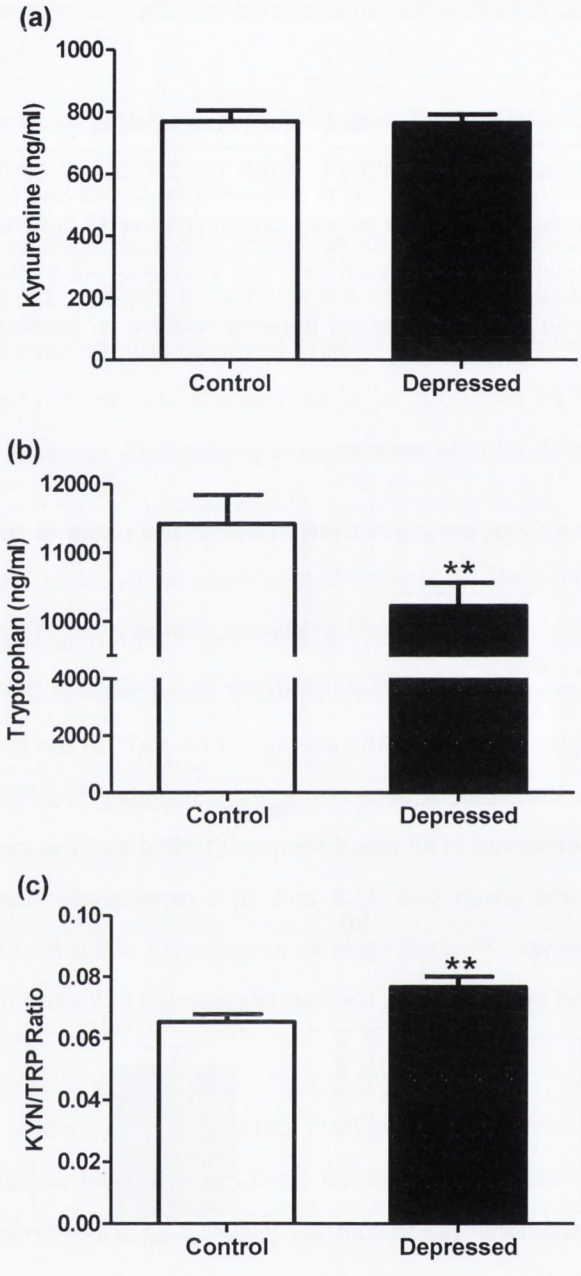

Figure 3.4 Kynurenine pathway activation profile in depressed patients compared with healthy controls. Plasma concentrations of (a) kynurenine, (b) tryptophan and (c) kynurenine/tryptophan (KYN/TRP) ratio in depressed patients compared with healthy controls. Data expressed as mean with SEM (n=39 per group). **$p \leq 0.01$ vs. control (Mann-Whitney U test).

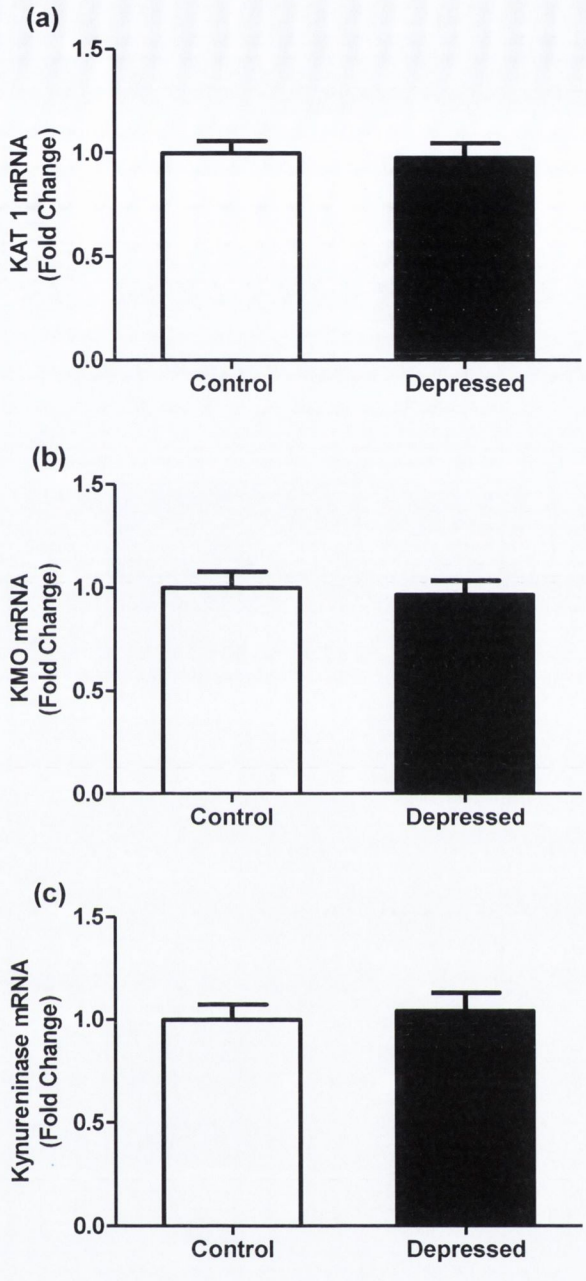

Figure 3.5 KAT, KMO and kynureninase mRNA expression in depressed patients compared with healthy controls. Relative mRNA expression for (a) KAT I, (b) KMO and (c) kynureninase expression in depressed patients compared with healthy controls. Data expressed as mean with SEM (n=39 per group). Mann-Whitney U test.

3.3.3.4 KYNA and 3-HAA concentrations in depressed patients compared with healthy controls

A Mann-Whitney U test revealed no significant difference in the concentration of the kynurenine metabolites KYNA (Z=-0.923, p=0.356) and 3-HAA (Z=-1.134, p=0.257) in depressed patients relative to healthy control subjects [Figure 3.6a and b respectively].

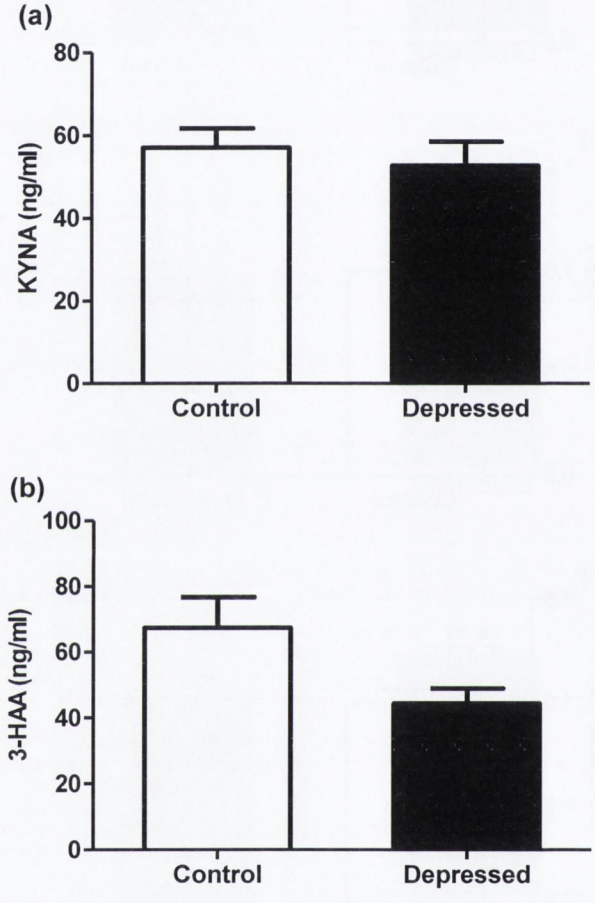

Figure 3.6 KYNA and 3-HAA concentrations in depressed patients compared with healthy controls. Plasma concentrations of (a) KYNA and (b) 3-HAA in depressed patients compared with healthy controls. Data expressed as mean with SEM (n=39 per group for 3-HAA; n=21-22 per group for KYNA), Mann-Whitney U test.

3.3.4 Evaluation of the relationship between HAM-D scores, the kynurenine pathway and circulating inflammatory markers

3.3.4.1 The association between HAM-D scores and kynurenine, tryptophan and the KYN/TRP ratio

Spearman's *rho* correlation analysis between HAM-D scores and plasma tryptophan concentrations revealed a significant negative correlation (r=-0.353, p=0.002) [Figure 3.7b]. This was accompanied by a significant positive correlation between HAM-D scores and the KYN/TRP ratio (r=0.254, p=0.027) [Figure 3.7c]. In contrast, no correlation exists between HAM-D scores and plasma kynurenine concentrations (Z=-0.049, p=0.673) [Figure 3.7a].

3.3.4.2 The association between HAM-D scores and plasma inflammatory markers CRP, IL-6 and IFN-γ

Correlational analysis revealed a significant positive correlation between HAM-D scores and plasma IL-6 concentrations (r=0.325, p=0.004) [Figure 3.8b]. In contrast, there was no significant association between HAM-D scores and plasma CRP concentrations (r=0.194, p=0.089) or HAM-D scores and plasma IFN-γ concentrations (r=0.206, p=0.071) [Figure 3.8a and c respectively].

3.3.4.3 The association between plasma inflammatory markers CRP, IL-6, IFN-γ and plasma tryptophan

Spearman's rho correlational analysis revealed a significant negative association between plasma tryptophan concentrations and CRP (r=-0.279, p=0.015) [Figure 3.9a]. In contrast, plasma tryptophan concentrations were not associated with either IL-6 (r=-0.126, p=0.280) or IFN-γ (r=-0.060, p=0.604) concentrations [Figure 3.9b and c respectively].

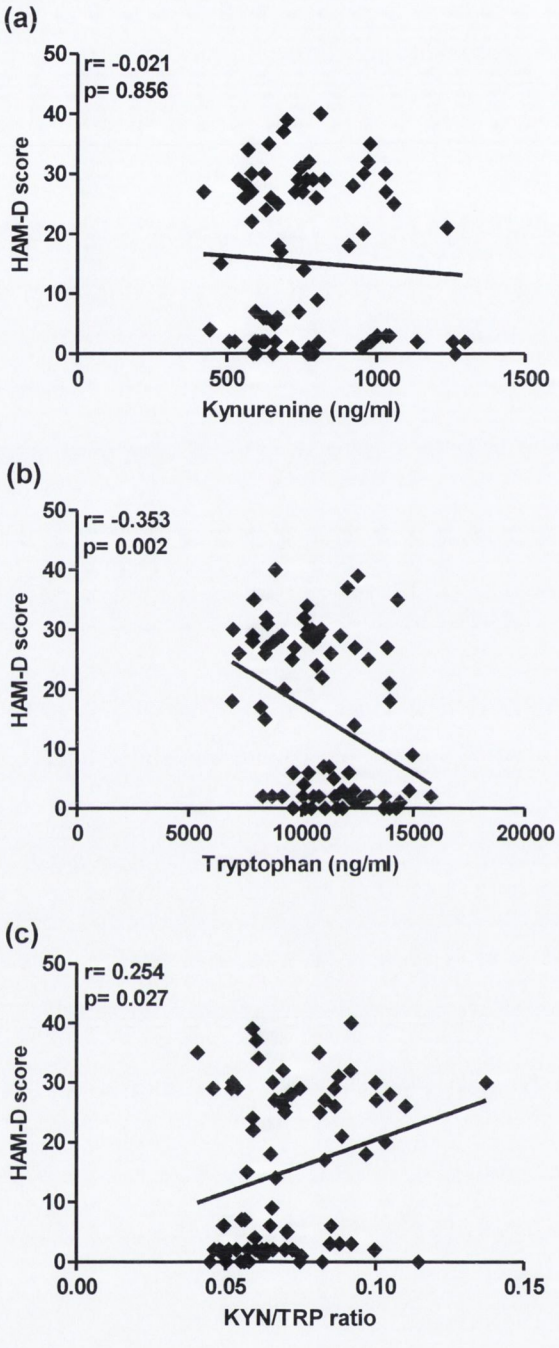

Figure 3.7 Correlational analysis between HAM-D scores and plasma kynurenine, tryptophan and the KYN/TRP ratio. (a) No correlation exists between HAM-D scores and plasma kynurenine, (b) A significant negative correlation exists between HAM-D scores and plasma tryptophan concentrations and (c) A significant positive correlation exists between HAM-D scores and the plasma KYN/TRP ratio.

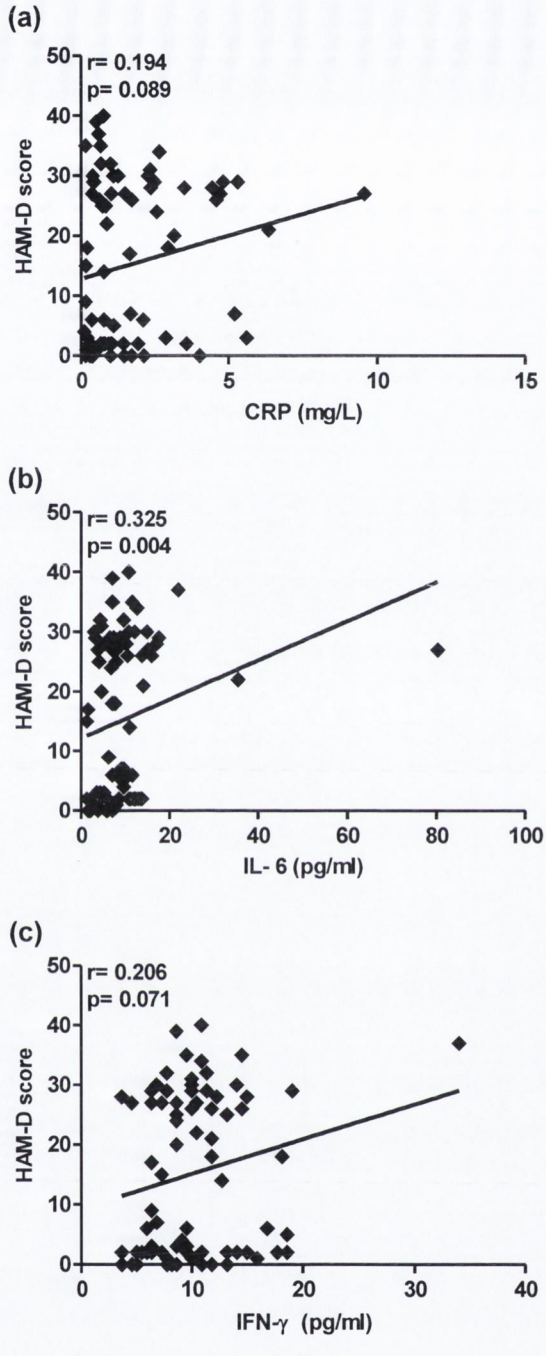

Figure 3.8 Correlational analysis between HAM-D scores and plasma CRP, IL-6 and IFN-γ concentrations. (a) No correlation exists between HAM-D scores and plasma CRP concentrations, (b) A significant positive correlation exists between HAM-D scores and plasma IL-6 concentrations and (c) No correlation exists between HAM-D scores and plasma IFN-γ concentrations.

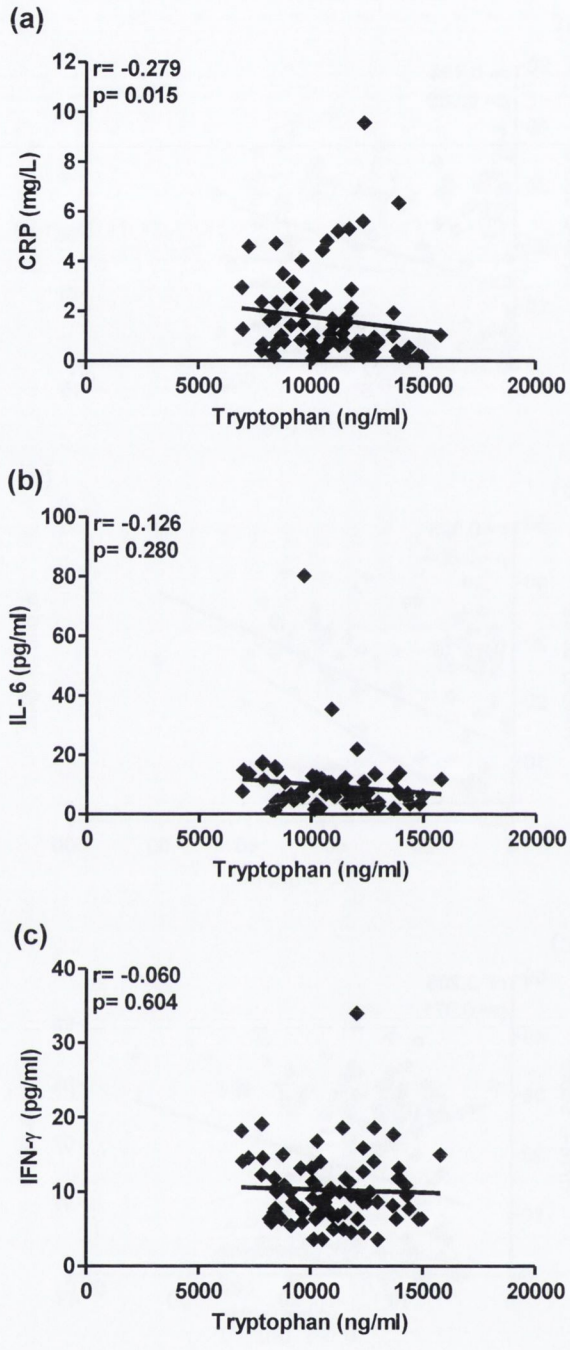

Figure 3.9 Correlational analysis between inflammatory markers (CRP, IL-6 and IFN-γ) and plasma tryptophan concentrations. (a) No correlation exists between HAM-D scores and plasma CRP concentrations, (b) A significant positive correlation exists between HAM-D scores and plasma IL-6 concentrations and (c) No correlation exists between HAM-D scores and plasma IFN-γ concentrations.

3.3.5 Analysis of SERT expression in depressed patients compared with healthy controls

Basal expression of SERT mRNA was observed in all whole blood samples studied and SERT mRNA in the control group had an average Ct of 34.4. The average Ct for the housekeeping gene GAPDH was 25 resulting in an average ΔCt of 9.4 for SERT. However, despite the presence of an inflammatory state in the depressed patients there was no significant difference between SERT expression in depressed patients and control subjects (Z=-0.495, p=0.621) [Figure 3.10].

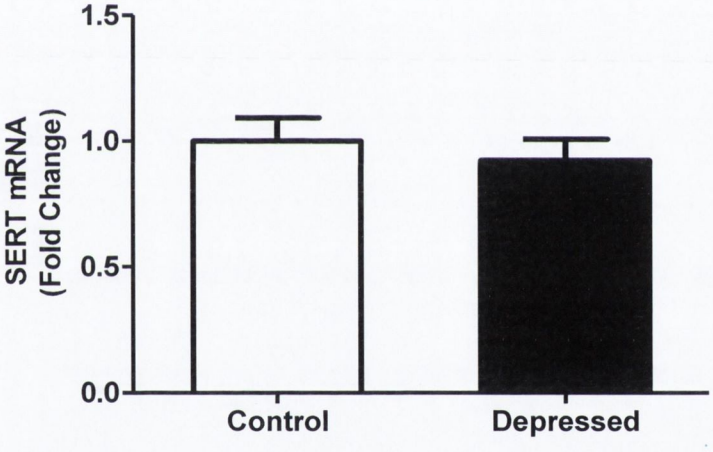

Figure 3.10 SERT mRNA expression in depressed patients compared with healthy controls. Relative mRNA expression for SERT in depressed patients compared with healthy controls. Data expressed as mean with SEM (n=39 per group), Mann-Whitney U test.

3.3.6 The impact of medication on biological variables in depressed patients

A Mann-Whitney U test revealed that there was no significant difference in any of the biological variables under investigation between antidepressant-treated patients with major depression (n=27) and un-medicated patients with major depression (n=12) ($p>0.05$).

Table 3.2 Analysis of the impact of medication on biological variables in depressed patients

Biological parameter	Antidepressant treated patients (n=27)	Un-medicated patients (n=12)	Statistics, p-value
CRP (mg/L)	2.05 (2.10)	2.28 (1.95)	Z=-0.730, p=0.480
IL-1β (pg/ml)	1.22 (1.36)	0.76 (1.01)	Z=-1.118, p=0.271
TNF-α (pg/ml)	111.0 (485.4)	186.6 (458.3)	Z=-0.000, p=1.000
IFN-γ (pg/ml)	11.69 (5.50)	9.64 (3.61)	Z=-1.357, p=0.178
IL-6 (pg/ml)	12.81 (14.28)	9.69 (8.91)	Z=-1.203, p=0.233
Kynurenine (ng/ml)	744.5 (168.8)	806.4 (193.1)	Z=-0.943, p=0.358
Tryptophan (ng/ml)	10133 (2053)	10442 (2207)	Z=-0.563, p=0.590
KYNA (ng/ml) *	56.57 (28.66)	40.70 (13.80)	Z=-1.156, p=0.275
3-HAA (ng/ml)	49.55 (31.43)	54.63 (46.57)	Z=-0.365, p=0.730
IDO1 mRNA	0.92 (0.80)	0.71 (0.27)	Z=-0.030, p=0.988
IDO2 mRNA	1.10 (0.96)	0.68 (0.49)	Z=-1.155, p=0.264
KAT I mRNA	0.96 (0.42)	1.00 (0.53)	Z=-0.030, p=0.988
KMO mRNA	0.99 (0.49)	0.91 (0.31)	Z=-0.183, p=0.869
Kynureninase mRNA	1.07 (0.61)	0.98 (0.42)	Z=-0.061, p=0.964
SERT mRNA	0.89 (0.49)	0.99 (0.63)	Z=-0.426, p=0.685

Data expressed as mean with SEM in parentheses and statistical analysis was performed using a Mann-Whitney U-test. *KYNA: Un-medicated patients with MDD n=5; Antidepressant-treated patients with MDD n=16).

3.4 Discussion

The present study sought to determine if an inflammatory signature in MDD patients was associated with activation of the kynurenine pathway and/or upregulation of SERT as mechanisms to link inflammation to the serotonergic deficit in depression. A mild circulating inflammatory phenotype, evidenced by increased concentrations of IL-6, IFN-γ and CRP, was observed in the MDD patients relative to healthy controls. Despite this, whole blood IDO mRNA expression or plasma kynurenine concentrations were not different between MDD patients and controls. In addition, there was no difference between depressed patients and controls in concentrations of the kynurenine metabolites, KYNA and 3-HAA, or in the expression of enzymes KAT, KMO or kynureninase that drive their production. Nonetheless, a depletion in tryptophan was evident in the depressed patients and was correlated with HAM-D scores. In addition, whole blood SERT mRNA expression was not increased in MDD patients relative to controls. These data support the idea that a mild inflammatory signature is evident in MDD and is accompanied by reduced circulating tryptophan concentrations. However, we found no indication of kynurenine pathway activation in the depressed cohort suggesting that an alternative mechanism mediates the depletion of tryptophan observed.

3.4.1 The association between inflammation and plasma tryptophan concentrations

Consistent with previous findings by Maes and colleagues, a significant reduction (12%) in circulating tryptophan concentrations was observed in depressed patients relative to control subjects and this reduction in plasma tryptophan concentration was correlated with an increase in the HAM-D score (Maes et al., 1990c, Maes et al., 1993b). The reduction in tryptophan concentrations observed in the depressed cohort was accompanied by an increase in plasma concentrations of the pro-inflammatory cytokines IL-6 (68%) and IFN-γ (20%) and the acute phase protein CRP (75%). Moreover, a reduced plasma tryptophan concentration was significantly correlated with an increase in CRP concentrations, but did not correlate with the increase in circulating concentrations of either IL-6 or IFN-γ. These data indicate that inflammation may be a contributor to the reduction in circulating tryptophan concentrations in depressed patients.

3.4.2 Kynurenine pathway activation profile in patients with MDD

Activation of the kynurenine pathway has been suggested as a mechanism by which inflammation in depression could result in a decrease in tryptophan availability for serotonin synthesis (Maes et al., 2011). Consequently, the transcriptional expression of IDO (IDO1 and IDO2) was analysed in association with the plasma KYN/TRP ratio in order to assess kynurenine pathway activation. IDO1 and IDO2 are expressed in distinct cell types suggesting they are not functionally redundant (Ball et al., 2009). Despite evidence of an inflammatory phenotype in the depressed cohort, and most particularly elevated circulating concentrations of the IDO inducing cytokines IFN-γ and IL-6, analysis revealed no significant difference in IDO mRNA expression (either IDO1 or IDO2) between the depressed cohort and control subjects. Of course, IDO expression is not limited to blood cells and therefore the possibility that IDO expression could be increased in other body tissues cannot be excluded.

However, HPLC analysis of plasma kynurenine concentrations revealed that there was no significant difference in kynurenine concentrations between depressed patients and control subjects and consequently kynurenine concentrations did not correlate with HAM-D scores. Whilst an increase in the KYN/TRP ratio, which is often used as an index of kynurenine pathway activation (Wichers et al., 2005, Suzuki et al., 2010), was observed in the depressed patient cohort, this increase was purely due to a decrease in tryptophan as opposed to any change in kynurenine concentrations, suggesting that the kynurenine pathway was not activated in these patients. As was the case for plasma tryptophan concentrations, an increase in the KYN/TRP ratio was correlated with an increase in HAM-D scores.

As kynurenine can undergo rapid metabolism by the enzymes KAT, KMO and kynureninase (Stone and Darlington, 2002), the concentrations of kynurenine metabolites KYNA and 3-HAA were measured as indicators of downstream activation of the neuroprotective and neurotoxic arms of the kynurenine pathway, respectively. However, there was no difference in the concentrations of KYNA or 3-HAA between the depressed patients and control subjects. Further to this, there was no difference in the transcriptional expression of KAT I, the enzyme that drives the neuroprotective arm of the kynurenine pathway, catalysing the conversion of kynurenine to KYNA, between depressed patients and control subjects. Moreover, the enzymes KMO and kynureninase which drive the neurodegenerative arm of the kynurenine pathway, catalysing the conversion of kynurenine into a range of downstream metabolites including 3-HK, 3-HAA and QUIN (Stone and Darlington, 2002), have similar expression levels between the depressed patients and control subjects. Taken together with IDO expression and plasma kynurenine concentration data, these results suggest that the kynurenine pathway was not activated in the

depressed cohort relative to controls. Therefore, it may be suggested that an alternative pathway/mechanism mediates the depletion of tryptophan observed in the depressed patients.

As BMI was matched between depressed patients and healthy controls, it is unlikely that a decrease in dietary tryptophan could account for the reduction in tryptophan observed in depressed cohort, however, as BMI does not provide any information on dietary intake this possibility cannot be completely ruled out. Whilst psychological stress has the propensity to reduce tryptophan availability via activation of the hepatic tryptophan degrading enzyme, tryptophan 2,3-dioxygenase (TDO) (Maes et al., 2011), one would expect to observe an increase in plasma kynurenine (or downstream kynurenine pathway metabolites) in parallel with a reduction in tryptophan if TDO were activated. As an increase in kynurenine or kynurenine pathway metabolites was not observed in this study, it is unlikely that the depletion in tryptophan observed resulted from a stress-related increase in TDO activity in these patients. Furthermore, antidepressant medication did not influence tryptophan concentrations (or indeed any inflammatory mediators) and therefore, is unlikely to account for the difference observed between depressed patients and control subjects. Consequently further study is required to determine the precise cause of the reduction in circulating concentrations observed in depressed patients.

These data indicate that the mild inflammatory phenotype observed in depressed patients is not sufficient to induce IDO, KMO or kynureninase expression and this is in contrast to the robust expression of these enzymes induced following exposure to inflammatory stimuli in the *in vitro* setting (Connor et al., 2008, Zunszain et al., 2012). This is an important finding that clearly differentiates idiopathic depression from depression induced by administration of exogenous cytokines such as IFN-α or IL-2 where kynurenine pathway activation is observed (Capuron et al., 2002b, Raison et al., 2010, Wichers et al., 2005), despite the fact that significant symptom overlap is observed between idiopathic depression and cytokine induced depression (Capuron et al., 2009).

Whilst the results presented are negative in terms of kynurenine pathway activation in depressed patients, it is important to note that the methods employed in the present study would have been able to pick up activation of the kynurenine pathway should it have been present. In this regard, the data from other studies, presented in Chapter 5, demonstrate a robust induction of IDO mRNA coupled with reduced tryptophan concentrations and increased kynurenine concentrations in blood cells stimulated with inflammatory stimuli (most particularly the T-cell activator anti-CD3).

3.4.3 SERT expression in MDD patients

Based on the results of the present study, the proven ability of *in vitro* exposure to inflammatory cytokines, or exposure of rodents to an in vivo inflammatory challenge, to upregulate SERT expression does not translate to the clinical situation of idiopathic depression where a much more subtle inflammatory state is observed. Specifically, these results indicate that the mild inflammatory state observed in depressed patients is not sufficient to upregulate expression of SERT.

3.4.4 The impact of a low-grade inflammatory phenotype in MDD

Overall, the data generated in this study question the biological relevance of the small magnitude of increase of IFN-γ (Control: 9.3 pg/ml vs. Depressed: 11.1 pg/ml) and IL-6 (Control: 7.1 pg/ml vs. Depressed: 11.9 pg/ml) observed in depressed patients. Similarly, it should be noted that while the increase in mean CRP from 1.2 mg/L in the control subjects to 2.1 mg/L in the depressed patients is statistically significant, both of these values are in the normal clinical range (below 6 mg/L) and do not indicate the presence of overt inflammation *per se.* Nonetheless, recent studies indicate that even mildly elevated IL-6 and CRP concentrations independently predict the subsequent development of depression over a decade or more, even in individuals with no history of depression at the time of sampling (Gimeno et al., 2009, Pasco et al., 2010). Whilst further study is required to provide a mechanistic basis for these findings, it is noteworthy that the lab has recently observed that elevated circulating IL-6 concentrations are associated with reduced hippocampal volumes in major depressive disorder (Frodl et al., 2012). These data may suggest that elevated IL-6 has the potential to impact on hippocampal neuroplasticity. In this regard, a potential role for IL-6 in the pathogenesis of depression is supported by the significant positive correlation between plasma IL-6 concentrations and HAM-D scores observed in the present study.

3.4.5 Conclusion

These data support the idea that a mild inflammatory signature is evident in depressed patients and that this is accompanied by a reduction in circulating tryptophan concentrations. However, we found no indication of kynurenine pathway activation in the depressed cohort suggesting that an alternative mechanism/pathway mediates the depletion of tryptophan observed in depressed patients. Taken together, these data question the ability of the mild inflammatory phenotype

observed in depression to induce molecules such as IDO and SERT that could negatively impact upon serotonergic functioning.

Chapter 4

Inflammatory and glucocorticoid response markers and their association with symptom clusters and history of childhood trauma in Major Depressive Disorder

4.1 Introduction

While the precise biological basis of depression remains elusive, a large body of evidence implicates a dysregulated endocrine and inflammatory response system in the pathogenesis of depression. Studies have investigated the co-occurrence of inflammation and depression with findings in support of an increased innate inflammatory profile in certain sub-groups of depressed patients evidenced by increased circulating pro-inflammatory cytokines, chemokines and acute-phase proteins (Maes et al., 1995b, Lanquillon et al., 2000, Cizza et al., 2008, Simon et al., 2008, Diniz et al., 2010, Hughes et al., 2012). Further to this, key innate immune signalling and activation markers have been implicated in the pathogenesis of major depression. Recent reports implicate the innate activating receptor triggering receptor expressed on myeloid cells 1 (TREM-1) in many chronic inflammatory disorders such as inflammatory bowel disease, rheumatic disease and cancer and more recently the psychiatric disorders, schizophrenia and bipolar disorder (Derive et al., 2010, Weigelt et al., 2011). While these findings are in support of the macrophage theory of depression originally proposed by Smith (1991), little attention has been paid to the role of T-cells and the adaptive immune response.

Until recently, the depression literature has focused on investigating the innate immune response, with a particular interest in the pro-inflammatory cytokines IL-6 and TNF-α and the acute phase protein, C reactive protein (CRP) (Howren et al., 2009, Dowlati et al., 2010, Liu et al., 2012). However, of late, there has been a re-emergence of reports in corroboration with early theories by Smith and Maes suggesting the involvement of the adaptive immune response and cell-mediated immune activation in the pathophysiology of major depression (Maes et al., 1995c, Miller, 2010). Some reports suggest an imbalance in T-cell subset cytokine production, with elevated levels of prototypical Th-1 cytokines such as IFN-γ, while anti-inflammatory IL-4 and TGF-β, produced by Th-2 and Th-3 cells, respectively, have been found to be significantly lower in depressed cohorts (Myint et al., 2005, Kim et al., 2007, Sutcigil et al., 2007). Literature directly assessing other T-cell subsets including T-regulatory (T-reg) cells and Th-17 cells in patients with major depressive disorder (MDD) is extremely limited.

Glucocorticoids have a crucial role in maintaining bodily homeostasis, a key component in the body's response to stress. In addition to regulating the inflammatory system in response to stress, glucocorticoids also have a key role in brain functioning, regulating neurogenesis and survival along with memory formation and the emotional appraisal of events and the volume of complex anatomical structures such as the hippocampus (as reviewed by Herbert et al., 2006). Consequently, dysregulation of this system has a crucial role to play in stress-induced disorders such as major depression. Impaired function of the glucocorticoid receptor (GR) is a hallmark for

glucocorticoid resistance (decreased responsiveness to glucocorticoids). This is one of the most widely reported and consistent findings in patients suffering from major depression with over 80% of participants studied showing GR resistance (Pace et al., 2007).

Given the heterogeneity of MDD, not all depressed patients exhibit dysregulation of the inflammatory and endocrine systems; 'Where there is depression, there is inflammation... sometimes!' (Glassman and Miller, 2007). However, there is evidence to suggest that inflammation is associated with depression in certain sub-groups of patients and that those who have experienced stressful life events, such as childhood trauma, may be at greater risk of developing depression (Batten et al., 2004, Danese et al., 2008). Stress is thought to be the trigger for the onset of a depressive episode and early life stress has been suggested to impact upon the development of neurobiological systems implicated in the stress and mood responses, thereby increasing ones vulnerability to stress and hence the risk of developing depression later in life, especially in response to secondary stress (Hammen et al., 2000, Mazure et al., 2000, Chapman et al., 2004, Heim and Binder, 2012). In support of this, pre-clinical research assessing the effects of maternal separation in adulthood have reported an altered stress response system in association with a pro-inflammatory phenotype similar to that evident in depressed patients (O'Mahony et al., 2009). Additionally, a clinical study by Heim et al. (2008a) demonstrated hyperactive hypothalamic-pituitary-adrenal (HPA) axis activity in adult men with a history of childhood trauma.

Alterations in the concentration and expression of the brain derived neurotrophic factor (BDNF) have also been reported in depressed patients (Lee et al., 2007). BDNF is a neurotrophin with a key role in neuronal proliferation, regeneration and survival (Lewin and Barde, 1996). It also has a critical role in synaptic plasticity and cognitive function, which have been shown to be disrupted in MDD. The hippocampus is a key limbic structure, which functions to regulate mood, sleep, learning and memory and the HPA axis. Preclinical evidence has shown that glucocorticoids reduce BDNF concentrations in the hippocampus, resulting in damage and atrophy of neurons (as reviewed by McEwen (1999) and Duman (2004)). In accordance with this, Mondelli et al. (2011) reported that early life adversity and recent stressors predict decreased BDNF expression in first episode psychosis, which in turn predicted smaller hippocampal volumes suggesting that stress related biological alterations may impact upon brain structure and function via an effect on BDNF. Further to this, Frodl et al. (2012) reported a decreased hippocampal volume in association with an altered glucocorticoid and inflammatory phenotype in depressed patients.

However, much of the evidence to date linking dysregulated biological systems with depression emerges from the study of circulating plasma/serum levels of inflammatory cytokines and

immune markers, while a small proportion of researchers report more specific CNS related variations in cytokine and metabolite concentrations through the use of cerebrospinal fluid (CSF) and post-mortem brain tissue, both of which have many limitations. As a result, many inconsistencies in the literature exist and we have yet to find specific biomarkers that may predict those at greater risk of depression or provide insight into treatment response, leading to the development of more efficient treatment strategies. However, the whole blood PAXgene system represents a novel approach in the search for specific biomarkers for MDD.

The primary measure of depression severity is the 21-item Hamilton Rating Scale for Depression (HAM-D 21) total score which is a routinely used, validated and standardised assessment tool for major depression (Hamilton, 1960). However, the total score merely provides insight into the global depressive state (Shafer, 2006). Others have demonstrated the use of the HAM-D subscale approach in genetics research on MDD and in the assessment of antidepressant medication (Seretti et al., 1999, Yu et al., 2002). The majority of research to date has focused on comparisons between depressed patients and healthy controls and correlations with total depression severity, however, the unidimensional scales enable the connection of biological parameters to individual symptom clusters of MDD, thereby assessing the contribution of each parameter to the individual components of the disorder (Lee et al., 2011). As depression is a heterogeneous disorder, I sought to assess specific symptoms associated with depression by examining refined clusters of the HAM-D scale to include core depression, anxiety, insomnia and somatic symptoms (Shafer, 2006). Previous studies have demonstrated that external measures have different association profiles with individual symptom clusters within a test (Williams and Richardson, 1993, Barefoot et al., 2000). Further to this, Faries et al. (2000) reported that core subscales were more efficient at detecting change than the HAM-D total score.

In addition, it is thought that specific types of childhood trauma may be related to the subsequent manifestation of particular disorders (Myers et al., 2002), and as a result, may be defined by different biological profiles. Emotional neglect, resulting in acute psychological stress, may increase vulnerability and sensitivity to later stressful life events, while various types of abuse are thought to contribute to emotional disturbance, distorted thinking patterns and the manifestation of post-traumatic stress disorder (Myers et al., 2002, Bowlby, 2008). Furthermore, given that reports on physical health outcomes are largely drawn from studies of abuse and not neglect, as it is thought that abuse represents more severe childhood traumatic events (Wegman and Stetler, 2009, Kiecolt-Glaser et al., 2011), the assessment of biological profiles in association with specific types of childhood trauma is of interest.

In light of this, the aims of the study were to profile the whole blood transcriptional expression of a range of markers from the inflammatory and glucocorticoid systems in depressed patients relative to control subjects. Further to this, I sought to determine whether patients characterised by individual symptom clusters could be distinguished on the basis of a specific inflammatory profile. Additionally, I sought to evaluate the impact of childhood trauma on the whole blood transcriptional expression of specific glucocorticoid related markers and inflammatory cytokines in depressed patients and healthy controls, with a particular interest in assessing the effect of specific types of childhood trauma on these parameters in depressed patients with a history of childhood trauma relative to patients who do not.

4.2 Methods

4.2.1 Participants

The study included 38 adult patients with MDD from the mental health services of the Adelaide and Meath Hospital, incorporating the National Children's Hospital, Dublin or St. James's Hospital, Dublin. The diagnosis of these patients with MDD was a clinical diagnosis based on Diagnostic and Statistical Manual for Mental Disorders IV (DSM-IV, American Psychiatric Association, 2000) criteria and confirmed by an independent psychiatrist using the HAM-D 21 (Hamilton, 1960, Hamilton, 1969). Thirty-eight healthy control subjects were recruited from the local community and the groups were balanced for age and sex. In the depressed group, 13 patients received mono-therapy with an SSRI and 13 with a dual acting antidepressant (mirtazapine or venlafaxine). Twelve patients were medication-free when they were transferred to the service and were investigated before restarting antidepressant therapy. Antipsychotics or mood stabilisers were exclusion criteria. Other exclusion criteria were age <18 or >65, history of neurological or comorbid psychiatric disorders (Axis I or Axis II), other severe medical illness, head injury or substance abuse. Demographic variables, inclusion and exclusion criteria were documented using a standardised questionnaire and through a structured interview by a psychiatrist. Written informed consent was obtained from all participants after being given a detailed description of the study which was designed and performed in accordance with the ethical standards laid out by the Declaration of Helsinki and was approved by the ethics committee of St. James and the Adelaide and Meath Hospitals, Dublin.

4.2.2 Clinical diagnostic tests

The Hamilton Rating Scale for Depression

Depression severity was assessed in patients and controls using the HAM-D 21 scale (Hamilton, 1960, Hamilton, 1969). This scale is the most frequently used, validated and standardised assessment for major depression and surveys the range of symptoms most commonly observed in depressed patients. While the HAM-D lists 21 items, the total score is the sum of the first consecutive 17 items and is indicative of global depressive burden. The remainder provide supplementary clinical information. Participants scoring between 0-7 fall within the normal range while scores of 8-13, 14-18, 19-24 and > than 25 are indicative of mild, moderate, severe and very severe depression, respectively (Ruhe et al., 2005). Depressive symptomology was assessed using the following sub-clusters of the HAM-D scale items.

(1) The Bech core depression subscale is a unidimensional subscale covering the affective core symptoms of depression severity and is defined by the following items; depressed mood, feelings of guilt, work activities, psychomotor retardation, anxiety (psychological) and somatic symptoms (general).

(2) Insomnia or sleep difficulty is a 3 item cluster defined by insomnia early, middle and late.

(3) Anxiety is a five item cluster defined by the following items; agitation, anxiety (psychological), anxiety (somatic), hypochondriasis and insight.

(4) The Somatic or vegetative symptom cluster is a 4 item cluster defined by, somatic (gastrointestinal), somatic (general), genital symptoms and loss of weight (Shafer, 2006). A total score was calculated for each of the subscales with higher scores indicative of greater symptom severity.

(5) The anxiety/ somatisation factor is a 6 item cluster used to define anxious depression (Farabaugh et al., 2010). A total score for the following items was calculated; anxiety (psychological), anxiety (somatic), somatic (gastrointestinal), somatic (general), hypochondriasis and insight. A total score of ≥ 7 was defined as anxious depression (Fava et al., 2008).

Childhood trauma questionnaire

Early life adversity was assessed using the Childhood Trauma Questionnaire (CTQ). This is a standardised, 28-item self-report instrument that assesses five types of childhood maltreatment: emotional, physical and sexual abuse and emotional and physical neglect. The total score is the sum of the first 25 consecutive items. The questionnaire also includes a 3 item minimalisation/denial scale for identifying participants who might be under reporting traumatic events. Each subscale has 5 items which are measured on a 5 point Likert scale, from 1 (never true) to 5 (very often true). The individual subscales are summed to give scores ranging from 5-25 and each have distinct cut-off points to define no adversity, low, moderate and severe adversity [Table 4.1] (Bernstein et al., 2003, Heim et al., 2009). Participants scoring in the moderate to severe range in at least one of the items were classified as having a positive history of childhood adversity. The cut-off scores used in this study were as follows: emotional abuse ≥ 13; physical abuse ≥ 10; sexual abuse ≥ 8; emotional neglect ≥ 15; and physical neglect ≥ 10. Reliability and validity of the CTQ has been established including measures of convergent and discriminative validity from structured interviews, stability over time and corroboration (Bernstein et al., 2003).

