Bipolar Disorder; not only in the Brain
Immunological Aspects

Bipolaire stoornis; niet alleen een stoornis in het brein
Immunologische aspecten

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General Introduction

1.1 Bipolar Disorder

1.1.1 Clinical Aspects

For nearly 2,500 years, mood disorders have been described as very serious and invalidating disorders. Mood disorders cover a large group of psychiatric disorders characterized by pathological mood states, often accompanied by psychomotor and vegetative disturbances. Within the group of mood disorders two major groups can be divided; major depressive disorders (unipolar depression) and bipolar disorders (previously called manic-depressive disorder).

Regarding the historical concept of bipolar disorders, the following can be mentioned. As early as the 1st century AD, a Greek physician, Aretaeus of Cappadocia, described mania and melancholia as two different phenomenological states of the same disease[1]. A more modern concept of this disease as a single cyclical disorder was introduced by Emil Kraepelin[2] in 1921 with the term manic depressive insanity. In the 1960s, a few psychiatrists showed independently that there are various clinical characteristics that account for manic depressive disorder as a distinct mood disorder from unipolar depression[3-5]. Modern classification systems, such as ICD-10[6] (International Classification of Diseases) and DSM-IV[7] (Diagnostic and Statistical Manual of Mental Disorders), describe bipolar disorder as an episodic, chronic illness, usually with full recovery between episodes. The diagnosis of bipolar disorder requires that a person has suffered of at least one episode of mania in their life with or without episodes of depression at other times. This requirement distinguishes bipolar disorder from unipolar depression, in which subjects suffer from at least one episode of depression without ever experiencing episodes of pathological elevated mood.

In DSM-IV[7] bipolar disorder is subdivided in different subtypes, depending on it's symptom profile. In *subtype I*, bipolar patients experience episodes of clear-cut mania, usually with psychotic symptoms. However, in *subtype II*, patients suffer from a disease course predominated by depressive episodes alternated with milder, short-lived periods of hypomania, rather than full-blown mania[8]. Some studies suggest that subtype II is more prevalent than subtype I[9].

Clinically, major depression frequently arises from a low-grade depressive mood state, in which no vital symptoms are present and psychosocial functioning is not seriously disturbed. This is known as *dysthymic disorder*. When patients go through more than 2 years of numerous brief periods of hypomanic symptoms and low-grade depression in a biphasic alternating pattern, they are classified as having

a *cyclothymic disorder*. Patients with cyclothymic disorder have a high risk of eventually developing more severe forms of bipolar disorder. In the case of *rapid cycling* bipolar disorder, patients have gone through more than 4 mood episodes in the past year[10]. Finally, patients with bipolar features that do not meet criteria for the aforementioned categories are classified as *Not Otherwise Specified* (NOS).

It is difficult to estimate the incidence of bipolar disorder, because the disease generally becomes apparent only over a period of years and a reliable diagnosis can only be made later in life. The first episode is often a depressive phase, which causes a diagnosis of bipolar disorder to be overlooked[11,12]. Therefore, incidence statistics are usually based on retrospective data[13]. Figures on lifetime prevalences are probably underestimated due to an over-diagnosis of unipolar depression and an under-diagnosis of subtype II[14]. The Netherlands Mental Health Survey and Incidence Study (NEMESIS) calculated the lifetime prevalence of bipolar disorder in The Netherlands at 1.9%[15]. This is consistent with figures in other Western countries, ranging from .3-3.7%. The disease rate is equally distributed between men and women[16]. Another difficulty by estimating the prevalence figures is caused by the usage of different diagnostic instruments, study populations, and classification systems with subtle differences in illness characteristics. When the whole bipolar spectrum disorder (DSM-IV; including cyclothymic disorder) is included, prevalence rates of 3-7% are estimated, both in an extensive review[17] and nationally in the NEMESIS study[18].

Bipolar disorder is associated with a high rate of morbidity and mortality[19]. Patients often have comorbid psychiatric conditions, such as panic disorders[20], alcohol or substance abuse[21,22], and eating disorders[23]. Moreover, somatic disorders, such as obesity[24,25], diabetes mellitus[26,27], hypothyroidism[28,29], and multiple sclerosis[30,31] are also more prevalent in patients with bipolar disorder compared to the general population. Unfortunately, next to the burden of the disease itself, this high prevalence of comorbidity is associated with a relatively low quality of life[32,33]. Along with mortality due to the somatic comorbidity, suicide plays an important role. Research reports on suicide estimate that 8-18% of all bipolar patients die by suicide, whereas 17-24% attempt suicide[34-36]. All together, bipolar disorder is one of world's top 10 most disabling conditions[37], which results in a heavy burden for the patients themselves and for their social environment[38-40]. The impact for society is also considerable, due to health service utilizations and high costs[41,42].

1.1.2 Etiology

In parallel with other psychiatric diseases, the exact pathophysiology of bipolar disorder is not yet unraveled. There is sufficient evidence to claim that the etiology of bipolar disorder is multifactorial, but it would carry too far in the introduction of this thesis to go into detail on the potential mechanisms. In the following section, some important factors will briefly be listed.

First of all, genetic factors play an important role. The lifetime risk of developing bipolar disorder for relatives of bipolar patients ranges from 40-70% for a monozygotic co-twin and 5-10% for a first degree relative[43-45]. Although bipolar disorder tends to aggregate in families, the pattern of inheritance is not clear-cut and is characterized by a complex relationship between gene susceptibility and clinical phenotypes. The majority of linkage studies are rather confusing but some point to associations of genes on chromosome 18, 21, or 22[46-48]. However, no consistent or specific polymorphism has been identified thus far. The overall consensus is that bipolar disorder is a polygenetic disease. Innovative and promising methods, such as microarrays, could provide interesting approaches to try to answer questions with regard to RNA expression, the interphase between gene expression and protein production[49,50].

Second, environmental factors are important, such as social environment, major life events, and stress[16,51-53].

Third, there are numerous biological mechanisms that may play a role in the pathophysiology. In addition to disturbances in the immune and the endocrine system, which will be covered extensively further on in this thesis, metabolic disturbances (e.g., fatty acid metabolism) also appear to be important. One could even carry this back to aberrancies within different molecular mechanisms, such as signal transduction pathways[53-55].

Finally, extensive studies have been performed on structural and functional abnormalities of the brain. Post mortem studies revealed, for example, abnormalities in frontal cortical interneurons[56], in the degree of myelination[57], and in neuronal size[58]. Magnetic resonance imaging (MRI) studies showed structural deficits in gray[59-61] and white matter[62,63], in the third and lateral ventricle, striatum, thalamus, amygdala, prefrontal cortex, cerebellum, and in the temporal lobe[63-66]. Magnetic resonance spectroscopy (MRS) visualizes functional abnormalities by using strong magnetic fields to measure and analyze the chemical composition of the brain. MRS-studies with bipolar patients gave indications for aberrancies in oxidative phosphorylation, phospholipid metabolism, choline metabolism, and second messenger systems[65-67].

1.1.3 Treatment

Either to recover from an acute episode or to maintain long-term stability, moodstabilizers are indicated. Lithium is the oldest and, according to various studies and meta-analyses, still the most effective mood stabilizer[68,69]. It even has anti-suicidal effects after long-term treatment[70]. Some anti-epileptics, such as valproate, carbamazepine, or lamotrigine, were found to have mood stabilizing activities as well[71]. Unfortunately, in many cases one or two moodstabilizers are not sufficient and therefore treatment of bipolar patients often entails relatively long-term, complex medication regimens that combine mood stabilizers, antipsychotic agents, antidepressants, and other medications, such as benzodiazepines[72-74]. Lately, antipsychotic agents, such as quetiapine and olanzapine, were also found to have some mood stabilizing potency. Although the role of antidepressants in the treatment of bipolar disorder has been rather controversial due to the possible triggering of mania or circadian cycling, recent findings suggest an important role in the treatment approach[71,75]. Noncompliance to treatment can be a substantial problem in bipolar disorder[76].

Next to the pharmacological treatment options, a range of psychosocial interventions, such as psycho-education or cognitive behavior therapy, appear to benefit patients with bipolar disorder[77,78].

1.2 Immune System

1.2.1 Innate/Adaptive Immune System

The main function of the immune system is to provide an effective response against harmful pathogens. To establish this, the immune system must distinguish between potential pathogenic material, such as viruses or bacteria, and material originating from its own body. To provide an effective defense mechanism, which differentiates between "self" and "non-self", a complex system is present with various components interacting with each other in a sequential and regulated manner.

The different cells of the immune system arise from pluripotent stem cells in the bone marrow and develop during the course of life by getting in touch with different self and non-self antigens mainly in the thymus, lymph nodes, and the periphery. Roughly, two systems can be defined, the innate and the adaptive immune system (Figure 1).

Innate Immune System: This is phylogenetically the oldest system. The first lines of defense are our natural barriers, such as the skin and

mucous membranes. Other important players are soluble mediators and phagocytic cells, like monocytes, macrophages, and polymorphonuclear neutrophils. Examples of soluble agents, belonging to the innate immune system, are cytokines like interleukin-8 (IL-8) or pro-inflammatory cytokines (IL-1β, IL-6, TNFα), chemokines like MCP-1, and complement factors, all of which provide essential communication between the different immune cells. The innate immune system acts quite fast (i.e., within hours), but the response is antigen non-specific. If this system fails to eliminate the pathogen, it can activate the adaptive immune system. The main players in this process are the antigen-presenting cells (APC; e.g., the dendritic cell), which take up the pathogen and process it in various compartments of the cell. Afterwards, APC can present the antigen to the cells of the adaptive immune system in a proper way with the right receptor and supporting co-stimulatory molecules on the cell surface[79].

Adaptive Immune System: This system acts more slowly than the innate immune system (i.e., after a few days), but the reaction is antigen-specific and more effective in terms of elimination of the pathogen. Next to the specificity, it also provides a memory function after repeated exposure. Roughly, two types of adaptive immune reactions can be distinguished. The first is a cell-mediated immune reaction and the second is an antibody-mediated immune reaction, also called humoral immunity. T and B cells, respectively, perform the key roles in these forms of immune reactions, but many other cells and soluble pleiotrophic factors, like the cytokines IL-10 and IL-12, and chemokines, are essential as well.

T cells form a heterogeneous group of cells with different functions. Some T cells take care of the previously mentioned memory function (memory T cells). Others destroy host cells, which are infected with intracellular pathogens, like viruses (cytotoxic $CD8^+$ T cells). The $CD4^+$ T-helper 1 (Th1) cells interact with mononuclear cells to destroy intracellular pathogens, and the $CD4^+$ T-helper 2 (Th2) cells interact with B cells and induce these cells to divide and differentiate into plasma cells. The balance of the cytokines produced by Th1 and Th2 cells determines the final outcome of the adaptive immune response (i.e., humoral or cellular; see also Figure 1).

B cells are genetically programmed to encode a surface receptor specific for a particular antigen. After encountering and recognizing such antigens, B cells differentiate into plasma cells. In this latter stage they produce large amounts of these receptor molecules in a soluble form, which are called antibodies. Antibodies specifically bind to the attacking antigens. In this way they, for instance, inactivate the antigens and provide extra signals for the innate immune system to attack these antigens by complement activation or by facilitated phagocytosis.

Figure 1. The Innate and Adaptive Immune System.

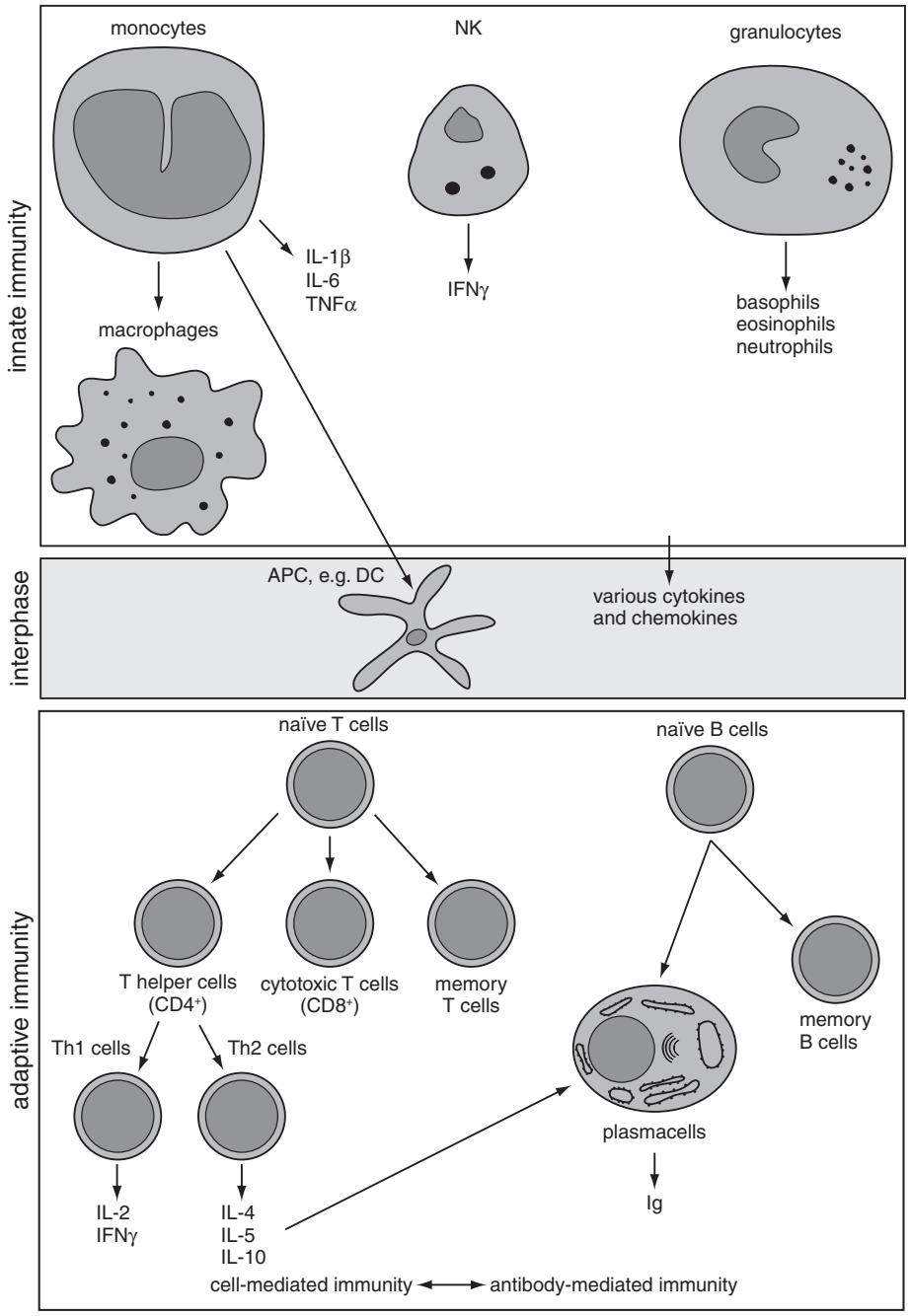


Figure 1. The Innate and Adaptive Immune System.
The main players in the innate immune system, the adaptive immune system, and the "interphase" between these two systems, as also described extensively in the text. Different systems will be activated, dependent on the type of pathogen encountered and the microenvironment.
IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; APC, antigen presenting cell; DC, dendritic cell; Th, T helper cells; Ig, immuneglobulins.

1.2.2 Autoimmunity

As mentioned before, one of the main functions of the immune system is to distinguish self and non-self. Generally this occurs by the induction of tolerance. Briefly, tolerance mechanisms cause a block in the development, growth, or differentiation of B and T cells that are autoreactive[80]. As a result, the remaining circulating lymphocytes react only with a wide pattern of foreign antigens.

Autoimmunity occurs as a result of the breakdown of tolerance mechanisms of autoreactive T and B cells, and the immune system starts to attack self-antigens. When parts of the body or specific organs are functionally damaged by these autoimmune mechanisms, a state of organ failure will eventually develop. One generally distinguishes organ specific autoimmune diseases, such as diabetes mellitus type I (confined to the beta cells in the pancreatic islets) or Hashimoto's thyroiditis (confined to the thyrocytes in the thyroid), and systemic autoimmune diseases, such as rheumatoid arthritis or systemic lupus erythematosus (SLE), which show autoreactivity towards antigens in multiple organ systems. Autoimmune diseases are present in approximately 3% of the Caucasian population[81]. The pathogenesis of the different autoimmune diseases is not yet exactly unraveled. Table 1 indicates that auto-antibodies directed against cellular compartments, such as enzymes or proteins, can be used as markers for autoimmune diseases. In a small part of the general population auto-antibodies are also present without having any clinical concequences. However, the majority of these subjects show signs of an already started autoimmune process often accompanied by a mild inflammation of the target organ. The presence of auto-antibodies in these "healthy" subjects induces an increased risk for autoimmune disease development.

Table 1. Auto-antibodies can be used as Markers for Autoimmune Diseases.

Target Organ	Disease	Associated with Abs against:	
Thyroid	Hashimoto's disease	TPO, Tg	Organ specific autoimmune disease
	Graves disease	TSH-R, TPO	
Stomach	Pernicious anemia	H^+/K^+ ATPase	
Islets of Langerhans	Diabetes mellitus type I	GAD65, IA-2, IAA	
Muscles	Myasthenia gravis	ACh-R	
Liver	Primary biliary cirrhosis	Mitochondrium, actin	
	Autoimmune hepatitis	Actin, SLA	
Exocrine glands, e.g. salivary gland	Sjögren's syndrome	ANA (SS-A/Ro, SS-B/La)	
Connective tissue, joints, blood vessels	Rheumatoid arthritis Systemic lupus erythematosus	Fc part of IgM (RF), CCP ANA (dsDNA, Sm)	Systemic autoimmune disease

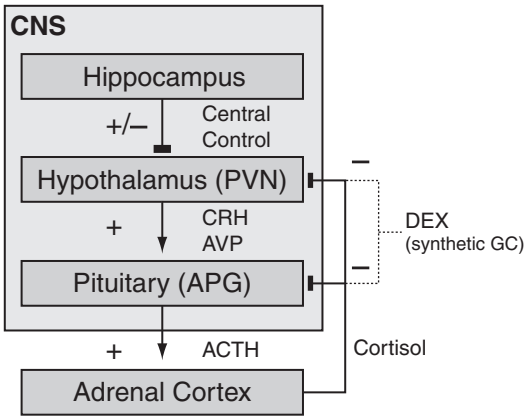
Abs, antibodies; TPO, thyroid peroxidase; Tg, thyreoglobulin; TSH, thyroid stimulating hormone; H^+/K^+ ATPase, H^+/K^+ adenosine triphosphatase; GAD65, glutamic acid decarboxylase; IA-2, tyrosine phosphatase-like protein; IAA, Abs against insulin; AChR, acetylcholine receptor; SAL, soluble liver antigen; ANA, antinuclear antibody; SS-A/Ro and SS-B/La, antibodies directed against nuclear targets; Fc, constant region of a immunoglobulin; Ig, immunoglobulin; RF, rheumatoid factor; CCP, cyclic citrullinated peptide; dsDNA, double stranded deoxynucleotide acid; Sm, Smith antigen.

1.3 Hypothalamus Pituitary Adrenal Axis

1.3.1 HPA-axis in General

The hypothalamus pituitary adrenal (HPA)-axis is a neuroendocrine system with an essential function in the regulation of stress-related responses. The anatomical mediators comprise the hippocampus, the hypothalamus (i.e., hypothalamic paraventricular nucleus), the anterior pituitary gland, and the adrenal cortex. These brain components excrete hormones, like corticotrophin-releasing hormone (CRH), vasopressin (AVP), and adrenocorticotrophin hormone (ACTH) (depicted in Figure 2), which finally regulates the release of cortisol, a glucocorticoid (GC), from the adrenal cortex. The basal state and the normal circadian rhythm of the HPA-axis are regulated via the hippocampus. The release of ACTH and cortisol is pulsatile. ACTH and cortisol also take care of negative feedback in the system to maintain homeostasis of the HPA-axis and to control the sensitivity of the stress response. Exogenous GCs, like dexamethasone (DEX), sort effects comparable with endogenous cortisol[82]. Next to the

Figure 2. Hypothalamus Pituitary Adrenal Axis.



CNS, central nervous system; +, positive feedback; -, negative feedback; CRH, corticotrophin releasing hormone; AVP, vasopressin; ACTH, adrenocorticotrophin hormone; GC, glucocorticoid.

effects on the metabolic status and neurodevelopment[83], GCs are well known for their inhibitory effects on virtually every aspect of the immune response[84-86].

1.3.2 HPA-axis Abnormalities in Mood Disorders

The HPA-axis as main regulator of the stress response has been a logical and major topic of interest in psychiatric research. Mood disorders have been the main subject of various HPA-axis related studies, although to a lesser extent also other disorders, such as schizophrenia[87], anxiety disorders[88], and eating disorders[89] have been investigated.

The amount of cortisol can simply be determined in blood and saliva, both with comparable diurnal patterns. The highest cortisol excretion can be measured early in the morning[90]. Different tests have been developed to investigate the function of the HPA-axis and its feedback mechanisms in more detail. The most frequently used tests are the dexamethasone suppression test (DST)[91] and the dexamethasone/corticotrophin-releasing hormone (DEX/CRH) test[92,93]. An aberrant response after the administration of DEX is generally caused by a blunted feedback mechanism (i.e., less negative feedback of this synthetic corticosteroid in the HPA-axis). During the DST, DEX is administered at night. The next morning, ACTH and cortisol are measured. If ACTH and cortisol

levels remain high, compared to the healthy response, this is caused by reduced cortisol suppression (feedback resistance). It is important to mention that DEX cannot easily pass the blood-brain barrier and thereby will not influence the hypothalamus. For this reason, the DST mainly measures the feedback route via the pituitary. On the contrary, the DEX/CRH *test* has been suggested as measuring more closely the actual HPA-axis activity[92,93]. During this test, DEX is administered at 11 PM (increasing a feedback loop via the pituitary) and subsequently CRH is administered the next day at 3 PM (giving the pituitary directly an extra impulse). When the feedback mechanism is disturbed, as is the case in mood disorders, peripheral measured ACTH and cortisol levels after these administrations are higher compared with healthy individuals.

The majority of studies on HPA-axis disturbances in mood disorders focus on unipolar depression, although bipolar disorder has also been studied. Endogenous hypercortisolemia was described in 25-30% of unipolar depressed patients[90,94-97], although these prevalence figures have also been disputed[98-100]. CRH levels are also increased[101,102]. With regard to ACTH levels, data are more inconsistent. Specifically, some investigators report increased ACTH levels[97,103], whereas others indicate normal to low levels[96,104,105]. Although the DST was initially considered a diagnostic tool specific to melancholic or endogenous depression[91,106-109], a disturbed test could also be found more generally in various sets of depressed patients[94,110-116]. In more recent papers on HPA-axis alterations a disturbed DEX/CRH test is taken by some investigators as a state marker of unipolar depression[117,118] and is suggested to be an easily accessible tool for monitoring and estimating the treatment response[119,120], the relapse risk[90,121], and the number of depressive episodes[122]. In contrast, suicidal behavior is associated with a less abnormal DEX/CRH test[123]. Others, however, consider a disturbed DEX/CRH test as a trait marker and found that these HPA-axis disturbances are already present in very young unipolar patients[124,125] and even in healthy family members at risk for developing mood disorders[126]. However, a recent study revealed that most disturbances in the HPA-axis only develop after the onset of the disease[127]. Presently, the discussion whether a disturbed DEX/CRH test is rather a state or a trait marker is still going on.

When we focus more specifically on the bipolar mood disorder, some publications suggest an even more profound dysregulated HPA-axis compared to its unipolar counterpart[128,129]. The basal cortisol levels, the DST, and the DEX/CRH test are abnormal in bipolar disorder[109,130]. Although some suggest that this HPA-axis dysfunction is a potential trait marker in bipolar disorder[130,131], others describe a state dependent effect either for mania[114,132], depression[109], or rapid cycling[133]. Clinically sufficient lithium

prophylaxis in some studies did not automatically prevent intermittent HPA dysregulation[134,135], whereas in another it did[136].

Intriguingly, steroid treatment can induce mood disorders with clinical features, such as manic episodes, psychosis, and recurrence[137-139]. On the other hand, patients with Cushing's disease and comorbid depressive symptoms experience antidepressive effects after using anti-glucocorticoid treatment[140].

The common mechanism for these HPA-axis disturbances in mood disorders is thought to be a decreased sensitivity for GCs in the GC sensitive areas in structures, such as the brain, which could explain the absence of Cushing-like features in patients with mood disorders in the presence of their elevated levels of cortisol[126]. Whether this is due to a decreased number of GC-receptors[141-144], polymorphisms at the receptor level[145-149], environmental factors[149-151], a disturbance in the centrally regulated set point[152,153], or other factors has not yet been resolved. Holsboer, one of the most important pioneers in this research area, hypothesized that an impaired GR signaling could be a key pathogenetic factor in depression, which is summarized in his "cortisol resistance hypothesis of depression"[154].

Both the DEX and the DEX/CRH test use peripherally produced hormones in the blood or saliva as their readout. However, GC also have effects on immune cells. Therefore, the different immune cells and their function could also act as a potent readout system and are easily accessible for *in vitro* tests. A meta-analysis from 2001[155] concluded that many of these immune assays are disturbed in mood disorders.

All these findings on HPA-axis disturbances in mood disorders have opened new avenues for potential new strategies with regard to the treatment of mood disorders and the development of animal models. With regard to new treatment modalities, usage of CRH-R antagonists is a promising approach. As GC receptor function is impaired in many patients with mood disorders, the central secretion of CRH is enhanced. CRH has all sorts of effects on mood regulation and anxiety. The disturbances in the HPA-axis, disturbed feedback mechanism and hypercortisolism, can be amplified with a CRH-R antagonist and thereby "reset" this disrupted set point. Although this treatment strategy is still under investigation in animal models[156], at least one clinical trial[157] has also showed beneficial effects. One can also influence the HPA-axis at other levels. GR antagonists[158] and steroid-synthesis inhibitors like metyrapone [140,159] also showed potentially beneficially effects for therapeutic purposes.

Regarding the development of animal models for mood disorders with the current knowledge on HPA-axis disturbances, transgenic mice overexpressing the GC receptor in their forebrain show an increased

emotional lability[160]. Long term exposure of exogenous administered corticosterone in rats results in a behavioral pattern comparable to depression. A decreased sensitivity at the receptor level in this rat model also resembles the findings in mood disorders[161]. Even in established stress models for mood disorders, such as the social disruption model, disturbances in the HPA-axis can be found[162].

In conclusion, in mood disorders abnormalities in the HPA-axis have been found, such as hypercortisolism, a disturbed DST and DEX/CRH test. Taken together, the aberrancies point in the direction of a decreased sensitivity for GCs. However the exact mechanism behind this alteration in sensitivity has not been resolved yet. Nevertheless, it opens new venues for treatment strategies and animal models for mood disorders.

1.4 Immune Abnormalities in Bipolar Disorder

1.4.1 Immune System and Psychiatric Disorders

There is also accumulating evidence that immune mechanisms play an important role in the pathophysiology of psychiatric disorders. Already in the late 1920s, the infectious theory was posed for some of the psychotic disorders[163] and the possibility of a pathophysiological role of the immune system for psychiatric disorders has been considered ever since. In the research field of immunopsychiatry two psychiatric disorders (i.e., major depression and schizophrenia) have in particular been the focus of attention. The consensus, recently reviewed for both major depression[155,164-166] and schizophrenia[167-170], is that different components of the immune system are in an activated state. But also other psychiatric disorders, such as autism[170,171], obsessive-compulsive disorder[172], and anxiety disorder[170,173], show aberrancies in immune function.

For this thesis we have concentrated on the immunopsychiatric findings in bipolar disorder, because within the continuum of mood disorders, bipolar disorder has up till now received less attention than unipolar depression[174].

1.4.2 Monocyte-/Macrophage-derived Cytokines in Bipolar Disorder

Cytokines contribute to the biology of depression, as evidenced in several studies. The leading part is for pro-inflammatory cytokines, which are mainly produced by monocytes or macrophages. Important

evidence for this statement can be found in the finding that an increased production of pro-inflammatory cytokines generally results in mild mood disturbances, next to the co-existence of fever. These cytokine-induced mood alterations are called "sickness behavior" and resemble several symptoms of depression, such as low mood, anhedonia, anorexia, and decreased physical and social activity[86,175]. The role of cytokines in this "sickness behavior" is demonstrated in various animal models[176-178], but also in humans by mimicking an inflammatory response via a low doselipopolisaccharide (LPS; endotoxin) injection [179] or a Salmonella typhi vaccination[180]. Even in patients spontaneously infected with common pathogens, like Epstein Barr Virus, the severity of sickness behavior is positively correlated with pro-inflammatory cytokine levels[181].

A large number of publications report an increased production of pro-inflammatory cytokines (i.e., IL-1β, IL-6, or TNFα) in different populations of depressed patients[182-196], although not all do so[197-201]. The involvement of cytokines is further substantiated by reports showing increased cytokine-inducible plasma levels of acute phase proteins, like C-reactive protein or haptoglobin[184,187,202-205].

This body of knowledge on pro-inflammatory cytokines in mood disorders resulted in 1991 in "the macrophage theory of depression", which proposes that an excessive secretion of monocyte/macrophage cytokines can be held responsible for symptoms of depression[206]. Many other researchers in the field adopted this hypothesis. Although adjusted in various ways, the main point that cytokines play a pivotal role in the development of mood symptoms is consistent in these reviews[165,207-212].

Several research groups tried to extrapolate the macrophage theory of depression to bipolar disorder and measured pro-inflammatory cytokines and other soluble immune factors in the serum of different groups of bipolar patients. Table 2 shows an overview of these cytokine related studies specific to bipolar disorder. Besides monocyte-/macrophage-derived pro-inflammatory cytokines, also soluble shed-of receptors (e.g., sIL-6R) and acute phase proteins (e.g., Hp, CRP) have been measured in bipolar patients. Reviewing the literature on monocyte-/macrophage-derived cytokines and acute phase proteins, as summarized in Table 2, a plethora of results has been obtained. With regard to the pro-inflammatory cytokines, increased serum levels has been associated with mania[213], but others have not been able to confirm this association[198,215]. Conflicting results have also been found regarding acute phase proteins[203,204]. These conflicting results are most likely caused not only by differences in methodological approaches and sample sizes, but also by the heterogeneity of the subject groups and differences in confounding factors, such as age and gender differences[198].

Table 2. Studies on Monocyte-/Macrophage-derived Cytokines and Acute Phase Proteins in Bipolar Disorder.

Reference	Subjects	Cytokines/ Soluble Factors	Results (focus on BD compared to HC)
Pro-inflammatory Cytokines			
Maes *et al* 1995[213]	Bipolar mania Schizophrenia Healthy controls	Plasma IL-6, sIL-6R	BD, SCH (psychosis): ↑IL-6, sIL-6R BD (mania): ↑sIL-6R Valproate treatment: no effect
Hornig *et al* 1998[204]	Bipolar disorder Unipolar depression	Plasma TNF	TNF: no aberrations, no Li effect
Haack *et al* 1999[198]	Bipolar disorder Schizophrenia Major depression Dysthymic patients Healthy controls	Plasma IL-1Ra, TNFα, sTNF-R p55 and p75, IL-6	BD: no evidence for aberrations in cytokine levels Various confounding factors play a role, e.g. age, gender, smoking Li treatment: ↑TNFα, IL-6
Rapaport *et al* 1999[214]	Rapid cycling (BD) Healthy controls	Plasma IL-6, sIL-6R	BD (RC at baseline): ↑sIL-6R, other cytokines ud Li treatment in RC: ↓sIL-6R Li treatment in HC: ↑sIL-6R
Boufidou *et al* 2004[215]	Bipolar disorder Healthy controls	Plasma IL-6 IL-6 ELISPOT on PBMC	BD (Li treated): ↓IL-6 (ELISPOT) Plasma IL-6: ud
Liu *et al* 2004[216]	Bipolar mania Healthy controls	Plasma IL-1RA	BD (mania): ↑IL-1RA
Acute Phase Proteins			
Maes *et al* 1997[203]	Bipolar mania Schizophrenia Major depression Healthy controls	Plasma Hp, Fb, complement 3 and 4 Hpx, alpha 1-AT	BD (mania): ↑Hp, Fb, alpha 1-AT, and Hpx After treatment: normaization of acute phase response
Hornig *et al* 1998[204]	Bipolar disorder Unipolar depression Unipolar depression	Plasma CRP	BD: normal CRP, but ↑in MD Li treatment: ↓CRP

IL, interleukin; R, receptor; BD, Bipolar disorder; SCH, schizophrenia; HC, healthy control subjects; RC, rapid cycling; ELISPOT, enzyme-linked immuno assay directly visualizing the secretory product of individual cells; TNF, tumor necrosis factor; Li, lithium; IFN, interferon; ud, undetectable; PBMC, polymorphonuclear cells.
Acute phase proteins (APP): Hp, Haptoglobin; TfRFb, fibrinogen; Hpx, hemopexin; alpha 1-AT, alpha 1-antitrypsin; CRP, C-reactive protein.

1.4.3 Lymphocytes in Bipolar Disorder

In the following paragraph the association between of T cells, B cells, their products (such as cytokines and shed-of receptors), and mood disorders will be discussed. Regarding this topic far more studies have been performed with unipolar depressed patients than with bipolar patients, identical to the previously mentioned studies on monocyte-/ macrophage-derived cytokine production. Since both mood disorders show a clear overlap in their symptomatology and perhaps even in their pathological background, it is likely that the findings in unipolar depression are also important for bipolar disorder. To our knowledge, two meta-analyses reviewed the reports on depression and immunity[155,164]. Both reported, quite consistently, the following alterations in the measurements on lymphocytes: a moderate decrease in lymphocyte mitogen-induced proliferative responses, an overall leukocytosis, and alterations in numbers of several white blood cell populations, like relative neutrophilia, lymphopenia, and a marginal increase of CD4/CD8 ratio. With regard to natural killer (NK) cells, they reported a decreased NK cell activity. However, the degree of heterogeneity between the individual study results still raises questions about their robustness. Age was in one meta-analysis determined to have clear effect on the immune parameters[164], but not in the other one[155].

In the previous paragraph the concept of "sickness behaviour" induced by monocyte-/macrophage-derived cytokines was described. There is also proof that interferon-alpha (IFNα) is involved in mood regulation, as evidenced in the treatment of hepatitis C patients with IFNα. This immunotherapy is complicated by high rates of depression (33-41%)[217,218]. This serious side effect can even lead to suicidal ideation[219] and can be reversed by antidepressive treatment or stopping the IFN treatment[220].

Table 3 shows a summary of studies on lymphocyte-derived immune parameters in bipolar disorder. The majority of the studies differed in the investigated immune parameters and the results are quite variable. Accordingly, it is difficult to draw a general conclusion. A fairly consistent finding is the increase of sIL-2R, a marker for T-cell activation[213,221,222], though one study could not replicate this finding in euthymic bipolar patients[223]. The raised sIL-2R level points to an association of bipolar disorder with an activated T-cell system, but more and controlled studies have to be performed to confirm such activation of the T-cell system. T helper(Th)1-derived (e.g., IFNγ, IL-2), and Th2-derived (e.g., IL-4, IL-10) cytokines have also been measured in bipolar patients. Again conflicting results have been found regarding cytokines influencing T-cell mediated immunity[215,216,224-226]. Additionally, the reported decrease

Table 3. Studies of Lymphocytes in Bipolar Disorder

Reference	Subjects	Immune Parameters	Results (focussed on BD)
Lymphocyte Subsets			
Darko *et al* 1988[228]	Bipolar depression Unipolar depression Healthy controls	Lymphocyte subsets	BD: no aberrations UP: lymphopenia, neutrophilia
Kronfol and House 1988[229]	Bipolar mania Schizophrenia Healthy controls	Lymphocyte subsets	BD, SCH: no aberrations in cell numbers and subsets
Rapaport 1994[223]	Bipolar disorder (euth) Healthy controls	Lymphocyte subsets	BD: no aberrations in cell numbers No effect of Lithium treatment
Breunis *et al* 2003[222]	Bipolar disorder (manic/depressed/ euthymic) Healthy controls	Lymphocyte subsets	BD (all moods): activated T cells and B cells
Functional Tests			
Albrecht *et al* 1985[230]	Bipolar depression Unipolar depression Healthy controls	Mitogen-induced lymphocyte blastogenesis	BD: no aberrations in blastogenesis/CMI
Kronfol and House 1988[229]	Bipolar mania Schizophrenia Healthy controls	Mitogen-induced lymphocyte proliferation	BD (mania): ↓ lymphoproliferation
Tsai *et al* 1999[221]	Bipolar mania (during acute episode and after remission) Healthy controls	Mitogen induced lymphocyte proliferation	BD (acute mania): ↑ lymphoproliferation
Barsi *et al* 1989[231]	Bipolar mania Major depression Healthy controls	Ab-dependent cytotoxicity against red blood cells	BD (mania): ↓ cytotoxicity
Sourlingas *et al* 1998[232]	Bipolar disorder (manic/depressed/ euthymic) Healthy controls	Lymphocyte cell cyclerelated properties: histone synthesis rate	BD (euthymic), HC: lymphocytes are in resting phase BD (manic/depressed): ↑ cycling lymphocytes (activated)
Sourlingas *et al* 2003[233]	Bipolar disorder Schizophrenia Healthy controls	Lymphocyte cell cyclerelated properties, e.g. histone synthesis rate, cellular protein, and DNA synthesis	BD and SCH: lymphocytes are in activated state, instead of a resting phase

Table 3. Studies of Lymphocytes in Bipolar Disorder (continued).