Table 4.1 Classification range for early life adversity scores

	No adversity	Low	Moderate	Severe
Emotional abuse	5-8	9-12	13-15	16-25
Physical abuse	5-7	8-9	10-12	13-25
Sexual abuse	5	6-7	8-12	13-25
Emotional neglect	5-9	10-14	15-17	18-25
Physical neglect	5-7	8-9	10-12	13-25

Cut-offs provided by Heim et al. (2009)

4.2.3 Measurement of whole blood inflammatory marker mRNA expression and circulating BDNF concentrations

4.2.3.1 Blood sampling

A 10 ml blood sample was taken from each subject in the early afternoon into a heparinised vacutainer and was centrifuged to generate plasma. Plasma was stored at -80°C until BDNF concentrations were assessed. A second blood sample (2.5 ml) was taken into a PAXgene blood RNA tube (Qiagen, UK) and used for whole blood RNA isolation. PAXgene tubes were stored at -20°C for 48 hours and then moved to -80°C until RNA extraction was performed. All blood samples were taken to the freezer within 1 hour after acquisition and were stored until the full sample set had been collected. All samples were processed together in the same batch with respect to the following methods.

4.2.3.2 Real-Time PCR analysis of mRNA expression of inflammatory markers in whole blood samples

RNA isolation was performed using a PAXgene blood RNA kit (Qiagen, UK) and was followed by off column DNase treatment in order to remove contaminating genomic DNA as previously described (see Chapter 2 section – 2.2.4). Following RNA quantification and equalisation, cDNA was synthesised using a cDNA archive kit (Applied Biosystems, UK).

Gene expression analysis was conducted using Real-Time PCR employing Taqman® Gene Expression Assays (Applied Biosystems, UK). To quantify expression of target genes of interest Taqman®Gene Expression Assays containing FAM-labelled probes were used [Table 4.2]. PCR reactions were in a duplex format also containing a Taqman® Gene Expression assay (primer-limited) containing a VIC-labelled probe for the endogenous control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH Assay ID: 4326317E). Samples were assayed using Applied

Biosystems universal cycling conditions using a fast protocol on the StepOnePlus™ Real-time PCR system (Applied Biosystems, UK). Fold change in gene expression from the control group was calculated using the ΔΔCt method and GAPDH served as endogenous control in the amplification system. GAPDH was chosen as an endogenous control gene for these analyses as it demonstrated the least variable expression profile among blood samples relative to another endogenous control gene human acid ribosomal protein (HUPO). Data are expressed as fold change in gene expression relative to the control group or the depressed cohort where appropriate.

4.2.3.3 Plasma BDNF measurements

Plasma BDNF concentrations were measured using a BDNF DuoSet (R&D systems, UK) according to manufacturer's instructions. Absorbance was read at 450 nm using a microplate reader (Sunrise Tecan, Reading, UK) and was then recalculated as a concentration (pg/ml) using a standard curve derived using GraphPad Prism Software Version 5.00 (GraphPad software, Inc). The limit of detection for the BDNF ELISA was 20 pg/ml.

4.2.4 Statistical analysis

Data are presented as mean with standard deviation (SD) in parentheses. All statistical analysis were considered significant when $p \leq 0.05$. Differences in demographic variables were tested using Student's *t*-test, Chi-square (χ^2) test and Mann-Whitney U test for differences in non-parametric clinical variables. Normality was tested using the Shapiro-Wilk test of normality which is most suitable for smaller datasets. Kruskal-Wallis non-parametric one-way analysis of variance (ANOVA) followed by Mann-Whitney pair-wise comparisons were used to test differences in clinical variables where appropriate. Spearman's *rho* correlation coefficients were used to assess the relationship between whole blood expression of inflammatory markers and HAM-D score and symptom subscales, childhood adversity and circulating BDNF concentrations. Overall, we assessed 5 different groups of biological targets (1. Inflammatory and immune activating; 2. T-cell; 3. Monocytic; 4. Glucocorticoid and 5. BDNF) for a range of different comparisons. As correcting across many hypotheses is arguable, the adjusted p-value was set at 0.05/5=0.01. However, given the novelty of this study and the exploratory nature of the correlational analyses, unadjusted p-values are also included throughout (Rothman, 1990, Perneger, 1998, Thompson, 1998). Graphs and statistics were generated using GraphPad Prism Software Version 5.00 (GraphPad software, Inc). All data were analysed using SPSS (version 16).

Table 4.2 List of genes used with the GenBank sequence ref numbers

Target Symbol	Target Name	Taqman Gene Assay ID
Cytokines		
IL-1β	Interleukin-1beta	Hs01555410_m1
TNF-α	Tumor Necrosis Factor alpha	Hs00174128_m1
IL-6	Interleukin-6	Hs00985639_m1
IFN-γ	Interferon gamma	Hs00989291_m1
IFN-inducible		
IP-10	Interferon gamma induced protein-10	Hs99999049_m1
SOCS-1	Suppressor of cytokines signalling-1	Hs00705164_s1
Immune activating		
TREM-1	Triggering receptor expressed on myeloid cells-1	Hs00218624_m1
DAP-12	DNAX activation protein of 12 kDa	Hs00182426_m1
TLR3	Toll-like receptor 3	Hs00152933_m1
TLR4	Toll-like receptor 4	Hs01061963_m1
PTX3	Pentraxin 3	Hs00173615_m1
PDE4B	Phosphodiesterase 4B	Hs00277080_m1
TRAF6	TNF receptor-associated factor 6	Hs00371512_g1
Monocytic markers		
EGR1	Early growth response protein 1	Hs00152928_m1
IL-12p40	Subunit beta of interleukin 12	Hs01011518_m1
iNOS	Inducible nitric oxide synthase	Hs01075529_m1
IFN-γR1	Interferon gamma receptor-1	Hs00988304_m1
COX-2	Cyclooxygenase-2	Hs00153133_m1
IκBα	I-kappa-B-alpha	Hs00153283_m1
PU.1	SFFV proviral integration 1 protein	Hs02786711_m1
ARG1	Arginase-1	Hs00968979_m1
IL-4R	Interleukin-4 receptor	Hs00166237_m1
MRC1	Mannose receptor-1	Hs00267207_m1
CD200R	Cluster of differentiation 200 receptor	Hs00793597_m1
T-cell markers		
IL-2	Interleukin-2	Hs00174114_m1
T-bet	T box expressed in T cell	Hs00203436_m1
IL-4	Interleukin-4	Hs00174122_m1
IL-13	Interleukin-13	Hs99999038_m1
GATA3	GATA-binding protein 3	Hs00231122_m1
TGF-β	Transforming growth factor beta	Hs00998133_m1
IL-10	Interleukin-10	Hs00961622_m1
FoxP3	Forkhead box P3	Hs01085834_m1
IL-17A	Interleukin-17A	Hs00174383_m1
IL-21	Interleukin-21	Hs00222327_m1
IL-22	Interleukin-22	Hs01574154_m1
RORC-γ	RAR-related orphan receptor gamma	Hs00172860_m1
Glucocorticoid markers		
GR	Glucocorticoid receptor	Hs00353740_m1
FKBP5	FK506 binding protein 5	Hs01561006_m1
SGK1	Serum/glucocorticoid regulated kinase 1.	Hs00178612_m1
GILZ	Glucocorticoid-induced leucine zipper	Hs00608272_m1

4.3 Results

4.3.1 Demographic data for patients with MDD and healthy controls

Thirty-eight severely depressed patients and 38 healthy controls were recruited. For participant demographic data see Table 4.3. Analysis revealed that there was no significant difference in age, gender or BMI, alcohol consumption or smoking between the depressed cohort and the control group ($p>0.05$) [Table 4.3].

Table 4.3 Demographic data for patients with MDD and healthy controls. Depicted are demographic variables and clinical depression severity measured with the HAM-D 21 scale

	Patients (n=38)	Controls (n=38)	Statistics (p-value)
Age [years]	41.6 (10.9)	37.6 (12.6)	t=-1.47,df=1,74, (p=0.147)
Gender (female/male)	22/16	21/17	χ^2=0.05, (p=1.000)
BMI	25.3 (3.8)	23.9 (3.6)	t=-1.63,df=1,74, (p=0.108)
Alcohol consumption (units per week)	4.1 (6.1)	4.8 (6.4)	t=-0.48,df=1,74, (p=0.636)
Smoking (Yes/No)	11/27	7/31	χ^2=1.17, (p=0.419)
Age of onset	25.7 (12.3)		
Cumulative illness duration	8.9 (9.2)		
Medication (free/SSRI/dual acting)	12/13/13		

Data expressed as mean with SD in parentheses and statistical analysis was performed using Student's t-test (Age, BMI, Alcohol consumption) and Chi squared (χ^2) test (Gender and Smoking).

4.3.2 Clinical Depression and symptom cluster severity

Consistent with the diagnosis of depression, patients with MDD had a significantly elevated total HAM-D score ($p<0.001$) compared to the control group (MDD: 27.7 (5.8) vs. Control: 2.4 (2.3)) [Table 4.4]. This pattern was also evident in each of the subscale scores with significant elevations in core depression, insomnia, anxiety and somatic symptomology in the depressed cohort ($p<0.001$) relative to controls. The median value for the anxiety/somatisation factor was significantly greater in the depressed participants ($p<0.001$) compared with healthy controls (MDD: 7.6 (1.9) vs. Control: 1.6 (1.4)) [Table 4.4].

Table 4.4 Clinical depression and symptom cluster severity for patients with MDD and healthy controls measured with the HAM-D 21 scale.

	Depressed (n=38)	Control (n=38)	Statistic (p-value)
Total HAM-D 21 score	27.68 (5.82)	2.42 (2.30)	Z=-7.539 (p<0.001)
HAM-D clusters			
Core Depression	11.13 (2.73)	0.53 (0.98)	Z=-7.670 (p<0.001)
Insomnia	3.84 (1.70)	0.24 (0.59)	Z=-7.239 (p<0.001)
Anxiety	5.97 (1.82)	1.79 (1.65)	Z=-6.795 (p<0.001)
Somatic	5.18 (1.80)	0.05 (0.23)	Z=-7.771 (p<0.001)
Anxiety/Somatization factor	7.63 (1.92)	1.68 (1.40)	Z=-7.452 (p<0.001)

Data expressed as mean and SD in parentheses. $p<0.001$ vs. control (Mann-Whitney U test).

4.3.3 Whole blood transcriptional inflammatory and glucocorticoid profile in depressed patients compared with healthy control subjects

4.3.3.1 Whole blood transcriptional levels of key inflammatory cytokines, interferon-inducible genes and general immune activation markers in depressed patients relative to control subjects

A Mann-Whitney U test revealed no significant difference in the transcriptional profile of key inflammatory cytokines IL-1β, TNF-α and IL-6 along with IFN-γ or its inducible genes IP-10 and SOCS-1 between the depressed cohort and the healthy controls ($p>0.05$) [Table 4.5]. Similarly, whole blood mRNA expression of the innate immune receptors TLR3, TLR4, TREM-1 and TREM-1's co-adaptor molecule DAP-12 were not found to be significantly different between the depressed patients and control subjects ($p>0.05$) [Table 4.5]. Comparable expression levels of the general immune activation markers PTX3, PDE4B and TRAF6 were also observed in the depressed cohort and control participants [Table 4.5].

4.3.3.2 Whole blood transcriptional expression of M1 and M2 monocytic markers in depressed patients relative to control subjects

A Mann-Whitney U test revealed no significant difference in the transcriptional expression of the M1 monocytic markers EGR1, IL-12p40, iNOS, IFN-γR1, COX-2 and IκBα in the depressed cohort compared with healthy controls ($p>0.05$) [Table 4.6]. Moreover, comparable expression levels of the M2 monocytic markers, PU.1, ARG1, IL-4Ra, MRC, and CD200R were observed in the depressed cohort and control participants [Table 4.6].

4.3.3.3 Whole blood T-cell subset gene expression in depressed patients relative to control subjects

A Mann-Whitney U test revealed no significant difference in the mRNA expression levels of the Th-1 cytokine IL-2 ($Z=-1.189$, $p=0.238$) and transcription factor T-bet ($Z=-0.966$, $p=0.339$) between depressed patients and control subjects [Table 4.7]. However, a significant decrease in the mRNA expression of the Th-2 cytokine IL-4 was observed in the depressed cohort relative to controls ($Z=-1.943$, $p=0.050$) [Table 4.7]. While the mRNA expression of IL-13 was undetectable in the PAXgene samples, no significant difference was observed in the expression of the Th-2 transcription factor GATA3 ($Z=-1.299$, $p=0.197$) between patients with MDD and healthy controls [Table 4.7]. Analysis of the whole blood transcriptional profile for T-regulatory cells revealed no significant difference in the mRNA expression of TGF-β ($Z=-0.457$, $p=0.653$) and the transcription factor FoxP3 ($Z=-0.779$, $p=0.441$) in the depressed patients relative to the controls, however, the MDD patients did have a significant elevation in the expression of IL-10 ($Z=-2.015$, $p=0.044$) at the message level when compared with controls [Table 4.7]. Whole blood mRNA expression of the Th-17 cell markers IL-17A and IL-22 was undetectable, while no difference was observed in the expression of IL-21 ($Z=-0.038$, $p=0.978$) or the transcription factor RORC-γ ($Z=-0.788$, $p=0.443$) between depressed patients and healthy controls [Table 4.7].

4.3.3.4 Whole blood mRNA expression of the GR and glucocorticoid-inducible genes in depressed patients relative to control subjects

A Mann-Whitney U test revealed no significant difference in the mRNA expression level of the GR or the glucocorticoid-inducible genes, FKBP5, SGK1 and GILZ between depressed patients and control participants [Table 4.8].

Table 4.5 Whole blood mRNA expression of inflammatory cytokines, interferon inducible genes and general markers of immune activation in depressed patients relative to healthy controls

PAXgene whole blood mRNA	Depressed (n=38)	Control (n=38)	Statistic (p-value)
Inflammatory cytokines			
IL-1β (fold change)	1.07 (0.46)	1.00 (0.34)	Z=-0.229 (p=0.824)
TNF-α (fold change)	0.97 (0.26)	1.00 (0.31)	Z=-0.229 (p=0.824)
IL-6 (fold change)	0.94 (0.45)	1.00 (0.73)	Z=-0.416 (p=0.683)
IFN-γ (fold change)	0.84 (0.63)	1.00 (0.88)	Z=-0.769 (p=0.447)
IFN-γ inducible genes			
IP-10 (fold change)	0.92 (0.62)	1.00 (0.94)	Z=-0.138 (p=0.895)
SOCS-1 (fold change)	0.91 (0.38)	1.00 (0.47)	Z=-0.675 (p=0.505)
Immune activation markers			
TLR3 (fold change)	1.12 (0.94)	1.00 (0.71)	Z=-0.150 (p=0.886)
TLR4 (fold change)	1.12 (0.36)	1.00 (0.31)	Z=-1.340 (p=0.183)
TREM-1 (fold change)	1.08 (0.39)	1.00 (0.45)	Z=-0.893 (p=0.268)
DAP-12 (fold change)	0.98 (0.29)	1.00 (0.35)	Z=-0.145 (p=0.889)
PTX3 (fold change)	1.14 (0.51)	1.00 (0.48)	Z=-1.184 (p=0.240)
PDE4B (fold change)	1.00 (0.33)	1.00 (0.28)	Z=-0.145 (p=0.889)
TRAF6 (fold change)	0.98 (0.34)	1.00 (0.29)	Z=-0.779 (p=0.441)

Data expressed as mean and SD in parentheses. Statistical analysis was performed using a Mann-Whitney U test, $p<0.05$ was considered significant, n=38 per group.

Table 4.6 Whole blood monocytic marker gene expression in depressed patients relative to healthy controls

PAXgene whole blood mRNA	Depressed (n=38)	Control (n=38)	Statistic (p-value)
Macrophage markers			
M1			
EGR1 (fold change)	0.98 (0.37)	1.00 (0.40)	Z=-0.467 (p=0.646)
IL-12p40 (fold change) [a]	1.14 (0.50)	1.00 (0.69)	Z=-0.558 (p=0.606)
iNOS (fold change) [b]	0.92 (0.60)	1.00 (0.78)	Z=-0.023 (p=0.991)
IFN-γR1 (fold change)	1.09 (0.34)	1.00 (0.22)	Z=-0.561 (p=0.580)
Cox-2 (fold change)	0.99 (0.39)	1.00 (0.38)	Z=-0.021 (p=0.988)
IκBα (fold change)	0.93 (0.27)	1.00 (0.31)	Z=-0.418 (p=0.423)
M2			
PU.1 (fold change)	0.92 (0.69)	1.00 (1.12)	Z=-0.332 (p=0.745)
ARG1 (fold change)	1.16 (0.73)	1.00 (0.59)	Z=-0.821 (p=0.417)
IL-4Ra (fold change)	1.12 (0.55)	1.00 (0.38)	Z=-0.779 (p=0.441)
MRC (fold change) [c]	1.19 (0.98)	1.00 (0.66)	Z=-0.229 (p=0.824)
CD200R (fold change)	0.90 (0.49)	1.00 (0.61)	Z=-0.603 (p=0.552)

Data expressed as mean and SD in parentheses. Statistical analysis was performed using a Mann-Whitney U test, $p<0.05$ was considered significant, n=38 per group. [a]IL-12p40: n=11 per group; [b]iNOS: n=22 per group; [c]MRC: Depressed (n=33); Controls (n=37).

Table 4.7 Whole blood T-cell subset gene expression in depressed patients relative to healthy controls

PAXgene whole blood mRNA	Depressed (n=38)	Control (n=38)	Statistic (p-value)
T-cell subsets			
Th-1			
IL-2 (fold change)[a]	0.88 (0.84)	1.00 (0.75)	Z=-1.189 (p=0.238)
T-bet (fold change)	0.95 (0.54)	1.00 (0.46)	Z=-0.966 (p=0.339)
Th-2			
IL-4 (fold change)	**0.90 (1.1)**	**1.00 (0.57)**	**Z=-1.943 (p=0.050)**
IL-13 (fold change)	Not detected	Not detected	—
GATA3 (fold change)	0.96 (0.44)	1.00 (0.34)	Z=-1.299 (p=0.197)
T-reg			
TGF-β (fold change)	1.02 (0.18)	1.00 (0.15)	Z=-0.457 (p=0.653)
IL-10 (fold change)	**1.25 (0.67)**	**1.00 (0.71)**	**Z=-2.015 (p=0.044)**
FoxP3 (fold change)	0.98 (0.53)	1.00 (0.38)	Z=-0.779 (p=0.441)
Th-17			
IL-17A (fold change)	Not detected	Not detected	—
IL-21 (fold change)[b]	0.90 (0.75)	1.00 (1.08)	Z=-0.038 (p=0.978)
IL-22 (fold change)	Not detected	Not detected	—
RORC-γ (fold change)[c]	0.87 (0.55)	1.00 (0.59)	Z=-0.788 (p=0.443)

Data expressed as mean and SD in parentheses. p≤0.05 vs. control (Mann-Whitney U test), n=38 per group. Significant findings do not survive correction for multiple testing, adjusted p-value: p≤0.01. [a] IL-2: Depressed (n=33); Controls (n=36). [b] IL-21: Depressed (25); Controls (n=26). [c]RORC-γ: Depressed (n=23); Controls (n=18).

Table 4.8 Whole blood expression of the GR and glucocorticoid-inducible genes in depressed patients relative to healthy controls

PAXgene whole blood mRNA	Depressed (n=38)	Control (n=38)	Statistic (p-value)
GR (fold change)	1.01 (0.26)	1.00 (0.17)	Z=-0.218 (p=0.832)
GC inducible genes			
FKBP5 (fold change)	1.18 (0.52)	1.00 (0.28)	Z=-1.257 (p=0.212)
SGK1 (fold change)	0.95 (0.25)	1.00 (0.24)	Z=-0.790 (p=0.435)
GILZ (fold change)	0.98 (0.33)	1.00 (0.37)	Z=-0.073 (p=0.946)

Data expressed as mean and SD in parentheses. Statistical analysis was performed using a Mann-Whitney U test, p<0.05 was considered significant, n=38 per group.

4.3.4 Circulating BDNF concentrations in depressed patients relative to control subjects

As the growth factor BDNF has been widely implicated in the biological basis of major depression, peripheral BDNF was measured in plasma using ELISA. Despite this, circulating BDNF concentrations were not altered in patients with MDD relative to control subjects ($Z=-0.629$, $p=0.533$) [Figure 4.1].

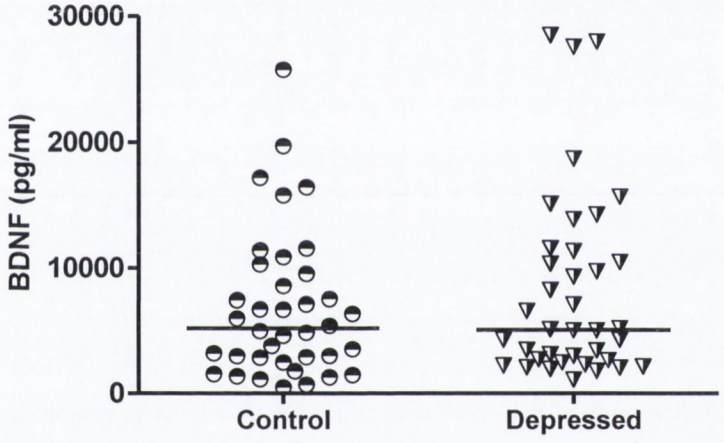

Figure 4.1 Circulating BDNF levels in depressed patients relative to control subjects. Plasma concentrations of BDNF in depressed patients relative to control subjects. Horizontal lines represent the median value. Statistical analysis was performed using a Mann-Whitney U test, n=38 per group.

4.3.5 The association between HAM-D total scores and individual symptom sub-scales and whole blood innate and monocytic inflammatory markers

Correlational analysis revealed that specific innate immune and monocytic markers were most closely associated with depression severity and symptomology. A significant positive correlation was observed between patient HAM-D total scores and the transcriptional expression of IL-1β (r=0.329, p=0.044), TREM-1 (r=0.330, p=0.043) and IFN-γR1 (r=0.400, p=0.013). Further to this, a significant positive relationship was observed between core depression symptoms and IL-1β (r=0.391, p=0.015), TREM-1 (r=0.396, p=0.014) and ARG1 (r=0.406, p=0.011) at the message level, with a strong trend towards an association with COX-2 mRNA expression (r=0.312, p=0.057) [Table 4.9]. Moreover, the anxiety/somatisation factor was found to positively associate with IL-1β (r=0.390, p=0.015), TREM-1 (r=0.404, p=0.012) and DAP-12 (r=0.360, p=0.027). Subsequent analysis revealed that the anxiety subscale alone was significantly and positively correlated with IL-1β (r=0.441, p=0.006), TLR4 (r=0.331, p=0.042), TREM-1 (r=0.491, p=0.002), DAP-12 (r=0.430, p=0.007), IκBα (r=0.363, p=0.025) and IL-4R (r=0.341, p=0.036) with a strong trend towards an association with COX-2 whole blood mRNA expression (r=0.312, p=0.056), while no association was revealed between any of the inflammatory markers and somatic symptomology alone (p>0.05) [Table 4.9]. This was also the case for the insomnia subscale which was found not to correlate with any of the inflammatory markers under investigation (p>0.05) [Table 4.9]. Spearman's *rho* correlational analysis also revealed that the associations between inflammation and depression severity and symptomology were unique to the depressed cohort and were not evident in the control subjects (p>0.05, data not shown).

Table 4.9 Correlational analysis between specific innate and monocytic inflammatory markers and HAM-D severity and symptomology in the depressed cohort

Biological parameter	Total HAM-D	Core Depression	Insomnia	Anxiety	Somatic	Anxiety/Somatisation factor
PAXgene mRNA						
Cytokine						
IL-1β	**r=0.329 (p=0.044)**	**r=0.391 (p=0.015)**	r=-0.055 (p=0.744)	**r=0.441 (p=0.006)***	r=0.257 (p=0.119)	**r=0.390 (p=0.015)**
Immune activation markers						
TLR4	r=0.174 (p=0.296)	r=0.138 (p=0.409)	r=-0.240 (p=0.147)	**r=0.331 (p=0.042)**	r=0.201 (p=0.227)	r=0.273 (p=0.097)
TREM-1	**r=0.330 (p=0.043)**	**r=0.396 (p=0.014)**	r=-0.081 (p=0.629)	**r=0.491 (p=0.002)***	r=0.241 (p=0.144)	**r=0.404 (p=0.012)**
DAP-12	r=0.282 (p=0.087)	r=0.231 (p=0.162)	r=-0.092 (p=0.584)	**r=0.430 (p=0.007)***	**r=0.320 (p=0.050)**	**r=0.360 (p=0.027)**
Monocytic markers						
M1						
IFN-γR1	**r=0.400 (p=0.013)**	r=0.186 (p=0.264)	r=0.031 (p=0.852)	r=0.288 (p=0.080)	r=0.220 (p=0.185)	r=0.255 (p=0.123)
COX-2	r=0.282 (p=0.086)	**r=0.312 (p=0.057)**	r=0.003 (p=0.985)	**r=0.312 (p=0.056)**	r=0.251 (p=0.128)	r=0.295 (p=0.072)
IκBα	r=0.266 (p=0.106)	r=0.271 (p=0.099)	r=-0.065 (p=0.699)	**r=0.363 (p=0.025)**	r=0.153 (p=0.360)	r=0.284 (p=0.084)
M2						
ARG1	r=0.166 (p=0.318)	**r=0.406 (p=0.011)**	r=-0.299 (p=0.068)	r=0.275 (p=0.095)	r=0.125 (p=0.455)	r=0.239 (p=0.148)
IL-4R	r=0.109 (p=0.514)	r=0.122 (p=0.467)	r=-0.107 (p=0.524)	**r=0.341 (p=0.036)**	r=0.133 (p=0.427)	r=0.229 (p=0.166)

Statistical analysis was performed using the Spearman *rho* correlation coefficient, p≤0.05 was considered significant (n=38). Bold type face represents significant findings. Asterisks indicate significant findings following correction for multiple testing, adjusted p-value: p≤0.01. No significant correlations were observed between HAM-D severity and symptomology and any of the other inflammatory cytokines, immune activation markers, monocytic and T-cell and glucocorticoid markers of interest in the depressed cohort. No significant correlations were detected between HAM-D severity and symptomology scores and these markers in the control group.

4.3.6 Childhood Trauma and its association with the inflammatory and glucocorticoid systems and circulating BDNF concentrations

4.3.6.1 Childhood trauma and individual subscale analysis

Participants in the depressed cohort reported more childhood traumatic events ($p<0.001$) than healthy control participants (MDD: 46.4 (21.1) vs. Control: 31.2 (5.8)). Of the 38 MDD patients, 21 reported a history of childhood trauma while 9 of the 38 healthy controls reported a history of childhood trauma. Breaking down the CTQ total score, 11 MDD patients reported moderate to severe emotional abuse, while no controls had a history of emotional abuse. Eleven patients and 3 controls reported a history of physical abuse, while 10 patients and 3 controls were sexually abused as children. Twelve patients and 1 control were emotionally neglected as children, while 10 MDD patients and 3 controls reported a history of physical neglect. A Kruskal-Wallis one-way ANOVA revealed a significant difference between all 4 groups for each of the 5 subscales ($df=3$, $p<0.001$) [Table 4.10]. At the level of the CTQ subscales, the n numbers were too small to carry out any further analysis on the controls subjects. However, a Mann-Whitney U test revealed that the depressed patients with emotional, physical and sexual abuse and emotional and physical neglect had significantly increased scores relative to depressed patients with no childhood trauma history ($p<0.001$) [Table 4.10].

Table 4.10 Childhood trauma individual sub-type breakdown in depressed patients and healthy controls measured using the 28-item CTQ

	Control without CT	n	Control with CT	n	MDD without CT	n	MDD with CT	n
CTQ sub-scales								
Emotional abuse	6.29 (1.75)	38	–	0	6.78 (2.30)	27	17.64 (3.72)***	11
Physical abuse	5.43 (0.95)	35	10.33 (0.58)	3	5.63 (1.25)	27	15.18 (5.06)***	11
Sexual abuse	5.20 (0.53)	35	9.67 (0.58)	3	5.29 (0.60)	28	16.50 (6.82)***	10
Emotional neglect	7.22 (2.61)	37	16.00 (0.00)	1	8.42 (3.04)	26	18.75 (3.44)***	12
Physical neglect	5.83 (1.22)	35	10.00 (0.00)	3	6.27 (1.54)	28	13.20 (2.10)***	10

Data expressed as mean and SD in parentheses. Statistical analysis was performed using a Kruskal Wallis one-way ANOVA followed by Mann-Whitney U tests. ***$p<0.001$ vs. MDD patients without childhood trauma (CT).

4.3.6.2 Whole blood transcriptional profile of specific markers of the inflammatory and glucocorticoid system in MDD patients and controls with and without childhood trauma

A Kruskal-Wallis one-way ANOVA revealed no significant effect of childhood trauma on the transcriptional expression of the glucocorticoid receptor or the glucocorticoid inducible genes FKBP5, SGK1 and GILZ in MDD patients and healthy controls (df=3, p>0.05) [Table 4.11]. A Kruskal-Wallis one-way ANOVA also revealed no significant effect of childhood trauma on the mRNA expression of the classical inflammatory cytokines IL-1β, TNF-α, IL-6 and IFN-γ in MDD patients and healthy controls (df=3, p>0.05) [Table 4.11]. A Kruskal-Wallis one-way ANOVA revealed no significant effect of childhood trauma on IL-4 mRNA expression in MDD patients and healthy controls (df=3, p>0.05) [Table 4.11]. However, the Kruskal-Wallis one-way ANOVA did detect a significant effect of childhood trauma on whole blood IL-10 expression at the message level in MDD patients and healthy controls (df=3, p=0.023) [Table 4.11]. To determine which groups were statistically different, a Mann-Whitney U test revealed a significant increase in IL-10 mRNA expression in controls with childhood trauma relative to controls without childhood trauma (Z=-2.009, p=0.045). A Mann-Whitney U test also revealed a significantly elevated expression in IL-10 mRNA in MDD patients without childhood trauma relative to healthy controls without childhood trauma (Z=2.355, p=0.019).

Table 4.11 Whole blood transcriptional profile of specific markers of the inflammatory and glucocorticoid systems in MDD patients and controls with and without childhood trauma

Biological parameter	Control without childhood trauma (n=29)	Control with childhood trauma (n=9)	MDD without childhood trauma (n=17)	MDD with childhood trauma (n=21)
Cytokines				
IL-1β (fold change)	1.00 (0.36)	1.03 (0.29)	0.98 (0.51)	1.16 (0.43)
TNF-α (fold change)	1.00 (0.27)	1.18 (0.45)	0.99 (0.26)	1.02 (0.28)
IL-6 (fold change)	1.00 (0.70)	1.18 (0.98)	0.97 (0.54)	0.98 (0.42)
IFN-γ (fold change)	1.00 (0.91)	1.57 (1.20)	0.95 (0.72)	0.96 (0.74)
Th2				
IL-4 (fold change)	1.00 (0.60)	1.03 (0.51)	0.76 (0.64)	1.03 (1.39)
T-reg				
IL-10 (fold change)	1.00 (0.53)	1.96 (1.31)*	1.66 (0.99)*	1.43 (0.67)
GR (fold change)	1.00 (0.18)	1.08 (0.13)	1.13 (0.31)	0.95 (0.19)
GC inducible genes				
FKBP5 (fold change)	1.00 (0.28)	1.14 (0.34)	1.40 (0.57)	1.07 (0.48)
SGK1 (fold change)	1.00 (0.25)	0.92 (0.21)	0.97 (0.25)	0.91 (0.24)
GILZ (fold change)	1.00 (0.36)	0.93 (0.40)	0.85 (0.28)	1.07 (0.33)

Data expressed as mean and SD in parentheses. Statistical analysis was performed using a Kruskal Wallis one-way ANOVA followed by Mann-Whitney U tests. *p<0.05 vs. controls without childhood trauma.

4.3.6.3 Circulating BDNF concentrations in MDD patients and controls with and without childhood trauma

A Kruskal-Wallis one-way ANOVA revealed a significant effect of childhood trauma on circulating BDNF concentrations in MDD patients and healthy controls (df=3, p=0.009) [Figure 4.2]. To determine which groups were statistically different a Mann-Whitney U test was carried out and revealed no difference in circulating BDNF levels in controls with childhood trauma relative to those without (Z=-2.009, p=0.061). A Mann-Whitney U test revealed a significant increase in BDNF concentrations in MDD patients without childhood trauma compared with healthy controls without childhood trauma (Z=2.572, p=0.010), while decreased circulating BDNF levels were detected in MDD patients with childhood trauma relative to healthy controls with childhood trauma (Z=-2.331, p=0.019). A Mann-Whitney U test also revealed a significant decrease in BDNF plasma concentrations in MDD patients with childhood trauma relative to patients without childhood trauma (Z=-2.642, p=0.007) [Figure 4.2].

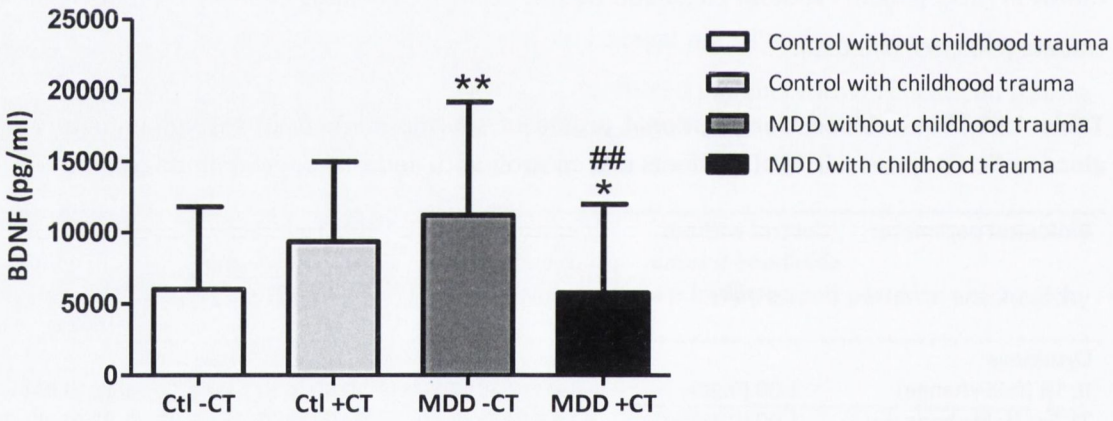

Figure 4.2 The effect of childhood trauma on circulating BDNF levels in depressed patients and healthy controls. Plasma concentrations of BDNF in depressed patients relative to control subjects. Data presented as mean with SD. Statistical analysis was performed using a Kruskal-Wallis one-way ANOVA followed by a Mann-Whitney U test for pair-wise analysis. **p≤0.01 vs. Control without childhood trauma (CT); *p≤0.05 vs. Control with childhood trauma; ##p≤0.01 vs. MDD without childhood trauma (CT). Ctl-CT (n=29); Ctl+CT (n=9); MDD-CT (n=17); MDD+CT (n=21).

4.3.7 The impact of distinct childhood trauma classifications on the inflammatory, glucocorticoid and BDNF marker profile in MDD patients

A Mann-Whitney U test revealed a significantly decreased GR mRNA expression in emotionally abused MDD patients relative to MDD patients with no history of emotional abuse (Z=-1.947, p=0.050) [Table 4.12]. A Mann-Whitney U test also revealed an elevated transcriptional expression of IL-1β in the emotionally abused MDD patients compared with MDD patients with no history of emotional abuse (Z=-2.173, p=0.030) [Table 4.12]. Further to this, a Mann-Whitney U test revealed a very strong trend towards a significant increase in whole blood transcriptional levels of IL-6 in physically abused MDD patients compared with MDD patients who were not physically abused (Z=-1.947, p=0.052) [Table 4.12]. A Mann-Whitney U test revealed a significant increase in whole blood transcriptional levels of IL-6 in sexually abused MDD patients compared with MDD patients who were not sexually abused (Z=-2.387, p=0.016) [Table 4.12]. A Mann-Whitney U test revealed a significant decrease in FKBP5 mRNA expression in physically neglected MDD patients relative to MDD patients who were not physically neglected (Z=-2.254, p=0.023) [Table 4.12]. A Mann-Whitney U test revealed no significant difference in the transcriptional expression of the PAXgene whole blood glucocorticoid and inflammatory cytokines under investigation along with circulating BDNF levels in emotionally neglected MDD patients relative to MDD patients without a history of emotional neglect (p>0.05). The transcriptional profile of other markers of interest along with circulating BDNF concentrations were not found to be statistically different between MDD patients with and without a history of the distinct childhood trauma classifications (p>0.05, data not shown).

Table 4.12 The impact of distinct childhood trauma classifications on the glucocorticoid, inflammatory and BDNF marker profile in MDD patients

Biological parameter	Depressed with no history of	n	Depressed with a history of	n	Statistic (p-value)
	Emotional abuse		**Emotional abuse**		
IL-1β (fold change)	1.00 (0.44)	27	1.32 (0.50)	11	**Z=-2.173 (p=0.030)**
GR (fold change)	1.00 (0.26)	27	0.83 (0.16)	11	**Z=-1.947 (p=0.050)**
	Physical abuse		**Physical abuse**		
IL-6 (fold change)	1.00 (0.53)	27	1.37 (0.48)	11	**Z=-1.947 (p=0.052)**
	Sexual abuse		**Sexual abuse**		
IL-6 (fold change)	1.00 (0.55)	28	1.41 (0.38)	10	**Z=-2.387 (p=0.016)**
	Physical Neglect		**Physical Neglect**		
FKBP5 (fold change)	1.00 (0.42)	28	0.67 (0.22)	10	**Z=-2.254 (p=0.023)**

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann-Whitney U test, p<0.05 was considered significant.

4.3.8 Correlational analysis between CTQ total scores and individual items on the childhood trauma scale and markers for the inflammatory and glucocorticoid systems and BDNF

Sprearman's *rho* correlational analysis revealed a significant negative correlation between the whole blood transcriptional expression of GR (r=-0.357, p=0.028) and its co-chaperone FKBP5 (r=-0.389, p=0.016) and the CTQ total score in the depressed cohort [Table 4.13]. A negative association between circulating BDNF levels and the CTQ total score was also detected (r=-0.411, p=0.010) [Table 4.13]. Assessment of the individual sub-scales of the CTQ revealed a significant negative association between emotional abuse scores from the depressed cohort and GR (r=-0.321, p=0.050) [Table 4.13]. No significant associations were observed between physical abuse scores and the biological markers of interest in the depressed cohort [Table 4.13]. Spearman's *rho* correlational analysis revealed trends towards a negative association between sexual abuse scores and FKBP5 (r=-0.313, p=0.055) and BDNF (r=-0.313, p=0.056) in the depressed cohort [Table 4.13]. Emotional neglect was strongly negatively correlated with GR in the depressed cohort (r=-0.430, p=0.007) and to a lesser extent with FKBP5 (r=-0.374, p=0.021). Correlational analysis also revealed a negative association between BDNF and emotional neglect in MDD patients (r=-0.354, p=0.029) [Table 4.13]. A negative association was also detected between physical neglect and FKBP5 in the depressed patients (r=-0.398, p=0.013) [Table 4.13]. No significant correlations were observed between CTQ total scores and individual items on the childhood trauma scale and the inflammatory cytokines IL-1β, TNF-α, IL-6, IFN-γ, IL-4 and IL-10.

Table 4.13 Correlational analysis between CTQ total scores and individual items on the childhood trauma scale and markers for the glucocorticoid system and BDNF in MDD patients

Biological parameter	Total CTQ	Emotional abuse	Physical abuse	Sexual abuse	Emotional neglect	Physical neglect
PAXgene mRNA						
GR	**r=-0.357 (p=0.028)**	**r=-0.321 (p=0.050)**	r=-0.053 (p=0.753)	r=-0.288 (p=0.080)	**r=-0.430 (p=0.007)***	r=-0.165 (p=0.322)
GR inducible genes						
FKBP5	**r=-0.389 (p=0.016)**	r=-0.277 (p=0.092)	r=-0.283 (p=0.085)	r=-0.313 (p=0.055)	**r=-0.374 (p=0.021)**	**r=-0.398 (p=0.013)**
SGK1	r=-0.086 (p=0.606)	r=0.023 (p=0.890)	r=-0.092 (p=0.583)	r=-0.193 (p=0.246)	r=-0.172 (p=0.301)	r=-0.093 (p=0.580)
GILZ	r=0.290 (p=0.077)	**r=0.312 (p=0.057)**	r=0.274 (p=0.096)	r=0.133 (p=0.427)	r=0.158 (p=0.342)	r=0.163 (p=0.329)
Plasma						
BDNF (pg/ml)	**r=-0.411 (p=0.010)***	r=-0.286 (p=0.081)	r=-0.289 (p=0.078)	r=-0.313 (p=0.056)	**r=-0.354 (p=0.029)**	r=-0.168 (p=0.315)

Statistical analysis was performed using the Spearman *rho* correlation coefficient, $p<0.05$ was considered significant (n=38). Bold type face represents significant findings. Asterisks indicate significant findings following correction for multiple testing, adjusted p-value: $p\leq0.01$. No significant correlations were observed between CTQ total scores and individual items on the childhood trauma scale and the specific inflammatory cytokines under investigation; IL-1β, TNF-α, IL-6, IFN-γ, IL-4 and IL-10 in the depressed cohort.

4.3.9 The impact of medication on biological variables in depressed patients

Antidepressant medication did not impact upon any of the biological variables found to be associated with major depression in the current study ($p>0.05$) [Table 4.14]. No significant difference was observed between un-medicated MDD patients and antidepressant treated patients with MDD for any of the other biological markers of interest ($p>0.05$, data not shown).