T-cell Activation (sIL-2R)			
Rapaport 1994[223]	Bipolar disorder (euthymic) Healthy controls	Plasma sIL-2R	BD (euthymic): no aberrations No effect of Lithium treatment
Tsai *et al* 1999[221]	Bipolar mania Healthy controls	Plasma sIL-2R	BD (acute mania): ↑ sIL-2R After remission; ↓ sIL-2R
Breunis *et al* 2003[222]	Bipolar disorder (manic/depressed/ euthymic) Healthy controls	Plasma sIL-2R	BD (all): ↑ sIL-2R (trait)
Maes *et al* 1995[213]	Bipolar mania Schizophrenia Healthy controls	Plasma sIL-2R	BD/SCH (psychosis): ↑sIL-2R BD (mania): ↑ sIL-2R Treatment (Valproate): no effect
Haack *et al* 1999[198]	Bipolar disorder Schizophrenia Major depression Healthy controls	Plasma sIL-2R	BD: no aberrations in sIL-2R No aberrations in other patient groups compared to HC
Rapaport *et al* 1999[214]	Rapid cycling (BD) Healthy controls	Plasma sIL-2R	RC (at baseline): ↑ sIL-2R Li treatment in RC: ↓ sIL-2R Li treatment in HC: ↑ sIL-2R
T-cell-derived Cytokines			
Rapaport *et al* 1999[214]	Rapid cycling (BD) Healthy controls	Th1: IL-2, IFNγ (plasma) Th2: IL-10, IL-4 (plasma)	BD (RC): ud BD (RC): ud
Kim *et al* 2002[224]	Bipolar disorder Schizophrenia Major depression Healthy controls	Th1: IL-12 (plasma)	BD, SCH: no IL-12 difference but MD: ↑ IL-12 In all groups: treatment ↓ IL-12
Su *et al* 2002[225]	Bipolar mania Healthy controls	Th1: IFNγ Th2: IL-10 (PHA-stim PBMC cultures)	BD (mania and remission): ↓ IFNγ, no IL-10 aberrations
Boufidou *et al* 2004[215]	Bipolar disorder Healthy controls	Th1: IL-2, IFNα Th2: IL-10 (PBMC ELISPOT/ plasma)	BD (Li treated): ↓ IL-2, IL-10, IFNα (ELISPOT) Plasma cytokines: ud
Kim *et al* 2004[226]	Bipolar mania Healthy controls	IFNγ, IL-4, and TGFβ (resp Th1, 2, 3, plasma)	BD (mania): ↑ IFNγ, ↑ IL-4, TGFβ Treatment: ↑ TGFβ
Liu *et al* 2004[216]	Bipolar mania Healthy controls	Th1: IFNγ, IL-2 Th2: IL-4, IL-10 (plasma)	BD (mania): ↓ IFNγ, no aberration of IL-2, 4, or 10

CMI, cell mediated immunity; BD, bipolar disorder; UP, unipolar depression; SCH, schizophrenia; HC, healthy control subjects; RC, rapid cycling; IL, interleukin; sIL-2R, soluble interleukin-2 receptor; PHA, phytohemagglitinin; TGF, transforming growth factor; Th, T helper cytokine (subtypes 1, 2, and 3: Th1: IL-12, IFNγ, IL-2, Th2: IL-4, IL-10, Th3: TGFβ).

in NK cell activity in major depression, could not be confirmed in bipolar disorder[227].

1.4.4 Autoimmune Disorders and Mood Disorders

The prevalence of mood disturbances in different autoimmune diseases has been the topic of many reports. SLE is probably the first autoimmune disease in which a connection with psychiatry has been found[234]. One of the reasons is that psychosis is one of the diagnostic criteria of SLE, but other psychiatric problems, such as mood symptoms, have also been reported in SLE[235]. The prevalence of psychiatric disorders in SLE varies from 40-80%[236-238].

Also other autoimmune diseases are accompanied by mood disturbances. In patients suffering from thyroiditis, mood symptoms can parallel the hypo- and hyperthyroid episodes[239,240]. For Sjögren's syndrome[241,242], rheumatoid arthritis[238], and diabetes mellitus[243] the same association with mood disturbances has been suggested. Although Kessing[244] proposed that the association is merely caused by the chronicity of the autoimmune diseases, others provided evidence that the mood symptoms are not only a response to coping with a chronic illness, but seem to occur more frequently than would be expected[245,246].

Previously in this chapter, auto-antibodies were portrayed as good markers for autoimmune diseases. In the remaining part of this chapter, the literature on the prevalence of auto-antibodies (Abs) in patients with mood disorders will be reviewed to provide additional evidence that the co-occurence of mood disorders and some of the autoimmune diseases is more than just a coincidence.

Endocrine Autoimmunity: Various papers report thyroid problems in mood disorders, usually (sub clinical) hypothyroidism[29,247-249], but one disputed this finding[250]. Treatment with thyroid hormone, like levothyroxine, is able to ameliorate mood symptoms in some depressed patients[249,251]. The oldest Ab-studies in mood disorders focus on anti-microsomal and anti-thyroglobulin Abs (Tg-Abs). Anti-thyroid peroxidase Abs (TPO-Abs) replace anti-microsomal Abs in the more recent studies, since it is known from the early 1980's onwards, that the major antigen in the thyroid microsomal fraction is TPO[252].

A variety of studies using unipolar depressed patients found an increased prevalence of the various anti-thyroid Abs, such as microsomal-Abs[253-258], Tg-Abs[253,256-258], and TPO-Abs[248,259] in the serum of patients. Nevertheless, some studies did not report this increased prevalence in unipolar depression compared to healthy control groups[100,260-262]. The percentage of Abs-positive patients ranged from 8-20%, but the

experimental setup varied between the different studies.

Although Haggerty and others suggest an even higher prevalence of Abs against the thyroid in bipolar disorder compared to the unipolar population[261,263], the overall figures of antibody-prevalences against the thyroid are comparable to it's unipolar equivalent. Again Abs against microsomes[258,261,263-266], Tg[258,261,263,265,266], and TPO[28,267] have been found increased, but controversies have also been reported[262,268]. Some studies suggested that the presence of autoAbs against the thyroid was associated with a rapid cycling pattern[267] or mainly present in bipolar patients suffering from a depressive mood episode[263], but a recent study from our group could not confirm such state-specificity[28]. Another interesting topic in bipolar disorder is treatment with lithium. One of the side effects of lithium treatment is hypothyroidism[258,269], but the exact mechanism is not known yet. Some studies indicate that lithium influences[265], increases[264,270,271], or induces[268] Abs against the thyroid, suggesting that these thyrotoxic effects could partly be due to autoimmune effects. Others dispute these findings by providing evidence that the increased level of Abs against the thyroid is unrelated to the usage of lithium[28,261,263,272].

Other organs, regularly affected in endocrine autoimmunity, are less well studied in mood disorders. Although increased prevalence figures on diabetes mellitus in patients with bipolar disorder have been reported[26,33,273], these studies hardly distinguish between type I (where the damage to the pancreas is autoimmune induced) and type II diabetes (characterized by insulin resistancy). Data on autoAbs against the pancreas in patients with mood disorders are lacking.

Systemic Autoimmunity: Antinuclear Abs (ANA) represent a group of auto-antibodies directed against nuclear antigens. As an example, Abs against double stranded DNA are a specific subgroup of ANA and can be used as marker for SLE. There is a strong relation between some of these systemic autoimmune diseases and psychiatry, mentioned previously in this chapter. Various investigators describe an increased ANA positivity (11-39%) in a broad group of psychiatric patients compared to the healthy population (3-16%)[274-277]. An increased ANA prevalence was also found for both bipolar and unipolar patients, suffering from depressive symptoms[278,279]. Maes and his group[280] even found 72% of major depressed patients to be positive for ANA. However, it should be noted that the different detection methods between the various papers are not identical. The inconsistency in methods could explain why results vary so much, even resulting in negative findings compared to other patient groups[258,262,281] or compared to healthy control subjects[262,282,283]. Furthermore, some types of medication, such as statins, antihypertensives[284], as well as lithium[285], are known to be able to induce

the production of ANA. Because the different types of ANA target different antigens (e.g., double stranded DNA [dsDNA]) or ribonuclear proteins (RNP), some studies tried to link ANA positivity to a specific ANA in mood disorder. These studies were not able to associate mood disorders with a higher seroprevalence of a specific ANA, such as Abs against dsDNA or one of the ENA[254,262,277,283]. With regard to rheumatoid factor (RF), no association with mood disorders has been found[254,277].

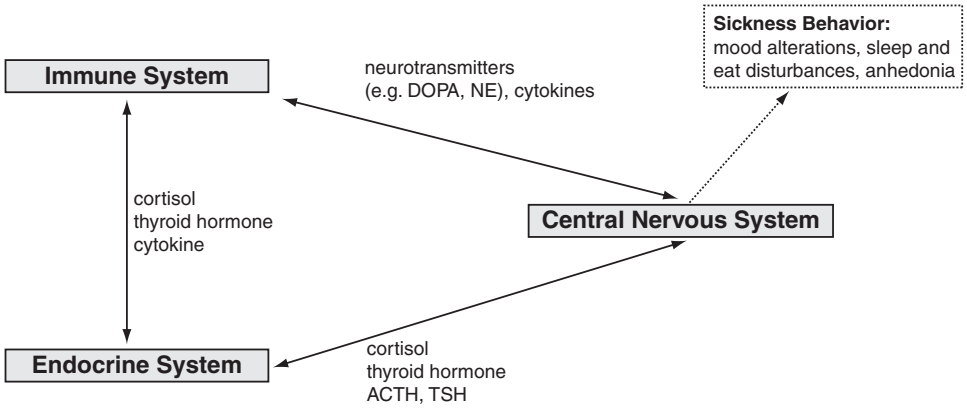
Antibodies against Brain: A logical organ in the search of auto-antibodies in psychiatric diseases is the brain. The initial studies in this field used indirect immunofluorescence employing patient serum and brain slides. A positive staining pattern was taken as evidence for anticerebral Abs in schizophrenic patients[286-288]. In these studies, patterns differed between the various slides and outcomes were difficult to repeat. Hence, unequivocal conclusions could not been drawn. Later studies noted increased antibody levels to specific antigens present in nervous tissue, such as somatostatin[289], gangliosides[290], and neurotransmitter receptors[291] in serum of patients with mood disorders. Taken together, data are too limited to make a statement on the role of Abs against nervous tissue in mood disorders.

In conclusion, literature data on the prevalence of auto-antibodies in mood disorders are difficult to evaluate, due to large variations between the various reports. The variations in outcomes are probably due to the large variability in methodology, sample size, different inclusion criteria for patients, and co-variables. Nevertheless, the association between mood disorders and the presence of auto-antibodies is quite striking and it seems unlikely that it is just a co-incidence of separate events.

1.4.5 Concept of Neuro-Immuno-Endocrinology

In this first chapter different "regulating" systems, i.e. immune, endocrine, and nervous systems are mentioned extensively. Nowadays it is widely accepted that interactions between the immune, endocrine, and central nervous system have critical importance in the understanding of many psychiatric disorders, especially mood disorders[292]. Accumulating evidence, as mentioned in the previous paragraphs and reviewed repeatedly[86,293-295], has indicated the existence of bidirectional communication pathways between the nervous, the endocrine, and the immune system. Evidence for these interactions arises from a number of experimental observations, such as the behavioral conditioning of immune responses (i.e. sickness behavior) or the effect of GCs on the immune system and behavior (i.e. anxiety). Main facilitators of this communication are soluble factors such as cytokines (e.g., IL-1β,

Figure 3. Neuro-Immuno-Endocrinology.



DOPA, dopamine; NE, norepinephrin; ACTH, adrenocorticotrophin hormone; TSH, thyroid stimulating hormone.

IL-6, TNFα), hormones (e.g., cortisol, TSH), and neurotransmitters (e.g., dopamine, norepinephrine). An overview of this concept of neuro-immuno-endocrinology is depicted in Figure 3. The importance of this concept in future research in mood disorders is significant and has to be taken into consideration during the interpretation of findings.

1.5 Lithium-induced Effects on the Immune System

Lithium is one of the most potent mood stabilizers. The mood stabilizing effect of this seemingly simple molecule was first reported in 1949[296] and the drug subsequently came into general use[297]. After various clinical trials, lithium remains in general the golden standard for long-term treatment of bipolar disorder[68,298,299].

Nevertheless, the precise working mechanism has not been elucidated. Different mechanisms probably underlie the therapeutic effects of this potent monovalent cation. First, lithium modulates the balance of various neurotransmitters[300], like dopamine[301,302] or serotonin[303-305]. Second, various key components of signal transduction pathways are influenced by lithium, as has been frequently reviewed[306-311]. These molecular mechanisms comprise the GSK3β/Wnt signaling pathway[312-314] and

G protein-coupled pathways[315-318].

Furthermore, lithium has -interestingly in the scope of this thesis- various effects on different parts of the immune system. Treating patients or healthy control subjects with lithium resulted quite consistently in a decrease in the plasma levels of acute phase proteins[203,204,319]. With regard to pro-inflammatory cytokines less consistent results have been reported, ranging from no effect[204], to an increase[320,321], or a decrease of production[215]. In this last study on a decrease of pro-inflammatory cytokines by Boufidou and others, the decrease in the production of IL-6, a pro-inflammatory cytokine, was paralleled by a decrease of IL-10, an anti-inflammatory cytokine. This suggests that the balance between pro- and anti-inflammatory cytokines remained the same, although the number of cytokine producing cells was decreased. Lithium did not influence the overall antibody production by B cells[322]. In contrast, the effect of lithium on the T cell activation marker, sIL-2R, was seemingly dependent on the group of individuals under investigation; whereas lithium treatment raised sIL-2R levels in healthy control subjects[214,323], it reduced these levels in bipolar patients[214]. Regarding other immune parameters, lithium treatment increased the white blood cell count[324,325] and the number of granulocytes[326]. More functionally, the natural killer cell activity is decreased by lithium[327].

To explore in more detail the lithium-induced effects on the immune function, several groups performed *in vitro* experiments with the drug. Some generalizations with regard to these *in vitro* effects of lithium can be drawn. *In vitro* addition resulted in an increase of immunoglobulins, mainly of IgG[328,329]. A few studies hypothesized that lithium sorts anti-inflammatory effects due to the increase of IL-10[330-332]. However, levels of pro-inflammatory cytokines are, at the same time, increased[330,333,334]. This finding is not consistent in all reports and for all pro-inflammatory cytokines[215,330,331]. *In vitro* addition of lithium also stimulates other indices of immune function, like the proliferation of lymphocytes in response to mitogens and the phagocytic capacity of macrophages[335-339]. It is not easy to translate the *in vitro* data to *in vivo* relevant effects, also because *in vitro* and *in vivo* data regarding lithium-induced effects are often in contrast. One disadvantage in interpreting the literature in this respect is that the methodology differs quite consistently between the various reports in read-out systems, lithium concentration, or the effects of acute versus chronic exposure to lithium. However, concerning immunological relevant side effects of lithium, it's antiviral capacity is mentioned consistently[320]. A few case studies reported a remission of recurrent herpes virus infection in patients taking lithium for treatment of their mood disorder[340-342]. *In vitro* studies found the same inhibition of

viral replication, uniquely for DNA viruses[343]. Unfortunately, lithium did not inhibit the viral titers of HIV[344].

The immune modulating effect of psychotropic agents is not confined to lithium alone. Antidepressants of several classes[345-347] and antipsychotics[348] also exert effects on the immune system, although results of studies to date are rather inconclusive. It is beyond the scope of this thesis to discuss this here in detail.

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Chapter 2
Aim of the Thesis

2.1 Stanley Medical Research Institute

The Stanley Medical Research Institute was founded in 1994 as the Stanley Foundation Bipolar Network (SFBN; this abbreviation is used throughout the thesis), to address many of the neglected areas of research in bipolar disorder as was emphasized in the National Institute of Mental Health (NIMH) Bipolar Disorder Workshop in 1989 and 1994[1,2]. The Stanley Medical Research Institute is a multi-center research program and consisted in origin of four sites in the United States (Los Angeles, Dallas, Cincinnati, and Bethesda), two in Germany (Freiburg and Munich), and one in The Netherlands (Utrecht). The methodology of inclusion and follow up is uniform between the different centers.

All the included outpatients with bipolar disorder (type I, II or NOS) were diagnosed by the Structured Clinical Interview for DSM-IV Axis I (SCID)[3] and a detailed illness history was assessed. During the monthly follow-up visits, a research clinician assessed cross sectional ratings for mania (Young Mania Rating Scale[4]), depression (Inventory of Depressive Symptoms[5]), and global illness severity (Clinical Global Impressions Scale modified for bipolar disorder[6]). The patients completed daily ratings of the severity of mania or depression and of the degree of functional impairment to gain a better insight into this heterogeneous bipolar population (NIMH-Life Chart Method)[7-10].

The main goal of the SFBN was to explore the different and novel treatment methods in bipolar disorder with clinical trials (open-label or blinded) and naturalistic case series. Furthermore, the SFBN aimed to define clinical parameters and longitudinal illness patterns, and to perform neurobiological studies in order to get new insight into the pathogenetic mechanisms of bipolar disorder. The studies described in this thesis were performed to gain more insight into the immune system of patients with bipolar disorder. The longitudinal methodology of the SFBN with its naturalistic setup allows a reliable association of the immune-parameter outcomes and the different co-variables. For the majority of these studies, we collected blood only from the SFBN-site in Utrecht because transportation of tubes from the United States with heparinized blood or with isolated PBMC would influence the immune cells too much. Blood was collected at 3 time points in Utrecht, in 1996, 1998, and 1999 from 81 patients in total. Patient material from the bleeding in 1996 and 1998 were used for previous projects.

2.2 Previous Immunological Studies in the SFBN

Kupka and others published the first immune paper on the SFBN sample. For this project, the prevalences of thyroperoxidase antibodies (TPO-Abs) and thyroid failure in outpatients with bipolar disorder were assessed and compared to a healthy control population and psychiatric inpatients of any diagnosis. The TPO-Abs were about three times more prevalent in patients with bipolar disorder (28%) than in the two control groups (3-18%). The presence of TPO-Abs in bipolar patients was associated with thyroid failure, but not with age, gender, mood state, rapid cycling, or lithium exposure. Thyroid failure was present in 17% of patients with bipolar disorder and more prevalent in women[11].

The second immune paper published previous to my PhD investigation, focused on cell mediated immunity in the same bipolar population, using fluorescence activated cell scanning (FACS) analysis of peripheral blood lymphocytes and enzyme linked immunosorbent assay (ELISA) of the serum to measure serum soluble IL-2 receptor (sIL-2R; a T-cell activation marker). Significantly higher numbers of circulating B cells, activated T cells, and raised sIL-2R levels were found in the patients with bipolar disorder when compared with healthy controls. Manic patients showed significantly higher levels of sIL-2R in comparison with depressed patients[12].

2.3 Present Research Questions

As described in the previous paragraphs, mood disorders most likely are accompanied by alterations in the immune and endocrine system. The majority of immune studies dealt with depressed patients. Patients with bipolar disorder are clearly underexposed in this respect.

The SFBN provides a group of clinically well-defined bipolar patients in a naturalistic setting. Two previous studies with bipolar patients from this SFBN cohort already dealt with aberrancies in the immune system. In the present studies we elaborated on these findings[11,12] and approached the following questions:

1. What is the capacity of monocytes of bipolar patients to produce IL-1β and IL-6 compared to healthy controls?

   We investigated whether the "macrophage theory of depression"[13] is also applicable for bipolar patients. We therefore measured the capacity of monocytes to produce IL-1β and IL-6 in bipolar patients and healthy controls.

2. What is the role of these pro-inflammatory cytokines in lithium-induced psoriasis?

   In this study we investigated the level of pro-inflammatory cytokines produced by monocytes in "lithium-induced" psoriasis. Lithium-induced psoriasis is a serious side effect of lithium[14]. We contrasted our findings to the production levels of monocytes of patients with "regular" psoriasis. It is well described that pro-inflammatory cytokines play an important role in the pathophysiology of psoriasis[15,16].

3. What is the ability of monocytes to differentiate into dendritic cells (DC) and how does the phenotype and functionality of the monocyte derived-DC of bipolar patients relate to those of healthy controls?

   Dendritic cells are aberrant in animal models for endocrine autoimmunity, such as in non-obese diabetic (NOD) mice[17] and biobreeding diabetic prone (BB-DP) rats[18]. Restoration of the DC function prevents autoimmunity[19]. Since a clear association was found between thyroid autoimmunity and bipolar disorder, we investigated if disturbances in monocyte-derived DC are also present in bipolar patients.

4. Are T cells of bipolar patients resistant to steroids?

   Since we found in a previous study a higher percentage of $CD25^+$ activated T cells in the peripheral blood of bipolar patients, we investigated whether this activation might possibly be explained by steroid-resistancy[20] and studied the in vitro dexamethasone-induced suppressive effects on the CD25 expression of T cells of bipolar patients and healthy controls.

5. Is the prevalence of increased endocrine auto-antibodies restricted to antibodies against the thyroid, or are antibodies against other endocrine organs, such as the pancreas and the parietal cells of the stomach also increased?

   Since we found in a previous study an increased prevalence of TPO-Abs in bipolar patients, we also determined auto-antibodies associated with other organspecific autoimmune diseases.
6. Is the increased seroprevalence of auto-antibodies restricted to organspecific autoimmune diseases or is there also an association with antibodies associated with systemic autoimmune diseases, such as antinuclear antibodies?

   Antinuclear antibodies (ANA) are associated with various systemic autoimmune diseases, such as systemic lupus erythematosus (SLE) and Sjögren's syndrome. We first screened for a broad range of ANA and subsequently determined the fine specificity of these ANA.

7. What is the role of lithium, either *in vivo* or *in vitro*, with regard to various immune measurements in bipolar disorder?

   Since lithium is frequently used in the treatment of bipolar patients and it is known for its immunomodulating capacity, we put emphasis on the role of lithium with regard to the immune measurements in virtually all of the above mentioned studies.

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Chapter 3
Innate Immune System

Chapter 3.1

An Imbalance in the Production of IL-1β and IL-6 by Monocytes of Bipolar Patients: Restoration by Lithium Treatment

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Submitted to Bipolar Disorders

Abstract

Aim

To study the *ex vivo* IL-1β and IL-6 production of monocytes of bipolar patients in the absence/presence of lithium.

Methods

Monocytes of outpatients with DSM-IV bipolar disorder (*n*=80, of whom 64 lithium treated) and of healthy controls (*n*=59) were cultured *in vitro* and exposed (24 hours) or not exposed to lipopolysaccharide (LPS) and/or graded concentrations of lithiumchloride (LiCl). The IL-1β and IL-6 production was assessed by ELISA (supernatants).

Results

LPS-stimulated monocytes of non-lithium treated bipolar patients were characterized by an abnormal IL-1β/IL-6 production ratio, i.e. a low IL-1β and a high IL-6 production. Lithium treatment increased IL-1β and decreased IL-6 production and thus restored the aberrant ratio. *In vitro* exposure of monocytes to LiCl did not have the same effects as lithium treatment: the procedure decreased IL-1β production and had minimal effects on IL-6 production.

Conclusion

Blood monocytes have an altered pro-inflammatory status in bipolar disorder. Lithium treatment restores this altered status. Short-time *in vitro* exposure of monocytes to lithium has other effects than lithium treatment.

Introduction

IL-1β and IL-6 are pro-inflammatory cytokines and important messenger molecules in the immune system, modulating T- and B-cell function. Monocytes and macrophages are major sources of these pro-inflammatory cytokines[1]. Besides having these immune functions, IL-1β and IL-6 also influence the brain and the neuro-endocrine system[2]. IL-1β and IL-6 are present in the central nervous system, can be produced by astrocytes and microglial cells, and receptors for these pro-inflammatory cytokines have been found in brain tissue[3,4]. Pro-inflammatory cytokines produced in the periphery are also capable of exerting their effect in the central nervous system[5,6]. This probably proceeds via two different routes. The neural route is represented by primary afferent neurons and the humeral route involves diffusion of the cytokines from the blood to brain target areas mainly via the circumventricular organs and the choroid plexus[5,7].

The effects of IL-1β and IL-6 on the central nervous system include an array of metabolic, endocrine and behavioral alterations, amongst which an activation of the hypothalamic-pituitary-adrenal (HPA) axis, thereby influencing the production of neurotransmitters such as norepinephrin (NE), tryptophan, dopamine (DA), and serotonin (5-hydroxytryptamine, 5-HT)[4,8,9].

In animal models behavioral changes, comparable to the symptoms of depressed patients and referred to as ''sickness behavior", can be induced by central or peripheral induction of pro-inflammatory cytokines, such as IL-1β and IL-6[4,5,10]. In humans low dosages (i.v.) of endotoxin increase the level of these cytokines and induce depressive symptoms[11].

IL-1β and IL-6 have extensively been studied in mood disorders, yet with a focus on (unipolar) major depressive disorder (MDD). With regard to the serum concentration of these cytokines, the majority of publications describe an increased level[12-19], although this could not be confirmed in all studies[20-22]. In studies on the *ex vivo* production of IL-1β and IL-6 by leukocytes of MDD patients, a more diverse picture has emerged. The majority of studies show an increased production of either IL-1β or IL-6[23-28], but decreased[29,30] and normal productions[31] have been documented as well. Measurements of IL-1β and IL-6 in cerebrospinal fluid in MDD patients have made the picture even more complex, showing lower levels of IL-6 and higher levels of IL-1β[32-34]. However, this latter observation has been disputed as well[35].

On the basis of a putatively raised production of pro-inflammatory cytokines in MDD the "macrophage theory of depression" was formulated, which proposes that an excessive secretion of pro-inflammatory

cytokines by monocytes/macrophages causes symptoms of depression. A presumed explanation is that these cytokines act as neuromodulators, becoming key factors in the mediation of the behavioral, neuroendocrine and neurochemical features of MDD[5,36-38]. Maes[39,40] has tried to extend the theory of cytokines acting as neuromodulators to bipolar disorder (BD), but so far studies on IL-1β and IL-6 production in BD patients are scarce[41,42].

The here-reported study involves a large group of well-defined outpatients with DSM-IV bipolar I and II disorder (*n*=80) and addresses the question whether the capacity of monocytes to produce pro-inflammatory cytokines is altered in BD. We therefore determined the production of IL-1β and IL-6 by monocytes of BD and compared this production to that of healthy control subjects (HC, *n*=59). Apart from studying the spontaneous IL-1β and IL-6 production by monocytes, we also studied the lipopolysaccharide (LPS)-induced production. The LPS-stimulated production was studied because previous research suggests that disturbances in pro-inflammatory cytokine synthesis are studied best under dynamic conditions[43,44].

Since decades lithium is known as an effective mood-stabilizing drug. Many consider it the drug of first choice in BD, particularly in long-term treatment[45]. The exact mode of action has not been elucidated yet, but it exerts complex effects on multiple biological systems such as signal transduction pathways. Reasonably it has multiple actions critical for its ultimate therapeutic effect[46,47]. Since lithium has also been recognized as an immune modulating drug, it is possible that some of its effects on affective disorders are mediated via altering the pro-inflammatory cytokine production[48,49]. We therefore additionally investigated the effect of lithium treatment on monocyte IL-1β and IL-6 production by comparing data in lithium users (*n*=64) versus non-users of lithium (*n*=16) and by studying the *in vitro* effects of lithium exposure to monocytes by adding lithium chloride to the monocyte culture systems.

Materials and Methods

Patients and Healthy Control Subjects

Patients: Heparinized blood was obtained from outpatients with DSM-IV bipolar I and II disorder (*n*=80) participating in the Stanley Foundation Bipolar Network (SFBN), a multi-center research program described elsewhere in detail[50,51]. For the here-described experiments all patients were recruited from the SFBN-site in The Netherlands. The

Table 1. Clinical and Demographic Characteristics.

		Bipolar Patients		Healthy Control Subjects	
Group size		80		59	
Age (years)[1]		44	(21-60)	38	(22-58)
Gender	Male	40	(50.0%)	26	(44.1%)
	Female	40	(50.0%)	33	(55.9%)
Psychotropic medication	Carbamazepin	19	(23.8%)		
	Valproate	28	(35.0%)		
	Thyrax	17	(21.3%)		
	Antidepressives	13	(16.3%)		
	Antipsychotics	19	(23.8%)		
	Benzodiazepins	20	(25.0%)		
Duration of treatment (years)[1]		13.5	(1-35)		
Lithium users		64	(80.0%)		
Li titer in serum (mM)[2]		.76 ± *.17*			
Cumulative period of Li treatment (months)[1]		74	(6-305)		
Patients without current Li		16	(20.0%)		
Period without Li (months)[1]		39	(6-118)		
Current mood state	Euthymic	50	(62.5%)		
	Depressed	15	(18.8%)		
	Manic	11	(13.8%)		
	Cycling	4	(5.0%)		
Diagnosis (DSM-IV)	Bipolar I disorder	61	(76.3%)		
	Bipolar II disorder	19	(23.8%)		
Duration of illness (years)[1]		19.4	(2-42)		

[1] mean (range)
[2] mean ± *SD*

Institutional Review Board (IRB) of the University Medical Center Utrecht had approved the study protocol and written informed consent was obtained from all patients.

A DSM-IV[52] diagnosis of bipolar disorder was made by means of the Structured Clinical Interview for DSM-IV Axis I (SCID)[53]. A detailed illness history, including past and present medication, was assessed at entry into the Network. Blood for the present study was drawn during one of

the monthly follow-up visits, when a research clinician also assessed the current mood state, i.e. euthymic, depressed, (hypo) manic or cycling, by means of a detailed interview including the Young Mania Rating Scale[54], the Inventory of Depressive Symptoms[55] and the Clinical Global Impressions Scale – Bipolar Version[56]. Clinical characteristics of the patients are shown in Table 1. Patients were included as "not using lithium" (nonLi-BD) when they had not used lithium for at least 6 months. The lithium users were on lithium for at least 6 months (Li-BD).

Blood was collected in sodium-heparin tubes (Becton and Dickenson, Franklin Lakes, New Jersey, USA), since lithium-anticoagulant for the collection of blood has been found to influence the *in vitro* cytokine production[57]. Blood was drawn in the morning and transported by courier to the laboratory in Rotterdam, lasting an acceptable 8-10 hours before further processing. If the samples are stored overnight, mainly the monocytes (needed for the production of pro-inflammatory cytokines) start to phagocytose the erythrocytes and become activated and less viable, as determined by trypan blue staining (unpublished results). As long as blood is collected into endotoxin-free anticoagulant, the cytokine measurements are relatively stable and reflect the *in vivo* status[57,58].

Healthy Control Subjects: Blood was collected from two healthy control groups:

1. Healthy blood bank donors (n=10). Blood was drawn and buffy coats were immediately prepared according to the standard protocols of the blood bank. Mononuclear blood cells were isolated from the buffy coats and subsequently frozen and stored within 2 hours. Monocytes prepared from these cell suspensions were used in a series of lithiumchloride (LiCl) dose–response experiments.
2. Healthy laboratory and hospital staff members of the Erasmus MC (The Netherlands) (n=59). Blood collections were performed on the same days as those of the patients, using the same procedures. All healthy control subjects (HC) gave written information about medication use and medical history and informed consent was obtained. The HC subjects needed to be both mentally and physically well and the exclusion criteria for this HC group were: any immune disorder, serious medical illness, recent infections, fever, any life-time major psychiatric disorder (including psychosis, mood disorders, anxiety disorders, or substance abuse disorders) or usage of any psychotropic or other medication (apart from anti-conceptive hormonal therapy).

Isolation of Mononuclear Cells

Monocyte-lymphocyte suspensions were prepared from heparinized blood via Ficoll (Pharmacia, Uppsala, Sweden; density 1.077 g/ml) gradient centrifugation. Firstly PBS (Bio Whittaker Europe, Verviers, Belgium) containing .1% BSA (Bayer, Kankakee, USA) (PBS/.1% BSA) was added to the heparinized blood sample in a dilution of 1:1. These diluted cells were distributed on Ficoll gradient and centrifuged for 15 min at 1000g. After this centrifugation, peripheral blood mononuclear cells (PBMC; i.e. lymphocytes and monocytes) were collected from the interface and washed with PBS/.1% BSA (centrifugation for 10 min at 760g). The PBMC were thereafter re-suspended in RPMI 1640 with 25mM HEPES and L-glutamine (Bio Whittaker Europe, Verviers, Belgium), additionally containing 10% inactive FCS (Bio Whittaker Europe, Verviers, Belgium; FCSi), penicillin/streptomycin (Bio Whittaker Europe, Verviers, Belgium; P/S) (P 100 U/ml, S 100 mg/ml) and extra ultraglutamine (Bio Whittaker Europe, Verviers, Belgium, 2mM; UG) (hereafter referred to as RPMI+).

Samples were frozen in 10% dimethylsulfoxide (DMSO) and stored in liquid nitrogen. This enabled us to test patient and control monocytes in the same series of experiments at appropriate times.

Cytokine Production by Monocytes

The frozen and stored PBMC suspensions were quickly thawed and directly diluted in RPMI+ to wash out the DMSO. The viability of the population was determined via trypan-blue exclusion staining. All populations contained at least 90% viable cells according to this staining. To separate monocytes from lymphocytes, PBMC suspensions were distributed on a Percoll gradient (Pharmacia; density 1.063 g/ml). Cells were thereafter centrifuged at 400g for 40 minutes. Monocytes were collected from the interface and the purity of the population was determined via a non-specific esterase staining. All populations contained at least 80% non-specific esterase positive cells.

The monocytes were placed in 24-wells flat-bottom plates (NUNC MaxiSorp™, Roskilde, Denmark) at a concentration of 5.10^5 cells/ml and cultured for 24 hours in RPMI+. The following factors were either added or not to the monocyte cultures:

- Lipopolysaccharide (LPS) of E. Coli (Difco Laboratories, Detroit, Michigan, USA) in a sub-optimal concentration of 1 µg/ml.
- Lithiumchloride (Sigma, Munich, Germany; LiCl) in concentrations of .2, 1.0, 2.0, or 5.0 mM.

The cells were cultured at 37°C in 5% CO_2. After 24 hours cells were spun down at 250g for 1 minute, supernatants were harvested and stored at minus 20°C until they were assayed for the concentration of IL-1β and IL-6.

Measurements of IL-1β and IL-6

Pro-inflammatory cytokine levels in supernatants of the monocyte cultures were measured via an IL-1β and IL-6 specific enzyme-linked immunosorbent assay (ELISA). A 96-well ELISA plate (NUNC, Roskilde, Denmark) was coated overnight at 4°C with a diluted monoclonal capture antibody specific for human IL-1β or IL-6 (Biosource Europe S.A., Nivelles, Belgium). After a blocking step with a PBS/.5% BSA solution, the supernatants (if required diluted to a measurable solution), a negative control-culture fluid sample (RPMI+), and standard solutions of IL-1β or IL-6 were aliquoted in duplicate in the wells. IL-1β or IL-6 detection antibodies (Biosource Europe S.A., Nivelles, Belgium) were thereafter added to the wells and plates were incubated for two hours. After washing, a Streptavidine-poly-HRP solution (horse-raddish peroxidase) was added to enhance the reaction, followed by incubation for 30 minutes and a second wash step. All incubations took place under continuous shaking.

Subsequently TMB substrate (KPL, Gaithersburg, Maryland, USA) was added and the reaction was developed in dark until the colour of the standard solution started to turn blue in the lower concentrations of the standard solution (5-10 min). The reaction was stopped with 1M H_3PO_4.

Absorbance at 450-650 nm was measured with an Easia reader (Medgenix Diagnostics, Brussels, Belgium). Using the standard titration curve, concentrations of IL-1β or IL-6 in unknown samples were calculated based on their optical density values (4-parameter fit). The inter-assay variation was less than 10%.

Statistical Analysis

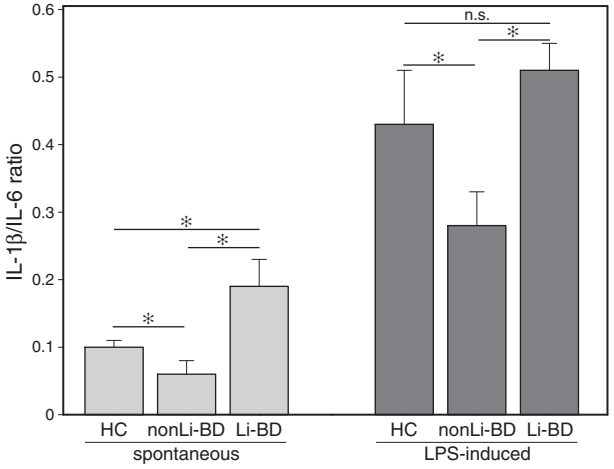
Statistical analysis was performed using the SPSS 11.0 package for Windows (SPSS, Chicago, Illinois, USA). Data were tested for their parametric distribution using the Kolmogorov-Smirnov test. Since the cytokine expression profiles did not show a parametric distribution, we decided to use non-parametric tests. We compared the different groups using a Kruskal-Wallis test, followed by a Mann-Whitney U test to determine differences between two groups. To analyze the *in vitro* data of LiCl we performed paired statistical analysis (0 vs. 1mM and 0 vs. 5mM LiCl) using the Wilcoxon signed-rank test. Correlations of the cytokine expression with different clinical parameters were examined using Spearman's rho test. Level of significance was set at the critical value of p=.05 (2 tailed).