Table 4.14 Analysis of the impact of medication on biological variables in depressed patients

Biological parameter	Anti-depressant treated patients with MDD (n=26)	Un-medicated patients with MDD (n=12)	Statistics (p-value)
Inflammatory cytokines			
IL-1β (fold change)	0.97 (0.43)	1.00 (0.44)	Z=-0.157 (p=0.875)
TNF-α (fold change)	0.93 (0.24)	1.00 (0.28)	Z=-0.754 (p=0.451)
IL-6 (fold change)	1.40 (0.61)	1.00 (0.54)	Z=-1.947 (p=0.053)
IFN-γ (fold change)	1.13 (0.91)	1.00 (0.61)	Z=-0.094 (p=0.925)
Immune activation markers			
TLR4 (fold change)	1.03 (0.34)	1.00 (0.30)	Z=-0.345 (p=0.730)
TREM-1 (fold change)	1.18 (0.42)	1.00 (0.39)	Z=-1.382 (p=0.167)
DAP-12 (fold change)	1.15 (0.37)	1.00 (0.20)	Z=-1.570 (p=0.116)
Macrophage markers			
M1			
IFN-γR1 (fold change)	1.21 (0.38)	1.00 (0.24)	Z=-1.507 (p=0.132)
COX-2 (fold change)	0.94 (0.39)	1.00 (0.36)	Z=-0.345 (p=0.730)
IκBα (fold change)	1.04 (0.31)	1.00 (0.26)	Z=-0.000 (p=1.000)
M2			
ARG1 (fold change)	1.00 (0.56)	1.00 (0.76)	Z=-0.377 (p=0.706)
IL-4Ra (fold change)	1.07 (0.55)	1.00 (0.48)	Z=-0.628 (p=0.530)
T-cell subsets			
Th2			
IL-4 (fold change)	1.19 (1.54)	1.00 (1.01)	Z=-0.188 (p=0.851)
T-reg			
IL-10 (fold change)	0.99 (0.52)	1.00 (0.59)	Z=-0.094 (p=0.925)
GR (fold change)	0.99 (0.28)	1.00 (0.21)	Z=-0.408 (p=0.683)
GC inducible genes			
FKBP5 (fold change)	1.13 (0.48)	1.00 (0.50)	Z=-0.754 (p=0.451)
SGK1 (fold change)	0.99 (0.27)	1.00 (0.25)	Z=-0.408 (p=0.683)
GILZ (fold change)	0.84 (0.30)	1.00 (0.28)	Z=-1.884 (p=0.060)
Plasma			
BDNF (pg/ml)	7901 (7606)	8835 (7511)	Z=-0.424 (p=0.672)

Data presented as mean with SD in parentheses. Statistical analysis was performed using a Mann-Whitney U test.

4.4 Discussion

In the present study, I profiled the expression of specific markers from the inflammatory and glucocorticoid systems in a cohort of depressed patients relative to age and sex matched healthy control subjects. The major findings from this study are that greater depression severity and affective symptoms of major depression, such as mood and anxiety, are positively associated with an inflammatory profile. To my knowledge, this is the first study to assess the relationship between PAXgene whole blood transcriptional expression of inflammatory markers and individual symptom clusters of major depression. Further to this, I observed that MDD patients with a history of childhood trauma exhibit a distinct and dysregulated biological profile relative to MDD patients with no history of early life stress, evident upon analysis of the individual traumatic subtypes of the childhood trauma questionnaire. While numerous studies have assessed the relationship between circulating BDNF concentrations and childhood trauma, this is the first to show a dysregulated inflammatory and glucocorticoid profile in depressed patients with early life stress using the PAXgene whole blood system.

4.4.1 PAXgene biomarker transcriptional profile in depressed patients relative to healthy controls

Despite previous findings presented in chapter 3, which show a mild circulating inflammatory phenotype in the depressed cohort relative to controls (Hughes et al., 2012), in the present study, we did not find any significant differences in the PAXgene expression of the classical inflammatory cytokines IL-1β, IL-6, TNF-α, IFN-γ and IFN-inducible genes, IP-10 and SOCS-1 at the message level. In addition, whole blood gene expression of the innate immune activation markers TLR3, TLR4, TREM-1, DAP-12 and PU.1 was not increased in the patient group relative to controls.

Whilst no changes in the transcriptional profile of cytokines and transcription factors for Th-1 and Th-17 cells were detected, a significantly decreased IL-4 mRNA expression was observed in the depressed subjects relative to the controls, indicative of decreased Th-2 cytokine expression. This is consistent with the literature and recent findings by Cattaneo et al. (2013) who also describe decreased levels of PAXgene whole blood IL-4 in a depressed population. Interestingly, we also identified an increased IL-10 whole blood mRNA expression in the depressed cohort relative to controls. This is in accordance with findings by Belzeaux et al. (2010) who show increased IL-10 expression in patient PBMCs. In the present study, increased IL-10 expression may be suggestive of regulatory T-cell (T-reg) cell activity, however in the absence of any changes in the transcription factor and master regulator of T-reg cells, Forkhead box P3 (FoxP3), this finding should be

interpreted with caution given that IL-10 is also produced by many cell types including monocytes and macrophages.

Cell-mediated immune activation relates to the interaction and cross talk between T-cells and monocytes (Maes, 2011). Production of pro- and anti-inflammatory cytokines by T-cells following antigen stimulation results in the acquired immune activation of distinct monocytic subsets that have roles in inflammation (M1), repair and regeneration (M2a), immunomodulation (M2b) and deactivation (M2c) (Christmann and Lafyatis, 2010, Chhor et al., 2013). As IFN-γ and TNF-α, the inducers of M1 activation, were not increased in patient whole blood, it was not surprising to find no changes in Th-1 stimulated M1 markers. However, the decreased expression of IL-4, a potent inducer of alternatively activated M2a monocytes did not appear to have any effect on the expression of either M1 or M2 monocytic markers. Further to this, increased patient mRNA expression of IL-10, an inducer of the M2c/ acquired monocytic deactivation state, also had no impact upon the expression of markers for monocytic activation. However in accordance with this, recent evidence by Zizzo et al. (2012) shows that IL-10 alone is unable to induce M2c activation and may require macrophage colony-stimulating factor (MCSF) which is also released by T-regs, to induce M2c activation.

Independent of IL-10, glucocorticoids are also involved in the polarisation of monocytes and the induction of the M2c activation state upon GR activation (Zizzo et al., 2012). However, no significant difference was observed in the whole blood transcriptional expression of GR or the glucocorticoid inducible genes FKBP5, GILZ and SGK1.

4.4.2 Assessment of the whole blood transcriptional profile in association with depression severity and depressive symptomology

While a decreased expression of the key Th-2 anti-inflammatory cytokine IL-4 and an increased expression in the immune-regulatory cytokine IL-10 was observed in the depressed cohort relative to controls, neither of these cytokines were found to be associated with depression severity or individual symptom clusters in the patient group, perhaps suggesting that these changes are collective of all symptoms which contrast to changes with MDD. Despite this, we did detect some interesting symptom-specific and depression severity correlational patterns with inflammatory markers from the innate and monocytic classifications. In the patient cohort alone, positive correlations were observed between whole blood transcriptional levels of IL-1β, TREM-1 and IFN-γR1 and total HAM-D scores (used as an indicator of depression severity), suggesting that amongst the depressed group, greater depression severity is associated with a pro-inflammatory

phenotype. While numerous studies have identified an association between inflammation and depression, few studies, if any, have addressed the relative correlation between inflammation and depressive symptomology. Concordant with the above findings, IL-1β, and TREM-1 were also significantly positively correlated to the core depression cluster. This highlights that depressed mood is a main contributing factor to the association between innate immune activity and depression in this particular cohort. However, the more sensitive symptom-wise approach also detected a significant positive association between core depression severity and ARG1. To my knowledge, this is the first report to show a positive association between patient whole blood ARG1 expression and core depression severity. This finding is in accordance with an earlier report, showing increased sera arginase activity in major depressed subjects relative to minor depressed patients (Elgun and Kumbasar, 2000). Further to this, Elgun and Kumbasar (2000) suggest that the positive association between arginase and depression severity may be a contributing factor to the symptomology of depression. This is highly plausible. In light of the suggestion that peripheral whole blood cells may act as 'sentinels' or as a 'surrogate for CNS expression', increased peripheral expression of ARG1 may reflect central arginase activity (Liew et al., 2006, Sullivan et al., 2006). The enzymatic actions of ARG1 are well characterised; ARG1 catalyses the hydrolysis of L-arginine thereby reducing its availability for nitric oxide synthesis (Albina et al., 1990, Mori et al., 1998). The pituitary and hypothalamus come into close contact with peripheral blood. Decreased production of nitric oxide in these brain regions may contribute to the loss of regulatory control of the HPA axis, resulting in increased corticotrophin-releasing hormone (CRH) production and an over active cortisol response, one of the most reproducible findings in depressed patients (Raadsheer et al., 1994, Bugajski et al., 2004, Pace et al., 2007). Evidently, this theory contrasts with the literature reporting increased levels of nitric oxide in depressed patients which also have toxic effects (Suzuki et al., 2001, Maes et al., 2009).

The symptom cluster approach also revealed that anxiety was closely associated with an inflammatory phenotype in the depressed cohort. 29 of the 38 patients assessed in the present study had a score greater than or equal to the cut-off score 7 on the anxiety/somatisation subscale and were classified as having anxious depression. While many studies have used this classification to assess treatment response to antidepressants, no study to my knowledge, has ever assessed the relationship between inflammation and anxious depression.

Further to the above findings associating the inflammatory response system with core depression severity, it also appears that the innate immune response has a key role to play in the anxiety symptoms of major depression, with significant positive associations between whole blood transcriptional expression of IL-1β, TREM-1 and its adaptor molecule DAP-12 and the

anxiety/somatisation factor. This is not unexpected however, as research into anxiety related disorders and particularly post-traumatic stress disorder has shown an association with a dysregulated innate immune response, evidenced by increased levels of nuclear factor-kappa B (NFκB) signalling and circulating pro-inflammatory cytokines such as IL-1β and IL-6 (Pace et al., 2012, Zimmerman et al., 2012).

Further analysis and the separation of anxiety and somatic factors into individual symptom clusters revealed that this association was purely driven by anxiety, with no correlation between any of the inflammatory markers and the somatic symptoms of depression. In fact, stronger correlations were revealed for IL-1β, TREM-1 and DAP-12 when correlated with the anxiety subscale alone. Additionally, a significant relationship between the transcriptional levels of the monocytic markers IκBα and IL-4R and anxiety was observed, while a strong trend for an association between whole blood COX-2 mRNA expression and anxiety severity also emerged. Although different monocytic markers were found to be associated with various symptom clusters, both core depression and anxiety subscales were positively associated with the transcriptional levels of the inflammatory cytokine IL-1β and the innate immune activation receptor TREM-1. Even following stringent correction for multiple testing, strong trends and significant positive associations were detected between IL-1β, TREM-1 and the affective symptom clusters, highlighting the importance of these findings. The discovery that both core depression and anxiety clusters are associated with a similar whole blood transcriptional inflammatory profile is interesting given the following findings. Musselman et al. (2001) have shown that 45% of cancer patients receiving high dose IFN-α treatment developed clinical depression. However, by pre-treating with SSRIs, certain cytokine-induced depressive symptoms were abolished. Interestingly, they found that SSRIs protected against the affective symptoms of depression to include depressed mood and anxiety, signifying a link between cytokine-induced depressive symptoms and the serotonin system. However, pre-treatment with SSRIs did not afford protection against the somatic and vegetative depressive symptoms, highlighting the heterogeneity of the biological basis of depression and the involvement of many distinct systems (Capuron et al., 2002a). This is in accordance with the findings in this study, which clearly associate the inflammatory process with affective depressive symptoms, including core depression and anxiety. However, different mechanisms are likely to be involved in the manifestation of somatic symptoms and insomnia, as no correlations were observed between any of the inflammatory markers and these depressive symptoms in this patient cohort.

While this analysis displays many novel findings in a system that may provide a better vantage point to assess the macro and micro changes which may be occurring within the CNS, we have

also clearly presented a case in favour of a symptom-wise approach to decipher the underpinning pathophysiological mechanisms of the individual symptoms that make up the heterogeneous disorder that is depression. This type of analysis may lend towards the discovery of biomarkers for depression and hence the development of a more targeted and personalised treatment approach.

4.4.3 The impact of early life stress upon glucocorticoid, inflammatory and BDNF markers in depressed patients and healthy controls

In the present study, 21 out of the 38 MDD patients and 9 healthy controls reported a history of childhood trauma. While there was no overall group difference in plasma BDNF concentrations between depressed patients and controls, circulating BDNF concentrations were increased in MDD patients with no history of childhood trauma relative to healthy controls with no history of childhood trauma. This is surprising as many reports suggest that BDNF is decreased in MDD patients (Lee et al., 2007, Sen et al., 2008, Satomura et al., 2011). Interestingly, MDD patients with a history of childhood trauma exhibited decreased circulating BDNF concentrations relative to MDD patients without childhood trauma. Further to this, a negative association was detected between BDNF and CTQ total scores in the MDD patients. It is noteworthy that all of these findings survive correction for multiple testing, highlighting the robust nature of these findings. No association was observed between BDNF and the healthy controls, perhaps suggesting that in the depressed population those who are exposed to more severe traumatic events as a child have decreased circulating BDNF concentrations. This is very interesting in light of findings by Elzinga et al. (2011), who show that MDD patients without a history of childhood trauma but who are carriers of the BDNF risk allele, Val^{66}Met, exhibit increased circulating BDNF levels, while those with the BDNF polymorphism in association with a history of childhood trauma have decreased serum BDNF levels relative to MDD patients carrying the normal Val/Val BDNF gene. This may represent a gene-stress interaction and may indicate the presence of a biologically distinct sub-group of MDD patients. Further to this, Grassi-Oliveira et al. (2008) have also observed decreased plasma BDNF levels in depressed patients that were physically neglected as children, relative to MDD patients with no history of childhood trauma.

In addition and consistent with earlier findings demonstrating increased IL-10 expression in the depressed cohort relative to controls, the transcriptional expression of IL-10 was significantly increased in the depressed cohort without childhood trauma relative to healthy controls with no history of childhood trauma. The expression of IL-10 at the message levels was also greater in controls with childhood trauma compared with controls without childhood trauma. While no

other significant changes were observed between patients and controls with and without a history of childhood trauma, correlational analysis detected significant negative correlations between the MDD patients and the GR and its co-chaperone FKBP5. This is interesting as both BDNF and the GR have key roles in regulating the stress response along with normal brain functioning. These findings are also in accordance with the literature, as exposure to early life stress is often characterised by a dysregulated HPA axis. Heim et al. (2008a) revealed that male MDD patients with a history of childhood trauma displayed a hyperactive HPA axis response to the dexamethasone/CRH (Dex/CRH) test relative to depressed men with no history of childhood trauma and healthy controls. These results suggest that MDD patients with a history childhood trauma may represent a biologically distinct subtype of depression (Heim and Binder, 2012).

Further to this, MDD patients were profiled based on the type of childhood trauma they experienced, breaking the total score down into the 5 categories; emotional, physical and sexual abuse and emotional and physical neglect. MDD patients with a history of moderate to severe emotional abuse had significantly decreased whole blood transcriptional levels of the GR along with increased IL-1β expression at the message level, compared with MDD patients without emotional abuse. Moreover, MDD patient emotional abuse scores were significantly negatively associated with the GR, further relating a disrupted glucocorticoid response system with severe early life emotional abuse. As a consequence of early life stress, neurobiological changes such as a dysregulated HPA axis appear to render individuals more vulnerable to subsequent stressful challenges and perhaps are responsible for the increased expression of the classical inflammatory cytokine IL-1β which may have in turn contributed the development of major depression. In addition, MDD patients with a history of moderate to severe physical or sexual abuse both express elevated whole blood transcriptional levels of IL-6 relative to non-abused MDD patients. These findings are in accordance with recent findings by our group showing an association between childhood maltreatment and circulating levels of the general inflammatory marker CRP (Frodl et al., 2012). No significant difference in the transcriptional expression of the targets of interest was observed between patients exposed to emotional neglect and MDD patients who were not. However, a significant negative correlation was revealed between patient emotional neglect scores and BDNF, GR and its co-chaperone FKBP5. FKBP5 was also decreased in MDD patients exposed to physical neglect compared with MDD patients without physical neglect and the decreased FKBP5 expression was found to associate with physical neglect severity in the depressed patients. It is well established that FKBP5 is an important regulator of GR sensitivity and can promote nuclear translocation of the inactive beta (β) isoform of the GR thereby reducing sensitivity (Maeng et al., 2008, Zhang et al., 2008). However, in agreement with our findings Menke et al. (2013) recently reported that MDD patients with an FKBP5 polymorphism show a

decreased induction of GR-induced FKBP5 expression in parallel with reduced plasma cortisol suppression following dexamethasone stimulation. Only patients with this polymorphism exhibited glucocorticoid resistance. Consequently, it is possible that the impact of early life stress on the endocrine system may induce epigenetics changes such as a change in the transcriptional regulation of FKBP5 gene resulting in a biologically distinct subset of depressed patients with a dysregulated stress response incapable of regulating the inflammatory response upon secondary stressful challenges.

4.4.4 Limitations

While these findings are very interesting and provide a new dimension to the literature, as research using the PAXgene system to study major depression is scarce, some of the significant findings highlighted in this study do not survive Bonferroni correction for multiple testing. However, while this reduces the risk of type I error, it has the potential to inflate type 2 error, thereby increasing the risk of false negative findings. These results are also limited by the fact that we have only assessed changes in the biological profile in the depressed cohort with and without a history of distinct types of early life stress. While these results would have been strengthened by the inclusion of a comparable healthy control group, this type of analysis was not possible due to the limited number of participants in the sub-groups and high variability amongst the clinical samples. However, mere assessment of the values suggests that with greater participant numbers, similar findings may be observed with increased inflammatory markers and decreased glucocorticoid marker mRNA expression in the MDD patients with a history of early life stress relative to healthy controls also. The lack of noticeable difference between the depressed patients relative to the healthy controls may be ascribable to the fact that major depression is a heterogeneous disorder and in order to detect specific biomarkers a cleaner phenotype maybe required. This argument is strengthened by the fact that we do detect associations between biological markers and greater depression severity, symptomology and individual childhood trauma subscales. Hence, future studies should endeavour to assess distinct subsets of MDD patients with a well characterised phenotype in association with a more sensitive symptom-wise approach.

4.4.5 Conclusion

In conclusion, despite the fact that the biological profile was largely unchanged in the depressed cohort compared with age and sex matched healthy controls, further assessment of the depressed cohort based on depression severity and depressive symptomology revealed a positive association between the innate inflammatory response and greater depression severity. Further to this, the affective symptoms of MDD such as core depression and anxiety were also found to positively correlate with the transcriptional levels of the classical pro-inflammatory cytokine IL-1β and the immune activating receptor TREM-1. Assessment of the biological profile in association with distinct traumatic early life events in the depressed cohort also revealed increased PAXgene transcriptional expression of IL-1β and IL-6 in patients reporting childhood abuse while decreased expression of the GR and its regulator FKBP5 were associated with neglect. Taken together, these data support previous literature associating the inflammatory and endocrine systems with major depression in certain sub-groups of depressed individuals.

Chapter 5

Inflammation, kynurenine pathway activation and tryptophan depletion - The relative contribution of innate vs. adaptive immune cells

5.1 Introduction

While the biological basis of depression remains to be elucidated, two main theories on the pathophysiology of the illness are the serotonin hypothesis and the inflammatory hypothesis of depression. With regard to the serotonin hypothesis of depression, deficits in serotonergic neurotransmission have long been associated with major depression with selective serotonin reuptake inhibitors (SSRIs) being the first line treatment choice for a major depressive episode (Thase, 2011). In association with this numerous studies report reduced tryptophan availability for serotonin synthesis in depressed cohorts (DeMyer et al., 1981, Cowen et al., 1989, Hughes et al., 2012). However, in accordance with the inflammatory hypothesis of depression, recent meta-analyses suggest that pro-inflammatory cytokines may also have a pivotal role to play in the biological basis of major depression (Dowlati et al., 2010, Haroon et al., 2012, Liu et al., 2012).

A proposed mechanism linking the serotonergic hypothesis with the inflammatory hypothesis of depression is the activation of indoleamine 2,3-dioxygenase (IDO), the rate-limiting, tryptophan degrading enzyme of the kynurenine pathway, which is up-regulated in response to elevated secretion of inflammatory cytokines, particularly IFN-γ or IL-6 and TNF-α or IL-1β in combination (Leonard and Maes, 2012). Upon IDO activation, tryptophan is shunted down the kynurenine pathway, thereby reducing its availability for serotonin synthesis and increasing the production of kynurenine (Moffett and Namboodiri, 2003). The ratio of kynurenine to tryptophan (KYN/TRP ratio) is used as a proxy measure of IDO activity (Christmas et al., 2011). Activation of the kynurenine pathway and subsequent tryptophan depletion has frequently been reported in cytokine-induced depression (Capuron et al., 2002b, Wichers et al., 2005, Raison et al., 2010). However, evidence to suggest activation of the kynurenine pathway in idiopathic depressed patients is scarce.

IDO is expressed in all organs of the body and in many cells types to include innate immune cells of the myeloid lineage such as monocytes and dendritic cells, along with cells from the adaptive immune response such as T-cells (Maes et al., 2011). However, while dysregulation of both the innate and adaptive immune response have been implicated in the pathogenesis of depression, the ability of innate vs. adaptive immune cells to deplete tryptophan has never been studied.

The bacterial endotoxin lipopolysaccharide (LPS) is widely used to induce an innate immune response in experimental research. LPS stimulation of peripheral blood mononuclear cells (PBMCs) occurs via interactions with Toll-like receptors (TLRs) expressed by innate immune cells. Specifically, LPS complexes with TLR4 and MD-2 which initiates the activation of the MyD88-dependent signal transduction pathway culminating in the activation of nuclear factor-κB (NFκB)

which induces the transcription of pro-inflammatory cytokines (Lu et al., 2008). T-cell activation and proliferation via anti-CD3 stimulation is commonly used in vitro to assess T-cell functional ability. The CD3 T-cell receptor is composed of a complex of integral membrane protein chains to include gamma, delta, zeta and epsilon. This CD3 receptor complexes with $CD4^+$ or $CD8^+$ co-receptors, which in turn are stimulated upon recognition of antigen bound to MHC complexes on antigen presenting cells (APCs). In the *in vitro* setting, anti-CD3 has the ability to bind to the epsilon subunit of the CD3 receptor which has an intracellular signalling domain and consequently can induce cell activation upon ligand binding. The presence of monocytes in culture provides the co-stimulatory signal for enhanced T-cell activation.

Therefore, the aim of this study was to compare inflammatory cytokine production, IDO mRNA expression and tryptophan depletion/kynurenine production in human PBMCs 6, 24, 48 hours following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3.

Further to this, I sought to assess if immune cells from severely depressed patients were more sensitive to the ability of inflammatory stimuli to induce pro-inflammatory cytokine production and kynurenine pathway activation compared with healthy controls. In addition, the circulating concentrations of kynurenine and tryptophan were assessed in the depressed patients and healthy controls.

Subsequently, I sought to assess the effect of therapeutic intervention with ECT on stimulated inflammatory profiles and the kynurenine pathway activation profile along with the impact of ECT on circulating measures of kynurenine pathway activity.

5.2 Methods

5.2.1 Experimental design

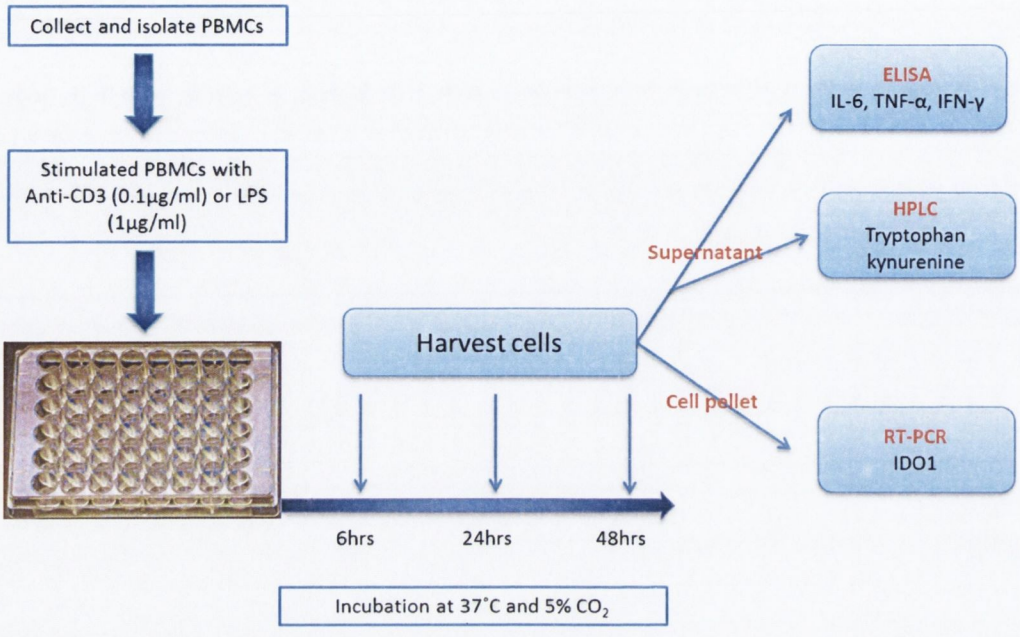

Figure 5.1 Diagrammatic representation of the experimental design

5.2.2 Experimental Procedure

5.2.2.1 Cell culture and preparation of treatment compounds

Culture Media: Using a 0.2 micrometre (µm) syringe filter, Fetal Bovine Serum (FBS) and penicillin-streptomycin were filter- sterilised and added to sterile RPMI 1640 1X containing L-Glutamine and 25 mM HEPES to give complete RPMI (cRPMI). Final concentrations of FBS and penicillin-streptomycin in the media were 10% and 1% respectively.

LPS: 25 mg LPS [Escherichia coli serotype 0111:B4] was dissolved in 1 ml d.d.H_2O. When fully dissolved 500 µl of this 25 mg/ml solution was added to 62 ml of cRPMI (1:125 dilution) to yield a 200 µg/ml stock solution that was then filter-sterilised using a 0.2 µm syringe filter. This stock solution was divided into 1 ml aliquots and stored at -20°C. Prior to use the stock solution was diluted to a working concentration of 1 µg/ml in pre-warmed cRPMI.

For LPS stimulation, 450 µl of a 1 x 10^6 cell suspension was added to the respective wells on a 48-well plate and the treated with 50 µl of 1 µg/ml LPS. This dose was selected based on previous experimental work carried out in the lab (Curtin et al., 2009, O'Sullivan et al., 2009).

Anti-CD3: 0.5 ml of a 1 mg/ml stock solution was stored at 2-8°C. Prior to use the stock was diluted to a working concentration in pre-warmed cRPMI.

For anti-CD3 stimulation, the treatment wells were coated with 200 µl of 0.1 µg/ml anti-CD3 and incubated overnight at 4°C. Following this, the excess anti-CD3 was removed and 450 µl of a 1 x 10^6 cell suspension was added to wells on a 48-well plate. A dose of 0.1 µg/ml for 48 hours was selected based on the results from the dose response and time-course study (Figure 5.2).

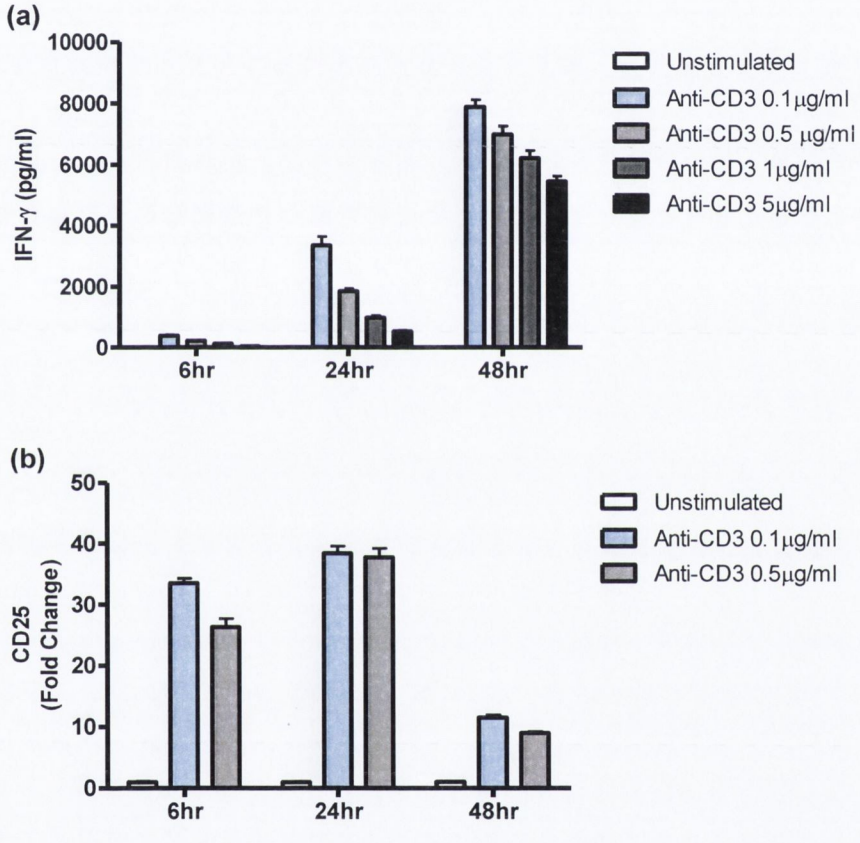

Figure 5.2 Anti-CD3 dose response over time. PBMCs isolated from healthy controls were stimulated for 6, 24 and 48 hours with a range of doses for anti-CD3 including: 0.1, 0.5, 1 and 5 µg/ml. (a) Anti-CD3 dose response over time for IFN-γ cytokine production in culture supernatant. (b) Relative mRNA expression for the T-cell activation marker CD25 following stimulation with anti-CD3 at 0.1 or 0.5 µg/ml. n=4 per group.

5.2.2.2 Real-Time PCR analysis of CD25 and IDO1

Total RNA was extracted from the PBMCs with the NucleoSpin®Total RNA isolation kit (Macherey-Nagel, Germany) and DNase treated per kit instructions. Following RNA quantification and equalisation, cDNA was synthesised using a cDNA archive kit (Applied Biosystems, UK) (see Chapter 2 section – 2.2.4).

Gene expression analysis was conducted using Real-Time PCR employing Taqman® Gene Expression Assays (Applied Biosystems, UK). To quantify expression of target genes of interest, Taqman® Gene Expression Assays containing FAM-labelled probes were used (Assay IDs: IDO: Hs00984148; CD25: Hs00907779_m1). PCR reactions were in a duplex format also containing a Taqman®Gene Expression Assay (primer-limited) containing a VIC-labelled probe for the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH assay ID:4326317E). Samples were assayed using Applied Biosystems universal cycling conditions using a fast protocol on the StepOnePlus™ Real-time PCR system (Applied Biosystems, UK). Fold change in gene expression from the unstimulated cells was calculated using the ΔΔCt method and GAPDH served as an endogenous control in the amplification system.

5.2.2.3 Inflammatory cytokine production in PBMC culture supernatants

PBMC supernatant concentrations of IL-6, TNF-α, IFN-γ were measured using ELISA MAX™ Deluxe kits (Biolegend, UK) and these immune assays were performed according to manufacturer's instructions. Absorbance was read at 450 nm using a microplate reader (Sunrise Tecan, Reading, UK) and was then recalculated as a concentration (pg/ml) using standard curves derived using GraphPad Prism Software Version 5.00 (GraphPad software, Inc.). Limits of detection for the ELISAs were 4 pg/ml for IL-6 and IFN-γ, 2 pg/ml for TNF-α.

5.2.2.4 Measurement of tryptophan and kynurenine metabolites using HPLC

Culture supernatant tryptophan and kynurenine concentrations were measured using HPLC. Kynurenine was measured at a wavelength of 250 nm by UV detection while tryptophan was measured fluorometrically at an excitation wavelength of 254 nm and an emission wavelength of 404 nm.

Homogenisation buffer was prepared with mobile phase containing 6% perchloric acid and 200 ng/20 µl of internal standard (N-methyl-serotonin) (Sigma Chemicals Co., UK). The mobile

phase contained 50 nM glacial acetic acid, 100 mM zinc acetate and 3% acetonitrile at pH 4.9. A 1:1 ratio of supernatant and homogenisation buffer was centrifuged at 12,800 rpm for 20 min at 4°C before being passed through a 0.45 µm syringe filter (Phenomenex UK). A 20 µl sample of the resultant filtrate was injected onto a C-18 reverse phase Kinetex core-shell technology column (Phenomenex, UK) fitted with a guard column for separation of metabolites in the sample (flow rate 0.8ml/min). Retention times and peak heights were recorded from the chromatographs generated and together with the data obtained for the standards, (5 ng/20 ul standard mix of tryptophan and kynurenine and N-methyl 5-HT) were used to calculate tryptophan and kynurenine concentrations expressed as ng/ml of plasma.

5.2.3 Participants

The study included 16 adult in-patients with severe treatment-resistant depression from the mental health services of St. Patrick's University Hospital, Dublin, and who were being referred for ECT. The diagnosis of these patients with severe depression was a clinical diagnosis based on Diagnostic and Statistical Manual for Mental Disorders IV (DSM-IV, American Psychiatric Association, 2000) criteria and confirmed by an independent psychiatrist using the 24-item Hamilton Rating Scale for Depression (HAM-D 24) (Hamilton, 1960). Sixteen healthy controls were recruited from the local community and the groups were balanced for age and sex. The study was conducted under real-life conditions and all patients were receiving antidepressant medication when recruited to the trial. To be included in the study, participants had to be over 18 years old, be referred for ECT for treatment of a major depressive episode as diagnosed by the Structured Clinical Interview for DSM-IV Axis I Disorders (First et al., 1996) and have a pre-treatment HAM-D 24 score of at least 21. Exclusion criteria were substance misuse in the previous 6 months, being medically unfit for general anaesthesia, ECT in the previous 6 months, dementia or other axis I diagnosis.

ECT was administered twice weekly (6-12 treatments on average) with hand-held electrodes, as previously described, using methohexitone (0.75–1.0 mg/kg) for anaesthesia and suxamethonium (0.5–1.0 mg/kg) as muscle relaxant (Eranti et al., 2007). The Spectrum 5000M device (Mecta Corp., Oregon, USA) was used according to the manufacturer's instructions. Seizure duration was measured by electroencephalography (EEG) monitoring. Seizure threshold was established by a method of limits after which treatments were given at 1.5 times seizure threshold for bitemporal ECT or 6.0 times seizure threshold for right unilateral ECT. Patients were maintained on their usual medication during the ECT course.

5.2.4 Clinical diagnostic test

The Hamilton Rating Scale for Depression

Depression severity was assessed in patients and controls using the HAM-D 24 (Hamilton, 1960). This scale is the most frequently used, validated and standardised assessment for major depression and surveys a range of symptoms most commonly observed in depressed patients. The HAM-D 24 item total score is the sum of all 24 items listed and was selected as it contains cognitive items which aid in the assessment of chronic major depression (Steidtmann et al., 2012) It is also most frequently used to assess depression severity in severely depressed patients receiving ECT. A HAM-D 24 rating was obtained at two evaluating time-points: 1-2 days before the first ECT treatment (Baseline) and 3-4 days after completing an adequate course of ECT (End of Treatment). Criteria for responders were a ≥60% decrease in HAM-D 24 score from baseline plus an end of treatment HAM-D score ≤16. Remission criteria were a ≥60% decrease in HAM-D 24 score from baseline plus an end of treatment HAM-D score ≤10. There was no prescribed minimum or maximum number of ECT sessions.

5.2.5 Procedures

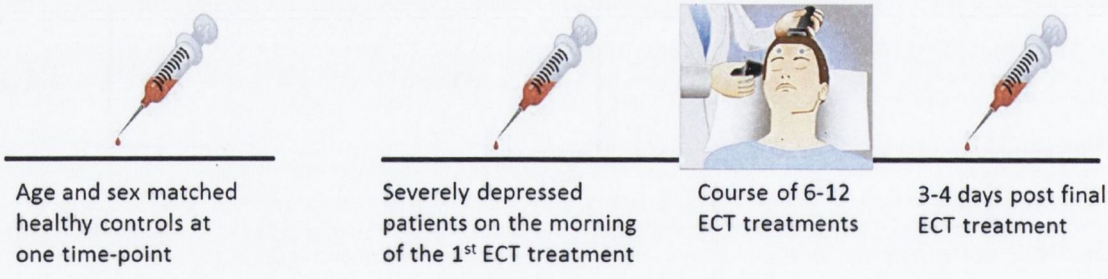

Figure 5.3 Diagrammatic representation of the recruitment and blood sampling process. A baseline blood sample was taken on the morning of the first ECT treatment and 3-4 days following the final ECT treatment. ECT was administered twice weekly with an average of 6-12 treatments. Age and sex matched healthy controls were assessed at one time point when a blood sample was acquired and clinical testing carried out.

5.2.5.1 Blood sampling and processing

Fasting whole blood samples were taken in sodium heparin tubes from the antecubital fossa between 07:30 am and 09:30 am. A 10 ml blood sample was taken from each subject into a K2 EDTA vacutainer and centrifuged at 2000 rpm for 10 minutes to separate plasma. Plasma was stored at -80˚C until inflammatory cytokine concentrations were assessed. A second blood sample was taken into two 9 ml heparinised vacutainers for PBMC isolation. Bloods were processed within 1 hour following blood draw and the PBMCs were isolated from the heparinised blood samples by Ficoll/Histopaque 1077 density gradient centrifugation. PBMCs were washed in RPMI 1640 medium, counted and resuspended in freezing solution (90% FBS + 10% DMSO), before being divided into 1 ml aliquots and cryopreserved in cryovials in liquid nitrogen storage (see Chapter 2 section2.2.3).

Prior to experimental procedure, PBMCs were thawed and washed in medium. Viability was assessed using trypan blue staining and the counted cells were adjusted to a concentration of 1 x 10^6 cells/ml in cRPMI (supplemented with 10% (v/v) FBS and antibiotics) prior to stimulation (see Chapter 2 section2.2.3).

5.2.6 Statistical analysis

Normally distributed data are presented as mean with standard error of the mean (SEM). Data were analysed using a two-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test. Differences in demographic and clinical variables were tested using student's *t*-test and Chi square (χ^2) test for gender distribution and smoking. Normally distributed circulating kynurenine and tryptophan concentrations were analysed using the student's *t*-test. Following Shapiro-Wilk test for normality, it was revealed that the clinical PBMC data were not normally distributed. As there is no equivalent non-parametric test to a two-way ANOVA, the data were log transformed. However, as this did not improve the distribution of the data, statistical analysis was carried out using a Kruskal-Wallis one-way ANOVA followed by either a Mann-Whitney U test for independent sample comparisons or a Wilcoxon-Signed Rank test for paired comparisons. All statistical analyses were considered significant when $p \leq 0.05$. Graphs and statistics were generated using GraphPad Prism Software Version 5.00 (GraphPad software, Inc). All data were analysed using SPSS (Version 16).

5.3 Results

5.3.1 Assessment of pro-inflammatory cytokine production in culture supernatants isolated from healthy human PBMCs following stimulation with LPS or anti-CD3

5.3.1.1 IL-6 production from healthy human PBMCs stimulated with LPS or anti-CD3

A two-way ANOVA for IL-6 revealed a significant main effect of treatment [$F_{(2,45)} = 82.59$, $p \leq 0.001$] and of time [$F_{(2,45)} = 6.290$, $p \leq 0.01$] [Figure 5.4a]. Bonferroni *post-hoc* analysis revealed that innate immune stimulation with LPS induced a robust increase in IL-6 at 6, 24 and 48 hours ($p \leq 0.001$) relative to concentrations from unstimulated cells, while a significant increase in the anti-CD3 stimulated IL-6 expression was observed at 24 ($p \leq 0.05$) and 48 hours ($p \leq 0.01$) compared with unstimulated IL-6 concentrations. The LPS induced increase in IL-6 was significantly greater than that observed following anti-CD3 stimulation at all three time points ($p \leq 0.001$) [Figure 5.4a].

5.3.1.2 TNF-α production from healthy human PBMCs stimulated with LPS or anti-CD3

A two-way ANOVA for TNF-α revealed a significant treatment x time interaction [$F_{(4,47)} = 7.889$, $p \leq 0.001$] and a main effect of treatment [$F_{(2,47)} = 19.31$, $p \leq 0.001$] and time [$F_{(2,47)} = 3.495$, $p \leq 0.05$] [Figure 5.4b]. While not statistically significant following Bonferroni *post-hoc* analysis, LPS did induce a 55-fold increase in TNF-α concentrations relative to unstimulated levels peaking at 6 hours and decreasing over time ($p > 0.05$ at each time point). In contrast, anti-CD3 induced a much more robust increase in TNF-α concentrations relative to unstimulated levels, which increased in a time-dependent manner from 24 ($p \leq 0.001$) to 48 hours ($p \leq 0.001$) [Figure 5.4b].

5.3.1.3 IFN-γ production from healthy human PBMCs stimulated with LPS or anti-CD3

A two-way ANOVA for IFN-γ revealed a significant treatment x time interaction [$F_{(4,47)} = 21.46$, $p \leq 0.001$] and a main effect of treatment [$F_{(2,47)} = 54.14$, $p \leq 0.001$] and of time [$F_{(2,47)} = 11.55$, $p \leq 0.001$] [Figure 5.4c]. While Bonferroni *post hoc* analysis revealed no significant differences in the LPS induced culture supernatant concentrations of IFN-γ ($p > 0.05$), a 3, 6 and 5 fold increase in LPS stimulated IFN-γ concentrations was observed at 6, 24 and 48 hours respectively. In contrast to this, anti-CD3 induced a robust time dependent increase in IFN-γ culture supernatant concentrations with a highly significant 244 fold increase at 24 hours ($p \leq 0.001$) and a 335 fold increase at 48 hours ($p \leq 0.001$) relative to concentrations from unstimulated PBMCs [Figure 5.4c].