Table 2. IL-1β and IL-6 Production in Different Subject Groups.

		HC	**nonLi-BD**	*stats*[1]	**Li-BD**	*stats*[1]	
		mean ± *SE*	mean ± *SE*	*HC vs. nonLi-BD*	mean ± *SE*	*HC vs. Li-BD*	*nonLi vs. Li-BD*
IL-1β	Spontaneous	2914 ± *742*	1350 ± *758*	*p=.066*	3708 ± *790*	*p=.871*	*p=.118*
(ng/ml)	LPS-induced	25845 ± *2107*	18767 ± *2659*	*p=.081*	27821 ± *2107*	*p=.549*	***p=.037***
IL-6	Spontaneous	22161 ± *4860*	12791 ± *6681*	*p=.249*	16378 ± *3258*	*p=.353*	*p=.541*
(ng/ml)	LPS-induced	87979 ± *10466*	99716 ± *16881*	*p=.347*	66863 ± *7262*	*p=.091*	***p=.045***

[1] represent statistical analysis using Mann-Whitney U test
HC, healthy controls; nonLi-BD, patients without current lithium treatment; Li-BD, patients with lithium treatment; stats, statistics.

Figure 1. Effect of Lithium Treatment on the IL-1β/IL-6 Production Ratio.



Data represent the production ratio IL-1β/IL-6. Means and SEM are given. **Light bars** represent spontaneous *ex vivo* production of monocytes, **dark bars** represent dynamic LPS stimulated conditions. In both conditions Kruskal-Wallis test *p* value <.01.
* Represents *p* value <.05 using Mann-Whitney U test

Results

LPS-stimulated Monocytes of BD show an Imbalance in the Production of Pro-inflammatory Cytokines in Favor of IL-6: Lithium Treatment restores this Imbalance

Table 2 shows that the production of IL-1β and IL-6 was, as expected, considerably higher if monocytes had been stimulated with LPS as compared to the spontaneous *in vitro* cytokine production. The table also indicates that monocytes of bipolar patients not being treated with lithium (nonLi-BD) show a trend for a reduced production of IL-1β and an enhanced production of IL-6, particularly under conditions of LPS stimulation (yet values did not reach statistical significance when compared to HC; Table 2). However expressed as IL-1β/IL-6 ratio (Figure 1), values did reach statistical significance, illustrating an imbalance in the production of IL-1β and IL-6 by monocytes of bipolar patients in favor of IL-6 and at the expense of IL-1β.

Table 3. Correlations of Clinical Characteristics with IL-1β and IL-6.

		nonLi-BD				Li-BD			
		IL-1β LPS	*stats*	IL-6 LPS	*stats*	IL-1β LPS	*stats*	IL-6 LPS	*stats*
Age	(r)	.126	*ns*[1]	.062	*ns*[1]	.036	*ns*[1]	.199	*ns*[1]
Duration of Li treatment	(r)	-		-		.061	*ns*[1]	.03	*ns*[1]
Duration of Illness	(r)	.053	*ns*[1]	.007	*ns*[1]	.069	*ns*[1]	.066	*ns*[1]
BMI	(r)	.198	*ns*[1]	.203	*ns*[1]	.205	*ns*[1]	.133	*ns*[1]
Gender	Male	16425 ± *3971*	*ns*[2]	77811 ± *25625*	*ns*[2]	25096 ± *2323*	*ns*[2]	67754 ± *9888*	*ns*[2]
(mean ± *SEM*)	Female	22572 ± *3050*		111884 ± *22048*		30908 ± *3604*		65882 ± *10864*	
Mood	Euthymic	18810 ± *3697*	*ns*[3]	90654 ± *20313*	*ns*[3]	29009 ± *2330*	*ns*[3]	67044 ± *8974*	*ns*[3]
(mean ± *SEM*)	Symptoms	18741 ± *3781*		106511 ± *26262*		25206 ± *2419*		66472 ± *12625*	
	Depressive	15574 ± *2867*	*ns*[4]	113164 ± *48522*	*ns*[4]	26111 ± *2447*	*ns*[4]	64667 ± *2213*	*ns*[4]
	Manic	13886 ± *1309*		94202 ± *2061*		24001 (*n*=1)			
	Cycling	24336 ± *7264*		101745 ± *40773*		25200 (*n*=1)			
Treatments (other than lithium)									
Carbamazepin	Yes	19846 ± *1799*	*ns*[2]	73067 ± *1296*	*ns*[2]	2495 ± *871*	*ns*[2]	14692 ± *5265*	*ns*[2]
(mean ± *SEM*)	No	27929 ± *2253*		72765 ± *7995*		3467 ± *818*		16046 ± *3468*	
Valproate	Yes	23707 ± *2377*	*ns*[2]	65817 ± *1099*	*ns*[2]	3593 ± *1012*	*ns*[2]	14407 ± *3807*	*ns*[2]
(mean ± *SEM*)	No	27249 ± *2467*		76846 ± *8667*		3044 ± *856*		16477 ± *4042*	
Thyrax	Yes	24507 ± *3496*	*ns*[2]	69874 ± *1207*	*ns*[2]	2440 ± *1508*	*ns*[2]	10720 ± *3558*	*ns*[2]
(mean ± *SEM*)	No	26415 ± *2102*		73613 ± *8014*		3451 ± *731*		17801 ± *3515*	
AD	Yes	21082 ± *4902*	*ns*[2]	56179 ± *1147*	*ns*[2]	3919 ± *2008*	*ns*[2]	18891 ± *9478*	*ns*[2]
(mean ± *SEM*)	No	26966 ± *1932*		76220 ± *7801*		3104 ± *687*		15102 ± *2978*	
AP	Yes	24172 ± *3455*	*ns*[2]	90460 ± *1509*	*ns*[2]	3172 ± *727*	*ns*[2]	10562 ± *5451*	*ns*[2]
(mean ± *SEM*)	No	26582 ± *2121*		67842 ± *7544*		3817 ± *819*		17175 ± *3404*	
benzodiazepin	Yes	24384 ± *2315*	*ns*[2]	73475 ± *1204*	*ns*[2]	3370 ± *1095*	*ns*[2]	16242 ± *5188*	*ns*[2]
(mean ± *SEM*)	No	26459 ± *2325*		73157 ± *8297*		3246 ± *812*		15838 ± *3548*	

[1] represent statistical analysis using Spearman's rho test
[2] represent statistical analysis using Mann-Whitney U test
[3] represent statistical analysis using Mann-Whitney U test between the patients with current mood symptoms and the euthymic patients
[4] we also checked with a Kruskal-Wallis test whether the four different subgroups differed, but again no statistical significant differences were found

nonLi-BD; patients without current lithium treatment, Li-BD; patients with lithium treatment, stats; statistics, ns; not statistical significant, r; correlation coefficient, BMI; body mass index (weight/length^2), symptoms; either depressed, manic, or cycling.

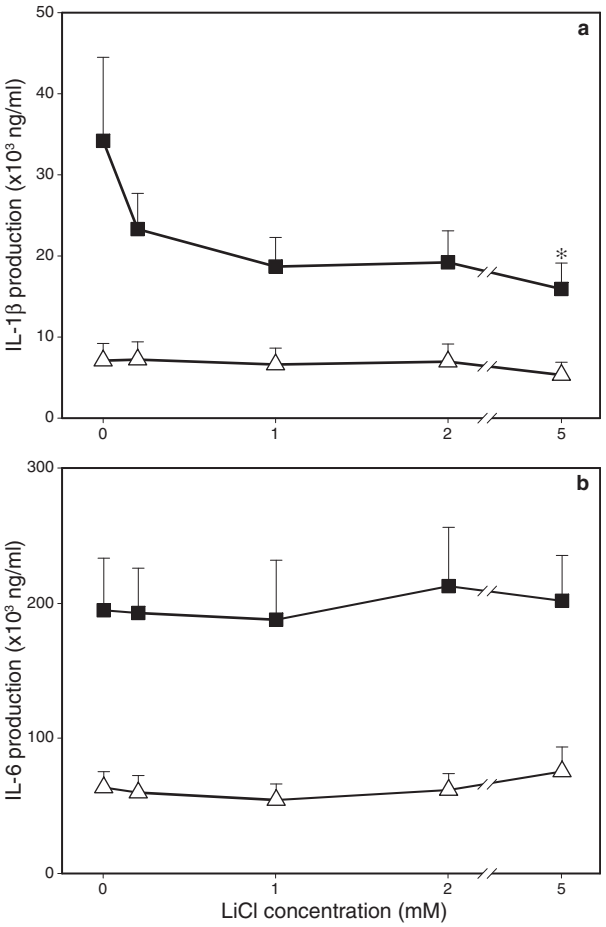
When patients were treated with lithium (Li-BD), monocytes showed an enhanced IL-1β production as compared to that of monocytes of nonLi-BD (Table 2) and a reduced IL-6 production, but the latter only under LPS-stimulated conditions. Lithium treatment thus results in a normalization of the IL-1β/IL-6 ratio, which became comparable to the ratio found in the HC both under LPS-stimulated and spontaneous conditions (Figure 1).

Table 3 shows the correlation between the IL-1β and the IL-6 production by patient monocytes and various clinical characteristics of the patients. We were not able to find statistically significant correlations neither between the studied cytokines separately (both spontaneous and LPS-stimulated) on the one hand and age, gender, duration of illness, psychotropic medication used (apart from lithium, see before), duration of lithium treatment, or diagnosis on the other hand, nor between these clinical parameters and the IL-1β/IL-6 ratio (data not shown). As weight can also be a confounder, we also checked for a possible association between body mass index (BMI) and the cytokine production. We only had the BMI data of 61 bipolar patients (no data on healthy controls, 19 missing values). The mean BMI was 25.2 ± 4.2 (Li-BD: 24.9 ± 3.3, nonLi-BD: 26.2 ± 6.6) and no significant associations with the cytokine production were found (Table 3). Regarding the psychopathological state of the patients, we could not find differences between the euthymic patients and the patients with mood symptoms, either depressed, manic, or cycling (Table 3). We also could not find a difference between the individual mood symptoms, which points more in the direction of a trait effect. This applied to both the entire dataset and the nonLi-BD and Li-BD subgroups of patients.

In vitro LPS-stimulated Monocytes reduce their IL-1β Production when exposed to LiCl.

In a first series of experiments we tested monocytes that had been isolated from frozen mononuclear cell suspensions prepared from buffy coats of healthy blood bank donors and performed a dose response study with different dosages of LiCl (0, .2, 1.0, 2.0 and 5.0 mM), searching for an effect on the *in vitro* production of IL-1β and IL-6. While the relatively low spontaneous production of IL-1β and IL-6 was not influenced by the addition of LiCl, the LPS-induced cytokine production was. The *in vitro* addition of LiCl dose-dependently down regulated IL-1β production, reaching statistical significance at 5.0 mM. It did not influence the IL-6 production by LPS-stimulated monocytes (Figure 2). It must be noted here that after 24 hours of culture all samples showed a viability of >90%

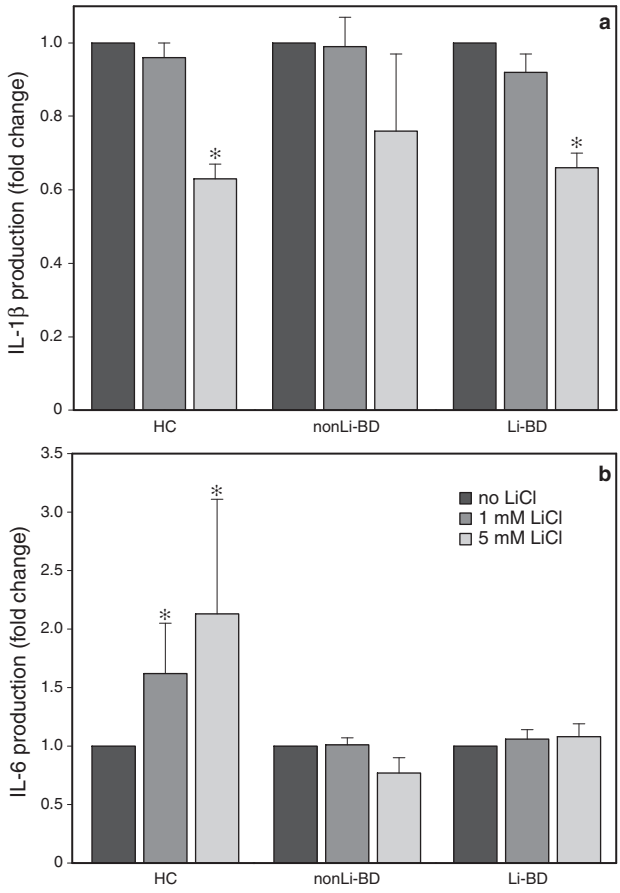
Figure 2. Effects of Graded Concentrations of Lithium Chloride on the Production of IL-1β and IL-6 from Monocytes of Healthy Blood Bank Donors.



Data represent the effects of graded concentrations of lithium chloride (LiCl) on the production of IL-1β (a) and IL-6 (b) of monocytes of healthy blood bank donors. Means and SEM are given. **Open triangles** represent spontaneous *ex vivo* production of monocytes, **black squares** represent dynamic LPS-stimulated conditions.

* Represents *p* values <.05 using Wilcoxon signed-rank test (LiCl concentration vs. no LiCl)

Figure 3. Effects of Lithium Chloride on the IL-1β and IL-6 Production on Monocytes of Different Subject Groups.



Data represent the change in IL-1β (a) or IL-6 (b) production of monocytes in healthy controls (HC), bipolar patients without lithium treatment (nonLi-BD), and with lithium treatment (Li-BD), due to *in vitro* lithium chloride (LiCl) addition in dynamic, LPS-stimulated conditions. Mean fold changes (compared to the condition without LiCl) and SEM are given. **Black bars** represent the condition without LiCl, the **gray bars** represent the change after 1mM (**dark**) or 5mM (**light**) LiCl relative to the condition without LiCl (fold change).

* Represents *p* values <.05 using Wilcoxon signed-rank test (LiCl concentration vs. no LiCl)

by trypan blue exclusion testing (data not shown), also in the highest concentration of 5mM LiCl. LiCl also did not affect the cell yield after culture. Both observations exclude a toxic effect of LiCl on monocytes even in the 5mM dose.

In a second series of experiments we used the monocytes of our actual patient populations, i.e. nonLi-BD (n=8) and Li-BD (n=8) patients and of the HC sampled at the same time as the patients (n=27). We used LiCl concentrations of 1mM, correlating with physiologic serum levels, and 5mM on the basis of the above described dose response experiment. First, we were able to confirm in this second set of experiments that LiCl did not affect the low spontaneous production of IL-1β and IL-6 (data not shown). Second, LiCl down regulated the production of IL-1β of LPS-stimulated monocytes of all groups, as in our first series of *in vitro* experiments. Third, in this second series LiCl stimulated the IL-6 production by HC monocytes to some extent (in contrast to the first series of experiments), but it did not affect the IL-6 production by monocytes of nonLi-BD and Li-BD patients (Figure 3).

Discussion

In our study on a large sample of 80 well-characterized bipolar patients, we were not able to find statistically significant increases in the IL-1β and/or IL-6 production by monocytes of bipolar patients. Therefore, we cannot support the concept that a high IL-1β and/or IL-6 production is an important (and perhaps even prime) abnormality in major affective disorders as postulated in the "macrophage theory of depression"[5,36-38]. However, we found that LPS-stimulated monocytes of bipolar patients had an aberrant pro-inflammatory status, i.e. the cells showed a relative high *ex vivo* production of IL-6 in comparison to a low production of IL-1β when compared to LPS-stimulated monocytes of healthy control subjects. Thus, our data do point in the direction of the existence of monocyte/macrophage aberrancies in bipolar disorder and more specifically of an imbalance in pro-inflammatory cytokine production under LPS-stimulated conditions. Our data also show that lithium treatment corrected this imbalance.

With regard to the validity of our data it must firstly be noted that the pro-inflammatory cytokine production from monocytes is sensitive to a number of confounding factors, such as age and gender[20]. We tested for these factors both across the entire data set and within the separate subgroups, but were neither able to find male-female differences nor associations between age and cytokine production profile. In addition,

there were no indications for a state dependent effect regarding the IL-1β and IL-6 production.

A major limitation of our study is its naturalistic set-up. Because of this naturalistic set-up, the majority of patients used various psychiatric co-medications (Table 1). Although we found lithium to be a prominent determinant in the IL-1β and IL-6 production by monocytes (see results), we could not find an effect of other medications in our sample. Nevertheless, such other medications are known to have immune modulating effects[59] and the considerable variability of the various (co-)medications in our patient group may have influenced our results. To answer the question, whether these various medications have an effect on the cytokine production, a longitudinal approach changing one such medication while keeping the others stable, would have been a better option, but again the naturalistic set-up of our study precluded such approach.

In addition smoking may have had an effect on the cytokine production in our patients. In contrast to our healthy control subjects, the majority of the bipolar patients were smokers, but details on smoking behavior of individual patients are lacking.

Moreover, psychological stress (both acute and chronic) can affect the immune system. A recent meta-analysis showed that stressors have an up-regulating effect of IL-1β and IL-6[60]. Unfortunately, no data were available on recent or more chronic stressful events in our cohort, so we could not correct for this potential confounding factor. Although none of the healthy control subjects suffered from a clear psychiatric or somatic disorder or used medication, we cannot rule out a low level of depressive symptoms in our HC subjects. We did not verify this by performing (semi-)structured psychiatric interviews. Finally, weight can also be a confounding factor[61]. Although the bipolar patients were slightly overweighed, we could not find any significant association between the BMI and the cytokine levels. No data were available on the BMI of our healthy controls.

What might be the relevance of the here found imbalance in IL-1β and IL-6 production of LPS-stimulated monocytes of non-lithium treated bipolar patients? IL-1β and IL-6 are both considered as pro-inflammatory cytokines playing a similar role in the acute phase response and the innate immune response, but IL-6 has an additional role in B cell stimulation[62]. In previous studies we found an increase in circulating B cells and a higher prevalence of auto-antibodies[63-65] in bipolar patients and it is tempting to speculate that such B cell/auto-antibody predominance is related to the here-described preferential production of IL-6 by bipolar monocytes.

With regard to the brain activities of IL-1β and IL-6, it is known that both

cytokines induce various neurotransmitter activities, yet their individual effects vary: Both stimulate the production of serotonin (5-HT)[8], but only IL-1β (not IL-6) stimulates the production of norepinephrin[8,66]. This implicates that the here reported imbalance in IL-1β and IL-6 production by monocytes under dynamic conditions (f.i. during infections) may also lead to imbalances in the neurotransmitter make-up in the brain, which in our opinion needs further exploration. A possible explanation for our finding that the individual cytokines did not show clear significant differences, but the IL-1β/IL-6 ratio did, is that primarily the interplay between the cytokines causes effects on the different systems, such as auto-antibody production or neurotransmitter balance instead of the individual cytokines.

One can hypothesize that the here reported results may be caused by counter-regulatory mechanisms within the serum influencing the monocytes. However, we could not find any proof for this assumption. During the first set of experiments we also measured the serum levels of IL-1β and IL-6 in a limited set of patients (n=39; with the same ELISA kits as described in the result section). In line with the findings of Boufidou *et al*[67], we could hardly detect any cytokine levels in the serum. Concerning IL-1β only one patient had a cytokine level just above the detection limit (10 pg/ml). As to IL-6, only in 7 patients cytokine levels above the detection levels were measured (mean 1.7 ± *4.3* pg/ml).

How do our data of IL-1β and IL-6 production by monocytes of bipolar patients and the effect of lithium treatment compare to those previously reported in the literature? Although there are several conflicting reports on the *ex vivo* production of IL-1β and IL-6 by circulating leukocytes of major depressed patients (see introduction), reports on the *ex vivo* production of these cytokines by leukocytes of non-lithium treated bipolar patients are scarce. There is one recent study[67] which investigates, as we do, the *ex vivo* production of IL-6 by immune cells in lithium treated and non-treated bipolar patients (IL-1β was not studied). In accord with our study, lithium treatment decreased the IL-6 production by stimulated immune cells. In contrast to the scarcity of studies on the *ex vivo* production of pro-inflammatory cytokines by immune cells, there is a reasonable number of reports on the serum level of cytokines in non-lithium treated bipolar patients. In particular the serum level of IL-6 has been studied and compared to that of healthy control subjects. IL-6 serum levels were undetectable[67,68] or not altered[20,39]. With regard to IL-6 in serum, it must be noted that this cytokine is not only derived from immune cells but also from thyroid cells[69], hence parallels between these serum studies and the here-described *ex vivo* production by monocytes/macrophages are difficult to draw. Interestingly lithium treatment increased serum

levels of IL-6[20,49,70] and IL-1β[49].

Unexpectedly we found that the *in vitro* exposure of monocytes to lithium had another effect than lithium treatment: while lithium treatment raised the IL-1β production of *ex vivo* cultured monocytes and lowered their IL-6 production, LiCl added *in vitro* dose-dependently reduced the capacity of LPS-stimulated monocytes to produce IL-1β. There is only one report, that -similar to this report- compares the *in vivo* treatment effects of lithium with the *in vitro* effects of the drug on the function of immune cells in the same patients and controls[67]. In that study lithium treatment and *in vitro* lithium exposure also had different effects: while lithium *in vitro* lacked any effects on the number of circulating cytokine secreting cells, lithium treatment of patients reduced the number of IL-2, IL-6, IL-10 an IFNγ secreting cells. These differences between lithium treatment and *in vitro* lithium exposure might be explained by the difference in concentration and duration to which the immune cells are exposed to lithium in the treatment as compared to the *in vitro* situation. However, also indirect effects of lithium treatment on the immune system may play a role in the treatment situation, such as effects exerted via the neuro-endocrine system. Obviously such indirect effects are lacking in the *in vitro* LiCl exposure situation. In this regard it is worth noticing that in our study the 1mM *in vitro* concentration (an effective serum level) had only minimal effects on the monocyte/macrophage pro-inflammatory cytokine production in the (be it short) *in vitro* exposure experiments (see results).

How do our data on the effects of *in vitro* lithium exposure on IL-1β and IL-6 production by monocytes in general relate to the effects of lithium exposure reported in the literature? There are many reports on the effects of *in vitro* lithium exposure on the cytokine production by immune cells, but these reports differ widely in experimental conditions, such as the actual cytokines under study, the studied immune cell populations, the state of stimulation of the immune cells, the readout systems and the concentration, duration and *in vitro* or *in vivo* character of lithium exposure[67,71-74]. The group of Kleinerman was the only group to determine the effect of LiCl on spontaneous IL-1β production. In line with our results, LiCl did not affect the spontaneous IL-1β production[71]. The effect of *in vitro* LiCl on IL-6 production is studied into more extent. Although two groups reported that the spontaneous IL-6 production was not influenced by LiCl[67,73], Arena *et al* reported an increased production[72]. Regarding the mitogen-stimulated IL-6 production, LiCl decreased the IL-6 production in one study[74] but LiCl did not affect it in two other studies[67,73]. The different reaction profile of IL-6 production of monocytes of bipolar patients and healthy control subjects, as found in our study

(respectively no difference after *in vitro* LiCl addition versus an increased production) was not found by others[67,72]. It is clear that a consistent picture has not been generated on the *in vitro* effects of lithium regarding IL-1β and IL-6 production.

In conclusion our study shows that monocytes of bipolar patients have an imbalance in their IL-1β/IL-6 production, which is corrected by lithium treatment. *In vitro*, lithium also had an effect on the IL-1β/IL-6 production ratio of monocytes, but different from that observed by lithium treatment. Our data do support the view expressed in the "macrophage theory of depression" that monocytes/macrophages have an altered status in affective disorders, but doubt the idea that a simple rise in the production of the pro-inflammatory cytokines IL-1β and IL-6 by monocytes/macrophages would be responsible for the changes in mood.

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Chapter 3.2

Evidence that the Immune Pathogenic Mechanism of Lithium-induced Psoriasis differs from that of Regular Psoriasis

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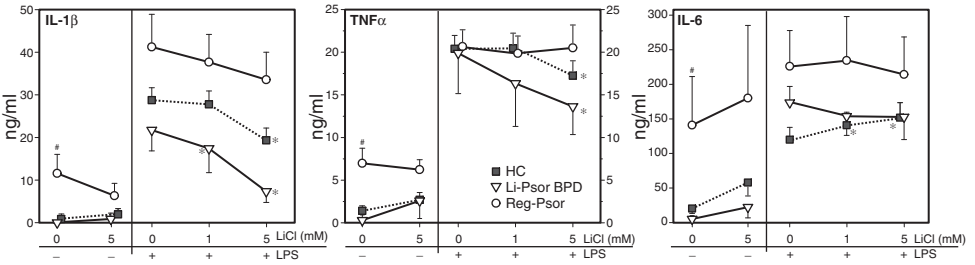
Letter to the Editor:

Lithium is known as an effective mood-stabilizing drug[1] with immune regulatory properties[2]. A well-known side effect of lithium treatment is the exacerbation of psoriasis[3]. Psoriasis is a mostly localized, chronic inflammatory skin disease, characterized by a strong increase in the proliferation of keratinocytes and an accumulation of T cells, monocytes, and monocyte-derived cells[4]. Pro-inflammatory cytokines such as IFN-γ, IL-1β, and TNFα, are thought to activate these inflammatory cells[5]. The pro-inflammatory cytokine network is not only disturbed in the immune and epithelial cells in the psoriatic lesion, but also in the circulating monocytes of the patients[6].

We investigated the basal and lipopolysaccharide (LPS, 1 µg/ml culture fluid) stimulated production of IL-1β, TNFα, and IL-6 by density gradient-purified peripheral blood monocytes of 27 healthy control subjects (HC: eight male/19 female, mean age 33.6 ± *8.5*), 16 patients with a "regular" psoriasis (Reg-Psor: 10 male/six female, mean age 45.8 ± *16.1*; all active lesions: body surface area 11.6%, no systemic medication), and six outpatients with DSM-IV bipolar disorder with lithium-induced psoriasis (Li-Psor BD: four male/two female, mean age 52.7 ± *8.5*, all active lesions: body surface area 4.3%, only psychotropic medication, 2 euthymic/4 symptomatic) in the absence or presence of 1 and 5 mM lithium chloride (LiCl) with previously described methods[7].

Figure 1 shows the IL-1β, TNFα, and IL-6 production of the blood

Figure 1. Pro-inflammatory Cytokine Production after Lithium and/or LPS-addition.



Means and SEM are given. **Black squares** represent healthy control subjects (HC), **open triangles** represent bipolar patients with lithium-induced psoriasis (Li-Psor BPD), and **open circles** represent patients with a regular psoriasis.

\# represent *p* values < .05 between the groups (Kruskal-Wallis test)

* represent *p* values < .05 comparing the cytokine production with LiCl added (1 or 5mM) versus 0mM (Wilcoxon signed-rank test)

monocytes of Reg-Psor, Li-Psor BD, and HC. Blood monocytes of Reg-Psor showed an enhanced production of the pro-inflammatory cytokines when compared with the values found in HC, particularly under non-LPS stimulated conditions. However, the production profiles of the cytokines of Li-Psor BD were in the same range as the HC, in sharp contrast to the high production range of Reg-Psor. When LiCl was added *in vitro* to LPS-stimulated monocytes, the production of IL-1β and TNFα of HC and Li-Psor BD was significantly down regulated, particularly in the case of Li-Psor BD having a significant decline at 1mM. This contrasted again to monocytes of Reg-Psor, where LiCl lacked a statistically significant dampening effect on the production of the pro-inflammatory cytokines. Further analysis did not show an association between the cytokine production and age or gender.

Our observation on the discrepancy between the pro-inflammatory cytokine production of monocytes of Reg-Psor and Li-Psor BD indicates that the pathogenesis of Li-induced psoriasis must be different from regular psoriasis. An excessive production of pro-inflammatory cytokines –as described for regular psoriasis[8]- is less likely to be an important pathogenic factor in lithium-induced psoriasis. Interestingly direct stimulating effects of lithium have been found on the proliferation of keratinocytes in in-vitro-cultured normal human skin explants[9], pointing to a non-immunological effect of the drug in its potency to elicit the psoriatic skin condition. Perhaps our observation of monocytes of patients at risk for a lithium-induced psoriasis being ultra-sensitive to the effects of lithium might also be extrapolated to their keratinocytes, which may be ultrasensitive and respond with an excessive proliferation to lithium exposure.

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Chapter 4
Antigen Presenting Cells

Chapter 4.1

Monocyte-derived Dendritic Cells in Bipolar Disorder

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Abstract

Background

Dendritic cells (DC) are key regulators of the immune system, which is compromised in patients with bipolar disorder.

Aim

To study monocyte-derived DC in bipolar disorder.

Methods

Monocytes purified from blood collected of DSM-IV bipolar disorder outpatients (*n=53*, 12 without lithium treatment) and healthy individuals (*n=34*) were differentiated into DC via standard granulocyte-macrophage colony-stimulating factor/interleukin-4 culture (with/without 1, 5, and 10 mM LiCl). DC were analyzed for DC-specific and functional markers and for T-cell stimulatory potency.

Results

Monocytes of bipolar patients showed a mild hampering in their differentiation into fully active DC, showing a weak residual expression of the monocyte marker CD14 and a relatively low potency to stimulate autologous T cells. Lithium treatment abolished this mild defect and monocyte-derived DC of treated bipolar patients showed signs of activation (i.e., an up regulated potency to stimulate autologous T cells and a higher expression of the DC-specific marker CD1a). This activated phenotype contrasted with the suppressed phenotype of monocyte-derived DC exposed to lithium *in vitro* (10 mM) during culture.

Conclusion

Dendritic cells show mild aberrancies in bipolar disorder that are fully restored to even activation after *in vivo* lithium treatment.

Introduction

We previously reported a raised prevalence of autoimmune thyroiditis, defined as a raised level of thyroperoxidase-antibodies (TPO-Abs), in bipolar patients[1]. In a consecutive study on the same cohort not only thyroid autoimmune reactivity but also autoimmune reactivity to pancreatic islets (GAD65-Abs) and to gastric mucosa (H^+/K^+ ATPase-Abs) were more prevalent in bipolar disorder[2]. The raised prevalence of TPO-Abs, GAD65-Abs, and H^+/K^+ ATPase-Abs was not associated with the current use of lithium.

To investigate whether a higher prevalence of autoimmunity in bipolar disorder (BD) was associated with a generalized activation of the T-cell system, we extended our studies and measured levels of serum-interleukin-2 receptor (sIL-2R) and numbers of activated T cells in the circulation of BD patients[3]. The T-cell system was found activated, as evidenced by a higher serum level of sIL-2Rand raised numbers of $CD3^+$ $CD25^+$ cells, in both symptomatic and euthymic patients; however, manic patients showed the highest state of T-cell activation. As with the auto-Abs, the T-cell activation was not associated with the use of lithium.

The stimulation of antigen-specific T cells and the production of antibodies, including that of TPO-Abs, GAD65-Abs, and H^+/K^+ ATPase-Abs, are under the control of dendritic cells (DC), a specialized group of immune cells. The DC are antigen-presenting cells (APC) par excellence and the only cells capable of stimulating naïve T cells and initiating an effective immune response towards foreign antigens[4-6]. Besides that, DC are also involved in preventing autoimmune responses by inducing tolerance to auto-antigens. To exert these functions, DC express major histocompatibility complex (MHC)-class II molecules and the so-called co-stimulatory molecules (CD80, CD86, and CD40) on their cell membrane to be able to strongly stimulate (auto-)antigen-specific T-helper and regulatory cells[6,7]. One of the main precursors of DC is the blood monocyte[6,8].

Our observation of an aberrant T-cell activation and a higher prevalence of thyroid, islet, and gastric-Abs in BD patients led to the hypothesis that DC might also be aberrant in these patients. To our knowledge, studies on the function of DC in bipolar disorder have not been performed. Therefore, we induced purified peripheral blood monocytes of 53 BD patients to differentiate into DC with a 6-day-culture of cells in the presence of the differentiating cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. The DC character of the cells was verified by the expression level of the DC-specific type II lectin DC-SIGN, of the DC-specific lipid antigen-presenting molecule CD1a, and of CD83 on the

cultured cells. The functional activity of the cells was determined by the capability of the generated DC to stimulate T cells to proliferate in mixed lymphocyte reactions (MLR). We also measured the expression levels of MHC-class II, CD80, CD86, CD40, and of molecules involved in APC/T-cell interactions (i.e., of the integrins CD11b, CD18, CD29, CD54). We investigated these functional and phenotypic DC parameters in BD patients with current lithium treatment and without lithium treatment separately, since this drug is known to be immune modulatory[9,10]. Outcomes were compared to those obtained using cells of healthy control subjects (n=34). We also investigated whether *in vitro* co-culture with lithium influenced the capacity of monocytes to differentiate into DC.

Materials and Methods

Patients and Healthy Control Subjects

Patients: Heparinized blood was obtained from outpatients with DSM-IV bipolar I and II disorder (n=53) participating in the Stanley Foundation Bipolar Network (SFBN), a multi-center research program described elsewhere in detail[11,12]. For these experiments, all patients were recruited from SFBN sites in The Netherlands. The Institutional Review Board of the University Center Utrecht had approved the study protocol and written informed consent was obtained from all patients. A DSM-IV[13] diagnosis of bipolar disorder was made by means of the Structured Clinical Interview for DSM-IV Axis I, Patient Edition (SCID-I/P) Research Vision[14]. A detailed illness history, including past and present medication, was assessed at entry into the Network. Blood for the present study was drawn during one of the monthly follow-up visits, when a research clinician also determined present mood (i.e., euthymic, depressed, [hypo-] manic or cycling) by means of a detailed interview including the Young Mania Rating Scale[15], Inventory of Depressive Symptoms[16], and Clinical Global Impressions Scale – Bipolar Version[17]. Patient clinical characteristics are shown in Table 1. Patients were included as "not using lithium" (nonLi-BD) when they were either lithium naive or did not use lithium for at least 12 months. The lithium users had effective lithium treatment for at least 6 months (Li-BD). It must be noted that Li-BD had comparable profiles of other psychotropic medication as the nonLi-BD.

Blood was collected in sodium-heparin tubes (Becton and Dickenson), since lithium-anticoagulant for the collection of blood has been found to influence the *in vitro* cytokine production[18]. Blood was drawn in the

Table 1. Clinical and Demographic Characteristics.

		Bipolar Patients	Healthy Control Subjects
Group size	Total	53	34
	1st Series	14	11
	2nd Series	15	9
	3rd Series	8	8
	4th Series	16	6
Age (years)[1]		45 (22-62)	38 (23-62)
Gender	Male	25 (47%)	12 (35%)
	Female	28 (53%)	22 (65%)
Psychotropic medication	Carbamazepin	35 (66%)	
	Valproate	16 (30%)	
	Thyrax	10 (19%)	
	Antidepressives	14 (26%)	
	Antipsychotics	9 (17%)	
	Benzodiazepins	18 (34%)	
Duration of treatment (years)[1]		14 (1-36)	
Lithium users		41 (77%)	
	Li titer in serum (mM)[2]	.78 ± *.17*	
	Cumulative period of Li treatment (months)[1]	76 (6-203)	
Patients never used Li (naïve)		6 (11%)	
Patients without current Li but with Li in past		6 (11%)	
	Period without Li (months)[1]	43 (12-114)	
Current mood state	Euthymic	31 (58%)	
	Depressed	12 (23%)	
	Manic	8 (15%)	
	Cycling	2 (4%)	
Duration of illness (years)[1]		21 (3-42)	
Hematological parameters	Hb[2,3]	8.7 ± *.8*	
	Leucocytes[2,4]	6.9 ± *1.4*	
	ESR[2,5]	5.8 ± *2.8*	

[1] mean (range)
[2] mean ± *SD*
[3] reference values: 7.4 – 9.6 mM
[4] reference values: 4.0 – 10.0*10^9/l
[5] reference values <10mm/hour
Li, lithium; Hb, hemoglobin; ESR, erythrocyte sedimentation rate.

morning and transported by courier to the laboratory in Rotterdam, lasting an acceptable 8-10 hours before further processing.

Healthy Control Subjects: Blood was collected from healthy laboratory and hospital staff members of the Erasmus MC (The Netherlands) (*n*=34) on the same days and at the same time points as the patients with the same procedures. All healthy controls (HC) gave written information about medication use and medical history. Informed consent was obtained. The exclusion criteria for this HC group were: any immune disorder, serious medical illness, recent infections, fever, psychiatric disorder, or use of any psychotropic or other medication (apart from anti-conceptive hormonal therapy).