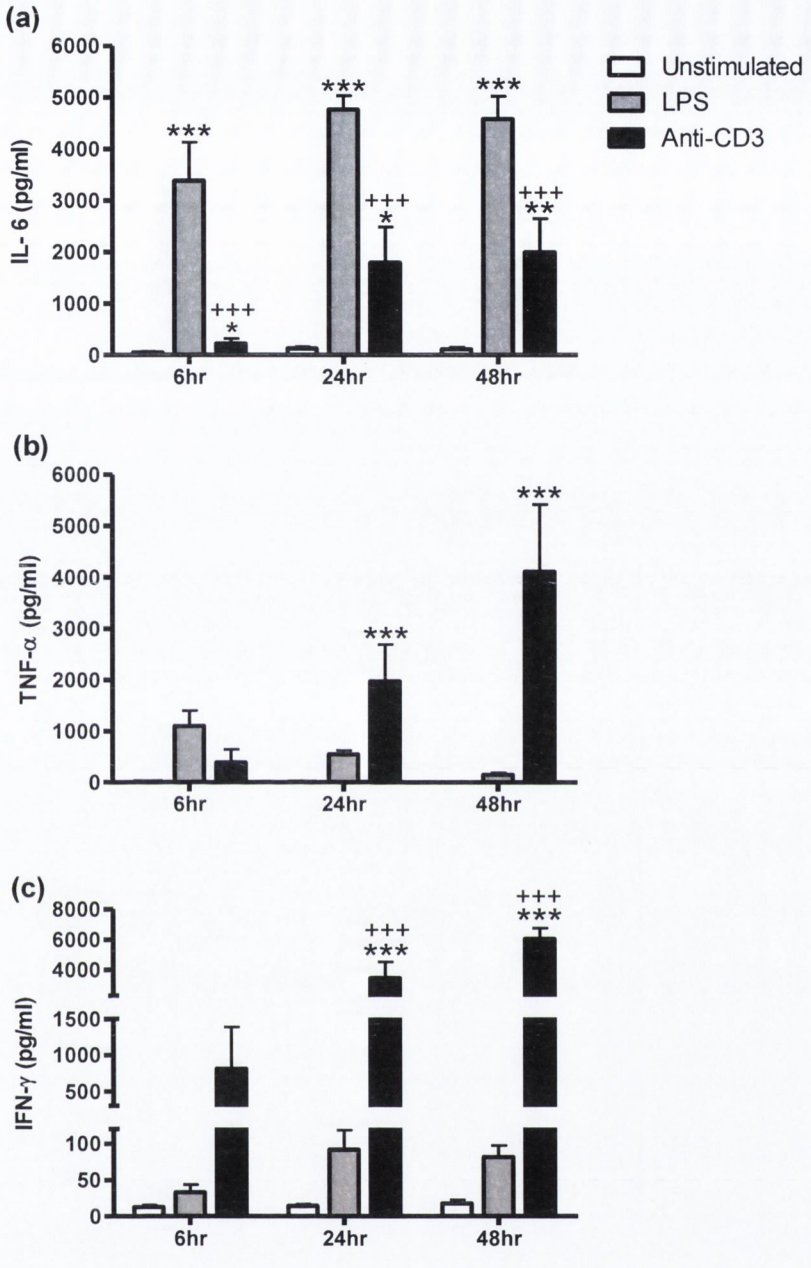

Figure 5.4 Pro-inflammatory cytokine production following stimulation with LPS or anti-CD3. Healthy human PBMC cell culture supernatant concentrations of (a) IL-6, (b) TNF-α and (c) IFN-γ at 6, 24 and 48 hours following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3. Data expressed as mean with SEM (n=5-7 per group). *p≤0.05, **p≤0.01, ***p≤0.001 vs. unstimulated human PBMC concentrations at each time-point; +++p≤0.001 vs. LPS stimulated control concentrations at each time-point (two-way ANOVA followed by Bonferroni *post-hoc* test).

5.3.2 Assessment of IDO mRNA expression in healthy human PBMCs following stimulation with LPS or anti-CD3

A two-way ANOVA for IDO revealed a significant treatment x time interaction [$F_{(4,46)}$ = 4.990, p≤0.01] and a main effect of treatment [$F_{(2,46)}$ = 23.32, p≤0.001]. However, the effect of time was not significant [$F_{(2,46)}$ = 1.338, p>0.05] [Figure 5.5]. Bonferroni *post hoc* analysis revealed that LPS stimulated IDO transcriptional expression was significantly elevated with respect to unstimulated IDO expression at 6 (p≤0.01), 24 (p≤0.05) and 48 hours (p≤0.001). In contrast to the sustained LPS stimulated increase in IDO mRNA expression over time, anti-CD3 induced an early but significant increase in the transcriptional expression in IDO mRNA at 6 hours (p≤0.001), which was decreased but remained significant at 24 hours (p≤0.05) and decreased further at 48 hours to a level that was not significantly different to that observed in the unstimulated PBMCs (p>0.05) [Figure 5.5]. Similar IDO expression levels were observed following stimulation with LPS and anti-CD3 at 6 and 24 hours. However, the LPS stimulated IDO mRNA expression was significantly greater at 48 hours than that induced by mitogen stimulation (p≤0.001) [Figure 5.5].

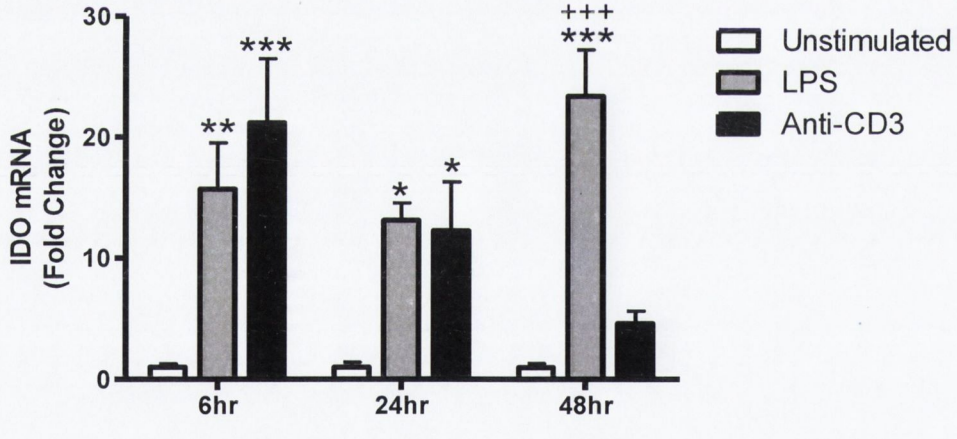

Figure 5.5 IDO mRNA expression following stimulation with LPS or anti-CD3 in healthy human PBMCs. Relative mRNA expression for IDO1 at 6, 24 and 48 hours following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3. Data expressed as mean with SEM (n=5-7 per group). *p≤0.05, **p≤0.01, ***p≤0.001 vs. unstimulated healthy control concentrations at each time-point; +++p≤0.001 vs. anti-CD3 stimulated control concentrations (Two-way ANOVA followed by Bonferroni *post-hoc* test).

5.3.3 Assessment of the kynurenine pathway activation in healthy human PBMCs following stimulation with LPS or anti-CD3

5.3.3.1 Kynurenine concentrations following stimulation with LPS or anti-CD3 in healthy human PBMCs

A two-way ANOVA for kynurenine revealed a significant treatment x time interaction [$F_{(4,43)}$ = 7.795, $p \leq 0.001$] and a main effect of treatment [$F_{(2,43)}$ = 17.50, $p \leq 0.001$] and of time [$F_{(2,43)}$ = 17.77, $p \leq 0.001$] [Figure 5.6a]. Bonferroni *post-hoc* analysis revealed that LPS stimulated kynurenine concentrations were not significantly different to the unstimulated concentrations observed at 6, 24 and 48 hours in healthy human PBMC culture supernatants ($p > 0.05$). In contrast to this, anti-CD3 induced a time dependent increase in kynurenine concentrations with a modest increase at 24 hours ($p \leq 0.05$) and a highly significant increase at 48 hours relative to unstimulated cells ($p \leq 0.001$) [Figure 5.6a]. Anti-CD3 also stimulated a significantly greater production of kynurenine in the PBMC culture supernatants relative to that induced by LPS at 48 hours ($p \leq 0.001$).

5.3.3.2 Tryptophan concentrations following stimulation with LPS or anti-CD3 in healthy human PBMCs

A two-way ANOVA for tryptophan revealed a significant treatment x time interaction [$F_{(2,43)}$ = 3.870, $p \leq 0.01$] and a main effect of treatment [$F_{(2,43)}$ = 9.455, $p \leq 0.001$] however, the effect of time was not significant [$F_{(2,43)}$ = 0.7025, $p > 0.05$] [Figure 5.6b]. Bonferroni *post-hoc* analysis revealed that treatment with the innate immune stimulus LPS had no effect on tryptophan concentrations in the culture supernatants relative to unstimulated tryptophan concentrations at any of the three time points ($p > 0.05$). In contrast to this, T-cell stimulation with anti-CD3 induced a significant decrease in tryptophan concentrations at 48 hours relative to unstimulated tryptophan concentrations ($p \leq 0.001$). The decreased tryptophan concentration was also found to be significantly reduced relative to that observed in the LPS stimulated culture supernatants ($p \leq 0.01$) [Figure 5.6b].

5.3.3.3 Kynurenine/tryptophan ratio following stimulation with LPS or anti-CD3 in healthy human PBMCs

A two-way ANOVA for the KYN/TRP ratio revealed a significant treatment x time interaction [$F_{(2,43)}$ = 7.620, p≤0.001] and a main effect of treatment [$F_{(2,43)}$ = 18.02, p≤0.001] and of time [$F_{(2,43)}$ = 12.39, p≤0.001] [Figure 5.6c]. Bonferroni *post-hoc* analysis revealed that treatment with the innate immune stimulus LPS did not induce a greater degree of IDO activity compared with unstimulated IDO activity in healthy human PBMCs at any of the 3 time points (p>0.05). In contrast to this, T-cell stimulation with anti-CD3 induced a significant increase in the KYN/TRP ratio at 24 hours (p≤0.05) which was further increased relative to unstimulated levels at 48 hours (p≤0.001). The KYN/TRP ratio was also significantly greater following anti-CD3 stimulation compared with LPS stimulation at 48 hours (p≤0.01) [Figure 5.6c].

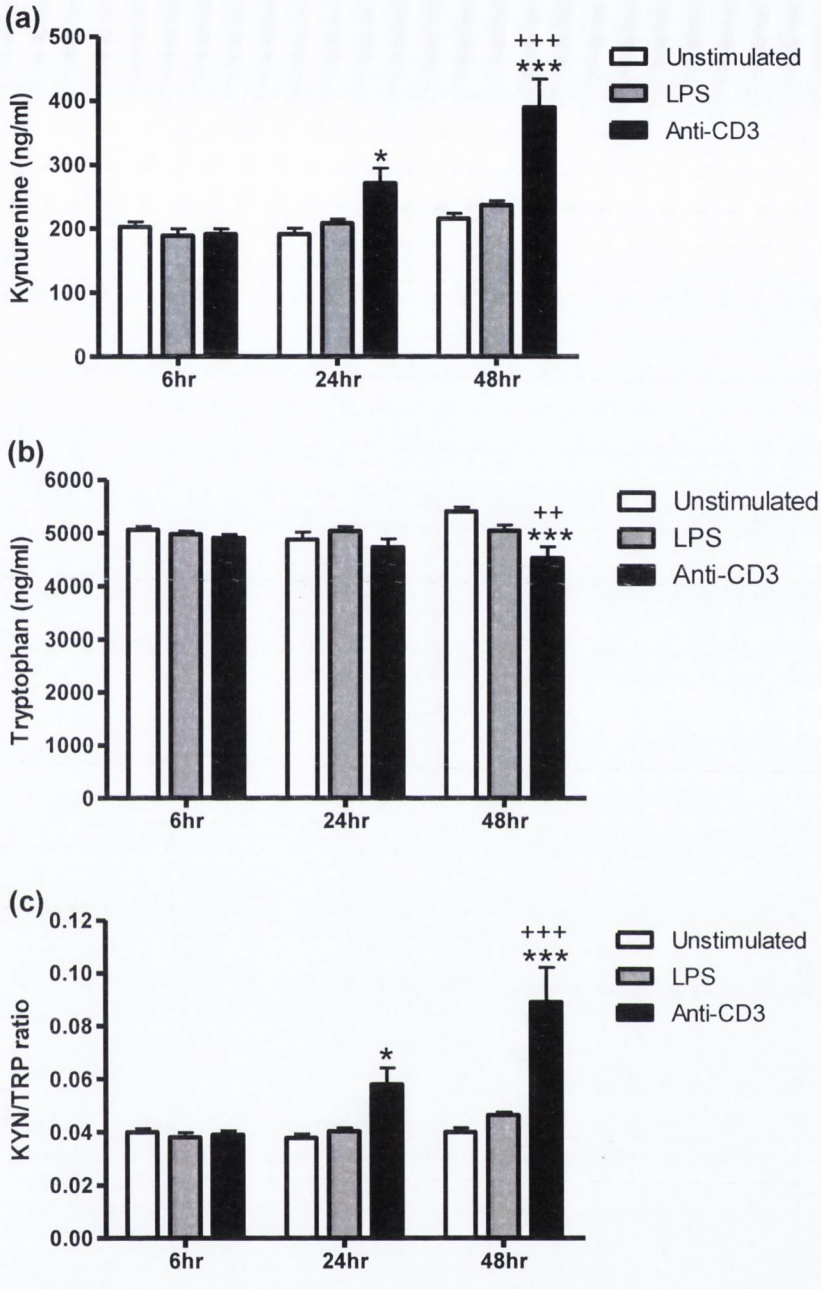

Figure 5.6 Kynurenine pathway activation profile in healthy human PBMCs following stimulation with LPS or anti-CD3. Healthy human PBMC cell culture supernatant concentrations of (a) kynurenine (b) tryptophan and (c) KYN/TRP ratio at 6, 24 and 48 hours following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3. Data expressed as mean with SEM (n=5-7 per group). *p≤0.05, ***p≤0.001 vs. unstimulated human PBMC concentrations at each time-point; +++p≤0.001 vs. LPS stimulated metabolite concentrations at each time-point (Two-way ANOVA followed by Bonferroni *post-hoc* test).

Subsequently, based on these findings and using the same experimental approach, I sought to assess if severely depressed patients are more sensitive to the ability of inflammatory stimuli to induce kynurenine pathway activation. Therefore, along with assessing circulating plasma kynurenine and tryptophan concentrations, I measured inflammatory cytokine secretion, IDO mRNA expression and kynurenine production coupled with tryptophan depletion in blood cells obtained from severely depressed patients compared to and age and sex matched healthy control subjects activated with the innate immune stimulus LPS or the T-cell stimulus anti-CD3.

5.3.4 Participant demographic data and patient treatment review

5.3.4.1 Demographic data for severely depressed patients and healthy controls

Sixteen severely depressed patients prior to therapeutic intervention with ECT and 16 healthy controls were recruited. For participant demographic data see Table 5.1. Consistent with a diagnosis of depression the depressed group had a significantly elevated HAM-D 24 score ($p \leq 0.001$) compared to the control group (MDD: 30.19 (6.34) vs. Control 4.00 (4.02)). Analyses revealed that there was no significant difference in age and gender ($p > 0.05$) while the depressed group had a significantly higher BMI ($p = 0.014$) [Table 5.1]. A significant difference was also revealed for smoking: 7 out of 16 patients smoked while no participants in the control group smoked. The control group consumed significantly more alcohol units per week prior relative to the depressed patients prior to their admission to hospital ($p \leq 0.001$) [Table 5.1].

Table 5.1 Demographic data for severely depressed patients and healthy controls. Depicted are demographic variables and clinical depression severity measured with the HAM-D 24 scale

	Control (n=16)	Depressed (n=16)	Statistic (p-value)
Gender (female/male)	14/2	14/2	$\chi^2 = 0.00$, (p=1.000)
Age [years]	59.81 (10.68)	57.25 (10.05)	t=0.699, df=1,30, (p=0.490)
BMI	22.11 (1.73)	25.63 (5.08)	t=-2.618, df=1,29, (p=0.014)
Alcohol consumption (units per week)	7.5 (4.0)	0.37 (1.08)	t=6.730, df=1,29, (p=0.000)
Smoking (Yes/No)	0/16	7/9	$\chi^2 = 8.96$, (p=0.003)
Number of previous episodes	–	7.56 (6.14)	–
Length of episode (weeks)	–	12.56 (8.20)	–
Number of ECT treatments	–	9.00 (2.25)	–
Hamilton Depression Score (HAM-D 24)	4.00 (4.02)	30.19 (6.34)	t=-13.961, df=1,30, (p=0.000)

Data expressed as mean with SD in parentheses and statistical analysis was performed using Student's t-test (Age, BMI, Alcohol consumption) and Chi squared (χ^2) test (Gender and Smoking).

5.3.4.2 Antidepressant medication review for the severely depressed patients

As this study was carried out under real-life conditions, 15 out of the 16 patients (93.75%) were mediated at the time of recruitment [Table 5.2]. Patients continued taking their usual medications during the course of ECT with little to no change in the treatment strategy over the course of ECT treatment [Table 5.2]. The pharmacotherapy received by these patients is routinely used in clinical practice and includes a range of antidepressants, antipsychotics, mood stabilisers and anxiolytics [Table 5.2]. None of the control participants were on any psychotropic medications during the course of the study.

Table 5.2 Medication review for severely depressed patients

Medication Review	Depressed (n=16)	
	n	%
Selective serotonin reuptake inhibitors	2	12.5
Serotonin-noradrenaline reuptake inhibitors	7	43.75
Tricyclic antidepressants	4	25
Melatonergic antidepressants	3	18.75
Noradrenergic and specific serotonergic antidepressants	5	31.25
Tetracyclic antidepressants	3	18.75
Any antidepressants	15	93.75
Antipsychotics	12	75
Mood stabilisers	11	68.75
Anxiolytics	12	75
Hypnotics	8	50
	Mean	SD
Baseline number of antidepressants	1.688	0.60
End of treatment number of antidepressants	1.625	0.62

Data expressed as the number (n) and percentage (%) of people on various classes of medication and the mean and SD for antidepressant treatment upon commencement of ECT treatment (baseline) and end of treatment.

5.3.5 PBMC integrity and white blood cell (WBC) Count

Trypan blue staining showed that between 90.3 and 97.6% of the blood mononuclear cells were viable in the healthy controls with an average white blood cell count of 2.06 x 10^6 [Table 5.3]. Trypan blue staining showed that between 89.7 and 97.8% of the blood mononuclear cells were viable in the severely depressed patients with an average white blood cell count of 2.75 x 10^6 [Table 5.3]. Trypan blue staining showed that between 90.9 and 99.2% of the blood mononuclear cells were viable in the severely depressed patients post ECT with an average white blood cell count of 3.56 x 10^6 [Table 5.3].

Table 5.3 PBMC integrity and WBC count in isolated PBMC from healthy controls, severely depressed patients and the patient cohort following ECT

	Mean	SD
Controls		
Cell Viability (%)	94.83	2.50
WBC count	2.06 x 10^6	1.12
Severely Depressed		
Cell Viability (%)	95.35	2.26
WBC count	2.75 x 10^6	1.58
Patients Post-ECT		
Cell Viability (%)	95.13	2.21
WBC count	3.56 x 10^6	1.85

Data expressed as mean with SD (n=16 per group)

5.3.6 Assessment of circulating concentrations of kynurenine and tryptophan in severely depressed patients and healthy controls

5.3.6.1 Circulating kynurenine concentrations in severely depressed patients relative to healthy controls

A student's *t*-test revealed no significant difference in the circulating concentrations of kynurenine in the severely depressed patients relative to healthy controls [t=1.252, df=24, p=0.223] [Figure 5.7a].

5.3.6.2 Circulating tryptophan concentrations in severely depressed patients relative to healthy controls

A student's *t*-test revealed a highly significant decrease in the circulating tryptophan concentrations in the severely depressed patients relative to healthy controls [t=4.206, df=24, p≤0.001] [Figure 5.7b].

5.3.6.3 Kynurenine/tryptophan ratio in severely depressed patients relative to healthy controls

A student's *t*-test revealed no significant difference in the KYN/TRP ratio between severely depressed patients and healthy controls [t=-1.046, df=24, p=0.306] [Figure 5.7c].

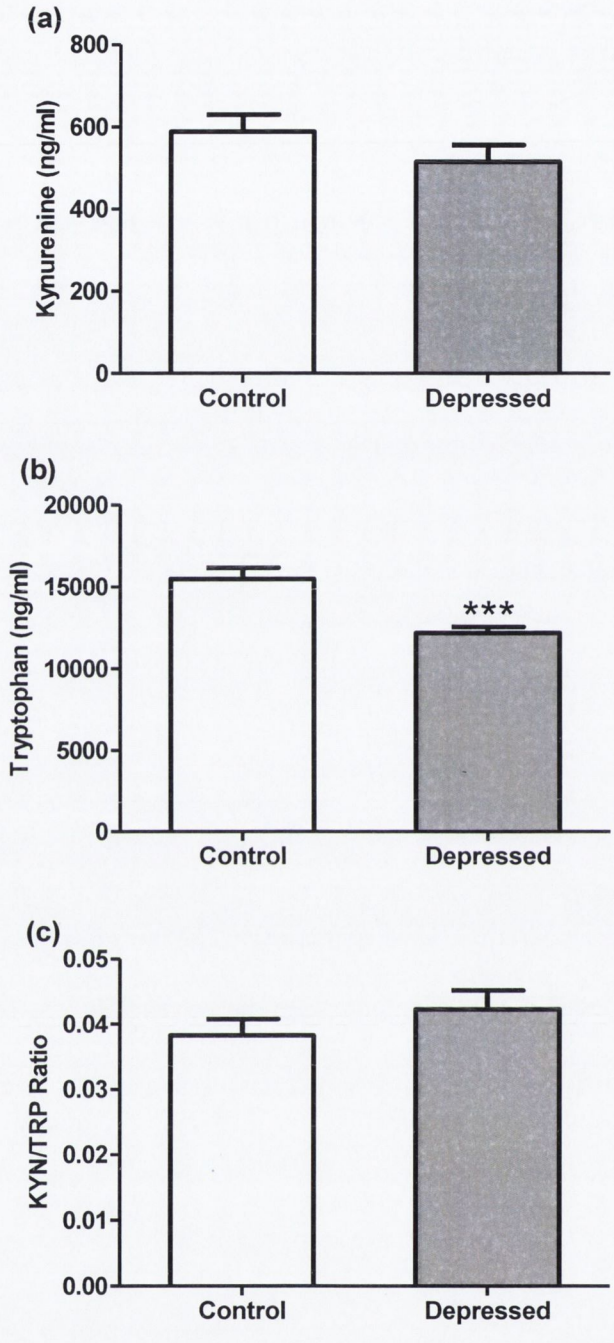

Figure 5.7 Circulating kynurenine pathway activation profile in severely depressed patients relative to healthy controls. Plasma concentrations of (a) kynurenine, (b) tryptophan and (c) KYN/TRP ratio in severely depressed patients relative to healthy controls. Data expressed as mean with SEM (n=13 per group). ***p≤0.001 vs. control (Student's *t* test).

5.3.7 Assessment of pro-inflammatory cytokine production following innate or adaptive immune cell stimulation in severely depressed patients and healthy controls

5.3.7.1 Culture supernatant IL-6 concentrations from severely depressed patients and healthy controls following stimulation with LPS or anti-CD3

A Kruskal-Wallis one-way ANOVA revealed a significant difference in IL-6 culture supernatant concentrations from a cohort of severely depressed patients and healthy control subjects following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 [χ^2=43.914, df=5, p<0.001] [Figure 5.8a]. Subsequent Mann-Whitney U tests revealed a robust increase in LPS stimulated IL-6 concentrations in the control samples (Z=-4.417, p<0.001) while anti-CD3 induced a more modest but yet significant increase in IL-6 relative to unstimulated control levels (Z=-3.422, p<0.001). The LPS induced IL-6 concentrations in the controls were significantly greater than that observed following anti-CD3 stimulation (Z=-2.302, p=0.021). Similarly, in the depressed cohort, LPS (Z=-4.086, p<0.001) and anti-CD3 (Z=-2.883, p<0.001) induced a significant increase in IL-6 relative to unstimulated patient concentrations. However, the LPS stimulated IL-6 concentrations in the depressed participants were significantly greater than that observed following anti-CD3 stimulation (Z=-1.970, p=0.049). There was no significant difference in the unstimulated (Z=-0.519, p=0.604), LPS stimulated (Z=-0.104, p=0.917) and anti-CD3 stimulated (Z=-0.539, p=0.590) IL-6 concentrations between the depressed cohort and the healthy controls [Figure 5.8a].

5.3.7.2 Culture supernatant TNF-α concentrations from severely depressed patients and healthy controls following stimulation with LPS or anti-CD3

A Kruskal-Wallis one-way ANOVA revealed a significant difference in TNF-α culture supernatant concentrations from a cohort of severely depressed patients and healthy control subjects following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 [χ^2=40.028, df=5, p<0.001] [Figure 5.8b]. Subsequent Mann-Whitney U tests revealed a robust increase in LPS stimulated TNF-α concentrations in the control samples (Z=-4.274, p<0.001) while stimulation with anti-CD3 also induced a highly significant increase in TNF-α relative to unstimulated control levels (Z=-3.677, p<0.001). No significant difference was revealed between the LPS and anti-CD3 stimulated TNF-α concentrations in the control samples (Z=-0.073, p=0.383). In the depressed cohort, stimulation with LPS (Z=-2.944, p=0.003) and anti-CD3 (Z=-3.817, p<0.001) induced a robust increase in TNF-α relative to unstimulated patient concentrations. However, the anti-CD3 stimulated TNF-α concentrations were significantly greater than those

observed following LPS stimulation in the depressed cohort (Z=-1.976, p=0.048). There was no difference in the unstimulated (Z=-0.598, p=0.550), LPS stimulated (Z=-0.735, p=0.462) and anti-CD3 stimulated (Z=-1.011, p=0.312) TNF-α concentrations between the depressed cohort and the healthy controls [Figure 5.8b].

5.3.7.3 Culture supernatant IFN-γ concentrations from severely depressed patients and healthy controls following stimulation with LPS or anti-CD3

A Kruskal-Wallis one-way ANOVA revealed a significant difference in IFN-γ culture supernatant concentrations from a cohort of severely depressed patients and healthy controls following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 [χ^2=56.252, df=5, p<0.001] [Figure 5.8c]. Subsequent Mann-Whitney U tests revealed a robust increase in LPS stimulated IFN-γ concentrations in the control samples (Z=-2.646, p<0.001) while stimulation with anti-CD3 also induced a highly significant increase in IFN-γ concentrations relative to unstimulated control levels (Z=-4.487, p<0.001). The anti-CD3 induced IFN-γ production was greater than that of LPS in the control subjects (Z=-4.229, p<0.001). In the depressed cohort, the LPS induced increase in IFN-γ concentrations was not significantly different to that observed in the unstimulated depressed samples (Z=-0.783, p=0.433), while anti-CD3 stimulated a robust increase in IFN-γ concentrations relative to unstimulated patient concentrations (Z=-4.513, p<0.001). Anti-CD3 induced significantly more IFN-γ production in the depressed cohort relative to that induced by LPS (Z=-4.005, p<0.001). There was no significant difference in the unstimulated (Z=-0.256, p=0.798), LPS stimulated (Z=-1.591, p=0.112) and anti-CD3 stimulated (Z=-0.069, p=0.945) IFN-γ concentrations between the depressed cohort and the healthy controls [Figure 5.8c].

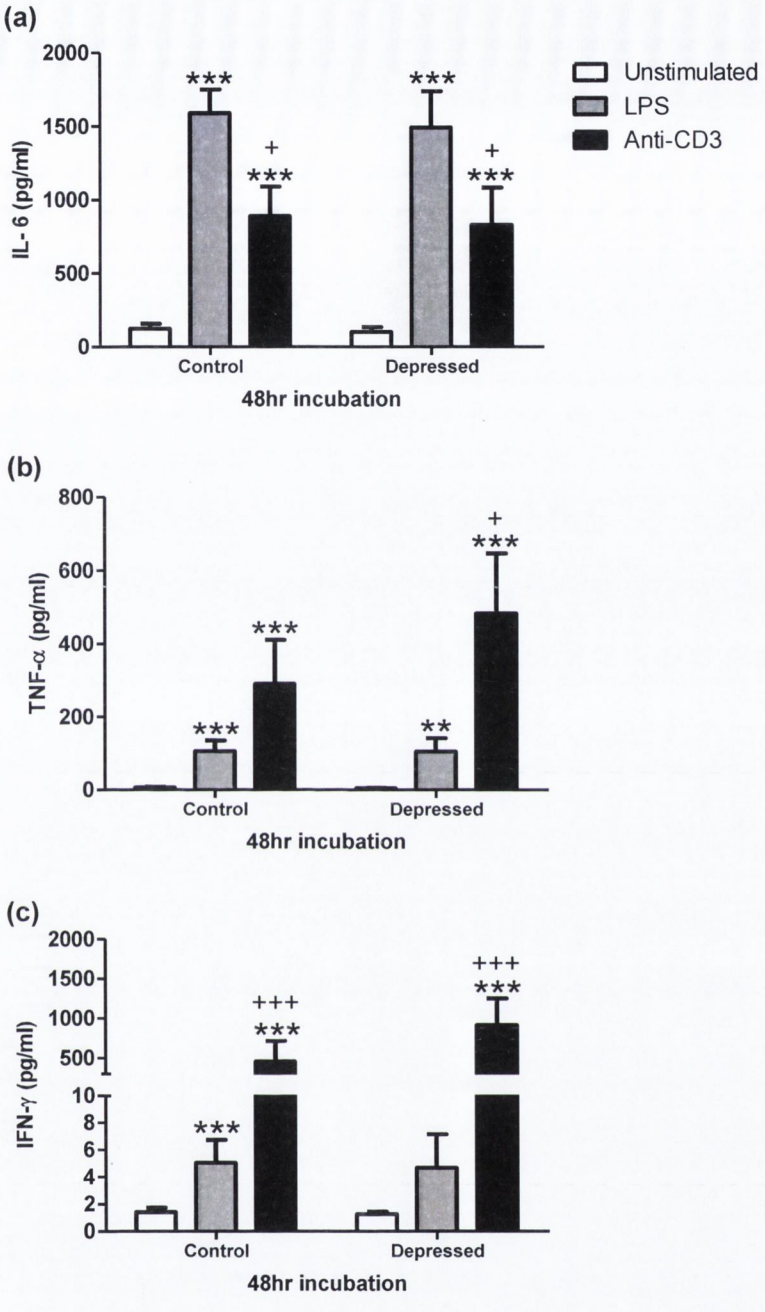

Figure 5.8 Culture supernatant pro-inflammatory cytokine profile in severely depressed patients and healthy controls following stimulation with LPS or anti-CD3. PBMCs isolated from severely depressed patients and age and sex matched healthy controls were incubated for 48 hours with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 and cell culture supernatant concentrations of (a) IL-6, (b) TNF-α and (c) IFN-γ were measured. Data expressed as mean with SEM (n=14-15 per group). **p≤0.01, ***p≤0.001 vs. unstimulated concentrations; +p≤0.05, +++p≤0.001 vs. LPS stimulated concentrations (Kruskal-Wallis one-way ANOVA followed by Mann-Whitney U tests).

5.3.8 IDO mRNA expression profile following innate or adaptive immune cell stimulation in severely depressed patients and healthy controls

A Kruskal-Wallis one-way ANOVA revealed a differential transcriptional IDO expression in PBMCs isolated from a cohort of severely depressed patients and healthy control subjects following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 [χ^2=42.816, df=5, p<0.001] [Figure 5.9]. Subsequent Mann-Whitney U tests revealed that in the control samples, LPS (Z=-3.923, p<0.001) and anti-CD3 (Z=-3.051, p=0.002) induced a significant increase in IDO mRNA expression relative to unstimulated control levels. However, the LPS induced increase in IDO transcriptional expression was significantly greater than that observed following anti-CD3 stimulation (Z=-2.590, p=0.010). In the depressed cohort, LPS (Z=-3.974, p<0.001) and anti-CD3 (Z=-3.615, p<0.001) induced a robust increase in IDO mRNA expression relative to unstimulated depressed patient expression levels. However, the LPS induced increase in IDO transcriptional expression was significantly greater than that observed following anti-CD3 stimulation in the depressed cohort (Z=-2.282, p=0.022). While no significant difference in the unstimulated expression of IDO between the depressed patients and controls was observed (Z=-0.128, p=0.898), a trend towards a significant increase in LPS stimulated IDO mRNA expression (Z=-1.769, p=0.077) and anti-CD3 (Z=-1.923, p=0.054) stimulated IDO mRNA expression in the healthy controls relative to the patient cohort was observed [Figure 5.9].

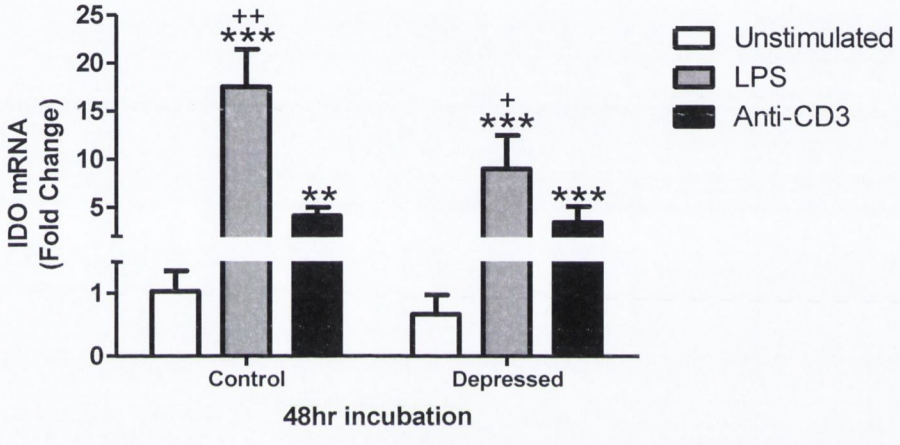

Figure 5.9 LPS and anti-CD3 stimulated IDO mRNA expression in PBMCs from severely depressed patients and healthy controls. PBMCs isolated from severely depressed patients and age and sex matched healthy controls were incubated for 48 hours with the innate immune stimulus LPS or the T-cell stimulus anti-CD3. Relative mRNA expression for IDO was quantified. Data expressed as mean with SEM (n=13 per group). **p≤0.01, ***p≤0.001 vs. unstimulated IDO mRNA expression; +p≤0.05, ++p≤0.01 vs. anti-CD3 stimulated IDO mRNA expression (Kruskal-Wallis one-way ANOVA followed by Mann-Whitney U tests).

5.3.9 Kynurenine pathway activation following innate or adaptive immune cell stimulation in severely depressed patients and healthy controls

5.3.9.1 Culture supernatant kynurenine concentrations from severely depressed patients and healthy controls following stimulation with LPS or anti-CD3

A Kruskal-Wallis one-way ANOVA revealed a significant difference in kynurenine culture supernatant concentrations from a cohort of severely depressed patients and healthy control subjects following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 [χ^2=31.383, df=5, p<0.001] [Figure 5.10a]. Subsequent Mann-Whitney U tests revealed that the LPS induced increase in kynurenine concentration in healthy controls was not significantly different from unstimulated control levels (Z=-1.732, p=0.083) while anti-CD3 induced a robust increase in kynurenine concentrations relative to unstimulated control levels (Z=-3.984, p<0.001). The LPS and anti-CD3 induced increase in kynurenine production were not significantly different in the healthy controls (Z=-1.674, p=0.094). In the depressed cohort, LPS did not increase the kynurenine concentrations relative to unstimulated concentrations from patient PBMCs (Z=-0.808, p=0.419); however, stimulation with anti-CD3 resulted in a robust increase in kynurenine production relative to unstimulated patient concentrations (Z=-3.522, p<0.001), and also in comparison to the LPS treated cells (Z=-2.771, p=0.006). There was no significant difference in the unstimulated (Z=-0.173, p=0.862), LPS stimulated (Z=-1.559, p=0.119) and anti-CD3 stimulated (Z=-1.386, p=0.166) kynurenine concentrations between the depressed cohort and the healthy controls [Figure 5.10a].

5.3.9.2 Culture supernatant tryptophan concentrations from severely depressed patients and healthy controls following stimulation with LPS or anti-CD3

A Kruskal-Wallis one-way ANOVA revealed a significant difference in tryptophan culture supernatant concentrations from a cohort of severely depressed patients and healthy control subjects following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 [χ^2=12.026, df=5, p=0.034] [Figure 5.10b]. Subsequent Mann-Whitney U tests revealed that the LPS induced decrease in tryptophan in healthy controls was not significantly different from unstimulated control levels (Z=-1.732, p=0.083). Stimulation with anti-CD3 did induce a significant decrease in tryptophan concentrations relative to unstimulated control levels (Z=-2.254, p=0.024). The LPS and anti-CD3 induced decrease in tryptophan concentrations were not significantly different in the healthy controls (Z=-1.155, p=0.248). In the depressed cohort, there was no significant difference between LPS stimulated and unstimulated tryptophan concentrations (Z=-0.520, p=0.603). However, anti-CD3 induced a decrease in tryptophan concentrations in the

depressed cohort relative to unstimulated patient concentrations (Z=-2.021, p=0.043). There was no significant difference in the unstimulated (Z=-0.577, p=0.564), LPS stimulated (Z=-0.751, p=0.453) and anti-CD3 stimulated (Z=-0.231, p=0.817) tryptophan concentrations between the depressed cohort and the healthy controls [Figure 5.10b].

5.3.9.3 Culture supernatant kynurenine/tryptophan ratio levels from severely depressed patients and healthy controls following stimulation with LPS or anti-CD3

A Kruskal-Wallis one-way ANOVA revealed a significant difference in the KYN/TRP ratio in culture supernatants from a cohort of severely depressed patients and healthy control subjects following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 [χ^2=30.676, df=5, p<0.001] [Figure 5.10c]. Subsequent Mann-Whitney U tests revealed that stimulation with LPS induced an increase in the KYN/TRP ratio in healthy controls (Z=-2.021, p=0.043). The KYN/TRP ratio was significantly increased following anti-CD3 stimulation in the healthy controls relative to unstimulated ratio levels (Z=-3.868, p<0.001). A weak trend towards a significant difference in the KYN/TRP ratio induced by LPS and anti-CD3 was revealed in the healthy controls (Z=-1.732, p=0.083). In the depressed cohort, there was no difference in the LPS and unstimulated KYN/TRP ratio levels (Z=-0.693, p=0.488). However, stimulation with anti-CD3 induced a robust increase in IDO activation relative to unstimulated patient levels (Z=-3.291, p=0.001) and LPS stimulated KYN/TRP ratio levels (Z=-2.944, p=0.003). There was no significant difference in the unstimulated (Z=-0.635, p=0.525), LPS stimulated (Z=-1.270, p=0.204) and anti-CD3 stimulated (Z=-1.097, p=0.273) IDO activity levels between the depressed cohort and the healthy controls [Figure 5.10c].

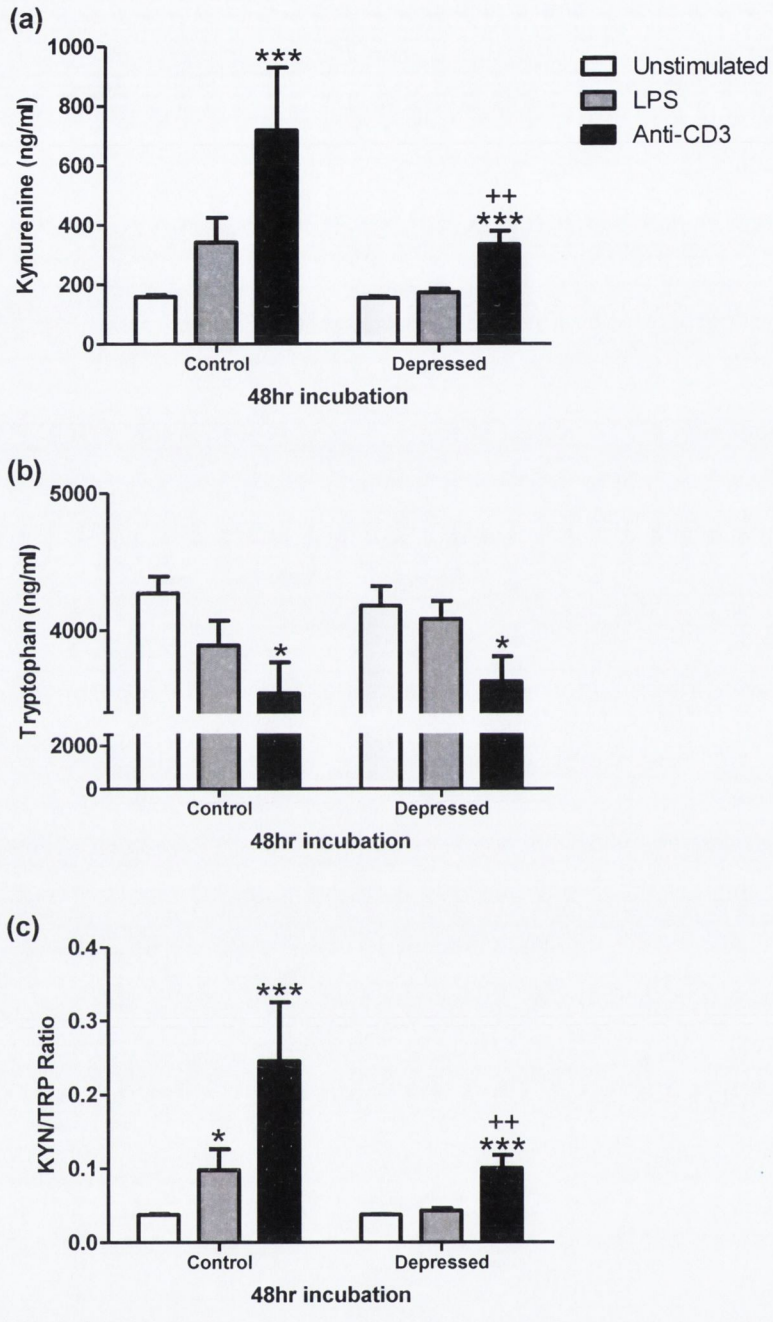

Figure 5.10 Culture supernatant kynurenine pathway activation profile in severely depressed patients and healthy controls following stimulation with LPS or anti-CD3. PBMCs isolated from severely depressed patients and age and sex matched healthy controls were incubated for 48 hours with the innate immune stimulus LPS or the T-cell stimulus anti-CD3. Cell culture supernatant concentrations for (a) kynurenine, (b) tryptophan and (c) KYN/TRP ratio were measured. Data expressed as mean with SEM (n=12 per group). *p≤0.05, ***p≤0.001 vs. unstimulated concentrations; ++p≤0.01 vs. LPS stimulated concentrations (Kruskal-Wallis one-way ANOVA followed by Mann-Whitney U tests).