Generation of DC

Peripheral blood mononuclear cells (PBMC; i.e., the lymphocytes and monocytes) were prepared from the heparinized blood via Ficoll (Pharmacia, Uppsala, Sweden) gradient centrifugation as has been described in detail before[19]. The PBMC were thereafter re-suspended in RPMI 1640 with 25 mM HEPES and L-glutamine (Bio Whittaker Europe, Verviers, Belgium), additionally containing 10% inactive fetal calf serum (Bio Whittaker Europe, Verviers, Belgium; FCSi), penicillin (100 U/ml)/ streptomycin (100 μg/ml; Bio Whittaker Europe, Verviers, Belgium; P/S), and extra ultraglutamine (Bio Whittaker Europe, Verviers, Belgium, 2mM; UG) (RPMI+). Samples were frozen in 10% dimethylsulfoxide and stored in liquid nitrogen. This enabled us to test patient and control monocytes in the same series of experiments at appropriate times. The frozen PBMC were quickly thawed and washed twice in RPMI+ (trypan blue exclusion: >85% viable cells). To separate monocytes from lymphocytes, Percoll (density 1.063 g/ml; Pharmacia) density gradient centrifugation (for 40 min, 400g) was used.

Monocytes were cultured in 4 series (see Results) at a concentration of $.5x10^6$ cells/ml on 24-well culture plates (Falcon) under plastic-adherent conditions in RPMI+ in the presence of GM-CSF 800 U/$.5x10^6$ cells/ml (series 1+2: Pepro Tech, Rockyhill, New Jersey, USA; series 3+4: Biosource, London, England) and IL-4 1000 U/$.5x10^6$ cells/ml (series 1+2: Biosource, London, England; series 3+4: Pepro Tech, Rockyhill, New Jersey, USA). In series 2-4 we added lithium chloride (LiCl) to the culture plates (1, 5, and 10 mM). Cells were thereafter incubated at 37°C, 5% CO_2 and 95% humidity. On day three, the culture fluid was refreshed with complete RPMI+, containing cytokines (and, where appropriate LiCl). After six days, DC were collected by re-suspending and washing the wells thoroughly with cold phosphate buffered saline (PBS) pH 7.4 (Bio Whittaker Europe, Verviers, Belgium) containing 3 mM ethylene

diamine tetra-acetic acid (Sigma-Aldrich, Steinheim, Switzerland). This culture method is well established[8].

T-cell Isolation

The pellet of the Percoll gradient was washed twice with PBS containing .1% bovine serum albumine (Bayer, Kankakee, Illinois, USA) (PBS/.1% BSA) and the cells were incubated with anti-CD3 microbeads (20µl/10^7 cells: Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 minutes at 4°C. A magnetic cell sorting system (autoMACS, Miltenyi Biotec) was used for the selection of CD3 positive T cells (positive selection). We isolated T cells from BD and HC to use in the autologous Mixed Lymphocyte Reaction (MLR) and from a cohort of HC to use in allogeneic MLR.

Fluorescence-activated Cell Sorting Analysis of DC

For analysis of marker expression by flowcytometry, all cell populations were stained by incubating for 10 minutes in the dark at room temperature with mouse anti-human FITC (fluorescein)- or PE (phycoerythrin)-conjugated monoclonal antibodies (mAbs), and washed afterwards. The following mAbs were used for flowcytometry: anti-IgG1 FITC (B&D), anti-IgG1 PE (B&D), anti-CD14 PE (Beckman Coulter [BC], Hialeah, Florida, USA), anti-DC-SIGN FITC (R&D systems, Minneapolis, Minnesota, USA), anti-CD83 PE (B&D), anti-HLA-DR FITC (B&D), anti-CD1a PE (BC), anti-CD80 PE (B&D), anti-CD86 FITC (Pharmingen, San Diego, California, USA), anti-CD40 FITC (Serotec, Oxford, England), anti-CD11b PE (B&D), anti-CD18 FITC (B&D), anti-CD29 FITC (BC), and anti-CD54 PE (B&D) (Table 2).

Immediately after staining, cells were subjected to fluorescence-activated cell sorting (FACS) on a FACScan flowcytometer (B&D). During the FACS measurement and analysis of the data, the researcher was blind to the identity (patient/control subject) of the sample. Debris and dead cells were gated out in the samples on the basis of their light scatter properties. The DC population was gated out by means of cell size (forward scatter) and irregular shape (side scatter). Because monocyte-derived cells often have a considerable amount of auto-fluorescence (granular aspects and lysosomes/vesicles) we used FACS instrument settings to correct for auto-fluorescence. As a positive control, we used beads with a standardized known fluorescence intensity (Spherotech, Libertyville, Illinois, USA) and expressed data as fluorescence intensities relative to the intensity of the beads (with excel-software by Spherotech). As a negative control, we determined the fluorescence

Table 2. Characterization of the Phenotype of the Monocyte-derived DC via CD Marker Determination.

CD Marker	Cell Specificity	Functions/Remarks
Monocyte/DC Marker Molecules		
CD14	monocytes	Receptor for LPS
DC-SIGN	dendritic cells	DC-specific type II lectin: capturing carbohydrate-bearing antigens
CD83	dendritic cells	Cell surface glycoprotein, member of Ig superfamily, DC maturation marker
Antigen Presenting Molecules		
HLA-DR	APC	MHC-class II molecule presenting peptide antigens
CD1a	APC	MHC-class I like molecule presenting lipid antigens
Co-stimulatory Molecules		
CD80	APC	Ligand for CD28 and CTLA-4
CD86	APC	Ligand for CD28 and CTLA-4
CD40	APC	Ligand for CD154
DC-/T-cell Adhesion Molecules/Integrins		
CD11b	No specificity	Forms Mac-1 together with CD18, ligand for ICAM-1
CD18	No specificity	Integrin β2 subunit associates with CD11a, b, c and d
CD29	No specificity	Integrin β2 subunit associates with CD49 in VLA integrins
CD54	No specificity	Intracellular adhesion molecule binds CD11a and Mac-1

Clusters of differentiation (CD) markers used in flow cytometric staining and their characteristics.
DC, dendritic cell; LPS, lipopolysaccharide; APC, antigen presenting cell; MHC, major histocompatibility complex; Ig, immuneglobulin; CTLA, cytotoxic T-lymphocyte associated antigen; ICAM, intercellular adhesion molecule; VLA, very late antigen.
Modified from Immunobiology, fifth edition, C.A. Janeway, *et al*.

intensity of the negative control antibodies IgG1-FITC or IgG1-PE. For analysis of the data, we used CellQuestPro software (B&D Pharmingen, Alphen aan de Rijn, The Netherlands). Data are generally expressed as mean fluorescence intensity (MFI). Our measuring method and the development of monocytes to DC is illustrated in Figure 1. Here, we show the CD14/DC-SIGN profile of monocytes and monocyte-derived DC; monocytes are CD14 high and DC-SIGN low (Figure 1A), monocyte-derived DC are CD14 low, DC-SIGN high (Figure 1B).

Mixed Lymphocyte Reaction

Autologous and allogeneic MLR were performed to measure the accessory capability of the DC. For these MLR, DC were irradiated with 20 Gy and placed in flat-bottom 96-wells-plates (NUNC, Roskilde, Denmark) in RPMI containing UG, P/S, and A^+-serum. We added 100 µl DC suspension with concentrations of $3x10^5$, $1.5x10^5$, $.75x10^5$ cells/ml and an equal volume of autologous or allogeneic T cells ($1.5x10^6$ cells/ml) to the wells, resulting in a total volume of 200 µl/well. As positive control, T cells were stimulated with the T-cell mitogen phytohaemagglutinin (PHA, 20 ng/ml, Sigma). As negative control, T cells were left unstimulated. Proliferation was determined after 16 hours of ^{3}H-thymidine addition (.5 µCi/well) on day 5. Cells were harvested on filter papers, and radioactivity was counted in a liquid scintillation analyzer (Microbeta; Wallac, Turun, Finland). The values are the mean of triplicates.

Auto-antibody and sIL-2R Determinations

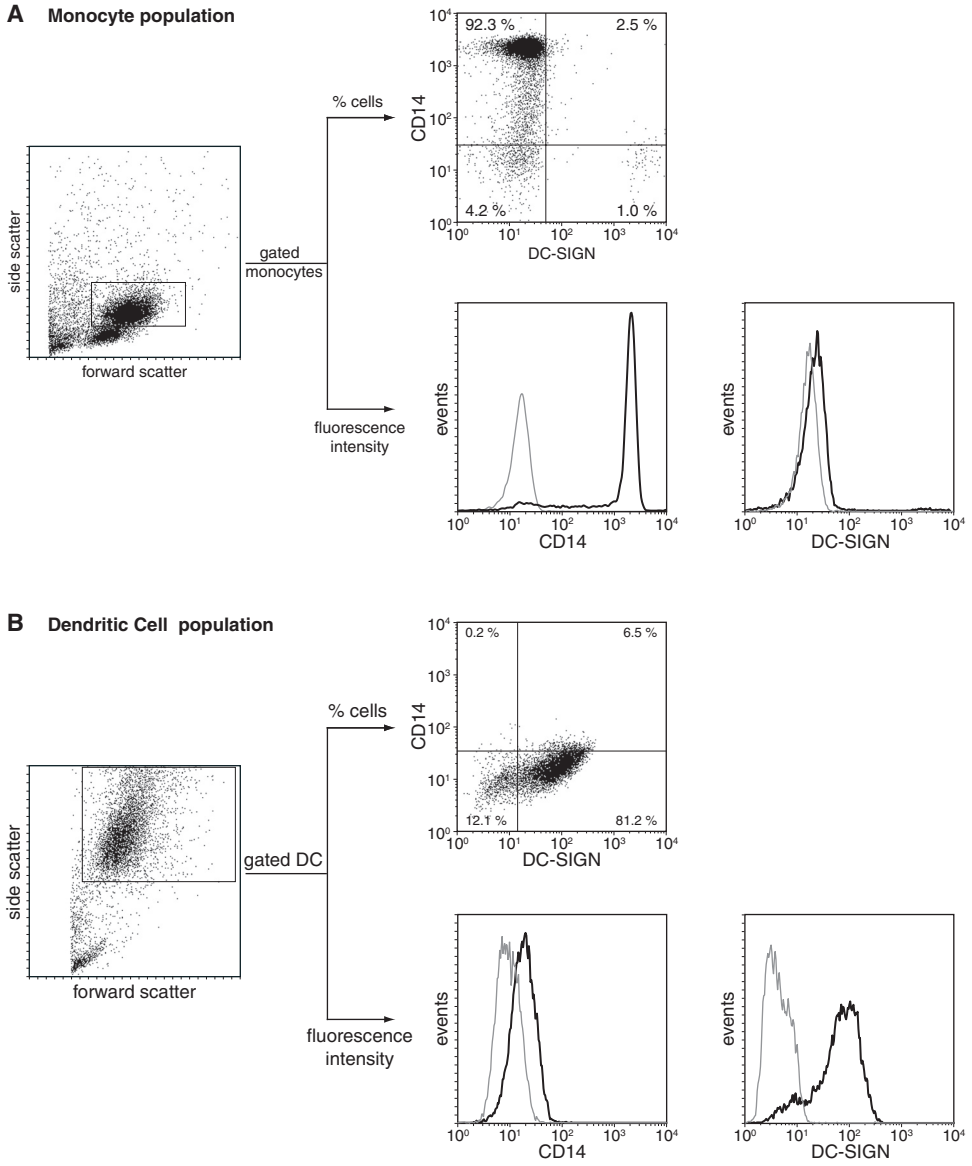
The levels of TPO-Abs, GAD65-Abs, and H^+/K^+-ATP-Abs have been measured in the same cohort and described previously[1,2]. The sIL-2R levels have also been measured previously[3].

Statistical Analysis

Statistical analysis was performed with the SPSS 11.0 package for Windows (SPSS, Chicago, Illinois, USA). Data were tested for their parametric distribution with the Kolmogorov-Smirnov test. Because this test showed a nonparametric distribution for the investigated data,

Figure 1. FACS Analysis of Monocytes and Dendritic Cells.
Monocytes (A: relatively small and regular shape) and dendritic cells (B: relatively large with irregular shape) were gated out on the basis of forward (size, FSC) and side scatter (shape, SSC). The CD14 (PE) and DC-SIGN (FITC) expression of the gated population is presented as both percentages of positive/negative cells and histograms showing the fluorescence intensity. The **gray lines** represent the isotype/negative control (IgG-FITC/-PE), the **black lines** represent the CD14 or DC-SIGN expression of the gated population (healthy control subject). Due to instrument settings (see text) relatively low fluorescence intensities are reached. As positive controls we used 8 graded peaks generated with commercially available beads with a predetermined/fixed fluorescence intensity. The values of minimum, medium and maximum peaks (peak 1,5,8) were with regard to FITC labeling 1, 20, and 388, and with regard to PE labeling 1, 29, and 744 (data not included in the Figures), hence the marker expression on the cells were all within the positive control range.

Figure 1. FACS Analysis of Monocytes and Dendritic Cells.



including marker expression and MLR, we decided to use nonparametric tests to analyze differences between groups. To analyze FACS and MLR data, statistical significance between the various groups was determined with a Kruskal-Wallis test, followed by a Mann-Whitney U test to determine differences between two groups. To analyze the *in vitro* data of LiCl (series 2-4) we performed paired statistical analysis (0 vs. 1 mM, 0 vs. 5 mM, and 0 vs. 10 mM LiCl) with the Wilcoxon signed-ranks test. Correlations of the various laboratory parameters with different clinical parameters were examined with Spearman's rho test. All data were tested for two-tailed significance. *P* values below .05 were considered to be statistically significant.

Results

Treatment with Lithium stimulates the Generation of Fully Active Dendritic Cells from Monocytes of Bipolar Patients

We carried out four separate series of culture experiments over time (first: January-February 2002; second: April-May 2002; third: September-December 2003; fourth: October-December 2004) to obtain monocyte-derived DC. These series of cultures differed regarding the FCS badges and the supplier of the cytokines used (see previous). In each series, cells were obtained with a clear phenotype of monocyte-derived DC when starting from monocytes of healthy control subjects (see Table 3) (i.e., cells with a very low CD14 expression, a positive expression for the DC-specific markers DC-SIGN, CD1a and also CD83, a positive expression of the T-cell stimulatory molecules MHC-class-II, CD86, CD40 and CD80 and a high expression of the integrins CD11b, CD18 and CD54). The range of expression levels of these various markers, however, clearly differed between the four series. This observation is congruent with our experience of a considerable inter-series variation in DC phenotype, depending on culture conditions, like FCS and cytokine-supplier (data not shown). Therefore, we decided to use a calculated normalizing factor to be able to compare data from the four series. We took the mean fluorescence intensity of a given marker obtained in the HC group of that series as 100% and calculated every single patient measurement relative to this 100% HC value.

Table 3 shows the data of this analysis and gives the expression levels of the various membrane markers on monocyte-derived DC of nonLi-BD

Table 3. Phenotype of Monocyte-derived Dendritic Cell of Healthy Control Subjects and Bipolar Patients with and without Lithium Treatment.

	Healthy Control Subjects (HC)	**Bipolar Patients (BD)**				
		nonLi-BD		**Li-BD**		
	MFI ± *SE*	Relative change versus HC (± *SE*)	*vs. HC*[2]	Relative change versus HC (± *SE*)	*vs. HC*[2]	*vs. nonLi-BD*[2]
Monocyte/DC molecules						
CD14[1]	10.5 ± *9.3*	123% ± *15*	*p<.05*	**63%** ± *11*	*p<.05*	*p<.05*
DC-SIGN	199.2 ± *111.7*	76% ± *11*	*ns*	111% ± *11*	*ns*	*ns*
CD83	39.62 ± *14.4*	114% ± *19*	*ns*	103% ± *17*	*ns*	*ns*
APC molecules						
HLA-DR	286.4 ± *177.2*	101% ± *20*	*ns*	85% ± *7*	*ns*	*ns*
CD1a[1]	95.25 ± *58.94*	83% ± *21*	*ns*	**200%** ± *28*	*p<.05*	*p<.05*
Co-stimulatory molecules						
CD80	47.8 ± *19.3*	98% ± *12*	*ns*	106% ± *13*	*ns*	*ns*
CD86	155.0 ± *61.1*	84% ± *13*	*ns*	106% ± *11*	*ns*	*ns*
CD40	337.1 ± *129.4*	92% ± *10*	*ns*	112% ± *13*	*ns*	*ns*
Adhesion molecules/ Integrins						
CD11b	735.9 ± *179.0*	84% ± *12*	*ns*	107% ± *9*	*ns*	*ns*
CD18	140.8 ± *50.2*	92% ± *13*	*ns*	103% ± *10*	*ns*	*ns*
CD29	38.26 ± *12.78*	91% ± *15*	*ns*	109% ± *11*	*ns*	*ns*
CD54	394.6 ± *99.9*	93% ± *16*	*ns*	120% ± *10*	*ns*	*ns*

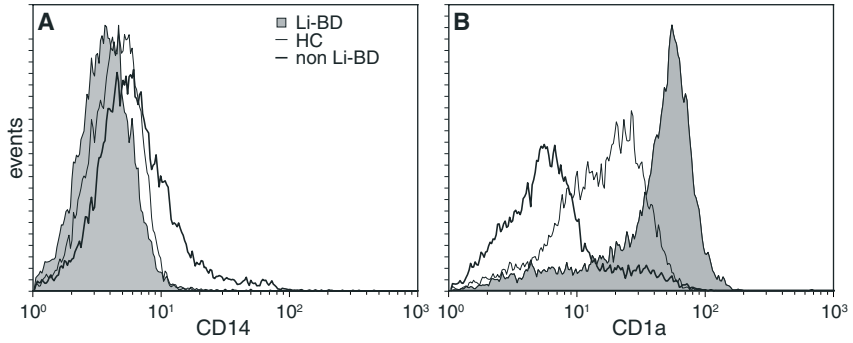
[1] statistical significant difference ($p<.01$) within the group with Kruskal-Wallis test
[2] represent statistical analysis between 2 groups with Mann-Whitney U test
Mean Fluorescence Intensity (MFI) of the monocyte-derived dendritic cells of healthy control subjects for the different markers, expressed as medians of the 4 different series of experiments (First column). The negative control antibody reaches a MFI of 7.0 (median for FITC) and 13.5 (median for PE). The positive controls are the 8 graded peaks generated with commercially available beads with a predetermined/fixed fluorescence intensity (see Material and Methods and legend of Figure 1). The median values of the different markers, shown in the table are higher than the negative control value and all within the range of the positive control beads. Fluorescence intensities for the DC of bipolar patients (without lithium treatment [nonLi-BD] and with [Li-BD]) are expressed as percentages relative to the healthy control value (set as 100%) obtained in the same series of experiments (see also text).

and Li-BD relative (in percentages) to the expression level of the marker found on HC DC of that series.