5.3.10 Assessment of clinical depression severity before and after ECT treatment

A Wilcoxon-Signed Rank test revealed a robustly significant difference ($Z=-4.640$, $p<0.001$) in the HAM-D 24 scores of the patient cohort following therapeutic intervention with ECT relative to their baseline HAM-D 24 scores prior to ECT treatment (Pre-ECT: 30.19 (6.34) vs. Post-ECT 10.06 (6.87)) [Table 5.4]. Further analysis revealed that 11 patients out of 16 responded to ECT treatment indicated by a ≥60% decrease in HAM-D 24 score from baseline plus an end of treatment HAM-D score ≤16 [6.18 (3.25)]. Ten of these responders were also classified as remitters as they had a ≥60% change in HAM-D 24 score from baseline and a post treatment score ≤10. Five of the patients did not respond to ECT treatment and with a mean HAM-D 24 score of 18.6 (4.22) were still moderately depressed post treatment with ECT. Given the limited number of samples, all further analysis will focus specifically on assessing differences before and after ECT in the complete patient cohort.

Table 5.4 Clinical depression severity assessed using the HAM-D 24 scale before and after ECT

	Pre ECT	Post ECT	n	Responder	n	Non-responder	n
HAM-D 24 score	30.19 (6.34)	10.06 (6.87)***	16	6.18 (3.25)	11	18.6 (4.22)	5

Data expressed as mean and SD in parentheses. ***p≤0.001 vs. Pre-ECT (Wilcoxon-Signed Rank test).

5.3.11 Assessment of circulating concentrations of kynurenine and tryptophan in depressed patients before and after ECT treatment

5.3.11.1 Circulating kynurenine concentrations in severely depressed patients before and after ECT treatment

A paired *t*-test revealed a significant increase in circulating kynurenine concentrations in the patient cohort following ECT treatment compared with baseline concentrations prior to ECT [t=2.995, df=12, p=0.011] [Figure 5.11a].

5.3.11.2 Circulating tryptophan concentrations in severely depressed patients before and after ECT treatment

A paired *t*-test revealed a significant increase in circulating tryptophan concentrations in the patient cohort following ECT treatment compared with baseline concentrations prior to ECT [t=2.259, df=12, p=0.043] [Figure 5.11b].

5.3.11.3 Kynurenine/tryptophan ratio levels in severely depressed patients before and after ECT treatment

A paired *t*-test revealed no significant difference in the KYN/TRP ratio between depressed patients prior to and following therapeutic intervention with ECT [t=1.692, df=12, p=0.116] [Figure 5.11c].

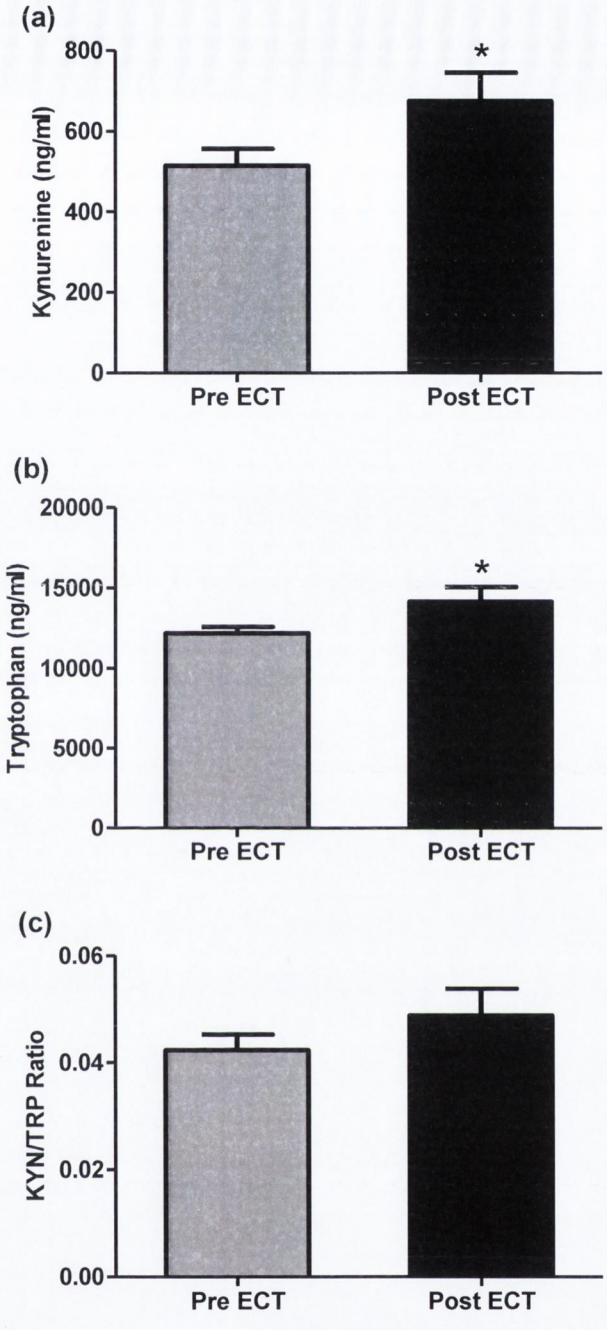

Figure 5.11 Circulating kynurenine pathway activation profile in severely depressed patients before and after ECT treatment. Plasma concentrations of (a) kynurenine, (b) tryptophan and (c) KYN/TRP ratio in the patient cohort following ECT treatment relative to pre ECT concentrations. Data expressed as mean with SEM (n=13 per group). *p≤0.05 vs. control (Student's *t* test).

5.3.12 Assessment of the pro-inflammatory cytokine production following innate or adaptive immune stimulation in severely depressed patients following therapeutic intervention with ECT

5.3.12.1 Culture supernatant IL-6 concentrations from depressed patients pre and post ECT treatment and following stimulation with LPS or anti-CD3

A Kruskal-Wallis one-way ANOVA revealed a significant difference in IL-6 culture supernatant concentrations from the cohort of severely depressed patients pre and post ECT treatment and following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 [χ^2=49.429, df=5, p≤0.001] [Figure 5.12a]. As previously described (section 5.3.7.1), a Mann-Whitney U test revealed a robust increase in LPS stimulated IL-6 concentrations in the depressed cohort (Z=-4.086, p<0.001) while anti-CD3 induced a more modest but yet significant increase in IL-6 concentrations relative to unstimulated patient IL-6 concentrations (Z=-2.883, p=0.004). The LPS stimulated IL-6 concentrations in the depressed patients were significantly greater than that observed following anti-CD3 stimulation (Z=-1.970, p=0.049). Treatment with ECT did not appear to alter the ability of the cells to respond to immune stimuli as both LPS (Z=-4.583, p<0.001) and anti-CD3 (Z=-3.953, p<0.001) induced robust increases in IL-6 concentrations relative to unstimulated concentrations. As was evident prior to ECT treatment, the LPS induced increase in IL-6 was much greater than that induced by anti-CD3 (Z=-3.546, p<0.001). A Wilcoxon Signed Rank test for paired comparisons revealed no significant difference between the unstimulated (Z=-1.477, p=0.140), LPS stimulated (Z=-0.057, p=0.955) or anti-CD3 stimulated (Z=-1.647, p=0.100) IL-6 concentrations before and after therapeutic intervention with ECT [Figure 5.12a].

5.3.12.2 Culture supernatant TNF-α concentrations from depressed patients pre and post ECT treatment and following stimulation with LPS or anti-CD3

A Kruskal-Wallis one-way ANOVA revealed a significant difference in TNF-α culture supernatant concentrations from the cohort of severely depressed patients pre and post ECT treatment and following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 [χ^2=36.596, df=5, p<0.001] [Figure 5.12b]. As described previously (section 5.3.7.2), subsequent Mann-Whitney U tests revealed that LPS induced a robust increase in TNF-α supernatant concentrations relative to unstimulated depressed patient concentrations (Z=-2.944, p=0.003), while anti-CD3 stimulated a more robust and highly significant increase in TNF-α concentrations relative to unstimulated patient concentrations (Z=-3.817, p<0.001). Anti-CD3 stimulated TNF-α concentration were significantly greater than those observed following LPS stimulation in the depressed cohort (Z=-1.976, p=0.048). Following successful treatment with ECT, significant

increases in the LPS (Z=-2.874, p=0.004) and anti-CD3 (Z=-3.953, p<0.001) stimulated TNF-α concentrations were observed relative to unstimulated TNF-α concentrations post treatment with ECT. The LPS and anti-CD3 induced increases were not different (Z=- 1.241, p=0.215). A Wilcoxon-Signed Rank test for paired comparisons revealed that the unstimulated (Z=-0.419, p=0.675) and LPS (Z=-1.503, p=0.133) stimulated TNF-α concentrations were not different prior to and following therapeutic intervention with ECT. However, the anti-CD3 stimulated TNF-α concentrations were considerably less in the patient cohort after ECT treatment relative to the anti-CD3 stimulated concentrations observed in the depressed patients prior to ECT treatment (Z=-2.551, p=0.010) [Figure 5.12b].

5.3.12.3 Culture supernatant IFN-γ concentrations from depressed patients pre and post ECT treatment and following stimulation with LPS or anti-CD3

A Kruskal-Wallis one-way ANOVA revealed a significant difference in IFN-γ culture supernatant concentrations from the cohort of severely depressed patients pre and post ECT treatment and following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 [χ^2=49.998, df=5, p<0.001] [Figure 5.12c]. As described previously (section 5.3.7.3), subsequent Mann-Whitney U tests revealed no significant effect of LPS on IFN-γ concentrations in the depressed cohort relative to unstimulated concentrations (Z=-0.783, p=0.433), while anti-CD3 stimulated a robust increase in IFN-γ relative to unstimulated patient concentrations (Z=-4.513, p<0.001) and relative to LPS stimulated IFN-γ patient concentrations (Z=-4.005, p<0.001). Similarly, LPS treatment had no significant effect on IFN-γ production in the patient cohort post ECT relative to unstimulated post ECT concentrations (Z=-1.153, p=0.249). However, the anti-CD3 stimulated IFN-γ concentration post ECT was significantly increased relative to unstimulated post ECT concentrations (Z=-4.514, p<0.001) and LPS stimulated post ECT concentrations (Z=-3.869, p<0.001). A Wilcoxon-Signed Rank test for paired comparisons revealed that the unstimulated (Z=-1.690, p=0.091) and LPS (Z=-0.981, p=0.327) stimulated IFN-γ concentrations were not different prior to and following therapeutic intervention with ECT. However, the anti-CD3 stimulated IFN-γ concentrations were decreased in the patient cohort after ECT treatment relative to the anti-CD3 stimulated concentrations observed in the depressed patients prior to ECT treatment (Z=-2.294, p=0.022) [Figure 5.12c].

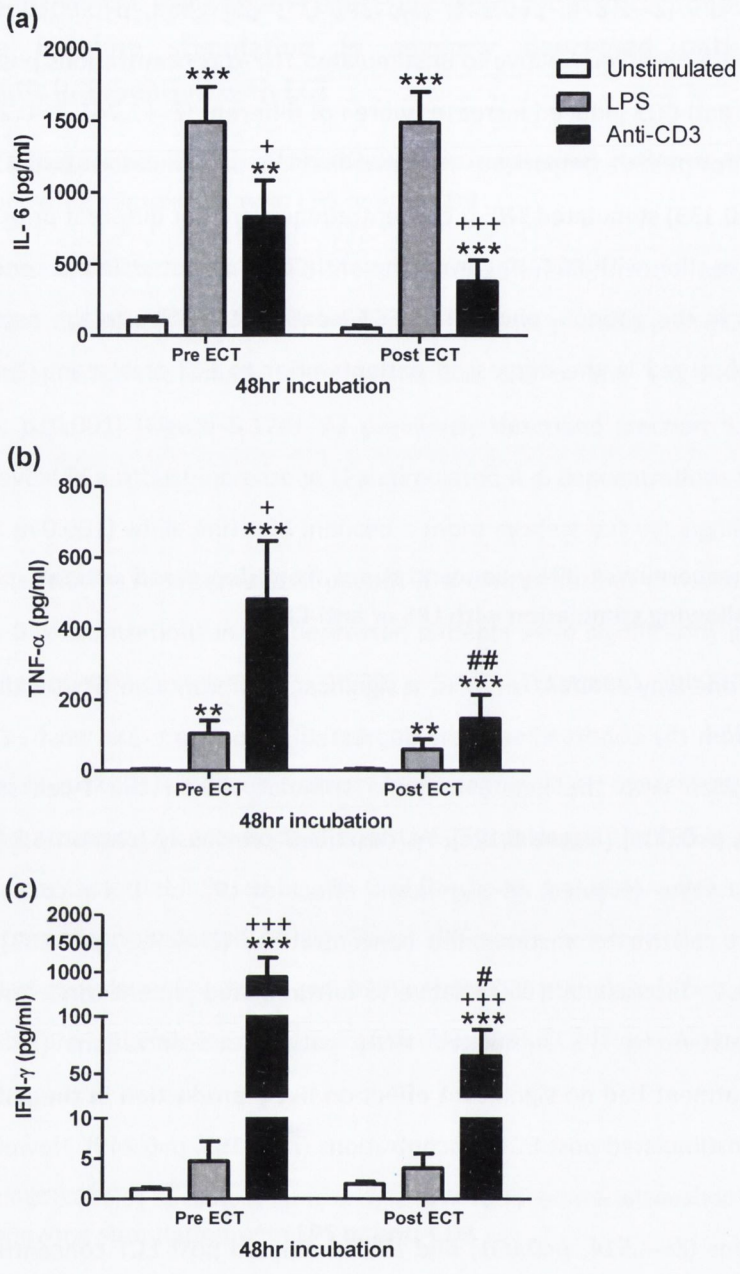

Figure 5.12 Culture supernatant pro-inflammatory cytokine concentrations in depressed patients pre and post ECT treatment and following stimulation with LPS or anti-CD3. PBMCs isolated from severely depressed patients before and after ECT were incubated for 48 hours with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 and cell culture supernatant concentrations of (a) IL-6, (b) TNF-α and (c) IFN-γ were measured. Data expressed as mean with SEM (n=14-15 per group). **p≤0.01, ***p≤0.001 vs. unstimulated concentrations; +p≤0.05, +++p≤0.001 vs. LPS stimulated concentrations; #p≤0.05, ##p≤0.01 vs. anti-CD3 stimulated pre ECT concentrations (Kruskal-Wallis one-way ANOVA followed by Mann-Whitney U tests or Wilcoxon Signed Rank tests for paired comparisons).

5.3.13 IDO mRNA expression following innate or adaptive immune cell stimulation in severely depressed patients prior to and following therapeutic intervention with ECT

A Kruskal-Wallis one-way ANOVA revealed a differential transcriptional IDO expression in PBMCs isolated from a cohort of severely depressed patients pre and post ECT treatment and following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 [χ^2=45.604, df=5, p<0.001] [Figure 5.13]. As described previously (section 5.3.8), subsequent Mann-Whitney U tests revealed that both LPS (Z=-3.974, p<0.001) and anti-CD3 (Z=-3.615, p<0.001) induced a significant increase in the transcriptional expression of IDO in the depressed patients. The LPS induced increase in IDO transcriptional expression was significantly greater than that observed following anti-CD3 stimulation in the depressed cohort (Z=-2.282, p=0.022). Similarly, in the patient cohort post treatment with ECT, IDO mRNA expression was increased following LPS (Z=-4.131, p<0.001) and anti-CD3 (Z=-4.080, p<0.001) stimulation. However, no difference was observed between LPS and anti-CD3 stimulated IDO mRNA post treatment with ECT (Z=-1.154, p=0.249). A Wilcoxon-Signed Rank test revealed that the unstimulated (Z=-1.712, p=0.087), LPS stimulated (Z=-0.105, p=0.917) and anti-CD3 stimulated (Z=-1.433, p=0.152) IFN-γ concentrations were not different prior to and following therapeutic intervention with ECT [Figure 5.13].

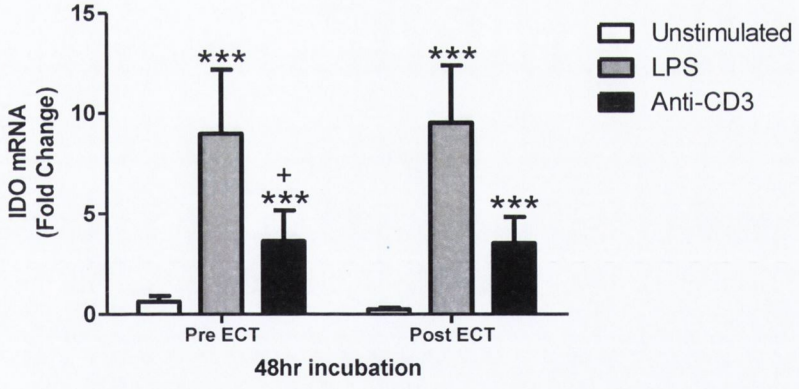

Figure 5.13 LPS and anti-CD3 stimulated IDO mRNA expression in severely depressed patients before and after ECT treatment. PBMCs isolated from severely depressed treatment-resistant patients prior to and following therapeutic intervention with ECT were incubated for 48 hours with the innate immune stimulus LPS or the T-cell stimulus anti-CD3. Relative mRNA expression for IDO was quantified. Data expressed as mean with SEM (n=13 per group). ***p≤0.001 vs. unstimulated IDO mRNA expression; +p≤0.05 vs. LPS stimulated IDO mRNA expression (Kruskal-Wallis one-way ANOVA followed by Mann-Whitney U tests or Wilcoxon Signed Rank tests for paired comparisons).

5.3.14 Kynurenine pathway activation following innate or adaptive immune cell stimulation in severely depressed patients before and after ECT

5.3.14.1 Culture supernatant kynurenine production from depressed patients pre and post ECT treatment and following stimulation with LPS or anti-CD3

A Kruskal-Wallis one-way ANOVA revealed a significant difference in kynurenine culture supernatant concentrations from a cohort of severely depressed patients pre and post ECT treatment and following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 [χ^2=15.483, df=5, p=0.008] [Figure 5.14a]. Subsequent Mann-Whitney U tests revealed that LPS did not induce a significant increase in kynurenine concentrations in the depressed cohort pre ECT(Z=-0.808, p=0.419) while stimulation with anti-CD3 induced a robust increase in kynurenine relative to unstimulated pre ECT patient concentrations (Z=-3.522, p<0.001) and relative to LPS stimulated pre ECT patient concentrations (Z=-2.771, p=0.006). However, in the patient sample post treatment with ECT, neither LPS (Z=-0.866, p=0.386) or anti-CD3 (Z=-1.270, p=0.204) induced a significant increase in kynurenine concentrations relative to unstimulated post ECT concentrations. A Wilcoxon-Signed Rank test for paired comparisons revealed that the unstimulated (Z=-1.712, p=0.087), LPS stimulated (Z=-0.105, p=0.917) and anti-CD3 stimulated (Z=-1.433, p=0.152) kynurenine production was not different prior to and following therapeutic intervention with ECT [Figure 5.14a].

5.3.14.2 Culture supernatant tryptophan concentrations from depressed patients pre and post ECT treatment and following stimulation with LPS or anti-CD3

A Kruskal-Wallis one-way ANOVA revealed a significant difference in tryptophan culture supernatant concentrations from a cohort of severely depressed patients pre and post ECT treatment and following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 [χ^2=18.066, df=5, p=0.003] [Figure 5.14b]. Subsequent Mann-Whitney U tests revealed no significant difference in the tryptophan concentrations between unstimulated and LPS stimulated PBMCs from pre ECT patients (Z=-0.520, p=0.603), while stimulation with anti-CD3 did induce a significant decrease in tryptophan concentrations relative to unstimulated pre ECT levels (Z=-2.021, p=0.043). No significant difference was detected between the LPS and anti-CD3 stimulated tryptophan concentrations in the pre ECT cohort (Z=-1.674, p=0.094). In the patient sample post treatment with ECT, neither LPS (Z=-0.231, p=0.817) or anti-CD3 (Z=-1.155, p=0.248) induced a significant decrease in tryptophan concentrations relative to unstimulated post ECT levels. However, a Wilcoxon-Signed Rank test for paired comparisons revealed a significant increase in tryptophan in the patient cohort post treatment with ECT relative to depressed

patients prior to ECT treatment under all three conditions: unstimulated (Z=-2.040, p=0.041), LPS stimulated (Z=-2.353, p=0.019) and anti-CD3 stimulated (Z=-2.432, p=0.015) [Figure 5.14b].

5.3.14.3 Culture supernatant KYN/TRP ratio from depressed patients pre and post ECT treatment and following stimulation with LPS or anti-CD3

A Kruskal-Wallis one-way ANOVA revealed a significant difference in the KYN/TRP ratio in culture supernatants from a cohort of severely depressed patients pre and post ECT treatment and following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 [χ^2=16.763, df=5, p=0.005] [Figure 5.14c]. Subsequent Mann-Whitney U tests revealed no significant difference in the LPS and unstimulated IDO activity levels in the depressed pre ECT cohort (Z=-0.693, p=0.488), however, stimulation with anti-CD3 induced a robust increase in the KYN/TRP ratio relative to unstimulated pre ECT patient levels (Z=-3.291, p=0.001) and LPS stimulated IDO activity levels in the patient cohort pre ECT (Z=-2.944, p=0.003). In the patient cohort post ECT, there was no difference in the KYN/TRP ratio following LPS stimulation (Z=-0.173, p=0.862) or anti-CD3 stimulation (Z=-1.039, p=0.299) relative to unstimulated levels. A Wilcoxon-Signed Rank test for paired comparisons, revealed a trend towards a significant decrease in the KYN/TRP ratio in the patient cohort post treatment with ECT relative to pre ECT levels (Z=-1.804, p=0.071). The LPS stimulated (Z=-0.157, p=0.875) and anti-CD3 stimulated (Z=-1.412, p=0.158) IDO activity level did not differ in the patient samples before and after ECT treatment [Figure 5.14c].

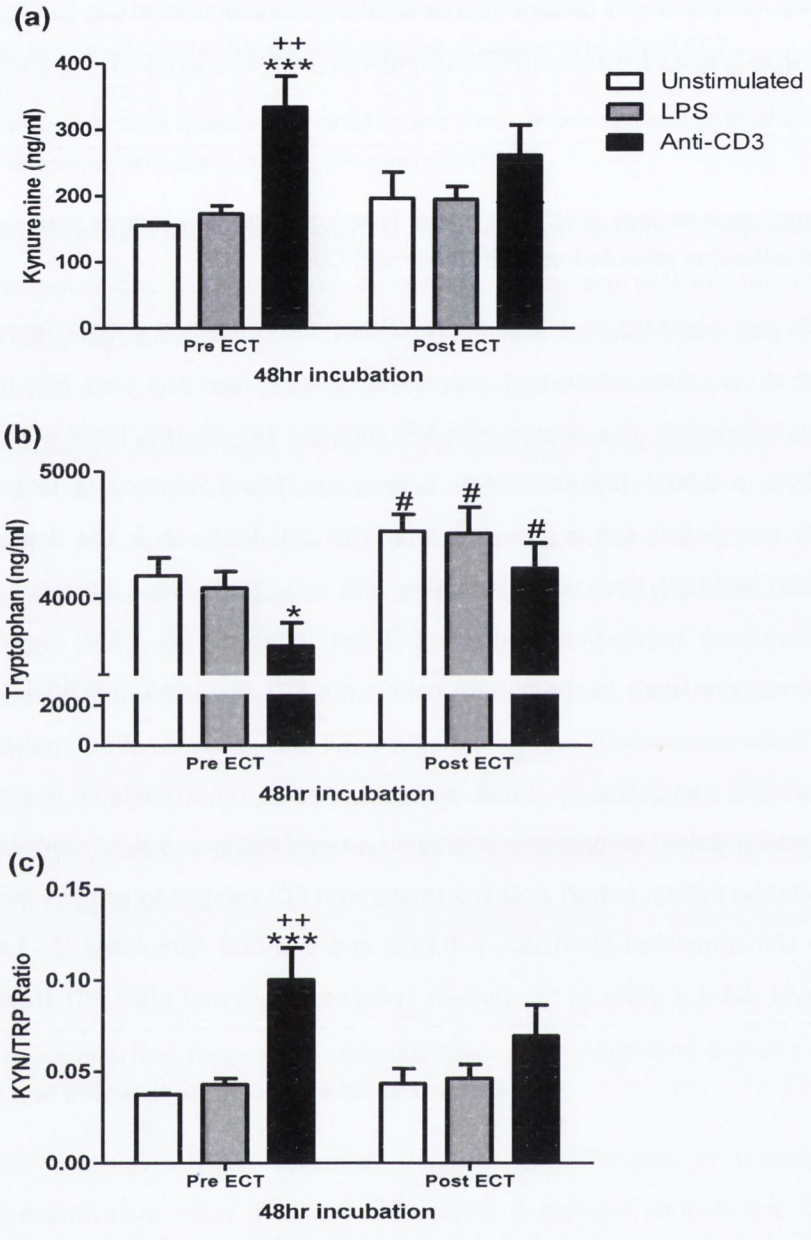

Figure 5.14 Comparison of the culture supernatant kynurenine pathway activation in depressed patients pre and post ECT treatment and following stimulation with LPS or anti-CD3. PBMCs isolated from severely depressed patients before and after ECT were incubated for 48 hours with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 and cell culture supernatant concentrations for (a) kynurenine, (b) tryptophan and (c) KYN/TRP ratio were measured. Data expressed as mean with SEM (n=12 per group).*p≤0.05, ***p≤0.001 vs. unstimulated concentrations; ++p≤0.01 vs. LPS stimulated concentrations; #p≤0.05 vs. unstimulated, LPS and anti-CD3 stimulated paired comparison (Kruskal-Wallis one-way ANOVA followed by Mann-Whitney U tests or Wilcoxon Signed Rank tests for paired comparison).

5.3.15 Controlling for the difference in BMI, smoking and alcohol use between depressed patients and control subjects

As reported in Table 5.1 the average BMI score in the depressed cohort [25.63 (5.08)] was significantly greater than that for the control participants [22.11 (1.73)] ($t=-2.618$, $df=1,29$, ($p=0.014$)). To control for this, a cut off score of 27 was applied for the depressed patients and control subjects combined. Only 3 patients had a BMI greater than 27 and a Mann-Whitney U test revealed no significant difference between the patients with a BMI >27 and the rest of the participants with a BMI <27 for any of the parameters under investigation ($p>0.05$).

As none of the control participants smoke, the effect of smoking was assessed in the depressed cohort alone. Depressed patients were divided into smokers ($n=7$) and non-smokers ($n=9$). A Mann-Whitney U test showed no significant effect of smoking on any of the parameters under investigation ($p>0.05$).

Fifteen out of 16 controls reported alcohol consumption compared with 2 patients, prior to starting the study and while a significant difference was observed between the number of alcohol units consumed a week, between controls and depressed patients, no participant consumed more than 14 standard units a week and therefore were all within the accepted safe range.

5.4 Discussion

In the present study, the relative ability of innate versus adaptive immune cells to induce kynurenine pathway activation and tryptophan depletion following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 was examined. The findings from this study revealed that activation of T-cells has a greater propensity to induce tryptophan depletion and kynurenine pathway activation than activation of innate immune cells. Further to this, I sought to assess if severely depressed patients were more sensitive to the ability of inflammatory stimuli to induce a pro-inflammatory response and kynurenine pathway activation. Interestingly, the stimulated production of the inflammatory cytokines IL-6, TNF-α and IFN-γ and mitogen stimulated kynurenine pathway activation profile in the depressed cohort, was comparable to that evident in healthy controls. However, circulating concentrations of tryptophan in the depressed patients were reduced relative to controls. Despite this, the circulating concentration of kynurenine and the KYN/TRP ratio were unaltered and comparable to those evident in the healthy control subjects, suggesting that the kynurenine pathway was not activated in the patient cohort. Assessment of the cellular response to immune stimulation following therapeutic intervention with ECT revealed a diminished activation of the kynurenine pathway, evidenced by a non-significant increase in mitogen stimulated kynurenine production and unaltered KYN/TRP ratio levels coupled with an increased concentration of tryptophan in unstimulated culture supernatants which were unaffected by immune stimulation. In accordance with this, circulating tryptophan concentrations were also increased following ECT treatment. Therefore, while PBMCs isolated from depressed patients are functionally capable of inducing the kynurenine pathway upon stimulation of the adaptive immune response, unstimulated and circulating measures of kynurenine pathway activation suggest that the pathway is not activated in depressed patients. In addition, the effect of ECT reduces T-cell mediated activation of the kynurenine pathway in association with increasing tryptophan availability both at a cellular level and in circulation.

5.4.1 The relative ability of innate vs. adaptive immune cells to induce kynurenine pathway activation

Stimulation of human PBMCs induced robust increases in the production of the pro-inflammatory cytokines IL-6, TNF-α and IFN-γ, with LPS being the more potent stimulus for IL-6 production while mitogen stimulation with anti-CD3 induced robust increases in TNF-α and IFN-γ. Further to this, while IDO mRNA expression was induced by both LPS and anti-CD3, increased kynurenine production coupled with a depleted tryptophan concentration was only evident following T-cell

stimulation. Therefore, it appears that activation of T-cells has a greater propensity to induce tryptophan depletion and kynurenine pathway activation than activation of innate immune cells.

The findings from this study are interesting and in accordance with reports by Maes and colleagues (1994; 2001) who suggest that decreased tryptophan availability in depression, potentially as a consequence of IDO activation, is related to T-cell mediated immune activation, evidenced by the detection of a significant negative correlation between decreased plasma tryptophan concentrations, increased stimulated IFN-γ secretion and elevated neopterin plasma levels - two key markers of cell mediated immune activation (Maes et al., 1994, Maes et al., 2011).

Consequently, I sought to investigate if depressed patients were more sensitive to the ability of immune cells to respond to inflammatory stimuli, in association with assessing the circulating levels of tryptophan and kynurenine prior to and following therapeutic intervention with ECT.

5.4.2 Inflammatory cytokine production in depressed patients and healthy controls following immune stimulation

Stimulation of PBMCs with the innate immune stimulus LPS or the T-cell mitogen anti-CD3 induced robust increases in IL-6, TNF-α and IFN-γ concentrations in culture supernatants with similar patterns of stimulated cytokine production evident in the depressed cohort and healthy controls. While the literature in this regard is limited and varied with respect to stimulus, dose and depression severity/sub-type, these results are consistent with the findings from a number of previous studies. Rothermundt et al. (2001) reported no significant difference in phytohaemagglutinin (PHA) stimulated (48 hours) whole blood IL-1β concentrations between depressed patients and healthy controls. This is in accordance with earlier findings by Seidel et al. (1995), who also report no difference in the mitogen stimulated whole blood IL-6 and IL-1β concentrations. In contrast, early observations by Maes et al. (1993c) found increased mitogen stimulated IL-6 production in depressed patients relative to controls, but only in those suffering from melancholic major depression. Kim et al. (2007) also reported an increased stimulated production of IL-6 and TNF-α concentrations in a depressed cohort relative to control subjects, however, this study is not directly comparable given that the cells were stimulated with a combination of PHA (4 μg/ml) and LPS at a concentration of 20 μg/ml, which is considerably larger than the 1 μg/ml dose used in the current study.

Therefore, in the present study it appears that the ability of severely depressed treatment-resistant patients to produce IL-6, TNF-α and IFN-γ in response to both innate and adaptive immune stimuli is comparable to that observed in healthy controls.

5.4.3 Kynurenine pathway activation profile in depressed patients and healthy controls following immune stimulation

Consistent with previous findings, LPS and anti-CD3 increased the transcriptional expression of IDO in the healthy control participants, with LPS inducing the more robust increase compared with that induced by anti-CD3. A similar IDO expression pattern was also observed in the depressed cohort; however, the LPS induced increase in the patient cohort appeared to be blunted by approximately 50% on average relative to healthy control expression levels.

Further to this, assessment of kynurenine pathway activation in severely depressed patients revealed that, similar to the healthy controls, T-cell stimulation had the ability to induce an increased concentration of the kynurenine coupled with a concomitant decrease in culture supernatant tryptophan concentrations which was reflected in the elevated KYN/TRP ratio.

A modest increase in the KYN/TRP ratio was evident following LPS stimulation in the control participants in the absence of significant changes in kynurenine and tryptophan alone. However, with greater participant numbers in the study, the LPS induced changes in kynurenine and tryptophan may reach statistical significance. While the effect of LPS on kynurenine pathway activation in the control participants is of a lower magnitude than that induced by anti-CD3, LPS had no effect on the kynurenine and tryptophan concentrations in the depressed cohort, which is reflected in the KYN/TRP ratio. This is an interesting finding given that it is stimulation with LPS that induced the most robust effects on IDO mRNA expression in both the depressed cohort and healthy controls.

Therefore, it would appear that while LPS induces a sustained increase in IDO mRNA expression in both the depressed patients and control subjects, the enzyme does not appear to be bioactive. While little is known about the regulation of transcriptional IDO expression, this appears to be a highly plausible suggestion given the following findings. During the process of dendritic cell maturation, induced by a cytokine maturation cocktail (TNFα, IL-6, IL-1β and PGE_2), IDO mRNA is increased by 100-fold (Braun et al., 2005). An investigation into the mechanisms involved in IDO expression and activity during dendritic cell maturation has shown that a 2-step induction process is necessary to activate the enzymatic activity of IDO in maturing dendritic cells (Braun et al.,

2005). While an increased IDO transcriptional expression is observed in the presence of the immunomodulatory agent prostaglandin E_2 (PGE_2), activation of the enzyme was only observed in response to a secondary signal through the Toll-like receptor (TLR) or the TNF receptor (TNF-R). LPS or TNF alone did not induce IDO activity in the dendritic cells (Braun et al., 2005). In contrast, IFN-γ which is robustly increased following mitogen stimulation with anti-CD3 in both the depressed patients and healthy controls in the present study, is a known inducer of IDO mRNA expression and activity (Odemuyiwa et al., 2004).

Further to this, to my knowledge, only one other study has assessed the stimulated kynurenine pathway activation profile in a depressed cohort relative to control subjects. Krause et al. (2012) reported an increased LPS stimulated kynurenine production in association with a decreased tryptophan concentration in a depressed cohort (n=21) and healthy controls (n=38) alike. While the findings in the present study with regard to the healthy controls are comparable to those reported by Krause and colleagues, I did not observe an LPS stimulated increased production of kynurenine in association with a reduced tryptophan concentration in severely depressed patients. The discrepancies between these two findings may lie in the fact that the depressed patients in the study by Krause and colleagues were only mildly depressed with an average HAM-D score of 13.93 while the severely depressed patients in the present study all had a HAM-D 24 score greater than 21 with an average of 30.19. Therefore, with greater depression severity the functional ability of the PBMCs to respond to stimulation with LPS appears to be reduced.

Taken together, it appears that while the innate immune cells have the ability to induce IDO activity under certain conditions, T-cell activation has the greater propensity to induce kynurenine pathway activation and tryptophan depletion. Further to this, it appears that depressed patients are not more sensitive to the ability of inflammatory stimuli to induce kynurenine pathway activation. The effect of LPS on the kynurenine pathway metabolites was completely abolished in the patient cohort while a similar kynurenine pathway activation profile was apparent following T-cell stimulation.

5.4.4 The effect of therapeutic intervention with ECT on the stimulated inflammatory profile

Similar to that evident prior to ECT, both LPS and anti-CD3 stimulated a robust increase in IL-6 production. However, while comparable patterns of stimulated TNF-α and IFN-γ production were observed following ECT treatment, the magnitude of the anti-CD3 induced TNF-α production was considerably reduced compared to that observed in the depressed cohort prior to ECT treatment. Additionally, a 14-fold decrease on average, in T-cell stimulated IFN-γ production post treatment with ECT was observed, indicating that following ECT treatment, T-cells have a reduced ability to respond to mitogen stimulation.

Studies assessing the effect of ECT on the immune or inflammatory profiles are extremely limited and tend to investigate acute rather than long term changes following a course of ECT treatment. Previous studies have assessed lymphocyte percentage and number, lymphocyte proliferative responses, circulating plasma/serum cytokine and molecular changes in response to ECT treatments (Albrecht et al., 1985, Fischler et al., 1992, Hestad et al., 2003, Stelzhammer et al., 2012). Only one study, to my knowledge, has assessed the effect of repeated ECT on the stimulated production of inflammatory cytokines (Fluitman et al., 2011). In this study, Fluitman and colleagues reported increased LPS stimulated monocytic IL-6 and TNF-α production following acute ECT treatment (n=12). However, this was merely a transient change as no difference in the stimulated monocytic production of IL-6 and TNF-α following repeated ECT treatments was observed. In accordance with these findings, the LPS stimulated IL-6 and TNF-α concentrations in the current study are not dissimilar to those observed at baseline. However, a decreased T-cell stimulated TNF-α production is evident in the patient cohort following a course of ECT compared with that observed prior to treatment with ECT. Fluitman et al. (2011) also reported decreased mitogen stimulated IFN-γ production following acute ECT only. In contrast, in the present study stimulated IFN-γ production is decreased following repeated ECT relative to mitogen stimulated baseline concentrations.

Taken together, it appears that the ability of T-cells to produce pro-inflammatory cytokines following immune stimulation is reduced following a successful course of ECT treatment relative to the stimulated concentrations observed at baseline. This decreased response following mitogen stimulation post ECT treatment may be related to the stress response. Repeated exposure to generalised seizures over the course of the ECT treatments may be considered a chronic physical stress while the accompanying cognitive impairment and short term working memory deficits associated with ECT may be psychologically stressful (Semkovska and McLoughlin, 2010). This suggestion may be plausible given the literature in clinical and preclinical

models which report a suppression of cellular immune responses following exposure to chronic stress (Segerstrom and Miller, 2004, Dhabhar and McEwen, 1997).

5.4.5 The effect of therapeutic intervention with ECT on immune stimulated kynurenine pathway activation

The stimulated expression of IDO at the message level was unaltered following ECT treatment. However, the significant increase in anti-CD3 stimulated kynurenine production observed prior to ECT was no longer evident following ECT treatment. Further to this, a global increase in tryptophan was observed in the unstimulated patient samples following ECT treatment relative to baseline concentrations, which was unaffected by immune stimulation. Consequently, the significant increase in the KYN/TRP ratio which was observed prior to ECT treatment was no longer evident following therapeutic intervention with ECT.

Taken together, it appears that ECT treatment reduces the ability of activated T-cells to induce kynurenine pathway activation in association with increasing the availability of tryptophan. The results from this study are noteworthy as no other study to my knowledge has assessed the impact of treatment with ECT on the functional ability of immune cells to induce tryptophan depletion and kynurenine pathway activation.

5.4.6 Circulating kynurenine and tryptophan concentrations in severely depressed patients and healthy controls

A deficit in serotonergic neurotransmission has long been associated with the pathogenesis of major depression, with numerous studies reporting decreased circulating concentrations of the dietary essential amino acid and serotonin precursor, tryptophan (DeMyer et al., 1981, Moller, 1985, Cowen et al., 1989, Hughes et al., 2012). In accordance with this, a decreased circulating tryptophan concentration was observed in the severely depressed patients relative to the healthy control subjects. However, while it has been proposed that tryptophan depletion in clinically depressed individuals may be a consequence of increased kynurenine pathway activation, comparable concentrations of plasma kynurenine between the depressed cohort and healthy controls in tandem with unaltered KYN/TRP ratio would suggest that the kynurenine pathway was not activated in this particular cohort. This very interesting finding strongly supports previous findings presented in Chapter 3, in which a decreased plasma tryptophan concentration in the absence of kynurenine pathway activation, in a completely distinct cohort of clinically depressed

patients, was also observed (Hughes et al., 2012). While it might be suggested that hepatic tryptophan 2,3-dioxygenase (TDO) activation may be responsible for the decreased tryptophan concentration in circulation, if that were the case one would expect to see a concomitant increase in kynurenine. In addition, as reviewed by Maes and colleagues, no associations have been found between reduced plasma tryptophan and a number of factors found to be altered in certain cohorts of depressed patients, including, catecholamines, BMI, sex hormone and the thyroid hormone (Maes et al., 2011). However, Maes does suggest that chronically elevated glucocorticoid concentrations, commonly observed in depressed patients, are associated with increased levels of free fatty acids that in turn are significantly correlated with decreased circulating tryptophan concentrations (Maes et al., 1990b).

The evidence in support of increased kynurenine pathway activation and subsequent tryptophan depletion in depression stems largely from the study of cytokine induced depression which occurs in 30-50% of medically ill patients being treated with IL-2 or IFN-α for cancer and hepatitis C (Hep C) (Musselman et al., 2001, Capuron et al., 2002b, Capuron and Miller, 2004). However, it has also been shown that cytokine induced depression severity is dose dependent, hence the larger the dose of IFN-α, the greater the depression severity. Therefore, while this is strong evidence in support of the proposed involvement of the kynurenine pathway in depression, idiopathic depression is largely characterised by a low grade inflammatory phenotype and it is questionable if the inflammatory profile observed in medically healthy depressed patients is robust enough to induce an elevated kynurenine pathway activation and subsequent tryptophan depletion.

It has also been suggested that depression is not associated with a decrease in tryptophan per se but rather the downstream catabolites of the kynurenine pathway. This theory is supported by findings by Raison et al. (2010) where they show that while tryptophan concentrations are depleted in the periphery of Hep C patients being treated with IFN-α, examination of cerebrospinal fluid (CSF) tryptophan concentrations revealed that the concentration of central tryptophan was normal. However, an increased kynurenine concentration observed in both the periphery and CNS and the associated elevations in CSF quinolinic acid (QUIN) and kynurenic acid (KYNA) were found to correlate with the elevated inflammatory profile and depressive symptomology. Consequently, it appears that while tryptophan is maintained in the CNS, the decreased concentration in the periphery may be reflecting changes in the kynurenine metabolites in the CNS. Further to this, it has been suggested that it is the kynurenines and perhaps an imbalance between the neurotoxic (QUIN) and neuro-protective (KYNA) catabolites that are responsible for the induction of depressive symptomology (Maes et al., 2011).

A study by Capuron et al. (2009) has shown that while there is considerable symptom overlap between cytokine induced depression and idiopathic major depression in medically healthy individuals, patients with IFN-α induced depression display greater somatic symptom severity in the form of psychomotor retardation and weight loss relative to medically healthy depressed individuals. This is very interesting in light of the recent report by Maes and Rief (2012) that concludes that kynurenine pathway activation is more pronounced in comorbid somatisation and depression compared with depression alone and suggest that changes in kynurenine pathway metabolites (tryptophan catabolites) are more closely associated with somatic symptomology rather than depression per se. In support of this, literature directly associating increased kynurenine pathway activation and idiopathic major depression is scarce. One cross-sectional study by Myint et al. (2007) report an increased KYN/TRP ratio in the absence of changes in kynurenine and tryptophan alone, and decreased neuroprotective kynurenic acid (KYNA) concentrations in the depressed patients relative to controls, while Gabbay et al. (2010) also report an increased KYN/TRP ratio in adolescents with melancholic depression. However, as there was no change in kynurenine, the increase in the KYN/TRP ratio appears to be solely as a consequence of the significant decrease in tryptophan. Yet, increased measures of kynurenine neurotoxicity were associated with depression severity in the melancholic depressed patients (Gabbay et al., 2010). Therefore, no study to date has reported an increase in kynurenine and a decrease in tryptophan in a cohort of idiopathic depressed patients (Maes and Rief, 2012). Furthermore, in accordance with our previous findings and the results of the present study, Maes and colleagues recently reported that while depression and somatic disorders are both characterised by decreased tryptophan concentrations, they did not find any evidence in support of increased IDO activity in depression (Maes et al., 2011, Maes and Rief, 2012).

5.4.7 Circulating kynurenine and tryptophan concentrations in severely depressed patients before and after ECT

Assessment of kynurenine and tryptophan concentrations in the depressed cohort following ECT revealed a significant increase in the circulating concentrations of both kynurenine and tryptophan relative to those observed in the patient cohort prior to ECT treatment. The ratio of kynurenine to tryptophan indicative of IDO activity was unaffected by successful treatment suggesting that the kynurenine pathway was not activated as seen in the patient cohort prior to ECT treatment. To my knowledge, this is the first study to assess evidence for kynurenine pathway activation in circulation following successful treatment of major depressive illness. A handful of studies have investigated the impact of ECT on tryptophan concentrations in association with

some other amino acids. However, most of these studies only assessed the acute effects of a single ECT treatment, which is unlikely to be associated with a major therapeutic response. As discussed by Palmio et al. (2005) Hoekstra and colleagues reported an increased tryptophan concentration in responders to ECT treatment 24 hour following treatment (Hoekstra et al., 2001). In accordance with this Palmio et al. (2005), showed that over a 2-24 hour period following a single ECT treatment, tryptophan levels increased. Our findings are in accordance with this and contribute to the literature further as we have shown that not only are the tryptophan concentrations normalised following a successful course of ECT treatments, this increase is evident in circulation 3-4 days post administration of the final ECT treatment, suggesting that the increased availability of tryptophan in circulation may be a longer lasting effect. However, in association with a normalisation of tryptophan, kynurenine concentrations are elevated in the patient cohort following ECT treatment relative to pre ECT levels. Despite this, the unchanged KYN/TRP ratio and increased tryptophan concentration suggest that the kynurenine pathway is not activated.

Therefore, in accordance with previous findings (Chapter 3), I have shown depleted tryptophan concentrations in depressed patients relative to healthy control subjects in the absence of kynurenine pathway activation. Further to this, successful antidepressant treatment with a course of ECT normalises tryptophan concentrations.

5.4.8 Limitations

Differences in sample number between variables measured are a consequence of patient compliance, sampling and experimental error. Consequently, this study is limited by the small sample size. Further studies should aim to increase the number of study participants, thereby reducing the high variability present in clinical cohorts, in order to assess associations between the inflammatory changes observed and the therapeutic efficacy of ECT. The study is also limited by the fact that all patients are medicated; however, it does represent real-life conditions. While an effect of medication cannot be ruled out, little to no change in patient antidepressant medication profiles were made throughout the duration of the study. Thus, the changes observed following intervention with ECT are likely to be as a result of its therapeutic effect and efficacy. Additionally, further studies should try to limit confounding factors such as differences in BMI, smoking and alcohol consumption between groups. However, in this study the variability in these factors had no effect on the significant findings reported throughout.

5.4.9 Conclusion

Taken together, the findings from this study clearly show that activation of T-cells has a greater propensity to induce tryptophan depletion and kynurenine pathway activation than activation of innate immune cells. Additionally, circulating concentrations of tryptophan are reduced in the depressed patients in the absence of kynurenine pathway activation, suggesting the involvement of alternative mechanism to deplete tryptophan. Interestingly, therapeutic intervention with ECT suppresses the response to mitogen stimulation evidenced by diminished activation of the kynurenine pathway in association with a global increase in tryptophan concentrations. The increased circulating tryptophan concentrations coupled with an unaltered KYN/TRP ratio further indicate that the kynurenine pathway is not activated. In conclusion, while depressed patients have the ability to induce kynurenine pathway activation in response to T-cell activation, there is no evidence to suggest that it is activated in this particular cohort. Further to this, ECT suppresses the ability of stimulated T-cells to activate the kynurenine pathway in association with increasing the availability of tryptophan. However, the mechanism by which these changes arise remains to be elucidated.

Tryptophan depletion has a role in T-cell regulation and the inhibition of T-cell proliferation, which may, in turn, contribute to immunodeficiency and the promotion of immune tolerance in situations of chronic low grade inflammation (Lee et al., 2002). In addition, decreased T-cell proliferative responses have been reported in depressed cohorts (Zorrilla et al., 2001). With this in mind and in light of the re-emerging interest in the role of the adaptive immune response in depression, the next chapter (Chapter 6) assesses T-cell subset activation in depressed patients relative to healthy controls and following therapeutic intervention with ECT.

Chapter 6

T-cell subset activation in depression and the impact of intervention with ECT

6.1 Introduction

Electroconvulsive therapy (ECT) is the most powerful antidepressant available. However, despite this, its mechanism of action of is largely unknown. While studies have tried to address the impact of ECT on neurotransmitters, glucocorticoids and brain derived neurotrophic factor (BDNF) (Wahlund and von Rosen, 2003), little attention has been paid to the impact of ECT on the inflammatory system in depression.

The consistent and reproducible finding that major depression and specifically treatment-resistant depression is accompanied by increased circulating concentrations of the pro-inflammatory cytokines IL-6 and TNF-α, in association with an elevated expression of the general immune activation marker and acute phase protein C reactive protein (CRP), has led to the belief that inflammation associated depression is characterised by activation of the innate immune response (Howren et al., 2009, Dowlati et al., 2010). As much of the depression literature has centred on the above mentioned findings, little attention has been paid to the role of T-cells and the adaptive immune response in major depression (Miller, 2010, Maes, 2011).

Activated T-cells are hallmarked by an increased expression of the IL-2 receptor (IL-2R) and specifically CD25, the IL-2 receptor alpha subunit. While not as extensively studied as the innate immune response, reports suggest that severely depressed patients are characterised by an increased activated T-cell presence evidenced by elevated numbers of $CD25^+$ cells along with increased circulating concentrations of the soluble IL-2R (sIL-2R) (Maes et al., 1990a, Maes et al., 1992). Further to this and in support of an inflammatory signature in depressed patients, reports suggest a shift in the balance between the pro-inflammatory Th-1 cells and the anti-inflammatory Th-2 cells, with a greater predominance of Th-1 cytokines such as IFN-γ and IL-2 relative to the anti-inflammatory and immunoregulatory mediators IL-4, IL-10 and TGF-β in depressed patients relative to healthy controls (Kim et al., 2007, Song et al., 2009). Thus, it appears that the dysregulated expression of T-cells in favour of a pro-inflammatory phenotype may contribute negatively to the pathogenesis of depression.

However, in recent years, studies assessing the involvement of autoreactive and regulatory T-cells in both a neuro-protective and pathogenic capacity have begun to emerge (Miller, 2010, Rook et al., 2011). This more active role for the adaptive immune response in depression contrasts with early findings by Kronfol et al. (1983) and Schleifer et al. (1984) who reported that depressed patients exhibited an immunosuppressed and impaired adaptive immune response, with decreases in T-cell proliferative responses upon mitogen stimulation (Zorrilla et al., 2001, Irwin and Miller, 2007). While an immunosuppressed T-cell response in the face of immune challenge

may appear to be in stark contrast to the pro-inflammatory signature that severely depressed patients are often characterised by, it is possible that inflammation itself and a dysregulated glucocorticoid response may be responsible for diminished T-cells responses in major depression (Miller, 2010). This is a reasonable suggestion given the findings from numerous studies investigating the disruptive impact of chronic TNF-α exposure on T-cell function and cytokine production (Cope et al., 1994, Cope et al., 1997, Lee et al., 2008, Nie et al., 2013). Further to this, glucocorticoid resistance, a key hallmark of major depression and thought to be a consequence of the chronic low grade inflammatory signature frequently observed in severely depressed patients may also be responsible for desensitizing T-cell responses in depressed and stressed individuals (Pace et al., 2007, Pariante and Miller, 2001). Despite increased levels of cortisol, T-cells may no longer be able to respond appropriately to neuroendocrine signals that normally function to mediate T-cell trafficking to the brain and in turn impart neuro-protective effects in response to stress (McEwen et al., 1997, Miller, 2010), thereby further contributing to the dysregulated inflammatory and stress response in depressed patients.

Additionally, it is thought that the immune modulating regulatory T-cells (T-reg cells) may have a role in suppressing the chronic inflammatory signature in major depression. T-reg cells, produced in the thymus but also generated from conventional $CD4^+$ cells in the periphery, are phenotypically described by their expression of the cell surface marker CD25 and the transcription factor and master regulator of T-reg cells, Forkhead box P3 (FoxP3) which is absolutely crucial to their development and function (Sakaguchi et al., 2009, Sakaguchi et al., 2013). With a key role in immunological defence with a potent suppressive capacity, it is thought that these cells may serve to protect the host from stress and inflammation via anti-inflammatory mechanisms (Miller, 2010). However, in depressed and stressed patients alike it is thought that T-reg cell presence and or function may be impaired or dysfunctional, yet there is little evidence in the depression literature to support or refute this theory.

Further to this, while the pro-inflammatory Th-17 cells, with effector functions similar to that of Th-1 cells, have a key role to play in the pathogenesis of many inflammatory disorders such as rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease, no study to date has assessed if Th-17 cells have a part to play in the biological basis of major depression (Wilson et al., 2007, Tesmer et al., 2008, Haroon et al., 2012).

In vitro T-cell activation and proliferation is commonly assessed by stimulating T-cells through the use of agonistic antibodies, such as anti-CD3. Stimulation of the CD3/TCR complex, in the presence of monocytes and macrophages that supply the co-stimulatory signal, results in the

initiation of multiple signalling pathways resulting in T-cell clonal expansion, up-regulated expression of T-cell activation markers, T-cell differentiation and cytokine production.

In light of this, the aims of the study were to characterise the circulating inflammatory profile in a cohort of severely depressed patients relative to age and sex matched healthy controls. Further to this and with a particular interest in T-cells, I sought to investigate the involvement of the various T-cells subsets, Th-1, Th-2, Th-17 and T-reg cells in depression through the assessment of unstimulated PBMC gene expression and cytokine production in culture supernatants. Additionally, I evaluated the ability of these cells to respond upon mitogen stimulation with anti-CD3. Moreover, I sought to assess potential variations in the circulating inflammatory profile and T-cell subset gene expression and cytokine production among patients at two time points: during a depressive episode and following an adequate course of 6-12 ECT treatments, with the hope of identifying specific biomarkers that relate to clinical staging and therapeutic efficacy.

6.2 Methods

A detailed description of the study participants, the ECT procedure, clinical diagnostic testing using the Hamilton depression rating scale and recruitment, blood sampling and processing are outlined in Chapter 5 - section 5.2.

6.2.1 Experimental procedures

6.2.1.1 Experimental design

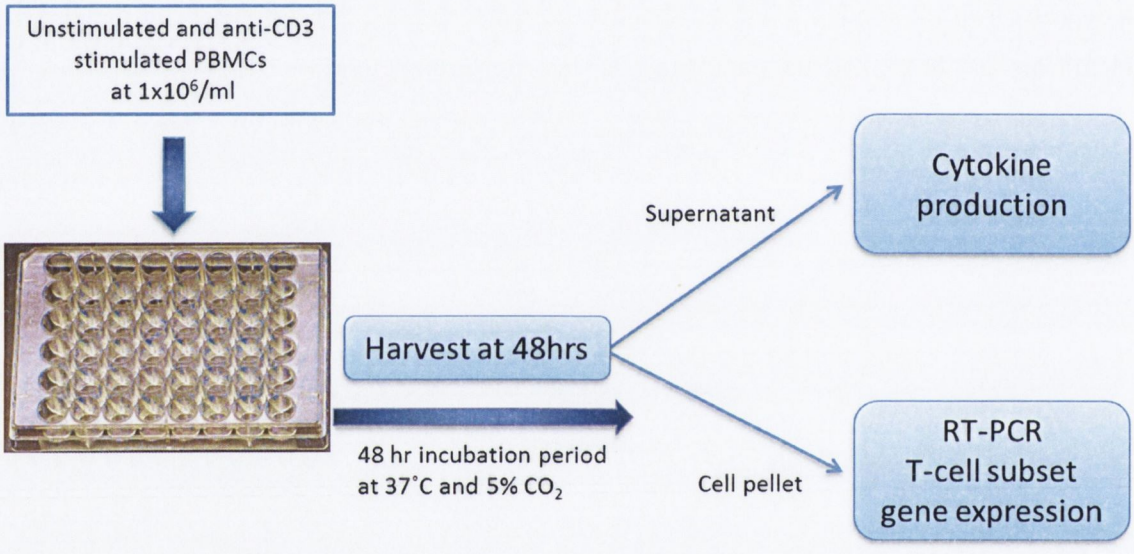

Figure 6.1 Diagrammatic representation of the experimental design

6.2.1.2 Cell culture and preparation of treatment compounds

Culture media and anti-CD3 were prepared as outline in Chapter 5 – section 5.2.2.1

For anti-CD3 stimulation, the treatment wells were coated with 200 µl of 0.1 µg/ml anti-CD3 and incubated overnight at 4°C. Following this, the excess anti-CD3 was removed and 450 µl of a 1 x 10^6 cell suspension was added to each well. The anti-CD3 concentration (0.1 µg/ml) and treatment time (48 hours) used, were determined based on results from the anti-CD3 dose response and time-course study outlined in Chapter 5 – section 5.2.2.1 (see Figure 5.2).

6.2.1.3 Real-time PCR analysis of T-cell subset mRNA markers in PBMCs

Total RNA was extracted from the PBMCs with the NucleoSpin®Total RNA isolation kit (Macherey-Nagel, Germany) and DNase treated as per kit instructions. Following RNA quantification and equalisation, cDNA was synthesised using a cDNA archive kit (Applied Biosystems, UK) (see Chapter 2 section – 2.2.4).

Gene expression analysis was conducted using Real-Time PCR employing Taqman® Gene Expression Assays (Applied Biosystems, UK). To quantify expression of target genes of interest, Taqman® Gene Expression Assays containing FAM-labelled probes were used [Table 6.1]. PCR reactions were in a duplex format also containing a Taqman®Gene Expression Assay (primer-limited) containing a VIC-labelled probe for the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH assay ID:4326317E). Samples were assayed using Applied Biosystems universal cycling conditions using a fast protocol on the StepOnePlus™ Real-time PCR system (Applied Biosystems, UK). Data were analysed using relative quantification (RQ) values and GAPDH served as an endogenous control in the amplification system.

Table 6.1 List of genes used with GenBank sequence ref numbers

Target Symbol	Target Name	Taqman Gene Assay ID
CD25	Interleukin-2 receptor alpha	Hs00907779_m1
Th-1		
IFN-γ	Interferon gamma	Hs00989291_m1
IL-2	Interleukin-2	Hs00174114_m1
T-bet	T-Box Expressed In T Cells	Hs00203436_m1
Th-2		
IL-4	Interleukin-4	Hs00174122_m1
IL-13	Interleukin-13	Hs99999038_m1
GATA3	GATA-binding protein 3	Hs00231122_m1
Th-17		
IL-17	Interleukin-17A	Hs00174383_m1
IL-21	Interleukin-21	Hs00222327_m1
IL-22	Interleukin-22	Hs01574154_m1
RORC-γ	RAR-related orphan receptor gamma	Hs00172860_m1
T-reg		
TGF-β	Transforming growth factor beta	Hs00998133_m1
IL-10	Interleukin-10	Hs00961622_m1
FoxP3	Forkhead box P3	Hs01085834_m1

6.2.1.4 Inflammatory cytokine, CRP and BDNF concentrations in plasma and PBMC culture supernatants

PBMC supernatant concentrations of IL-6, TNF-α, IFN-γ, IL-17 and IL-10 were measured using ELISA MAX™ Deluxe kits (Biolegend, UK), and plasma CRP and BDNF concentrations were measured using ELISA DuoSet kits (R&D systems, UK); these immune assays were performed according to manufacturer's instructions. Absorbance was read at 450 nm using a microplate reader (Sunrise Tecan, Reading, UK) and was then recalculated as a concentration (pg/ml for IL-6, IFN-γ, TNF-α, IL-10 and IL-17A and mg/L for CRP) using standard curves derived using GraphPad Prism Software Version 5.00 (GraphPad software, Inc.). Limits of detection for the ELISAs were 4 pg/ml for IL-6 and IFN-γ, 2 pg/ml for TNF-α, IL-10 and IL-17A, 5 pg/ml for CRP and 20pg/ml for BDNF.

Plasma IL-1β, IL-6, TNF-α and IFN-γ concentrations were measured using the MesoScale Discovery (MSD) multi-spot assay system. The plates were analysed using the Sector Imager 2400 and the MSD Discovery workbench software (MesoScale Diagnostics, Gaitherburg, MD). Multi-plex systems have demonstrated good correlations with the traditional method of quantifying biomarkers using ELISAs (Khan et al., 2004, Elshal and McCoy, 2006, Tighe et al., 2013). Furthermore, multi-plex platforms have a lower limit of detection, and thus higher sensitivity, than mono-plex ELISAs (Fu et al., 2010, Malekzadeh et al., 2012). The limits of detection for IL-1β, IL-6, TNF-α and IFN-γ were, 0.5 pg/ml; 0.22 pg/ml; 0.49 pg/ml and 0.4 pg/ml, respectively.

6.2.2 Statistical analysis

Data are presented as mean with standard deviation (SD) in parentheses. All statistical analyses were considered significant when $p \leq 0.05$. Normality was tested using the Shapiro-Wilk test of normality which is most suitable for smaller datasets. Differences in demographic variables were tested using Student's *t*-test, Chi-square (χ^2) test and Mann-Whitney U test. Differences in non-parametric clinical variables in independent sample sets were measured using the Mann-Whitney U test, while the Wilcoxon Signed Rank test was employed when comparing paired samples (for example, depressed patients' pre and post ECT). Spearman's *rho* correlation coefficient was used to assess the correlation between CD25 and FoxP3 mRNA expression. Graphs and statistics were generated using GraphPad Prism Software Version 5.00 (GraphPad software, Inc). All data were analysed using SPSS (Version 16).

6.3 Results

6.3.1 Participant demographic data and patient treatment review

6.3.1.1 Demographic data for severely depressed patients and healthy controls

As previously described (Chapter 5 – section 5.3.4.1), 16 severely depressed patients prior to therapeutic intervention with ECT and 16 healthy controls were recruited. For participant demographic data see Table 6.2. Consistent with a diagnosis of depression the depressed group had a significantly elevated HAM-D 24 score ($p<0.001$) compared to the control group (MDD: 30.19 (6.34) vs. Control 4.00 (4.02)). Analysis revealed that there was no significant difference in age and sex ($p>0.05$) while the depressed group had a significantly higher BMI ($p=0.014$) [Table 6.2]. A significant difference was also revealed for smoking: 7 out of 16 patients were smokers while no participants in the control group smoked. The control group consumed significantly more alcohol units per week relative to the depressed patients ($p<0.001$) [Table 6.2].

Table 6.2 Demographic data for severely depressed patients and healthy controls. Depicted are demographic variables and clinical depression severity measured with the HAM-D 24 scale

	Control (n=16)	Depressed (n=16)	Statistic (p-value)
Gender (female/male)	14/2	14/2	χ^2= 0.00, (p=1.000)
Age [years]	59.81 (10.68)	57.25 (10.05)	t=0.699, df=1,30, (p=0.490)
BMI	22.11 (1.73)	25.63 (5.08)	t=-2.618, df=1,29, (p=0.014)
Alcohol consumption (units per week)	7.5 (4.0)	0.37 (1.08)	t=6.730, df=1,29, (p=0.000)
Smoking (Yes/No)	0/16	7/9	χ^2= 8.96, (p=0.003)
Number of previous episodes	–	7.56 (6.14)	–
Length of episode (weeks)	–	12.56 (8.20)	–
Number of ECT treatments	–	9.00 (2.25)	–
Hamilton Depression Score (HAM-D 24)	4.00 (4.02)	30.19 (6.34)	t=-13.961, df=1,30, (p=0.000)

Data expressed as mean with SD in parentheses and statistical analysis was performed using Student's *t*-test (Age, BMI, Alcohol consumption) and Chi squared (χ^2) test (Gender and Smoking).

6.3.1.2 Antidepressant medication review for the severely depressed patients

As previously described (Chapter 5 – section 5.3.4.2), as this study was carried out under real life conditions 15 out of the 16 patients (93.75%) were medicated at the time of recruitment [Table 6.3]. Patients continued their usual medications during the course of ECT with little to no change in the treatment strategy over the course of ECT treatment [Table 6.3]. The pharmacotherapy received by these patients is routinely used in clinical practice and include a range of antidepressants, antipsychotics, mood stabilisers and anxiolytics [Table 6.3]. None of the control participants were on any mood altering treatments during the course of the study.

Table 6.3 Medication review for severely depressed patients

Medication Review	Depressed (n=16)	
	n	%
Selective serotonin reuptake inhibitors	2	12.5
Serotonin-noradrenaline reuptake inhibitor	7	43.75
Tricyclic antidepressants	4	25
Melantonergic	3	18.75
Noradrenergic and specific serotonergic antidepressant	5	31.25
Tetracyclic antidepressants	3	18.75
Any antidepressants	15	93.75
Antipsychotic	12	75
Mood stabilisers	11	68.75
Anxiolytic	12	75
Hypnotic	8	50
	Mean	SD
Baseline number of antidepressants	1.688	0.60
End of treatment number of antidepressants	1.625	0.62

Data expressed as the number (n) and percentage (%) of people on various classes of medication and the mean and SD for antidepressant treatment upon commencement of ECT treatment (baseline) and end of treatment.

6.3.2 Circulating inflammatory profile in severely patients and healthy controls

6.3.2.1 Circulating inflammatory cytokine profile in severely depressed patients relative to healthy controls

A Mann-Whitney U test revealed a significant increase in the plasma concentrations of IL-6 (Z=-2.241, p=0.024) and TNF-α (Z=-2.665, p=0.008) in the severely depressed patients relative to healthy control participants [Figure 6.2b and 6.2c]. No significant difference in the circulating concentrations of IL-1β (Z=-1.598, p=0.110) and IFN-γ (Z=-0.488, p=0.626) were observed between depressed patients and control subjects [Figure 6.2a and 6.2d].

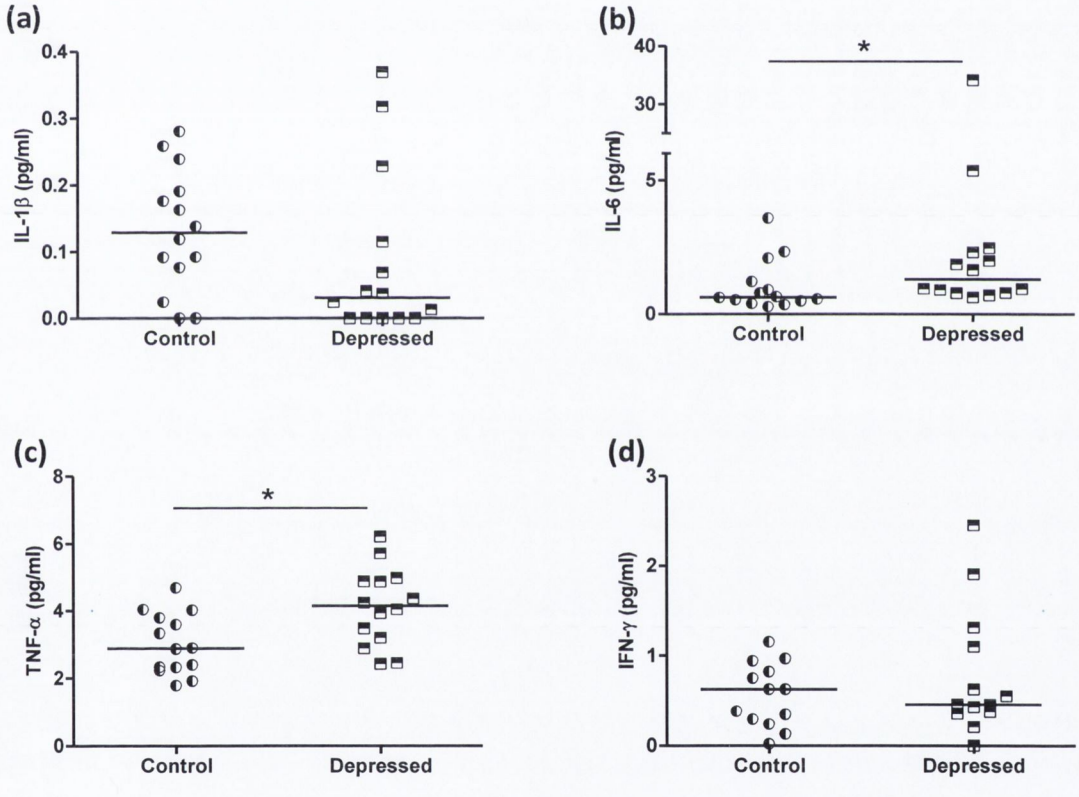

Figure 6.2 Plasma inflammatory cytokine profile in severely depressed patients relative to healthy controls. Circulating concentrations of (a) IL-1β, (b) IL-6, (c) TNF-α and (d) IFN-γ in severely depressed patients relative to healthy controls. Horizontal lines represent the median value for each group (n=13-14 per group). *p≤0.05 vs. Control (Mann-Whitney U test).

6.3.2.2 Circulating concentrations of the acute phase protein CRP in severely depressed patients relative to healthy controls

A Mann-Whitney U test revealed that the circulating concentration of CRP was significantly higher in the depressed cohort relative to control participants (Z=-2.390, p=0.017) [Figure 6.3].

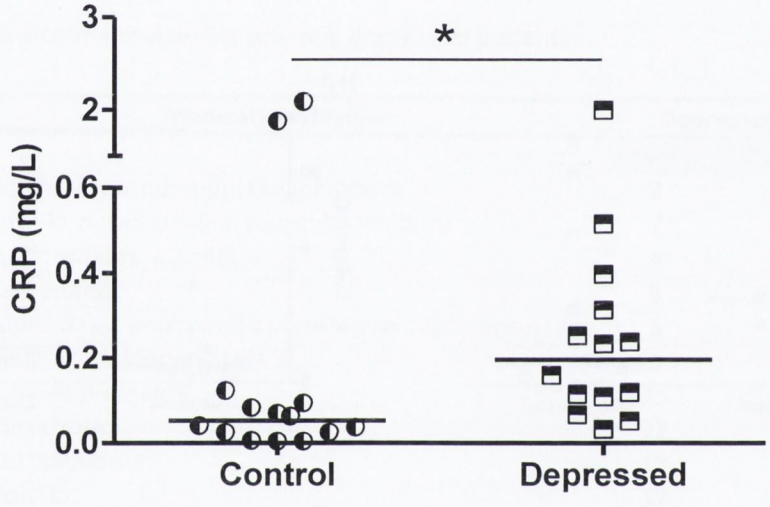

Figure 6.3 Circulating CRP concentrations in treatment-resistant depressed patients relative to healthy controls. Plasma CRP concentrations in depressed patients relative to control subjects. Horizontal lines represent the median value for each group (n=14). *p≤0.05 vs. Control (Mann-Whitney U test).

6.3.2.3 Circulating concentrations of BDNF in severely depressed patients relative to healthy controls

A Mann-Whitney U test revealed no significant difference in circulating BDNF concentrations between depressed patients and control subjects ($p>0.05$) [Figure 6.4].

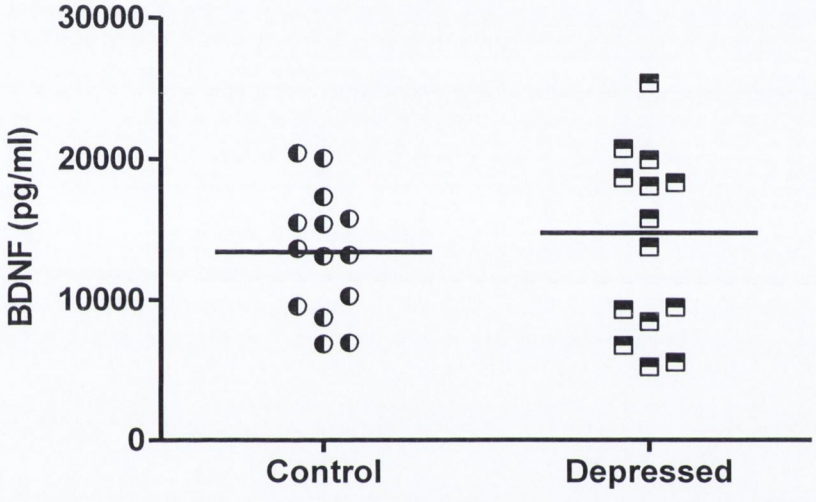

Figure 6.4 Circulating BDNF concentrations in severely depressed patients relative to healthy controls. Plasma concentrations of BDNF in severely depressed patients relative to control subjects. Horizontal lines represent the median value for each group (n=14). Statistical analysis was performed using a Mann-Whitney U test.

6.3.3 PBMC integrity and white blood cell (WBC) Count

As previously described (Chapter 5 section – 5.3.5), trypan blue staining showed that between 90.3 and 97.6% of the blood mononuclear cells were viable in the healthy controls with an average white blood cell count of 2.06 x 10^6 [Table 6.4]. Trypan blue staining showed that between 89.7 and 97.8% of the blood mononuclear cells were viable in the severely depressed patients with an average white blood cell count of 2.75 x 10^6 [Table 6.4]. Trypan blue staining showed that between 90.9 and 99.2% of the blood mononuclear cells were viable in the severely depressed patients post ECT with an average white blood cell count of 3.56 x 10^6 [Table 6.4].

Table 6.4 PBMC integrity and WBC count in isolated PBMC from healthy controls, severely depressed patients and the patient cohort following ECT

	Mean	SD
Controls		
Cell Viability (%)	94.83	2.50
WBC count	2.06 x 10^6	1.12
Severely Depressed		
Cell Viability (%)	95.35	2.26
WBC count	2.75 x 10^6	1.58
Patients Post-ECT		
Cell Viability (%)	95.13	2.21
WBC count	3.56 x 10^6	1.85

Data expressed as mean with SD. (n=16 per group)

6.3.4 Unstimulated PBMC T-cell subset gene expression in severely depressed patients and healthy controls

A Mann-Whitney U test revealed a significant increase in unstimulated PBMC CD25 mRNA expression in the severely depressed patients compared with healthy controls (Z=-2.426, p=0.015) [Table 6.5]. No significant difference was observed in the unstimulated mRNA expression of the Th-1 markers, IL-2 (Z=-1.443, p=0.149), IFN-γ (Z=-0.368, p=0.713) and T-bet (Z=-0.892, p=0.373), between severely depressed patients and healthy controls [Table 6.5]. Th-2 markers IL-4 and IL-13 were undetected in the unstimulated sample set and no significant difference in the mRNA expression of GATA3 was observed between depressed patients and controls subjects (Z=-0.188, p=0.851) [Table 6.5]. mRNA expression of the Th-17 cell markers; IL-17, IL-21, IL-22 and RORC-γ were undetectable in the unstimulated PBMCs. A Mann-Whitney U test revealed no significant difference in the unstimulated PBMC mRNA expression of T-reg cell markers, TGF-β (Z=-0.726, p=0.468) and IL-10 (Z=-0.184, p=0.854) between the depressed cohort and controls, however, a highly significant increase in Foxp3 was detected in the treatment-resistant depressed patients relative to healthy control subjects (Z=-2.883, p=0.004) [Table 6.5].

Table 6.5 Unstimulated PBMC T-cell subset gene expression in severely depressed patients and healthy controls

Biological parameter	Control	Depressed	n	Statistic (p-value)
Activation marker				
CD25 (RQ)	3.72 (2.03)	5.35 (2.37)	15	**Z=-2.426 (p=0.015)**
T-cell subsets				
Th-1				
IL-2 (RQ)	7.44 (9.33)	3.37 (2.76)	12	Z=-1.443 (p=0.149)
IFN-γ (RQ)	5.48 (4.61)	5.91 (4.37)	14	Z=-0.368 (p=0.713)
T-bet (RQ)	5.45 (6.83)	3.76 (3.10)	15	Z=-0.892 (p=0.373)
Th-2				
GATA3 (RQ)	1.90 (2.73)	1.68 (1.73)	16	Z=-0.188 (p=0.851)
T-reg				
TGF-β (RQ)	1.07 (0.28)	1.00 (0.28)	15	Z=-0.726 (p=0.468)
IL-10 (RQ)	1.29 (0.76)	1.27 (0.93)	14	Z=-0.184 (p=0.854)
Foxp3 (RQ)	6.17 (2.24)	10.06 (3.83)	15	**Z=-2.883 (p=0.004)**

Data expressed as mean and SD in parentheses. PBMCs were cultured for 48 hours. p≤0.05 was considered significant (Mann-Whitney U test).

6.3.5 The association between CD25 and Foxp3 mRNA expression in unstimulated PBMCs from severely depressed patients

An increased expression of CD25 and FoxP3 was observed in unstimulated PBMCs from the patient cohort [see Table 6.5]. As CD25 and FoxP3 are expressed by activated T-reg cells, correlational analysis was carried to examine if the expression of these T-cell markers were correlated.

Spearman's *rho* correlational analysis revealed a significant positive association between the T-cell activation marker CD25 and the master regulator of T-reg cells FoxP3 in severely depressed patients (r=0.557, p=0.031) [Figure 6.5]. A significant association between CD25 and FoxP3 was not observed in the healthy controls (p>0.05, data not shown).

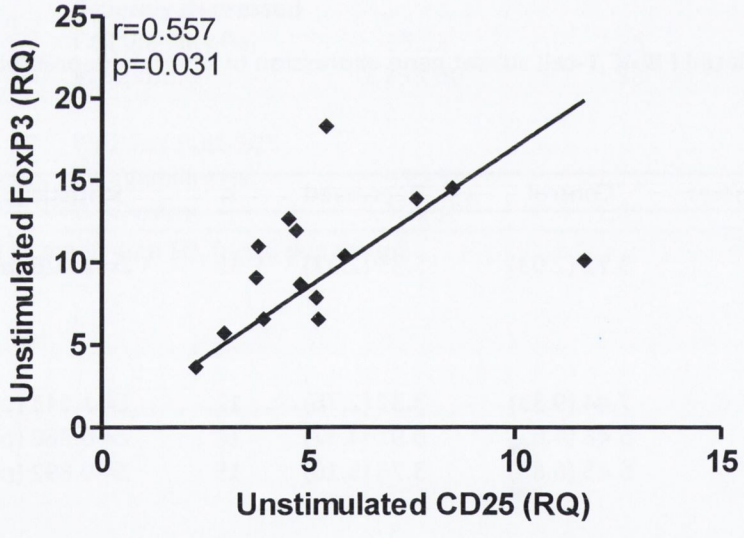

Figure 6.5 Correlational analysis between CD25 and FoxP3 mRNA expression in unstimulated PBMCs from severely depressed patients. Unstimulated PBMCs were cultured for 48 hours. p≤0.05 was considered significant (Spearman's *rho* correlation coefficient) n=15.

6.3.6 Cytokine production in unstimulated PBMC culture supernatants isolated from severely depressed patients and healthy controls

Inflammatory cytokine production was examined in PBMC supernatants cultured for 48 hours. A Mann-Whitney U test revealed no significant difference in the production of the pro-inflammatory cytokines IL-6 (Z=-0.519, p=0.604), TNF-α (Z=-0.598, p=0.550), IFN-γ (Z=-0.256, p=0.798) and IL-17 (Z=-0.156, p=0.876) and the anti-inflammatory cytokine IL-10 (Z=-0.207, p=0.836) in the severely depressed patients compared with control subjects (p>0.05) [Table 6.6].

Table 6.6 Cytokine production in unstimulated PBMC culture supernatants from severely depressed patients and healthy controls

Target	Control	Depressed	n	Statistic (p-value)
Supernatants				
IL-6 (pg/ml)	123.4 (131.3)	102.9 (121.9)	15	Z=-0.519 (p=0.604)
TNF-α (pg/ml)	6.23 (7.03)	4.14 (5.21)	14	Z=-0.598 (p=0.550)
IFN-γ (pg/ml)	1.41 (1.21)	1.25 (0.75)	14	Z=-0.256 (p=0.798)
IL-17 (pg/ml)	3.07 (3.78)	2.57 (1.95)	16	Z=-0.156 (p=0.876)
IL-10 (pg/ml)	28.85 (37.16)	22.85 (30.37)	16	Z=-0.207 (p=0.836)

Data expressed as mean and SD in parentheses. PBMCs were cultured for 48 hours. Control vs. Depressed (Mann-Whitney U test).

6.3.7 Assessment of clinical depression severity before and after ECT

As previously described (Chpater 5 – section 5.3.10), a Wilcoxon-Signed Rank test revealed a robustly significant difference ($Z=-4.640$, $p<0.001$) in the HAM-D 24 scores of the patient cohort following therapeutic intervention with ECT relative to their baseline HAM-D 24 scores prior to ECT treatment (Pre-ECT: 30.19 (6.34) vs. Post-ECT 10.06 (6.87)) [Table 6.7]. Further analysis revealed that 11 patients out of 16 responded to ECT treatment indicated by a ≥60% decrease in HAM-D 24 score from baseline plus an end of treatment HAM-D score ≤16 [6.18 (3.25)]. Ten of these responders were also classified as remitters as they had a ≥60% change in HAM-D 24 score from baseline and a post treatment score ≤10. Five of the patients did not respond to ECT treatment and with a mean HAM-D 24 score of 18.6 (4.22) were still moderately depressed post treatment with ECT. Given the limited number of samples, all further analysis will focus specifically on assessing differences before and after ECT in the complete patient cohort.

Table 6.7 Clinical depression severity assessed using the HAM-D 24 scale before and after ECT

	Pre ECT	Post ECT	n	Responder	n	Non-responder	n
HAM-D 24 score	30.19 (6.34)	10.06 (6.87)***	16	6.18 (3.25)	11	18.6 (4.22)	5

Data expressed as mean and SD in parentheses. ***p≤0.001 vs. Pre-ECT (Wilcoxon-Signed Rank test).

6.3.8 Assessment of the inflammatory profile prior to and following therapeutic intervention with ECT in severely depressed patients

While ECT is the most effective treatment available for severely depressed patients its mechanism of action remains elusive. Studies assessing the impact of ECT on the inflammatory profile in major depression are extremely limited. In the present study, following an average course of 6-12 ECT treatments (twice weekly) the inflammatory profile in the patient cohort was assessed and compared to the signature observed prior to ECT treatment.

6.3.8.1 The impact of ECT on the circulating inflammatory profile and BDNF in severely depressed patients

A Wilcoxon-Signed Rank test revealed no significant difference in the circulating concentration of the pro-inflammatory cytokines IL-1β (Z=-0.524, p=0.600), IL-6 (Z=-0.722, p=0.470), TNF-α (Z=-0.094, p=0.925) and IFN-γ (Z=-0.035, p=0.972) between severely depressed patients prior to and following therapeutic intervention with ECT [Table 6.8]. Further to this, circulating concentrations of the acute phase protein CRP were not found to be different post-ECT relative to those observed in severely depressed patients prior to ECT treatment (Z=-1.287, p=0.198) [Table 6.8]. A Wilcoxon-Signed Rank test confirmed that therapeutic intervention with ECT had no effect on circulating BDNF concentrations in the depressed cohort (Z=-0.722, p=0.470) [Table 6.8].

Table 6.8 Circulating inflammatory cytokine profile and BDNF concentrations in severely depressed patients before and after ECT

Target	Pre ECT	Post ECT	n	Statistic (p-value)
Plasma				
IL-1β (pg/ml)	0.09 (0.13)	0.10 (0.09)	14	Z=-0.524 (p=0.600)
IL-6 (pg/ml)	3.97 (8.77)	3.41 (7.18)	14	Z=-0.722 (p=0.470)
TNF-α (pg/ml)	4.12 (1.15)	4.31 (1.55)	14	Z=-0.094 (p=0.925)
IFN-γ (pg/ml)	0.79 (0.72)	0.71 (0.51)	13	Z=-0.035 (p=0.972)
CRP (mg/L)	0.33 (0.50)	0.46 (0.50)	14	Z=-1.287 (p=0.198)
BDNF (pg/ml)	13931 (6465)	15365 (5996)	14	Z=-0.722 (p=0.470)

Data expressed as mean and SD in parentheses. Pre ECT vs. Post ECT (Wilcoxon-Signed Rank test).

6.3.8.2 Unstimulated PBMC T-cell subset gene expression in severely depressed patients before and after ECT

Relative mRNA expression for the T-cell activation marker CD25 and the T-cell subsets, Th-1, Th-2, Th-17 and T-reg in unstimulated PBMCs isolated from severely depressed patients was assessed prior to and following therapeutic intervention with ECT. A Wilcoxon-Signed Rank test revealed a significant decrease in CD25 mRNA expression in the depressed cohort following ECT treatment relative to Pre-ECT expression levels (Z=-1.988, p=0.047) [Table 6.9]. No significant differences were detected in the mRNA expression of the Th-1 markers; IL-2 (Z=-1.255, p=0.209), IFN-γ (Z=-0.220, p=0.826) and T-bet (Z=-1.136, p=0.256) between patients before and after ECT treatment. The Th-2 markers IL-4 and IL-13 were undetected in the unstimulated sample set and no significant difference was observed in the mRNA expression of GATA3 between patients before and after ECT treatment (Z=-1.551, p=0.121)) [Table 6.9]. mRNA expression of the Th-17 cell markers; IL-17, IL-21, IL-22 and RORC-γ was undetected in the unstimulated sample set. A Wilcoxon-Signed Rank test revealed no significant difference in the expression of the Th-2 markers TGF-β (Z=-1.533, p=0.125) and IL-10 (Z=-1.350, p=0.177) at the message level, however, a significant decrease in FoxP3 was observed in the Post ECT group relative to Pre ECT mRNA expression levels (Z=-2.272, p=0.023)) [Table 6.9].

Table 6.9 Unstimulated T-cell subset gene expression in severely depressed patients before and after ECT

Biological parameter	Pre ECT	Post ECT	n	Statistic (p-value)
Activation marker				
CD25 (RQ)	5.35 (2.37)	4.19 (2.26)	15	**Z=-1.988 (p=0.047)**
Th-1				
IL-2 (RQ)	3.37 (2.76)	5.59 (4.18)	12	Z=-1.255 (p=0.209)
IFN-γ (RQ)	5.91 (4.37)	5.90 (4.96)	14	Z=-0.220 (p=0.826)
T-bet (RQ)	3.76 (3.10)	6.11 (7.53)	15	Z=-1.136 (p=0.256)
Th-2				
GATA3 (RQ)	1.68 (1.73)	3.70 (4.86)	16	Z=-1.551 (p=0.121)
T-reg				
TGF-β (RQ)	1.00 (0.28)	1.08 (0.28)	15	Z=-1.533 (p=0.125)
IL-10 (RQ)	1.27 (0.93)	1.01 (0.56)	14	Z=-1.350 (p=0.177)
FoxP3 (RQ)	10.06 (3.83)	8.11 (2.93)	15	**Z=-2.272 (p=0.023)**

Data expressed as mean and SD in parentheses. PBMCs were cultured for 48 hours. p≤0.05 was considered significant (Wilcoxon-Signed Rank test).

6.3.8.3 Inflammatory cytokine production in unstimulated PBMCs from severely depressed before and after ECT

Inflammatory cytokine production was examined in unstimulated PBMC supernatants cultured for 48 hours. A Wilcoxon-Signed Rank test was used to assess the difference in inflammatory cytokine production in the patient cohort prior to and following therapeutic intervention with ECT. While no significant differences were detected in the concentrations of the pro-inflammatory cytokines IL-6 (Z=-1.477, p=0.140), TNF-α (Z=-1.456, p=0.649) and IL-17 (Z=-0.140, p=0.889), a significant increase in IFN-γ was observed following ECT treatment compared with pre-treatment levels (Z=-1.964, p=0.050) [Table 10]. Further to this, IL-10 was significantly decreased following ECT treatment compared with pre-treatment concentrations (Z=-2.999, p=0.003)) [Table 6.10].

Table 6.10 Inflammatory cytokine production in unstimulated PBMC culture supernatants in severely depressed patients before and after ECT

Biological parameter	Pre ECT	Post ECT	n	Statistic (p-value)
Supernatants				
IL-6 (pg/ml)	102.9 (121.9)	49.10 (75.61)	15	Z=-1.477 (p=0.140)
TNF-α (pg/ml)	4.14 (5.22)	4.77 (6.90)	14	Z=-1.456 (p=0.649)
IFN-γ (pg/ml)	1.24 (0.76)	1.84 (1.17)	14	**Z=-1.964 (p=0.050)**
IL-17 (pg/ml)	2.57 (1.95)	2.56 (1.80)	16	Z=-0.140 (p=0.889)
IL-10 (pg/ml)	22.85 (30.37)	6.57 (7.70)	16	**Z=-2.999 (p=0.003)**

Data expressed as mean and SD in parentheses. PBMCs were cultured for 48 hours. p≤0.05 was considered significant (Wilcoxon-Signed Rank test).

6.3.9 The effect of stimulation with anti-CD3 on T-cell subset gene expression and cytokine production in healthy controls, severely depressed patients prior to ECT treatment and the patient cohort post treatment with ECT

6.3.9.1 Transcriptional expression of T-cell activation marker CD25 and the T-cell subset markers following PBMC stimulation with anti-CD3 relative to unstimulated PBMC expression

PBMCs isolated from depressed patients before and after ECT and healthy controls were treated with the T-cell stimulus anti-CD3 for 48 hrs. A Wilcoxon-Signed Rank test for paired comparisons was used to assess the impact of anti-CD3 stimulation relative to unstimulated mRNA expression in the healthy controls, severely depressed patients prior to ECT treatment and the patient cohort post treatment with ECT. Expression of the T-cell activation marker CD25 and markers for the T-cell subsets, Th-1, Th-2, Th-17 and T-reg were assessed at the message level.

A Wilcoxon-Signed Rank test revealed that anti-CD3 stimulation robustly increased the expression of CD25 in healthy controls and depressed patients prior to and following therapeutic intervention with ECT relative to unstimulated expression levels in each of the 3 groups ($p \leq 0.001$) [Table 6.11]. Assessment of the effect of anti-CD3 on the mRNA expression of the Th-1 markers IL-2, IFN-γ and the transcription factor T-bet in the control group, the depressed group prior to ECT and the patients cohort post-ECT treatment revealed a significant elevation in the expression of the Th-1 markers relative to unstimulated expression levels ($p \leq 0.01$) [Table 6.11]. As the unstimulated mRNA levels of the Th-2 cytokines IL-4 and IL-13 were undetectable, assessment of the impact of anti-CD3 relative to unstimulated expression levels was not possible. However, a Wilcoxon Signed Rank test revealed that anti-CD3 stimulation for 48 hours had no significant effect on the expression of GATA3 in the control participants, the severely depressed patients prior to ECT and the patient cohort post-ECT treatment ($p > 0.05$) [Table 6.11]. As the expression of the markers for the Th-17 cells, IL-17, IL-21, IL-22 and RORC-γ were undetectable in the unstimulated sample set, assessment of the effect of anti-CD3 stimulation relative to unstimulated levels was not possible. A Wilcoxon-Signed Rank test revealed a significantly decreased expression in the T-reg markers TGF-β and IL-10 in all 3 groups following anti-CD3 stimulation relative to unstimulated mRNA expression levels ($p \leq 0.001$) [Table 6.11]. However, while anti-CD3 induced a significant increase in the expression of FoxP3 at the message level relative to unstimulated transcriptional levels in the healthy controls and the patient cohort post ECT ($p \leq 0.01$), a lack of response was observed between the unstimulated and anti-CD3 stimulated PBMC transcriptional expression of FoxP3 in the severely depressed patients prior to ECT treatment ($Z = -1.193$, $p = 0.233$) [Table 6.11].

Table 6.11 The effect of anti-CD3 on T-cell activation and T-cell subset transcriptional expression relative to unstimulated mRNA expression in healthy controls, severely depressed patient prior to ECT and the patient cohort post ECT

Biological parameter	Group	n	Unstimulated	Anti-CD3	Statistics (p-value)
Activation marker					
CD25 (RQ)	Control	15	3.72 (2.03)	794.9 (224.9)	Z=-3.408 (p=0.001)
CD25 (RQ)	Depressed	15	5.35 (2.37)	724.9 (254.8)	Z=-3.408 (p=0.001)
CD25 (RQ)	Post-ECT	15	4.19 (2.26)	793.9 (278.3)	Z=-3.408 (p=0.001)
T-cell subsets					
Th-1					
IL-2 (RQ)	Control	12	7.44 (9.33)	50.70 (27.62)	Z=-3.059 (p=0.002)
IL-2 (RQ)	Depressed	12	3.37 (2.76)	29.14 (22.89)	Z=-3.059 (p=0.002)
IL-2 (RQ)	Post-ECT	12	5.59 (4.18)	60.78 (38.76)	Z=-3.059 (p=0.002)
IFN-γ (RQ)	Control	14	5.48 (4.61)	1496 (1440)	Z=-3.296 (p=0.001)
IFN-γ (RQ)	Depressed	14	5.91 (4.37)	908 (1063)	Z=-3.296 (p=0.001)
IFN-γ (RQ)	Post-ECT	14	5.90 (4.96)	884.9 (630.9)	Z=-3.296 (p=0.001)
T-bet (RQ)	Control	15	5.45 (4.61)	15.24 (10.48)	Z=-2.726 (p=0.006)
T-bet (RQ)	Depressed	15	3.76 (3.10)	11.11 (4.22)	Z=-3.237 (p=0.001)
T-bet (RQ)	Post-ECT	15	6.11 (7.53)	15.27 (6.19)	Z=-2.897 (p=0.004)
Th-2					
GATA3 (RQ)	Control	16	1.88 (0.28)	2.65 (3.88)	Z=-1.138 (p=0.255)
GATA3 (RQ)	Depressed	16	1.68 (1.73)	1.68 (1.14)	Z=-1.241 (p=0.215)
GATA3 (RQ)	Post-ECT	16	3.70 (4.86)	3.10 (3.80)	Z=-0.776 (p=0.438)
T-reg					
TGF-β (RQ)	Control	15	1.07 (0.28)	0.27 (0.18)	Z=-3.351 (p=0.001)
TGF-β (RQ)	Depressed	15	1.00 (0.28)	0.24 (0.12)	Z=-3.408 (p=0.001)
TGF-β (RQ)	Post-ECT	15	1.08 (0.28)	0.26 (0.10)	Z=-3.408 (p=0.001)
IL-10 (RQ)	Control	14	1.29 (0.76)	0.11 (0.12)	Z=-3.296 (p=0.001)
IL-10 (RQ)	Depressed	14	1.27 (0.93)	0.05 (0.42)	Z=-3.233 (p=0.001)
IL-10 (RQ)	Post-ECT	14	1.01 (0.56)	0.06 (0.05)	Z=-3.296 (p=0.001)
FoxP3 (RQ)	Control	15	6.14 (2.24)	10.93 (3.88)	Z=-3.408 (p=0.001)
FoxP3 (RQ)	**Depressed**	**15**	**10.06 (3.83)**	**12.11 (6.44)**	**Z=-1.193 (p=0.233)**
FoxP3 (RQ)	Post-ECT	15	8.11 (2.93)	13.74 (6.71)	Z=-2.726 (p=0.006)

Data expressed as mean and SD in parentheses. PBMCs were cultured for 48 hours. p≤0.05 was considered significant (Wilcoxon Signed Rank test for paired comparisons).

6.3.9.2 Inflammatory cytokine production following PBMC stimulation with anti-CD3 relative to unstimulated PBMC cytokine expression

A Wilcoxon-Signed Rank test revealed a robustly increased production of the inflammatory cytokines IL-6, TNF-α, IFN-γ, IL-17 and IL-10 in the PBMC culture supernatants following anti-CD3 stimulation for 48hrs relative to the unstimulated concentrations observed in the healthy controls, severely depressed medication-resistant patients prior to ECT treatment and the patients cohort post treatment with ECT ($p \leq 0.01$) [Table 6.12].

Table 6.12 The effect of treatment with the T-cell stimulus anti-CD3 on inflammatory cytokine production in healthy controls, severely depressed patient prior to ECT and the patient cohort post ECT treatment

Biological parameter	Group	n	Unstimulated	Anti-CD3 stimulated	Statistics (p-value)
Supernatants					
IL-6 (pg/ml)	Control	15	123.4 (131.3)	888.7 (776.4)	Z=-3.408, (p=0.001)
IL-6 (pg/ml)	Depressed	15	102.9 (121.9)	827.6 (985.2)	Z=-3.010, (p=0.003)
IL-6 (pg/ml)	Post-ECT	15	49.10 (75.61)	378.8 (569.7)	Z=-3.294, (p=0.001)
TNF-α (pg/ml)	Control	14	6.23 (7.03)	290.4 (447.7)	Z=-3.296, (p=0.001)
TNF-α (pg/ml)	Depressed	14	4.14 (5.22)	483.1 (611.7)	Z=-3.233, (p=0.001)
TNF-α (pg/ml)	Post-ECT	14	4.77 (6.90)	147.3 (251.7)	Z=-3.233, (p=0.001)
IFN-γ (pg/ml)	Control	14	1.42 (1.21)	454.0 (961.6)	Z=-3.296, (p=0.001)
IFN-γ (pg/ml)	Depressed	14	1.24 (0.76)	910.2 (1266)	Z=-3.296, (p=0.001)
IFN-γ (pg/ml)	Post-ECT	14	1.84 (1.17)	65.46 (85.62)	Z=-3.297, (p=0.001)
IL-17 (pg/ml)	Control	16	3.07 (3.78)	120.9 (173.3)	Z=-3.516, (p=0.000)
IL-17 (pg/ml)	Depressed	16	2.57 (1.95)	169.5 (174.0)	Z=-3.516, (p=0.000)
IL-17 (pg/ml)	Post-ECT	16	2.56 (1.80)	60.31 (78.76)	Z=-3.516, (p=0.000)
IL-10 (pg/ml)	Control	16	28.85 (37.16)	153.2 (135.7)	Z=-3.361, (p=0.001)
IL-10 (pg/ml)	Depressed	16	22.85 (30.37)	153.5 (172.3)	Z=-3.103, (p=0.002)
IL-10 (pg/ml)	Post-ECT	16	6.57 (7.67)	75.43 (121.4)	Z=-3.516, (p=0.000)

Data expressed as mean and SD in parentheses. PBMCs were cultured for 48 hours. $p \leq 0.05$ was considered significant (Wilcoxon Signed Rank test for paired comparisons).

6.3.10 Anti-CD3 stimulated T-cell subset gene expression and cytokine production in severely depressed patients and healthy controls

6.3.10.1 Anti-CD3 stimulated PBMC T-cell subset gene expression in severely depressed patients and healthy controls

A Mann-Whitney U test revealed no significant difference in the stimulated expression of the T-cell activation marker CD25, between the severely depressed patients and healthy controls (Z=-1.016, p=0.310) [Table 6.13]. However, assessment of the Th-1 cytokine markers revealed a significant decrease in the mitogen stimulated proliferation markers IL-2 in the depressed cohort relative to controls (Z=-1.963, p=0.050) [Table 6.13]. No significant difference was observed in the transcriptional expression of IFN-γ (Z=-0.965, p=0.335) or T-bet (Z=-1.431, p=0.152) in the depressed patients compared with the control subjects following Mann-Whitney U analysis [Table 6.13]. A Mann-Whitney U test revealed no significant difference in the anti-CD3 stimulated mRNA expression of the Th-2 markers IL-4 (Z=-1.451, p=0.147), IL-13 (Z=-0.184, p=0.854) and GATA3 (Z=-0.490, p=0.624) between depressed patients and healthy controls [Table 6.13]. A Mann-Whitney U test also revealed that there was no significant difference in the anti-CD3 stimulated expression of the Th-17 cell markers IL-17 (Z=-0.786, p=0.432), IL-21 (Z=-1.589, p=0.112) and IL-22 (Z=-0.079, p=0.937) in the severely depressed patients relative to the control participants [Table 6.13]. No significant difference was observed in the stimulated expression of the immunoregulatory mediator TGF-β (Z=-0.311, p=0.756) between the depressed patient and control subjects, however, a Mann-Whitney U test revealed a robustly decreased expression of IL-10 in the depressed cohort compared with control subjects (Z=-2.573, p=0.010) [Table 6.13]. A Mann-Whitney U test revealed no significant difference in the stimulated expression of the T-reg cell master regulator FoxP3 in the severely depressed patients relative to control participants (Z=-0.104, p=0.917) [Table 6.13].

6.3.10.2 Anti-CD3 stimulated cytokine production in severely depressed patients and healthy controls

Mitogen stimulated inflammatory cytokine production was examined in PBMC supernatants cultured for 48 hours. A Mann-Whitney U test revealed no significant difference in the production of the pro-inflammatory cytokines IL-6 (Z=-0.539, p=0.590), TNF-α (Z=-1.011, p=0.312), IFN-γ (Z=-0.069, p=0.945) and IL-17 (Z=-0.829, p=0.407) and the anti-inflammatory cytokine IL-10 (Z=-0.452, p=0.651) in the severely depressed patients compared with control subjects [Table 6.14].

Table 6.13 Anti-CD3 stimulated PBMC T-cell subset gene expression in severely depressed patients relative to healthy controls

Biological parameter	Control	Depressed	n	Statistic (p-value)
Activation marker				
CD25 (RQ)	794.9 (224.9)	724.9 (254.8)	15	Z=-1.016 (p=0.310)
T-cell subsets				
Th-1				
IL-2 (RQ)	50.70 (27.62)	29.14 (22.89)	12	**Z=-1.963 (p=0.050)**
IFN-γ (RQ)	1496 (1440)	908 (1063)	14	Z=-0.965 (p=0.335)
T-bet (RQ)	15.24 (10.48)	11.11 (4.22)	15	Z=-1.431 (p=0.152)
Th-2				
IL-4 (RQ)	3.61 (3.50)	2.77 (3.37)	15	Z=-1.451 (p=0.147)
IL-13 (RQ)	27.90 (22.22)	50.52 (69.36)	14	Z=-0.184 (p=0.854)
GATA3 (RQ)	2.65 (3.88)	1.68 (1.14)	16	Z=-0.490 (p=0.624)
Th-17				
IL-17 (RQ)	15.54 (14.50)	13.31 (15.76)	15	Z=-0.786 (p=0.432)
IL-21 (RQ)	9.23 (7.82)	5.00 (3.95)	13	Z=-1.589 (p=0.112)
IL-22 (RQ)	26.10 (19.16)	36.35 (44.07)	16	Z=-0.079 (p=0.937)
T-reg				
TGF-β (RQ)	0.27 (0.18)	0.24 (0.12)	15	Z=-0.311 (p=0.756)
IL-10 (RQ)	0.11 (0.12)	0.05 (0.42)	14	**Z=-2.573 (p=0.010)**
Foxp3 (RQ)	10.93 (3.88)	12.11 (6.44)	15	Z=-0.104 (p=0.917)

Data expressed as mean and SD in parentheses. PBMCs were cultured for 48 hours. p≤0.05 was considered significant (Mann-Whitney U test).

Table 6.14 Anti-CD3 stimulated PBMC cytokine production in severely depressed patients relative to healthy controls

Target	Control	Depressed	n	Statistic (p-value)
Supernatants				
IL-6 (pg/ml)	888.7 (776.4)	827.6 (985.2)	15	Z=-0.539 (p=0.590)
TNF-α (pg/ml)	290.4 (447.7)	483.1 (611.7)	14	Z=-1.011 (p=0.312)
IFN-γ (pg/ml)	454.0 (961.8)	910.2 (1266)	14	Z=-0.069 (p=0.945)
IL-17 (pg/ml)	120.9 (173.3)	169.5 (174.0)	16	Z=-0.829 (p=0.407)
IL-10 (pg/ml)	153.2 (135.7)	153.5 (172.3)	16	Z=-0.452 (p=0.651)

Data expressed as mean and SD in parentheses. PBMCs were cultured for 48 hours. Controls vs. Depressed (Mann-Whitney U test).

6.3.11 Assessment of the anti-CD3 stimulated inflammatory profile in severely depressed patients before and after ECT

6.3.11.1 Anti-CD3 stimulated PBMC T-cell subset gene expression in severely depressed patients before and after ECT

Relative mRNA expression for the T-cell activation marker CD25 and the T-cell subsets, Th-1, Th-2, Th-17 and T-reg in mitogen stimulated PBMCs (48 hours) isolated from severely depressed patients before and after ECT.

A Wilcoxon-Signed Rank test revealed no significant difference in the expression of CD25 in the depressed cohort prior to and following therapeutic intervention with ECT (Z=-0.682, p=0.496) [Table 6.15]. However, upon analysis of the Th-1 cell markers, a Wilcoxon-Signed Rank test revealed a robust increase in the expression of mitogen stimulated IL-2 post treatment with ECT relative to mRNA expression levels in the patient cohort prior to ECT treatment (Z=-2.432, p=0.015) [Table 6.15]. No significant difference was observed in the anti-CD3 stimulated mRNA expression of IFN-γ in the patient cohort following ECT treatment relative to transcriptional expression levels observed prior to ECT treatment (Z=-0.220, p=0.826). However, a significant increase in anti-CD3 stimulated T-bet mRNA expression was observed post ECT relative to pre ECT expression levels (Z=-2.953, p=0.003) [Table 6.15]. Further to this, a Wilcoxon-Signed Rank test revealed no significant difference in the expression of theTh-2 markers, IL-4 (Z=-0.966, p=0.334), IL-13 (Z=-0.534, p=0.594) and GATA3 (Z=-1.603, p=0.109) or the expression of the Th-17 markers, IL-17 (Z=-1.533, p=0.125), IL-21 (Z=-0.035, p=0.972) and IL-22 (Z=-0.103, p=0.918) between the patient cohort prior to and following therapeutic intervention with ECT [Table 6.15]. Additionally a Wilcoxon-signed Rank test revealed that the stimulated expression of the T-reg cell markers TGF-β (Z=-1.079, p=0.281), IL-10 (Z=-0.847, p=0.397) and FoxP3 (Z=-0.966, p=0.334) were not differentially expressed prior to and following therapeutic intervention with ECT [Table 6.15].

Table 6.15 Anti-CD3 stimulated T-cell subset gene expression in severely depressed patients before and after ECT

Biological parameter	Pre ECT	Post ECT	n	Statistic (p-value)
Activation marker				
CD25 (RQ)	724.9 (254.8)	793.9 (278.3)	15	Z=-0.682 (p=0.496)
Th-1				
IL-2 (RQ)	29.14 (22.89)	60.78 (38.76)	12	**Z=-2.432 (p=0.015)**
IFN-γ (RQ)	908 (1063)	884.9 (630.9)	14	Z=-0.220 (p=0.826)
T-bet (RQ)	11.11 (4.22)	15.27 (6.19)	15	**Z=-2.953 (p=0.003)**
Th-2				
IL-4	2.77 (3.37)	2.41 (1.96)	15	Z=-0.966 (p=0.334)
IL-13	50.52 (69.36)	41.85 (47.48)	14	Z=-0.534 (p=0.594)
GATA3 (RQ)	1.68 (1.14)	3.10 (3.80)	16	Z=-1.603 (p=0.109)
Th-17				
IL-17	13.31 (15.76)	7.81 (10.46)	15	Z=-1.533 (p=0.125)
IL-21	5.00 (3.95)	6.22 (7.73)	13	Z=-0.035 (p=0.972)
IL-22	36.35 (44.07)	34.21 (42.06)	16	Z=-0.103 (p=0.918)
T-reg				
TGF-β (RQ)	0.24 (0.12)	0.26 (0.10)	15	Z=-1.079 (p=0.281)
IL-10 (RQ)	0.05 (0.42)	0.06 (0.05)	14	Z=-0.847 (p=0.397)
FoxP3 (RQ)	12.11 (6.44)	13.74 (6.71)	15	Z=-0.966 (p=0.334)

Data expressed as mean and SD in parentheses. PBMCs were cultured for 48 hours. p≤0.05 was considered significant (Wilcoxon-Signed Rank test for paired comparisons).

6.3.11.2 Anti-CD3 stimulated PBMC cytokine production in severely depressed patients prior to and following therapeutic intervention with ECT

Mitogen stimulated inflammatory cytokine production was examined in PBMC supernatants cultured for 48 hours. A Wilcoxon-Signed Rank test revealed no significant difference in the anti-CD3 stimulated production of IL-6 prior to and following therapeutic intervention with ECT (Z=-1.647, p=0.100) [Table 6.16]. However, a Wilcoxon-Signed Rank test revealed a significant decrease in the stimulated cytokine production of the pro-inflammatory cytokines TNF-α (Z=-2.551, p=0.011), IFN-γ (Z=-2.294, p=0.022) and IL-17 (Z=-3.124, p=0.002) in association with a decreased stimulated production of the anti-inflammatory cytokine IL-10 (Z=-2.120, p=0.034) in the patient cohort following therapeutic intervention with ECT relative to pre ECT cytokine supernatant concentrations [Table 6.16].

Table 6.16 Anti-CD3 stimulated PBMC cytokine production in severely depressed before and after ECT

Biological parameter	Pre ECT	Post ECT	n	Statistic (p-value)
Supernatants				
IL-6 (pg/ml)	827.6 (985.2)	378.8 (569.7)	15	Z=-1.647 (p=0.100)
TNF-α (pg/ml)	483.1 (611.7)	147.3 (251.7)	14	Z=-2.551 (p=0.011)
IFN-γ (pg/ml)	910.2 (1266)	65.46 (85.62)	14	Z=-2.294 (p=0.022)
IL-17 (pg/ml)	169.5 (174.0)	60.31 (78.76)	16	Z=-3.124 (p=0.002)
IL-10 (pg/ml)	153.5 (172.3)	75.43 (121.4)	16	Z=-2.120 (p=0.034)

Data expressed as mean and SD in parentheses. PBMCs were cultured for 48 hours. p≤0.05 was considered significant (Wilcoxon-Signed Rank test for paired comparisons).

6.3.12 Controlling for the difference in BMI, smoking and alcohol use between severely depressed patients and control subjects

As reported in Table 6.2 the average BMI score in the depressed cohort [25.63 (5.08)] was significantly greater than that for the controls participants [22.11 (1.73)] (t=-2.618, df=1,29, (p=0.014). To control for this a cut off score of 27 was applied for the depressed patients and control subjects combined. While only 3 patients had a BMI greater than 27, a Mann-Whitney U test revealed a greater increase in circulating TNF-α concentrations in the patients with a BMI >27 [5.13 (1.452)] compared with all participants with a BMI <27 [3.34 (0.998)] (Z=-1.929, p=0.054). However despite this, a Mann-Whitney U test comparing the TNF-α concentration between the depressed cohort with a BMI <27 [3.80 (0.991)] and the control participants [3.02 (0.903)] also revealed an increased circulating TNF-α concentration in the patient cohort (Z=-2.108, p=0.035). Consequently, while BMI is associated with increased plasma TNF-α concentration, this is not a confounding factor as an increased circulating level of TNF-α is also observed in the depressed cohort independent of BMI. None of the other significant findings observed in this study were affected by BMI.

As none of the control participants smoke, the effect of smoking was assessed in the depressed cohort alone. Depressed patients were divided into smokers (n=7) and non-smokers (n=9). A Mann-Whitney U test revealed that anti-CD3 stimulated transcriptional expression of IL-10 was significantly less in the patient smokers [0.019 (0.008)] compared with patient non-smokers (0.075 (0.043)] (Z=-2.969, p=0.003). Additionally, a Mann-Whitney U test comparing stimulated IL-10 mRNA expression between depressed non-smokers [0.75 (0.043)] with healthy controls [0.110 (0.122)] revealed no significant difference [Z=-0.522, p=0.602]. Consequently, smoking is a confounding factor for stimulated IL-10 production in the patient cohort.

Fifteen out of 16 controls reported alcohol consumption compared with 2 patients, prior to starting the study and while a significant difference was observed between the number of alcohol units consumed a week, between controls and depressed patients, no participant consumed more than 14 standard units a week and therefore are all within the accepted safe range.

6.4 Discussion

In the present study, I sought to investigate the inflammatory profile, with a particular interest in T-cell subset gene expression, in a cohort of severely depressed patients relative to age and sex matched healthy control participants. Additionally, I evaluated the variation in PBMC gene expression and cytokine production in the patient cohort at two time points: during the acute phase of illness and following a course of ECT treatment. The major findings from this study suggest therapeutic intervention with ECT does not alleviate the increased circulating concentrations of the pro-inflammatory cytokines IL-6, TNF-α and CRP in the patient cohort, despite, a robust decrease in HAM-D 24 scores. This is an interesting and novel finding as this is the first study to profile the circulating concentrations of a range of inflammatory cytokines in a severely depressed patient cohort following a course of ECT.

Further to this, assessment of unstimulated PBMC transcriptional levels revealed a potential role for T-reg cells in severe depression. The elevated expression of T-reg cell phenotypic markers may be representative of a state marker for severe depression as the increased expression of CD25 and FoxP3 observed during a depressive episode are decreased following ECT treatment. This is an extremely novel finding given the limited research concerning T-reg cells and their potential involvement in the biological basis of major depression. Additionally, decreased stimulated PBMC transcriptional expression of IL-2 was observed, which is indicative of a reduced lymphocyte proliferative response. This, in association with an apparent blunted immune response upon mitogen stimulation in the severely depressed patients was normalised following ECT treatment. Interestingly, however, a robust decrease in the stimulated pro-inflammatory cytokine production in the patient group following ECT was also observed relative to the concentrations detected pre ECT. This is the first study to date, to characterise T-cell subset gene expression and cytokine production in a cohort of severely depressed patients and demonstrate that treatment with ECT has an impact upon PBMC gene expression and cytokine production.

6.4.1 The circulating inflammatory profile in severe depression

As previously discussed, major depression is associated with inflammation in certain sub-groups of depressed patients and those suffering from severe, treatment-resistant depression are often characterised by an increased inflammatory phenotype (Sluzewska et al., 1997, Lanquillon et al., 2000, Fitzgerald et al., 2006). In the present study, the severely depressed patients have significantly elevated circulating concentrations of the pro-inflammatory cytokines IL-6 and TNF-α along with the acute phase protein CRP compared with healthy controls. This is in accordance

with the findings reported in Chapter 3 in which severely depressed patients were characterised by a mild circulating inflammatory phenotype (Hughes et al., 2012). Further to this, meta-analyses by Zorrilla et al. (2001) and Dowlati et al. (2010) highlight that increased circulating concentrations of the innate inflammatory cytokines IL-6, TNF-α and the acute phase protein, CRP, are some of the most consistent and reproducible findings implicating the inflammatory response system in the pathogenesis of major depression (Haroon et al., 2012). While no significant changes were observed in the circulating concentrations of the key inflammatory cytokine IL-1β, this is not surprising given that it is very difficult to detect and not always measureable. Moreover, similar circulating concentrations of the T-cell cytokine IFN-γ were also observed in the severely depressed patients and healthy control subjects. However, in a recent review by Maes (2011), it is suggested that this is not always a reliable and sensitive measure of T-cell activation.

6.4.2 Unstimulated PBMC T-cell subset gene expression and cytokine production in severe depression

A more sensitive and widely reported marker for T-cell activation is the IL-2 receptor alpha subunit, CD25, which is expressed on the surface of activated lymphocytes and is a marker for cell-mediated immune activation (Maes et al., 1995a, Maes, 2011). In the present study, an increased expression of CD25 mRNA was observed in unstimulated PBMCs isolated from severely depressed patients compared with healthy controls. This is in accordance with the literature, as increased expression of CD25 and circulating levels of the soluble IL-2 receptor (sIL-2R) are also thought to be a hallmark for major depression (Maes et al., 1992, Maes et al., 1996). Subsequent gene expression analysis revealed no significant difference in the expression of unstimulated Th-1 and Th-2 cell markers between depressed patients and control subjects. Additionally, given the limited research into Th-17 cell expression in major depression, it was disappointing to find that the gene expression of the Th-17 markers; IL-17, IL-21, IL-22 and the transcription factor RORC-γ were all undetectable in the unstimulated PBMC sample set. However, some very interesting and novel findings did emerge upon examination of the T-reg cell markers at the message level. While unstimulated PBMC mRNA expression of the immunoregulatory cytokines IL-10 and TGF-β were not differentially expressed between the severely depressed patients and healthy controls, a surprising and robustly significant increase in the master regulator of T-reg cell expression, FoxP3, was observed in the depressed cohort compared with control subjects. Additionally, while CD25 is expressed on approximately 20% of all $CD4^+$ T cells when activated, high CD25 expression in association with the expression of the key T-reg cell regulator and phenotypic marker FoxP3, is

indicative of the presence of T-reg cells (as reviewed by Coleman et al. (2007)). In accordance with this, correlational analysis revealed a significant positive association between CD25 and FoxP3 expression in the depressed cohort alone perhaps indicative of increased T-reg cell expression in the severely depressed patients.

While there is little evidence, the general consensus in the literature suggests that depressed patients may be characterised by a decreased T-reg cell expression, perhaps, contributing to the loss of immunoregulatory control. However, as reviewed recently by Haroon et al. (2012), much of the evidence with regard to T-reg cells and major depression is based on indirect evaluations with studies suggesting that decreased circulating concentrations of IL-10 and TGF-β are representative of decreased T-reg cell expression and functionality. Specifically, Sutcigil et al. (2007) report decreased TGF-β concentrations in depressed patients while Dhabhar et al. (2009) observed an increased IL-6/IL-10 ratio in a depressed cohort. These results may be representative of decreased T-reg cell functional ability. However, as IL-10 and TGF-β are also produced by monocytes and macrophages, the implication of decreased T-reg cell expression and functionality based on these findings alone should be interpreted cautiously.

To my knowledge, apart from the present study, only two other studies have directly assessed the presence of T-reg cells in a depressed cohort. Li et al. (2010) report a decreased expression of $CD4^{+}CD25^{+}$ T-reg cells in unmedicated, first episode melancholic major depressed patients relative to healthy age and sex matched control subjects. Further to this, Himmerich et al. (2010) were the first to show that, in accordance with a decreased expression of IL-1 and IL-6 during antidepressant therapy, expression of T-reg cells was increased in patients who had been suffering from a mild depressive episode, while a significantly larger increase in CD4CD25hi cells post treatment was evident in the more severely depressed patients with a HAM-D 21 greater than 21 at onset. While these studies are in accordance with a decreased T-reg cell hypothesis and are in contrast to the findings in this present study, they are also not directly comparable given that the patients in this study are severely depressed and currently receiving pharmacotherapy compared to mildly depressed, first episode, unmedicated patients.

Despite the presence of markers for T-reg cells at the message level in the severely depressed patients, further analysis and assessment of unstimulated cytokine production in the culture supernatants revealed no significant difference in IL-10 production or in the concentrations of the other inflammatory markers IL-6, TNF-α, IFN-γ and IL-17 in the severely depressed patients relative to healthy controls. In the absence of increased concentrations of immunoregulatory cytokines it is questionable if T-reg cells are activated. Interestingly, however, recent studies investigating T-cell and monocytic inflammatory systems in bipolar and schizophrenia patients on

pharmacotherapy also report an elevated presence of T-reg cells in association with a monocytic inflammatory signature (Drexhage et al., 2011a, Drexhage et al., 2011b). Further to this, and in accordance with the findings in the present study, despite the increased occurrence of $CD4^{+}CD25^{+}$FoxP3 T-reg cells, the concentration of the immunoregulatory cytokine IL-10 was not dissimilar between bipolar patients and healthy controls (Drexhage et al., 2011b). While it is possible that antidepressant medication in the absence of therapeutic efficacy, in the severely depressed patients, may be contributing to the increased expression of T-reg cell markers, the absence of increased immunoregulatory cytokines may be attributable to the increased circulating levels of the pro-inflammatory cytokines, predominantly TNF-α. Earlier this year, Nie et al. (2013) demonstrated the disruptive effects of TNF-α on the functional suppressive ability of FoxP3 in rheumatoid arthritis. While the levels of T-reg cells and expression of FoxP3 were unaltered, a loss of T-reg suppressive function in the presence of TNF-α was observed. Further assessment revealed that TNF-α was responsible for decreasing the phosphorylation of FoxP3 on activated T-cell with a subsequent loss of suppressor activity.

In addition, it is noteworthy, that the patients in this study were chronically and severely depressed. Chronic depression is often associated with atypical depressive symptoms such as hypersomnia, increased appetite and weight gain (Thase, 2007). In the present study, the high BMI score observed in a portion of the depressed cohort may indicate the presence of atypical features. Interestingly, it has been suggested that different biological profiles may characterise various sub-types of depression (Gold and Chrousos, 2002, O'Keane et al., 2012). This theory is supported by findings by Lamers et al. (2013) who report elevated concentrations of IL-6, TNF-α and CRP along with higher BMI scores in patients with atypical depression relative to melancholic depressed patients and healthy controls. Higher circulating concentrations of TNF-α were observed in depressed patients with higher BMI scores in the present study. In light of this and the discussion above, it is possible that the increased inflammatory profile associated with atypical features of depression may be contributing to the manifestation of a distinct, sub-type specific, atypical adaptive immune response.

At first glance it appears that the severely depressed patients are characterised only by a pro-inflammatory phenotype and an activated innate immune response. However, further analysis highlighted a potential role for T-cells in an immunosuppressive capacity, suggesting that severe depression may be in part, a consequence of a dysregulated innate and adaptive immune response rather than a pro-inflammatory state.

6.4.3 The impact of ECT on the circulating inflammatory profile in severe depression

As discussed above the severely depressed patients in the present study were characterised by a mild circulating inflammatory phenotype evidenced by increased plasma concentrations of IL-6, TNF-α and CRP. Reassessment of this inflammatory signature 3-4 days after a course of ECT treatment revealed that ECT had no significant impact upon the circulating concentrations of the inflammatory cytokines under investigation. Therefore, despite a dramatic decrease in the HAM-D 24 score post ECT, patients still displayed elevated circulating concentrations of IL-6, TNF-α and CRP. While comparable studies are extremely limited, Hestad et al. (2003) have reported conflicting findings showing decreased TNF-α concentrations 24 hours and 1 week following a successful course of 6-12 ECT treatments. It should be noted however, that the 15 ECT patients studied had various types of depression, including a severe depressive episode, recurrent and dysthymic depression and bipolar depression whilst only patients with a clear and specific severe depressive phenotype without comorbidities were recruited for this study. As Hestad et al. (2003) did not investigate the impact of ECT on the concentrations of any other cytokines, it would appear that this is the first study to examine the long term impact of ECT on the circulating concentrations of IL-1β, IL-6, IFN-γ and the acute phase protein CRP.

Additionally, further to the observed comparable BDNF concentrations between the depressed cohort and control subjects, no significant difference in the circulating concentration of BDNF was detected in the patient cohort following therapeutic intervention with ECT relative to pre ECT concentrations. This was surprising given the wealth of literature reporting increased BDNF in serum and plasma post ECT, although Stelzhammer et al. (2012) recently reported decreased serum BDNF following acute ECT (Bocchio-Chiavetto et al., 2006, Okamoto et al., 2008, Piccinni et al., 2009). The comparable concentrations of BDNF between groups in the present study might be explained by the fact that all of the patients recruited to the study were receiving antidepressant medication. While others have shown decreased circulating BDNF levels in a depressed cohort relative to controls (Karege et al., 2002), treatment with antidepressants and ECT has been shown to normalise or increase circulating BDNF concentrations (Sen et al., 2008, Okamoto et al., 2008, Piccinni et al., 2009).

Despite the robust clinical improvement observed in the patient cohort following ECT treatment, the circulating inflammatory signature remained elevated. This is perhaps representative of a state independent marker which is not linked to the clinical stage of the disorder but perhaps an indicator of the condition itself. While this is a very interesting finding, it is also possible that decreases in the circulating inflammatory profile in accordance with clinical improvement may be masked by the limited sample size. Future studies should endeavour to assess the inflammatory

profile in a larger cohort of patients who respond to antidepressant treatment relative to a matched cohort who do not respond. The limited number of participants and highly variable nature of the clinical samples prohibited that type of analysis in this particular study.

6.4.4 The impact of ECT on the unstimulated inflammatory profile in severe depression

While ECT failed to alleviate the increased circulating inflammatory profile in the patient cohort, significant changes were observed in gene expression, with distinct differences in the unstimulated transcriptional expression of specific T-cell markers following therapeutic intervention with ECT. As previously mentioned, the T-cell activation marker CD25 was highly elevated in the severely depressed patients relative to healthy controls. Interestingly, evaluation of the transcriptional expression of CD25 3-4 days post treatment with ECT revealed a significant decrease and normalisation of unstimulated CD25 mRNA levels in the patient cohort. Further to this, the increased unstimulated expression of the master regulator of T-reg cells, FoxP3, in the severely depressed cohort was also decreased significantly following therapeutic intervention with ECT. While no other study has investigated the impact of ECT on T-cell subset gene expression, Drexhage et al. (2011a) do suggest that a higher T-reg cell presence at admission in psychiatric patients was associated with better clinical outcome at discharge. No other significant changes were observed in the transcriptional expression of the T-cell subset markers following ECT treatment. However despite this, the normalisation of the unstimulated expression levels of CD25 and FoxP3 following ECT treatment is extremely novel and very interesting.

Additionally, assessment of the unstimulated culture supernatants from the patient cohort post ECT also revealed some other interesting findings. While ECT did not impact upon the unstimulated PBMC production of the inflammatory cytokines IL-6, TNF-α and IL-17, a surprising and significant increase in IFN-γ was observed in the patient cohort post ECT relative to the observed concentrations prior to ECT treatment. However, mere observational analysis of the data suggest that it is actually the ECT non-responders who are responsible for driving this significant increase in IFN-γ production post ECT, while the ECT responders remained largely unchanged. Contrastingly, successful ECT treatment induced a robustly decreased production of the immunoregulatory cytokine IL-10 in the patient cohort which appeared to be more pronounced in the responders compared with the non-responders. Given that depression is largely associated with a pro-inflammatory profile, an increased expression of the anti-inflammatory cytokine IL-10 may be expected in association with effective antidepressant

treatment (Dowlati et al., 2010). However, this finding may be confounded by the considerable number of smokers in the depressed cohort.

6.4.5 Anti-CD3 stimulated T-cell subset gene expression in severe depression

While some of the earliest findings assessing the involvement of the immune system in the pathogenesis of major depression report differences in the numbers of white blood cells (neutrophils, NK cells, T and B cells) present in circulation, these findings are questionable given the inconsistencies between studies and the heterogeneity of the disorder itself (Zorrilla et al., 2001). However, early assessment of T-cell responses to mitogen stimulation in bereaved and severely depressed patients did reveal that stressed and depressed individuals were characterised by a suppressed immune-proliferative response upon mitogen stimulation in vitro (Kronfol et al., 1983, Schleifer et al., 1983, Schleifer et al., 1984, Zorrilla et al., 2001). In the present study, the mitogen used to stimulate the T-cells was anti-CD3. Although commonly used in combination with its co-stimulatory molecule CD28, by carrying out our *in vitro* stimulations purely with anti-CD3 and total PBMCs, we were dependent on monocytic antibody presentation and co-stimulation, which most closely represents the *in vivo* physiological activation process via antigen bound to MHC complexes on APCs (Popmihajlov and Smith, 2008).

Stimulation with anti-CD3 resulted in a robust increase in the T-cell activation marker CD25 in the depressed patients and control subjects. However, assessment of stimulated transcriptional expression of the Th-1 cytokine IL-2 between the depressed patients and control subjects revealed that the severely depressed patients had decreased IL-2 mRNA expression levels relative to the stimulated expression observed in healthy controls. As IL-2 is responsible for promoting lymphocyte proliferation, increased expression would be expected following an immune challenge as is evident in the healthy control participants (Blume et al., 2011). However, the blunted lymphocyte proliferative response observed in the depressed patients is in keeping with the early finding by Kronfol et al. (1983) and Schleifer et al. (1984) and is in accordance with the reported decrease in phytohaemagglutinin (PHA) stimulated IL-2 production from depressed patient culture supernatants (Anisman et al., 1999).

Additionally, anti-CD3 stimulation induced a decrease in the transcriptional expression of IL-10 relative to unstimulated levels in both the control and depressed cohorts. A comparison of the stimulated IL-10 mRNA expression between healthy controls and depressed patients revealed that the depressed patients displayed a greater decrease in IL-10 mRNA expression compared

with controls subjects. However, following correction for smoking, the stimulated decrease in IL-10 no longer remained significant relative to stimulated control IL-10 expression levels.

Interestingly, while stimulation with anti-CD3 induced a robust increase in FoxP3 at the message level in the control participants, mitogen stimulation with anti-CD3 had no impact upon transcriptional FoxP3 expression in the depressed cohort relative to unstimulated expression levels. However, no significant difference was observed on comparison of the stimulated FoxP3 mRNA expression between depressed patients and control subjects. Therefore, it is possible that FoxP3 in the unstimulated sample set from the depressed cohort is already at a maximal expression level which does not respond further upon secondary stimulation with anti-CD3.

While no other significant findings were observed in the expression of the T-cell subset markers between the depressed patients and control subjects, a distinct pattern for the majority of markers was observed with a decreased inflammatory signature and blunted immune response following mitogen stimulation in the depressed cohort relative to healthy controls. Thus, the T-cells in the depressed cohort appear to be in a refractory state, potentially down-regulated or impaired by the chronic low grade circulating inflammatory concentrations of the pro-inflammatory cytokines IL-6, TNF-α and CRP.

6.4.6 The impact of ECT on the stimulated inflammatory profile in severe depression

Assessment of the stimulated inflammatory profile in the patient cohort following therapeutic intervention with ECT revealed some very exciting and novel findings implicating ECT or its positive therapeutic efficacy in the normalisation of the apparent blunted mitogen stimulated immune-proliferative transcriptional profile, evident in the severely depressed patients prior to ECT treatment. As detected in the controls and depressed cohort prior to ECT, a robust expression of the T-cell activation marker CD25 was observed at the message level in the depressed cohort following a course of ECT treatment; the expression of CD25 was comparable to that observed prior to ECT. Interestingly, however, successful treatment with ECT appears to have reversed the decreased mitogen-stimulated expression of the lymphocyte proliferation marker IL-2 in the patient cohort, with the detection of a normalised response in the patient cohort 3-4 days after completing a course of ECT treatments. While this is the first study to assess the long-term impact of ECT on the transcriptional expression of IL-2 in a severely depressed cohort, this finding is in accordance with an early report by Weizman et al. (1994) who also observed a restoration and normalisation of mitogen-stimulated IL-2 production following successful antidepressant treatment with clomipramine. Additionally, while not significantly different, observational analysis

indicated that the blunted expression pattern of IFN-γ and IL-4 observed in the depressed cohort prior to ECT treatment appeared to be normalising post treatment with ECT. Larger sample sizes with greater power, in future studies will be necessary to confirm this. By contrast, the decreased stimulated mRNA expression of IL-10 in the depressed cohort relative to the healthy controls remained unchanged in the patient cohort post ECT. While this finding maybe confounded by smoking, the decreased stimulated expression of IL-10 in these patients, prior to, and following, ECT treatment may reflect a state phenomenon that is not related to any clinical stage. This is not the case however, for the T-reg cell master regulator, FoxP3, which is restored to a normal transcriptional expression in the unstimulated patient sample set post ECT with a robustly elevated transcriptional expression following anti-CD3 stimulation, similar to that observed in the healthy control participants.

Therefore, it would appear that successful antidepressant treatment with ECT restores the blunted mitogen stimulated response observed in the depressed patients prior to treatment, with the exception of IL-10.

However, while mitogen stimulation with anti-CD3 robustly increased the production of the inflammatory cytokines IL-6, TNF-α, IFN-γ, IL-17 and IL-10 in culture supernatants post ECT relative to unstimulated levels, on comparison of the stimulated inflammatory production from the depressed cohort culture supernatants pre and post ECT, a robustly significant decrease in TNF-α, IFN-γ, IL-17 and IL-10 was observed in the clinically improved patient cohort following successful treatment with ECT. This is perhaps related to a possible normalisation of the stress response system and increased glucocorticoid receptor functioning, thereby inhibiting NFκB mediated cytokine production. However, further data analysis in this study is required to elucidate this fully.

6.4.7 Summary

Taken together, the unstimulated elevated transcriptional expression of CD25, indicative of T-cell activation, in association with an elevated expression of the master regulator of T-reg cells, FoxP3, may suggest the presence of an immunosuppressive T-cell subset. Further to this, secondary stimulation with anti-CD3 revealed a decreased expression of IL-2, indicative of decreased T-cell proliferative ability in the severely depressed cohort. This decreased expression of IL-2 in the face of an immune challenge, in association with an immunosuppressive T-cell phenotype and in the presence of chronic low grade circulating inflammation may be representative of a T-cell phenotype similar to that of 'T-cell exhaustion'. Exhausted T-cell are primarily observed during

chronic viral infections and cancer, the severity of which is associated with increased inhibitory receptors and immunoregulatory cells and cytokines, which in turn potentially prolong the infection and the chronic inflammatory signature (Wherry et al., 2007, Wherry, 2011, Utzschneider et al., 2013). However, successful treatment with ECT appears to stabilise the expression of the T-reg cell markers, in association with increasing the stimulated transcriptional expression of IL-2, to a level comparable with that evident in the healthy controls. In essence, ECT appears to reboot an exhausted and dysregulated cellular immune response, perhaps as a consequence of a normalisation of the glucocorticoid and neurotransmitters systems in the CNS. However, the high relapse rate that is commonly associated with ECT may be as a consequence of the unresolved chronic low grade circulating inflammatory profile which appears to be a trait marker in this cohort of severely depressed patients. Future longitudinal studies should endeavour to assess a cohort of patients over a one year time-frame following successful therapeutic intervention with ECT, with a specific interest in changes in their inflammatory profile prior to and following relapse.

6.4.8 Limitations

The findings from this study are extremely novel and provide greater insight into the involvement of the various T-cell subsets, specifically T-reg cells in the pathogenesis of severe depression. Additionally, this is the first study to assess the long-term impact of the therapeutic intervention with ECT on the transcriptional expression of T-cell subset markers and inflammatory cytokine production in parallel with a robust decrease in HAM-D 24 scores. However, this study is limited by the small number of participants and high variability in the clinical samples. Differences in sample number between variables measured are a consequence of patient compliance, sampling and experimental error. In future studies, with a greater number of participants, it may be possible to assess the inflammatory signature in patients who remit following ECT treatment relative to those who do not. Additionally, as this study was carried out under real life conditions, all patients were medicated. While an effect of mediation cannot be ruled out, little to no change in patient antidepressant medication profiles were made throughout the duration of the study. Thus, the changes observed following ECT treatment are likely to be as a result of its therapeutic effect. In addition, Stelzhammer et al. (2011) report that certain proteomic inflammatory and biological molecules are altered by anaesthesia and muscle-relaxant pre-treatment and not specifically by ECT. As such, the possibility that this short-acting medication contributes to the observed biological changes post treatment with ECT cannot be ruled out. However, given that the altered cellular transcriptional profiles in depressed patients are normalised in responders to

ECT, it is more likely that these changes are related to the therapeutic effects of ECT. In addition, this study would have been strengthened by specifically identifying the numbers and percentages of the distinct T-cell subsets present in the patient and control groups. Future studies should consider carrying out detailed flow cytometric analysis to define the number and percentages of the various T-cell subsets in the participants under investigation, as well as carrying out proliferation assays on the anti-CD3 stimulated PBMCs to further examine the apparent blunted proliferation response. The isolation of individual cell types and subsequent assessment of the unstimulated and stimulated inflammatory profile would also provide further clarity and specifically pinpoint the source of the inflammatory cytokines under investigation, as it is possible that acquired immune monocytic activation, downstream of T-cell activation, may also be contributing to the inflammatory profile.

However, despite this, much of the research assessing the involvement of T-cells in major depression is based on circulating serum and plasma cytokine measures. While circulating concentrations along with cytokine production in culture supernatants have been assessed, I have also characterised the transcriptional expression of the T-cell subset markers in PBMCs, which offer the possibility of linking specific biological observations with clinical stages of the depressive disorder as is particularly evident in the unstimulated mRNA expression of CD25 and FoxP3 which were robustly elevated in the acute phase of illness but returned to control levels following cessation of the course of ECT during which time a decrease in HAM-D scores was also apparent (Le-Niculescu et al., 2007). Further to this, the comparable transcriptional expression observed between blood cells and the brain has led to the suggestion that PBMCs may act as sentinels for CNS related disorders (Gladkevich et al., 2004, Liew et al., 2006, Sullivan et al., 2006, Iga et al., 2008). With this in mind and given that mRNA levels are a direct reflection of gene-environment interactions and environmental events regulate transcription, the use of mRNA in this ECT study is particularly pertinent (Belzeaux et al., 2010).

Furthermore, this study provides evidence contrary to the suggestion by Maes (2011) that 'stimulated cytokine levels – under standardised conditions – reflect the basal production of those cytokines'. Certainly, in this study it seems more likely that the stimulated cytokine production is representative of cellular immune competence or the ability of the immune cells to respond in the face of immune challenge rather than a measure of the current inflammatory status which may be better represented by the unstimulated cytokine levels.

6.4.9 Conclusion

In conclusion, severely depressed patients are characterised by a mild circulating inflammatory signature in association with the presence of T-reg cell markers in the absence of an immunosuppressive response. Stimulation with the T-cell mitogen anti-CD3 revealed an impaired proliferative response and a refractory immune response in the face of an immune challenge. However, while ECT did not reduce the elevated circulating cytokine inflammatory profile, it did have an impact upon the dysregulated transcriptional expression of the T-cell subset markers, normalising the increased unstimulated expression of CD25 and FoxP3 and restoring the blunted immune response to mitogen stimulation at the message level. Therefore, it may be suggested that severely patients are characterised by a dysregulated innate and adaptive immune response which at the level of gene expression is normalised by the therapeutic effects of ECT.

Chapter 7

General Discussion and Future Directions

The present thesis examines the relative contribution of the inflammatory and stress response systems and activation of the kynurenine pathway and tryptophan depletion to the biological basis of major depression, in an effort to identify biomarkers that may be used in the diagnostic process and, in turn, facilitate a more targeted and fast acting approach to treating major depression, and particularly depression associated with inflammation.

7.1 Inflammation associated depression and the benefits of a targeted symptom-wise approach

Given the highly complex and heterogeneous nature of major depression, inflammation is only associated with certain subtypes of the depressive condition. Specifically, numerous studies have highlighted a role for inflammation in the biological basis of severe or treatment resistant depression. In line with this, elevated circulating concentrations of the inflammatory cytokines IL-6, IFN-γ and the acute phase protein CRP were observed in the first cohort of severely depressed patients under investigation (Chapter 3). Additionally, a comparable circulating pro-inflammatory phenotype was observed in the severely depressed patients referred for ECT (Chapter 6), evidenced by elevated plasma concentrations of IL-6, TNF-α and CRP. The similar pro-inflammatory signature observed between the two independent studies is in accordance with findings from recent meta-analyses that report increased concentrations of IL-6 and CRP as some of the most reproducible and consistent findings associated with depression (Howren et al., 2009, Dowlati et al., 2010, Liu et al., 2012). However, given the heterogeneity and comorbid nature of psychiatric disorders, these markers are not specific enough to contribute to the diagnosis of major depression (Mossner et al., 2007). Consequently, in an effort to determine more specific biomarkers for depression, further assessment of a range of transcriptional whole blood inflammatory markers and glucocorticoid inducible genes in depressed patients, in association with depression severity and specific symptom clusters, revealed a positive correlation between the pro-inflammatory markers IL-1β, TREM-1 and IFN-γR1 and depression severity. In addition, affective symptoms such as core depression and anxiety were both found to positively correlate with IL-1β and TREM-1 mRNA expression while positive associations between the macrophage marker arginase-1 and core depression and between the inflammatory and monocytic markers IkB-α, IL-4R and TLR-4 and anxiety were also detected in the depressed cohort. This highlights an association between inflammation and specific symptom clusters such as core depression and anxiety, while no association was observed between inflammation and the somatic symptoms of depression. In accordance with this, Maes and Rief (2012) suggest that the manifestation of somatic symptomology is more closely related to kynurenine pathway activation.

In addition, stress is commonly associated with an increased inflammatory signature and is thought to be a precipitating factor for the onset of major depression in vulnerable individuals. Specifically, those exposed to early life adversity are thought to be at greater risk of developing depression later in life (Batten et al., 2004, Danese et al., 2008). Depressed patients with a history of childhood trauma are often characterised by an elevated inflammatory signature in association with glucocorticoid resistance and altered BDNF concentrations both thought to be as a consequence of the persistent chronic low grade inflammatory phenotype (Heim et al., 2008b, Mondelli et al., 2011). In accordance with this, significant negative correlations were detected between BDNF, the GR and its co-chaperone FKBP5 and the severity of early life adversity in the depressed cohort. Assessment of the biological profile in association with distinct traumatic early life events in the depressed cohort also revealed increased whole blood transcriptional expression of IL-1β and IL-6 in patients reporting childhood abuse while decreased expression of the GR and its regulator FKBP5 were associated with neglect.

Taken together, these data support previous literature implicating the inflammatory and endocrine systems in the pathophysiology of major depression in certain sub-groups of depressed individuals. In addition, the study of whole blood mRNA expression is a novel method in the search for specific biological markers, in an effort to establish a more personalised treatment approach for major depressive disorders. Given the crosstalk between circulating peripheral blood cells and the CNS, assessment of mRNA levels which directly reflect gene-environment interactions may provide a better vantage point to assess the macro and micro changes that might be occurring within the CNS. Further to this, given the variety of symptoms that characterise depression and the large number of biological systems implicated in the aetiology of depressive disorders, the use of a more symptom-wise approach (as demonstrated in Chapter 4) to decipher the underpinning pathophysiological mechanisms of individual symptoms may lead towards the development of specific biomarkers and hence the development of a more targeted and personalised treatment approach.

7.2 Kynurenine pathway activation and tryptophan depletion - Its relevance to the biological basis of idiopathic major depression

Over the past number of years, a great deal of interest has centred on investigating the potential involvement of the kynurenine pathway in the pathogenesis of major depression and as a potential link between the cytokine hypothesis of depression and the serotonergic hypothesis of depression. In support of this, numerous preclinical reports studying animal models of depression

and clinical studies assessing cytokine-induced depression have highlighted a possible role for kynurenine pathway activation in the biological basis of depression (O'Connor et al., 2009a, O'Connor et al., 2009b, Wichers et al., 2005, Raison et al., 2010). However, studies directly assessing IDO activity and kynurenine pathway activation in idiopathic depressed patients are few and limited. In this regard, only two studies to date have assessed kynurenine pathway activation in a cohort of idiopathic depressed patients. Myint et al. (2007) reported negative alterations in the neuroprotective branch of the kynurenine pathway, reflected as reduced circulating concentrations of kynurenic acid, while Gabbay et al. (2010) observed a positive association between depression severity in melancholic patients and neurotoxic kynurenine pathway activity reflected by the 3-HAA/KYN ratio. However, despite this, neither study observed direct evidence of kynurenine pathway activation demonstrated by the absence of an increased concentration of kynurenine in association with a concomitant decrease in tryptophan in the depressed cohort. Consequently, one of the main goals of this thesis was to assess the inflammatory profile in association with IDO expression and activity, kynurenine pathway activation and tryptophan depletion in major depression. In contrast to the theory implicating a role for kynurenine pathway activity in the pathogenesis of depression, two independent studies presented in this thesis clearly demonstrate that while patients with major depression are characterised by a mild circulating inflammatory phenotype and a decreased concentration of total plasma tryptophan, there was no evidence of kynurenine pathway activation. This was verified by similar whole blood transcriptional levels of IDO expression in depressed patients and control subjects and the absence of an increase in kynurenine and the kynurenine/tryptophan ratio, which is thought to be indicative of IDO activation, in the depressed cohort. Whilst the results from Chapters 3 and 5 are negative in terms of kynurenine pathway activation in the depressed patients, it is important to note that the methods employed in these studies would have been able to pick up kynurenine pathway activation should it have been present.

The discrepancies in the activation of the kynurenine pathway observed between the major depressed patients under investigation in this thesis and cytokine-induced depression described in the literature, are most likely to be attributable to the divergent inflammatory profiles between these two distinct conditions. A mild inflammatory signature was observed in both of the depressed cohorts under investigation, with both cohorts displaying elevated circulating concentrations of the inflammatory cytokine IL-6 and the acute phase protein CRP. However, as described in Chapter 3, although key inflammatory markers were significantly greater relative to controls, the level of inflammation in the depressed cohort still fell within the normal range. Consequently, this low grade inflammatory profile appears to be insufficient to drive the peripheral activation of the kynurenine pathway compared with the overt inflammation and

consequent IDO activity observed in medically ill patients undergoing immunotherapy in the form of IL-2 or IFN-α.

The dietary amino acid tryptophan is the essential precursor to serotonin production in the brain. As such, decreased circulating tryptophan availability, commonly observed in patients with depression, is thought to have a negative impact on central serotonin production, culminating in the altered regulation of mood, sleep, cognition and emotion, core symptoms that feature heavily in the diagnosis of major depression. Consistent with the literature, circulating tryptophan concentrations were reduced in both of the depressed cohorts under investigation. However, in the absence of kynurenine pathway activation, the mechanism(s) responsible for the depletion of tryptophan in patients with major depression remain to be elucidated. Interestingly, successful therapeutic intervention with ECT resulted in the restoration and maintenance of peripheral tryptophan concentrations over a 3-4 day period (Chapter 5). Previous findings have suggested positive effects of ECT on neurotransmitter systems and receptor expression and function both in the periphery and the CNS (see for review Wahlund and von Rosen (2003)). In addition, while decreased circulating concentrations of tryptophan have been observed in IFN-α induced depression, tryptophan concentrations are maintained in the CNS (Raison et al., 2010). It is possible that the reductions in circulating tryptophan concentrations reflect or indicate altered processes in the CNS related to inflammation or kynurenine pathway activity, which in turn may be positively regulated following repeated treatment with ECT.

Further to this, and given the inflammatory phenotype often associated with major depression, secondary immune stimulation might be expected to produce an exaggerated inflammatory response in association with kynurenine pathway activation in the depressed cohort. This, however, was not the case, with comparable, if not blunted production of inflammatory cytokines observed in depressed patients when compared to control subjects. In addition, while isolated cells from depressed patients were capable of inducing kynurenine pathway activation, stimulation of T-cells had a greater propensity to induce an increased production of kynurenine and depleted tryptophan concentration in the depressed cohort compared with innate immune stimulation. However, similar to the production of inflammatory cytokines, the ability of patient cells to induce kynurenine pathway activation was of a lower magnitude relative to that observed in the control participants. Interestingly, however, assessment of stimulated immune responses following ECT treatment revealed a decreased stimulated inflammatory profile in accordance with the complete abolition of IDO activity and kynurenine pathway activation. This suggests that the effect of ECT has the potential ability to regulate the activity of the kynurenine pathway and

increase tryptophan concentrations, which, even when subjected to secondary immune stimulation, remain unaltered and elevated with respect to pre ECT concentrations.

7.3 T-cells and depression – A role for T-reg cells?

T-cells and the adaptive immune response are responsible for orchestrating specific immune responses and targeted cell-mediated immune activation with a central role in immune defence. In addition, emerging evidence in recent years highlights an important role for T-cells in a neuroprotective and immunosuppressive capacity in relation to stress and inflammation (Lewitus and Schwartz, 2009, Lewitus et al., 2009, Rook et al., 2011). This is an interesting concept as the earliest reports investigating the association between inflammation and depression are in direct contrast to a more active T-cell role, with the suggestion that stress and severe depression negatively affect T-cell function with reduced responses upon mitogen stimulation (Irwin and Miller, 2007, Zorrilla et al., 2001). As research into acquired immune responses and particularly T-cell expression and function has been extremely limited in major depression (Miller, 2010), it was interesting to find a decreased mitogen stimulated IL-2 mRNA expression in the depressed cohort (Chapter 6). Given that IL-2 is essential for T-cell proliferation, decreased IL-2 mRNA expression in the face of an immune challenge suggests an altered T-cell response and a reduced proliferative capacity in depressed patients, which is in agreement with early findings from the 1980's (Kronfol et al., 1983, Schleifer et al., 1983, Schleifer et al., 1984). Additionally, the decreased mitogen stimulated IL-2 expression was also accompanied by a non-significant but apparent blunted inflammatory response, specifically in relation to IFN-γ and IL-4 at the message level. These findings, are perhaps in line with the observed reduction in circulating tryptophan availability in the depressed cohort given its importance in normal T-cell proliferative responses and evidence of T-cell apoptosis during periods of tryptophan deficiency (Lee et al., 2002). Reduced T-cell proliferation and function may also be a consequence of the immunosuppressive effects of T-reg cells.

While some reports suggest alterations and an imbalance in T-cell subset cytokine production in depressed patients with findings in favour of an elevated presence of Th-1 cell cytokine production such as IFN-γ, in association with a decreased production of the Th-2 cytokine IL-4 or immunoregulatory mediator TGF-β, there was no difference in the unstimulated PBMC expression of cytokine and transcription factor markers for Th-1 or Th-2 cells or in the unstimulated production of pro or anti-inflammatory cytokines in the depressed cohort.

However, in accordance with more recent findings highlighting a potential role for T-reg cells in a protective capacity, it was also interesting to discover a robust increase in the transcriptional expression of T-reg cell markers CD25 and FoxP3, in unstimulated PBMCs isolated from severely depressed patients. While an elevated presence of T-reg cells has been reported in bipolar and schizophrenia patients (Drexhage et al., 2011a, Drexhage et al., 2011b), the general consensus in the literature suggests that T-reg cells and their immunosuppressive capacities may be down-regulated in severely depressed patients which may occur in combination with an increase in the presence of pro-inflammatory Th-17 cells, given that severe depression is often characterised by a circulating pro-inflammatory phenotype. However, while Th-17 cells are highly implicated in the pathogenesis of numerous inflammatory and autoimmune disorders, no published report to date has assessed their potential contribution to the biological basis of major depression. Consequently, there is no direct clinical evidence to date to support or refute the imbalance of T-regs and Th-17 cells in major depression.

One mechanism by which T-reg cells exert their inhibitory effects is via the production of immunoregulatory cytokines IL-10 and TGF-β. However, elevations in the unstimulated expression of the immunoregulatory and anti-inflammatory cytokines IL-10 and TGF-β were not observed in the depressed cohort. Alternatively, T-reg cells can also exert their immunosuppressive effects and alter immune cell function, through the expression of Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) which acts to obstruct the CD28-B7 co-stimulatory pathway between antigen presenting cells and T-cells and in turn promote the production of IFN-γ by antigen presenting cells and the depletion of tryptophan through the induction of the kynurenine pathway (Fallarino et al., 2003). However, despite the presence of T-reg cell markers, in the absence of kynurenine pathway activation and a lack of evidence to support immune suppression, it is questionable as to whether these cells expressing T-reg markers were functional. Furthermore, secondary immune stimulation did not increase the expression of FoxP3 in the depressed cohort, with unstimulated mRNA expression of FoxP3 in the depressed cohort comparable with that observed in the control samples following anti-CD3 stimulation. Consequently, it appears that the expression of the T-reg cell master regulator in the depressed cohort is at a maximal expression level in the depressed cohort. In light of data which demonstrates T-reg cell induced immunosuppression via the depletion or uptake of the immunoproliferative stimulus IL-2, the decreased transcriptional presence of IL-2 following mitogen stimulation with anti-CD3 may be related to the presence of regulatory T-cells. However, there is no direct evidence in support of functional T-reg cell activity. This, in association with a blunted immunoproliferative response following mitogen stimulation, may also be as a result of inflammation itself, considering the effects of chronic TNF-α exposure on T-cell proliferation, cytokine production and apoptosis and its disruptive effects on T-cell

receptor signalling and NFκB activation, culminating in non-responsive T-cells (Cope et al., 1994, Cope et al., 1997, Lee et al., 2008, Nie et al., 2013). Furthermore, chronic inflammation is also thought to impair glucocorticoid receptor expression and function and the disruption of MAPK signalling and STAT-mediated GR translocation to the nucleus, in addition to promoting a steroid refractory phenotype via the expression of the inactive beta isoform of the glucocorticoid receptor (Goleva et al., 2002, Wang et al., 2004, Schewitz et al., 2009). As a consequence, it is thought that T-cells are no longer capable of responding to vital neuroendocrine trafficking signals and the mobilisation of T-cells to the brain, where, in times of stress, they impart neuroprotective effects (Miller, 2010). Specifically, while glucocorticoids act to dampen the inflammatory response, they also promote a shift in T-cell subtype, from pro-inflammatory Th-1 cells to anti-inflammatory IL-4 expressing Th-2 cells (Elenkov and Chrousos, 1999, El-Etr et al., 2005). Kipnis and colleagues have highlighted a vital role for adaptive immune responses in the maintenance of normal cognitive function, learning and memory (Kipnis et al., 2004), while other preclinical studies have demonstrated the importance of the adaptive immune response and the positive effects of IL-4 on cognition in models of aging and Alzheimer's disease (Cao et al., 2009, Loane et al., 2009). Additionally, preclinical evidence has demonstrated severe cognitive impairment in association with alterations in BDNF concentrations and an elevated pro-inflammatory signature in the absence of functional T-cell activity and normal trafficking of IL-4+ T-cells to the CNS via meningeal spaces and the choroid plexus (Derecki et al., 2010). As such, alterations in T-cell function and trafficking as a consequence of inflammation and glucocorticoid resistance may have detrimental consequences and contribute to impaired cognitive responses commonly observed in psychiatric and neurodegenerative disorders.

Consequently, while a deficiency in T-reg cell presence and/or function may contribute to the inflammatory signature in depression as well as alterations in monoaminergic neurotransmission, culminating in depressive like behaviour as demonstrated pre-clinically by Kim et al. (2012), an elevated presence of T-reg cells may also have unfavourable consequences by interfering and suppressing normal functioning of the adaptive immune response. This debate regarding the relative contribution of T-reg cells to the progression or protection of immune related disorders is also of particular interest in cancer research at present (Banerjee et al., 2013). Consequently, it appears that depression and other immune related conditions are perhaps in part a consequence of a dysregulated immune response rather than a condition mainly typified by an altered expression and production of innate immune markers in favour of a pro-inflammatory signature.

In line with this thought, this thesis presents data that clearly demonstrate a normalisation of the transcriptional expression of immune markers in patients who respond therapeutically to ECT.

While the mechanism of ECT and its impact on immune function remain largely unknown, it is clear from this study (Chapter 6) that the effect of ECT has a strong positive influence, resulting in the restoration of altered PBMC immune function. This was evidenced by the re-established stimulated transcriptional expression of IL-2 along with normalisation of the unstimulated expression of the T-reg cell markers CD25 and FoxP3, which when stimulated with anti-CD3 post ECT, respond in a manner comparable to that observed in control participants. Thus, it appears that ECT has the ability to reboot a refractory immune cell response, the effects of which can be seen 3-4 days following the cessation of the course of ECT. Despite this, the increased circulating inflammatory signature remains unaltered, which may represent a trait marker for depression in these patients. It would be very interesting to follow up on these patients over a one year time-frame and assess if this persistent inflammatory signature is a predictor of relapse in this patient cohort.

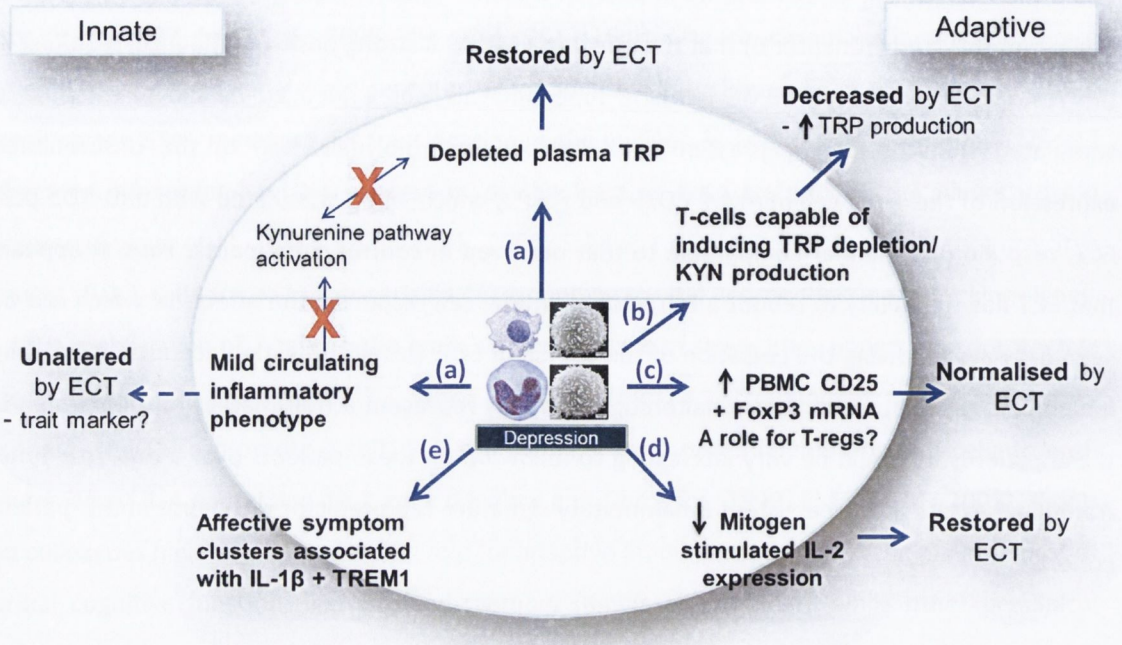

Figure 7.1 Thesis summary. Inner circle: (a) Both the cross sectional cohort of severely depressed patients and the independent ECT cohort, under investigation in this thesis, were characterised by a mild circulating inflammatory phenotype and depleted tryptophan availability, but in the absence of any evidence to suggest that the kynurenine pathway was activated. (b) However, despite this, *in vitro* immune stimulation studies, in PBMCs isolated from the patient cohort prior to ECT treatment, revealed that isolated PBMCs from severely depressed patients were capable of inducing kynurenine production and tryptophan depletion, particularly following mitogen stimulation with anti-CD3 (T-cell stimulus). (c) Severely depressed patients prior to ECT were also characterised by an elevated unstimulated PBMC expression of the T-cell activation marker CD25 and the master regulator for T-cells, FoxP3, perhaps indicating a role for T-reg cells? (d) In addition to this, the decreased anti-CD3 stimulated expression of IL-2 in the depressed cohort prior to ECT may be indicative of a decreased immunoproliferative response. (e) Analysis of the cross-sectional cohort using a more specific symptom-wise approach revealed that affective symptom clusters, such as, core depression and anxiety, were positively associated with the innate immune cytokine IL-1β and activating receptor, TREM-1. Outer circle – assessing the effect of ECT: Despite a robust decrease in depression severity for ECT treatment, the circulating inflammatory measures remained elevated, perhaps representative of a state-independent marker. However, with effective ECT treatment, tryptophan availability, in circulation and at a cellular level, was restored, the ability of stimulated T-cells to induce kynurenine pathway activity was reduced and the abnormal cellular expression of adaptive immune markers was normalised following successful ECT treatment. ECT: electroconvulsive therapy; FoxP3: Forkhead box P3; IL: Interleukin; KYN: kynurenine; PBMC: peripheral blood mononuclear cell; TREM-1: triggering receptor expressed on myeloid cells-1; TRP: tryptophan.

7.4 Future Directions

Alterations in the stress response system are some of the most reproducible findings observed in depressed populations with a large percentage of depressed patients characterised by elevated circulating concentrations of cortisol in association with diminished glucocorticoid receptor expression, sensitivity and function otherwise known as glucocorticoid resistance. Consequently, further investigations should assess circulating cortisol concentrations in severely depressed patients referred for ECT. Given that cortisol is commonly elevated in depressed populations one may expect to find elevated plasma cortisol concentrations. While cortisol is a potent immunosuppressive agent that acts to restore bodily homeostasis in response to stress, to date there is no direct evidence linking increased cortisol concentrations and decreased T-cell responses in depression. Consequently, it would be interesting to assess if increased cortisol concentrations were negatively associated with the diminished T-cell proliferative response evidenced by the decreased transcriptional expression of IL-2 in the depressed cohort prior to ECT treatment. However, the persistent presence of chronic low grade inflammation is also thought to be responsible for the manifestation of an altered stress response system and glucocorticoid resistance. To investigate this, *in vitro* exposure of isolated patient PBMCs to the synthetic glucocorticoid dexamethasone for 24 hours would permit the assessment of glucocorticoid resistance via the transcriptional expression of glucocorticoid inducible genes, with a particular interest in the glucocorticoid receptor and its co-chaperone FKBP5. In light of data in support of an altered response to dexamethasone and glucocorticoid resistance in the patient cohort, further assessment of the altered inhibitory effects of dexamethasone could be assessed by co-incubating patient PBMCs with dexamethasone and immune stimuli such as LPS or anti-CD3, with the expectation that the ability of dexamethasone to suppress proliferation and immune response would be reduced. While previous studies have reported data in support of this theory (Pariante and Miller, 2001), to my knowledge, no study has assessed the impact of ECT treatments on PBMC glucocorticoid sensitivity in severely depressed patients.

As described in the introduction (Section 1.9) preclinical studies indicate that psychological stress increases gut permeability thereby enabling gut flora to access the systemic system. In line with this observation, Maes et al. (2008) have reported the presence of antibodies against endotoxin from a number of commensal bacteria in plasma from depressed patients. Consequently, it is possible that LPS from commensal bacteria could stimulate a systemic low grade inflammatory response in depressed patients however this remains to be fully elucidated. To investigate this hypothesis, future studies should measure circulating endotoxin concentrations in depressed patients and control subjects.

The adaptive immune response, and specifically the relative contribution of T-cells with a particular interest in T-reg cells and Th-17 cells, is an exciting area of interest in depression and certainly warrants further investigation. While Th-17 cells have been identified as major contributors to the pathogenesis of many inflammatory and autoimmune disorders their presence in psychiatric disorders or their contribution to the biological basis of major depression remains to be determined. Future studies should endeavour to identify both Th-17 and T-reg cell populations using flow cytometric analysis. The literature assessing the relative contribution of T-reg cells in depression is sparse and varied and given the complexity of regulatory T-cells and their diverse mechanisms of action, future studies should profile a greater range of T-reg cell markers and other FoxP3 inducible genes such as CTLA-4 and glucocorticoid-induced TNFR family related gene (GITR) as well as the expression of programmed cell death protein (PD)-1 and T cell immunoglobulin mucin (Tim)-3 which are also found on exhausted T-cells and T-reg cells. It would also be of interest to assess the presence or absence of CD127, as the absence of CD127 in association with increased expression of CD25 and FoxP3 on immune cells confers potent immunosuppressive capacities. Consequently, the presence or absence of these markers may provide a greater indication into the role regulatory T-cells are playing, if any, and subsequently whether they are contributing in a neuroprotective or immunopathological capacity.

In addition, given that IL-2 is a potent inducer of T-reg cells, an induction of which has been observed following IL-2 therapy for the treatment of cancer (Ahmadzadeh and Rosenberg, 2006), it would be extremely interesting to assess if this robust induction of regulatory T-cells has a role to play in the manifestation of cytokine induced depression. This is even more pertinent given that cytokine induced depression is strongly associated with kynurenine pathway activation and tryptophan depletion, which is also a potent stimulus for T-reg cell expansion. In turn, increased expression and function of the inhibitory receptor CTLA-4 may result in the suppression of T-cell responses in association with the activation of IDO and the subsequent further depletion of tryptophan. Consequently, it is possible that the immunosuppressive effects of T-reg cells could be a contributing factor to the manifestation of cytokine induced depression. This is an exciting area of interest which warrants further investigation.

With regard to the kynurenine pathway, while no direct peripheral evidence indicates kynurenine pathway activation in idiopathic major depression, no study to date has assessed central kynurenine pathway activation. Consequently, future studies should try to avail of CSF from severely depressed idiopathic major depressives and in a similar manner to studies by Raison and colleagues, evaluate the activity of the kynurenine pathway in the CNS. In turn, this may shed light on the mechanisms behind the diminished peripheral availability of tryptophan often observed in

depressed patients as it is possible that the decreased tryptophan concentrations are associated with altered inflammatory processes.

While numerous facets of the inflammatory response system have been assessed in this thesis and particularly at the message level using the whole blood PAXgene system which presents a novel approach in the search for biomarkers of depression, it is devoid of assessment of whole blood expression of chemokines and their receptors. Given the strong evidence implicating chemokines in the pathogenesis of depression (Rajagopalan et al., 2001, Suarez et al., 2003), future studies should assess PAXgene chemokine expression as a potential biomarker for depression in association with the assessment of circulating chemokine concentrations. Given their role in the mediation of immune cell trafficking to sites of inflammation or injury, chemokine medicated immune cell trafficking to the brain in patients with major depression should be explored.

Given that depression is primarily a disorder of the CNS, future studies in this field should endeavour to combine the advances in imaging and PET scanning with alterations in biological markers in depression in an effort to gain a more comprehensive view of the changes that are occurring in the depressed brain and how changes in peripheral biological systems and immune markers are impacting, contributing to, or are as a consequence of, altered CNS functioning in living patients. This type of analysis would be most interesting prior to and following therapeutic intervention with ECT. The combination of these methods may provide a more targeted approach in the search for biomarkers with an ultimate goal to develop personalised treatment strategies for patients with major depressive disorders.

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VIII. Publication history

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Tryptophan depletion in depressed patients occurs independent of kynurenine pathway activation

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The kynurenine pathway (KP) and its rate-limiting tryptophan degrading enzyme indolamine 2,3-dioxygenase (IDO) have been implicated in the pathogenesis of depression. IDO expression is driven by inflammatory cytokines, and has been suggested as the link between inflammation and a serotonergic deficit in depression. Studies also indicate that inflammatory cytokines upregulate the serotonin transporter (SERT), representing another mechanism by which inflammation could influence serotonin availability. Here we examined circulating concentrations of inflammatory cytokines (IFN-γ, TNF-α, IL-1β, IL-6), and the acute phase protein CRP alongside plasma tryptophan, kynurenine, kynurenic acid (KYNA) and 3-hydroxyanthranilic acid (3-HAA) concentrations, and whole blood mRNA expression of IDO, kynurenine aminotransferases (KAT I and II), kynurenine-3-monooxygenase (KMO), kynureninase and SERT in patients with major depressive disorder (MDD) compared with age and sex-matched controls. Whilst no changes in TNF-α or IL-1β were observed, plasma concentrations of IL-6, IFN-γ and CRP were increased in the depressed cohort. Despite this inflammatory phenotype, IDO expression or plasma kynurenine were not significantly different between MDD patients and controls. In addition, there was no difference between controls and depressives in concentrations of KYNA and 3-HAA, or in expression of enzymes KAT, KMO or kynureninase that drive their production. Nonetheless, a depletion in tryptophan was evident in depressed patients and was correlated with HAM-D scores. In addition, we failed to observe any difference in SERT mRNA expression in the blood cells from patients with MDD relative to controls. These data support the idea that a mild inflammatory signature is evident in MDD and is accompanied by reduced circulating tryptophan concentrations. However, we found no indication of KP activation in the depressed cohort suggesting that an alternative mechanism mediates the depletion of tryptophan observed. Taken together these data question the ability of the mild inflammatory phenotype observed in depression to induce molecules such as IDO and SERT that could negatively impact upon serotonergic functioning.

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Reduced expression of glucocorticoid-inducible genes GILZ and SGK-1: high IL-6 levels are associated with reduced hippocampal volumes in major depressive disorder

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Neuroplasticity may have a core role in the pathophysiology of major depressive disorder (MDD), a concept supported by experimental studies that found that excessive cortisol secretion and/or excessive production of inflammatory cytokines impairs neuronal plasticity and neurogenesis in the hippocampus. The objective of this study was to examine how changes in the glucocorticoid and inflammatory systems may affect hippocampal volumes in MDD. A multimodal approach with structural neuroimaging of hippocampus and amygdala, measurement of peripheral inflammatory proteins interleukin (IL)-6 and C-reactive protein (CRP), glucocorticoid receptor (GR) mRNA expression, and expression of glucocorticoid-inducible genes (glucocorticoid-inducible genes Leucin Zipper (GILZ) and glucocorticoid-inducible kinase-1 (SGK-1)) was used in 40 patients with MDD and 43 healthy controls (HC). Patients with MDD showed smaller hippocampal volumes and increased inflammatory proteins IL-6 and CRP compared with HC. Childhood maltreatment was associated with increased CRP. Patients with MDD, who had less expression of the glucocorticoid-inducible genes GILZ or SGK-1 had smaller hippocampal volumes. Regression analysis showed a strong positive effect of GILZ and SGK-1 mRNA expression, and further inverse effects of IL-6 concentration, on hippocampal volumes. These findings suggest that childhood maltreatment, peripheral inflammatory and glucocorticoid markers and hippocampal volume are interrelated factors in the pathophysiology of MDD. Glucocorticoid-inducible genes GILZ and SGK-1 might be promising candidate markers for hippocampal volume changes relevant for diseases like MDD. Further studies need to explore the possible clinical usefulness of such a blood biomarker, for example, for diagnosis or prediction of therapy response.

List of poster/conference presentations

- PNIRS 2013, Stockholm, Sweden: Poster presentation - Early life adversity and depression are associated with a dysregulated inflammatory profile.
- Neuroscience Ireland 2012, RCSI, Dublin: Poster presentation - Inflammation, kynurenine pathway activation and tryptophan depletion - The relative contribution of adaptive v's innate immune cells.
- CINP World Congress of Neuropsychopharmacology 2012, Stockholm, Sweden: Poster presentation - Depressed patients with a mild inflammatory phenotype display robust tryptophan depletion in the absence of kynurenine pathway activation.
- PNIRS 2011, Chicago, Illinois, USA: Poster presentation and data blitz - Comparison of the kynurenine pathway activation and tryptophan depletion induced by activation of human T-cells and innate immune cells.

  I was awarded a $1,000 NIH funded travel bursary to attend the PNIRS conference in Chicago in 2011.
- Neuroscience Ireland 2011, NUI Maynooth, Kildare: Poster presentation - Tryptophan depletion in depressed patients occurs independent of kynurenine pathway activation.
- PNIRS 2010, Trinity College Dublin: Poster presentation - Inflammatory gene expression profile in treatment resistant depression.