With regard to the nonLi-BD, the expression levels of the various markers were practically identical to the monocyte-derived DC populations of HC, albeit the monocyte specific marker CD14 was slightly higher on nonLi-BD DC (see Table 3 and Figure 2a; giving a representative example). The presence of this residual monocyte molecule on nonLi-BD DC indicated to us that a full transition of monocytes into DC had most likely not completely taken place.

To determine the functional capacity of such not-fully developed DC of BD patients, we performed an MLR with ^{3}H-thymidine incorporation into autologous and allogeneic T cells as a readout system to test the capacity of the DC, being excellent APC, to stimulate such T cells to proliferate. The autologous T-cell stimulation by APC is generally regarded as reflecting a T-cell reaction towards auto-antigens presented by the APC[20,21], whereas the allogeneic T-cell stimulation represents the reaction towards foreign antigens (i.e., mismatched transplantation) on the APC. First of all, it should be noticed (and relevant for the outcomes of the autologous MLR) that the T cells of the BD patients and HC did not show the same intrinsic proliferation capacity when stimulated with the mitogen PHA (Figure 3). The T cells of nonLi-BD showed an intrinsically enhanced proliferation, whereas those of Li-BD had a proliferation capacity comparable to that of HC. Such an intrinsic activation of the T-cell system in BD patients has

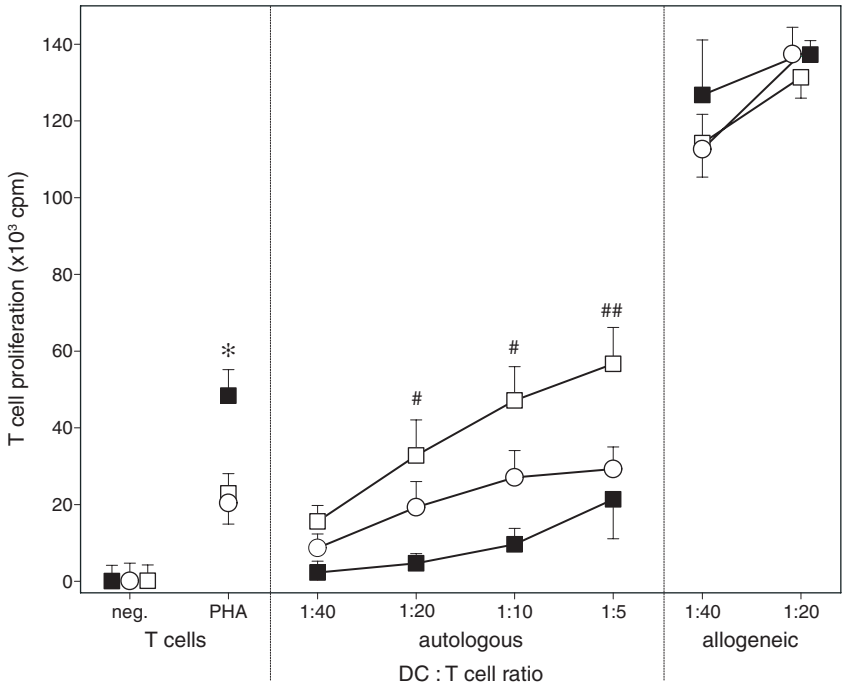
Figure 2. Fluorescence Intensity of CD14 and CD1a on DC.



Representative examples of the actual fluorescence intensity of CD14 (A) and CD1a (B) on monocyte-derived dendritic cells (DC). The **thin black lines** represent a healthy control sample (HC), the **thick black lines** represent a bipolar patient sample without lithium therapy (nonLi-BD), and the **gray lines** represent a bipolar patient sample with lithium therapy (Li-BD).

been noticed by others and us previously[3,22-24]. With regard to the actual stimulation by DC, autologous T cells were less stimulated to proliferate by their DC in the nonLi-BD, despite the intrinsically higher T-cell proliferation capacity of such T cells (however, not statistically significantly different from HC). This tendency for a reduced stimulatory capacity of DC of nonLi-BD for autologous T cells is in accord with the mild differentiation

Figure 3. Autologous MLR of Monocyte-derived DC.



Autologous mixed lymphocyte reaction (MLR) of monocyte-derived dendritic cells (DC) of bipolar patients versus healthy control subjects (HC), representing the capacity of monocyte-derived DC to stimulate T cells to incorporate ^{3}H-thymidine, given as counts per minute (cpm). **Open circles** represent T-cell stimulatory capacity of DC of HC, **filled squares** that of bipolar patients without lithium treatment (nonLi-BD), and **open squares** that of patients with lithium treatment (Li-BD). The figure shows T-cell proliferation without any stimulus or DC added (negative control sample), with the addition of only phytohaemagglutinin (PHA: positive control sample), T-cell proliferation with autologous DC, and with allogeneic DC. Data are expressed as mean values ± *SEM* and various ratios of DC versus T cells are shown.

* $p<.05$ using Kruskal-Wallis test, # $p<.05$ of Li-BD versus nonLi-BD users using Mann-Whitney U test, ## $p<.05$ of Li-BD versus nonLi-BD and HC using Mann-Whitney U test.

defect of DC noticed in the marker analysis previously mentioned (increased CD14). The T-cell stimulation capacity of the DC was much higher for allogeneic T cells, not disturbed, and equal to that of HC DC (Figure 3).

When evaluating the expression level of the marker molecules on the monocyte-derived DC of Li-BD, it seemed that the expression level of the monocyte marker CD14 was now absent and that of the DC-specific marker CD1a significantly higher as compared with DC of HC and nonLi-BD (Table 3, Figure 2B). This indicated that there are signs of an enhanced differentiation of prototypic DC from monocytes in Li-BD. It must be noted, however, that the expression levels of HLA-DR, the tested co-stimulatory molecules, and integrins were the same as those of DC of HC and nonLi-BD. In accord with the view of an improved DC differentiation from monocytes of lithium-using BD patients, the DC of Li-BD also showed a statistically higher capability to stimulate autologous T cells as compared with the DC of nonLi-BD and HC. The allogeneic T-cell stimulation capacity of the monocyte-derived DC of Li-BD was, however, equal to that of nonLi-BD and HC (Figure 3).

It is also relevant to note that the DC yields harvested after culture for both BD groups (Li-BD: $7.2x10^5 \pm 3.6x10^5$, nonLi-BD: $7.4x10^5 \pm 3.2x10^5$) were comparable to yields in HC ($9.1x10^5 \pm 4.9x10^5$) (p=.23).

Collectively our data indicate that the DC of nonLi-BD are mildly defective, leading to a relatively low autologous MLR (despite the higher intrinsic proliferative capability of T cells of nonLi-BD) and that, in lithium using patients, the DC are actually activated, leading to a stronger proliferation of their own T cells in an autologous MLR (overcoming the intrinsically lower T-cell stimulation of DC of Li-BD).

Because we found effects of the use of lithium on DC development, we correlated the expression of CD14 and CD1a with the duration of lithium treatment. A significant negative correlation was found for CD14 expression (r=-.43, p<.05), meaning that the longer patients were treated with lithium, the lower CD14 was expressed on the monocyte-derived DC; CD1a expression was not correlated to the duration of lithium treatment (r=-.11). With regard to a possible effect of the other psychotropic medication on *in vitro* DC differentiation from monocytes, we were unable to find statistically significant associations (data not shown).

We were also not able to find any statistically significant correlation between the differentiation of monocyte-derived DC and mood state (either between the four mood states or between symptomatic and euthymic BD patients), duration of illness (CD14: r=-.26, CD1a: r=.08), age (CD14: r=-.30, CD1a: r=.07), and gender (CD1a male: 1.8 ± *.32* versus female: 1.6 ± *.34*). This was the case both in the entire dataset and within the different subgroups. We also tested the possible

associations with the other phenotypic markers, but again without significant correlations (data not shown).

With regard to a possible correlation between the DC phenotype and other markers of immune activation in the patients, we were again unable to find a correlation with either sIL-2Rlevel (CD14: r=.15, CD1a: r=.04) or auto-antibodies against the thyroid (TPO-Abs) (CD14: r=.04, CD1a: r=.11). With regard to GAD65-Abs and H^+/K^+ ATPase-Abs, numbers in this sample of antibody-positive patients were too low to draw valid conclusions.

In Vitro Exposure to LiCl hampers the Generation of Fully Functional Dendritic Cells from Monocytes

On the basis of a limited dose-response study on the effects of various dosages of LiCl (.2 mM, 1.0 mM, 2.0 mM, 5.0 mM, 10.0 mM) on the *in vitro* generation of DC from monocytes of six HC, we decided to culture the monocytes of the BD patients and HC in 0, 1, 5, and 10 mM LiCl. The 1 mM was chosen because it equals the therapeutic levels reached in serum; 5 and 10 mM were chosen because these concentrations showed clear effects in our limited dose-response study.

Lithium chloride did not affect the viability of the generated DC and after 6 days of culture, trypan blue staining showed a viability of >90% in all cases, including in the highest concentration of 10 mM LiCl; LiCl also did not affect the cell yield after culture. This observation excluded a toxic effect of LiCl on the cells, even in the high dose of 10 mM (data not shown).

Lithium chloride exposure *in vitro* led to a relatively diverse picture, regarding the up or down regulation of the various markers and functional molecules on the monocyte-derived DC related to the dose of LiCl used (Figure 4). Nevertheless, generalizations can be made; the low 1 mM dose of LiCl only had very limited effects, whereas the higher dosages of 5 and 10 mM had clear effects. Such clear effects were, in particular: a dose-dependent down regulation of the DC-specific marker DC-SIGN and also of CD1a, and a slight up regulation of CD14 on the DC, indicating that the generation of prototypic DC from monocytes was hampered, owing to *in vitro* LiCl exposure. The antigen-presenting molecule HLA-DR and the co-stimulatory molecule CD86 were, however, up regulated on these LiCl-exposed monocyte-derived DC, whereas the co-stimulatory molecule CD40 was down regulated. This indicates a complex and different effect on molecules that are normally regulated in a coordinated fashion. Lithium chloride *in vitro* also affected the integrin expression on the monocyte-derived DC in a complex manner: CD54 and CD29 were up regulated, whereas CD11b and CD18 were in general down regulated.

Figure 4. *In Vitro* Effect of Lithium Chloride on Monocyte-derived DC.

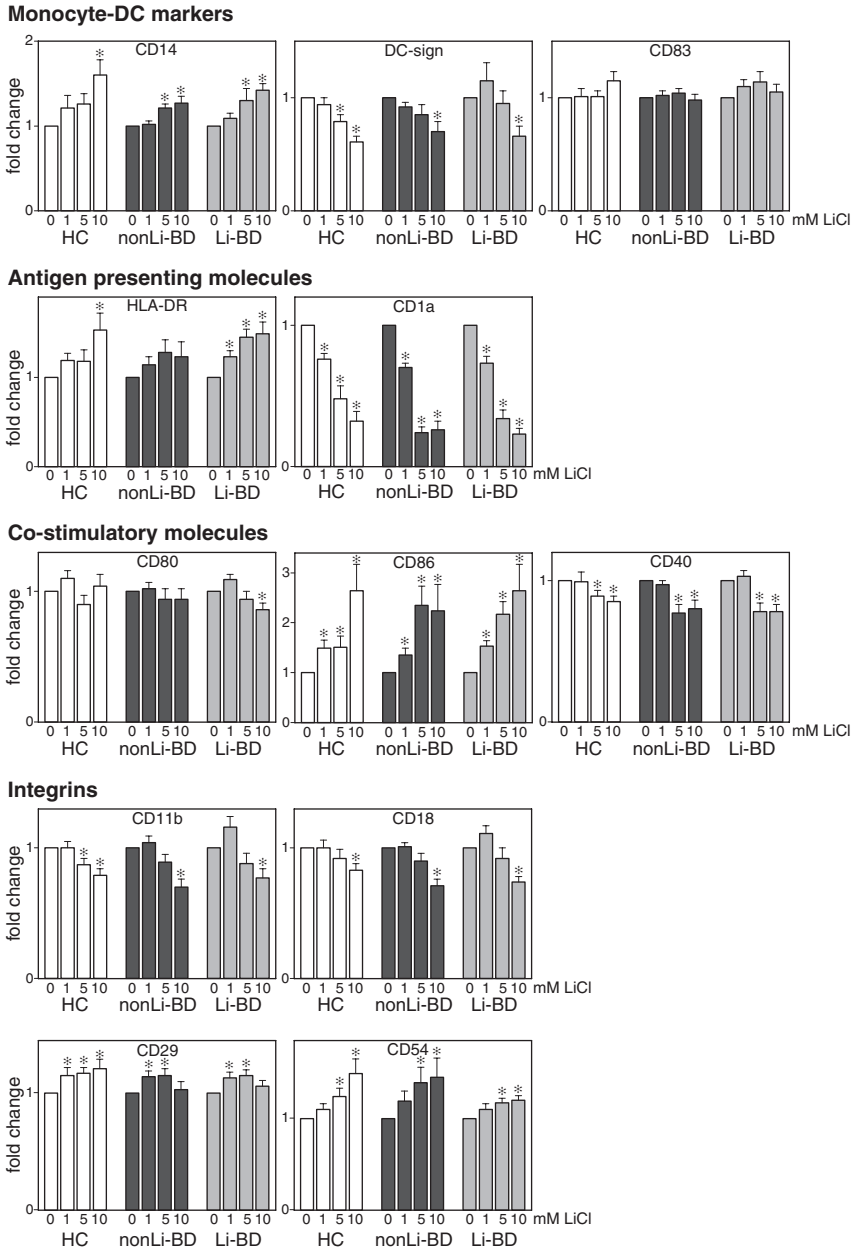
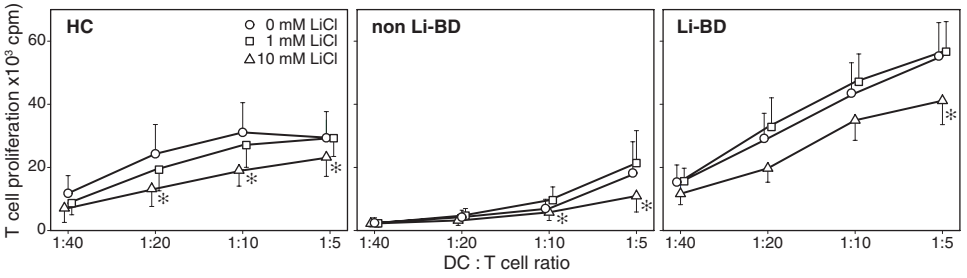


Figure 4. *In Vitro* Effect of Lithium Chloride on Monocyte-derived DC.
In vitro effect of lithium chloride (LiCl) on monocyte-derived dendritic cells of healthy controls (HC: **white bars**), bipolar patients without lithium (nonLi-BD: **black bars**) and with lithium treatment (Li-BD: **gray bars**) from series 2-4. Data are expressed as the mean fold change (compared to the 0 mM LiCl condition) ± *SEM* of the fluorescence intensity, corrected with pre-labeled beads (CFI). The phenotypic membrane markers are (in order of appearance): monocyte-DC markers (CD14, DC-sign, CD83), antigen presenting molecules (HLA-DR, CD1a), and co-stimulatory molecules (CD80, CD86, CD40), and integrins (CD11b, CD18, CD29, CD54).
* $p<.05$ of the LiCl versus the non-LiCl condition using Wilcoxon signed-rank test

With regard to the functionality of the monocyte-derived DC generated in the presence of LiCl, we used again the ^{3}H-thymidine incorporation of T cells in an autologous MLR as a read-out system for the T-cell stimulatory capacity of the DC, but only with 1 and 10 mM LiCl. Figure 5 shows that monocyte-derived DC, cultured in the presence of 1 mM LiCl were not affected, but that 10 mM LiCl resulted in a reduced potency of monocyte-derived DC to stimulate the proliferation of T cells. These findings are generally in accord with the marker profiles of the DC as described previously, showing a hampering of the generation of prototypic DC due to an *in vitro* exposure of lithium.

Figure 5. *In Vitro* Effect of LiCl on Autologous MLR.



The *in vitro* lithium chloride (LiCl)-induced effects on autologous mixed lymphocyte reaction (MLR) of monocyte-derived dendritic cells (DC) of bipolar patients and healthy control subjects (HC). MLR (see also Figure 3) represents the capacity of monocyte-derived DC to stimulate T cells after *in vitro* LiCl exposure measured by their proliferation in counts per minute (cpm). **Circles** represent condition without LiCl, **squares** represent condition with 1 mM LiCl, and **triangles** represent condition with 10 mM LiCl. Data are expressed as mean values ± *SEM*.
* $p<.05$ of the 10 mM versus the 0 mM LiCl condition using Wilcoxon signed-rank test

Discussion

Reported signs of an aroused immune system in BD patients comprise a rise in serum levels of acute phase proteins[9,25], a higher prevalence of thyroid, gastric, and islet antibodies[1,2], an enhanced activity and level of B cells[3], an activated T-cell system[3,23,24], and an altered cytokine production[22,26,27]. There are, however, also reports that refute such activation[28-30]. It is also not clear whether the immune aberrancies are linked to the mood state of the patients, genetically determined, or due to environmentally determined influences such as the use of lithium, which is known to be an immune modulating drug.

We report here that DC cultured from monocytes of nonLi-BD show a mild hampering in the differentiation into fully active DC: the cells showed an increased residual expression of the monocyte marker CD14 after one week culture in GM-CSF and IL-4 and had a relatively low capability to stimulate T cells in autologous MLR.

Lithium treatment of BD resulted in a complete restoration of this mildly hampered *in vitro* differentiation defect and, in fact, the treatment activated monocyte-derived DC: the cells possessed an enhanced capability to stimulate autologous T cells, had an up regulated expression of the DC-specific marker CD1a, and completely lost the weak residual expression of the monocyte marker CD14, the loss of which was correlated with the duration of lithium treatment.

With regard to the validity of our data, it must be noted that monocyte function is sensitive to age and gender[31] and these variables might, indeed, have affected the DC generation from monocytes. We tested for these variables, however, and were neither able to find male-female differences nor associations between age and the different characteristics of monocyte-derived DC. A clear limitation of our study is its naturalistic set up and, hence, the majority of patients used various psychiatric medications (Table 1). Although we found lithium to be a prominent determinant in the DC generation, we could not find an effect of the other medications in this sample. Literature suggests a potential immune-modulating role for such other non-lithium medications[32]; however, the variability of these medications in our patient group was enormous, and numbers of patients per therapeutic group small. This might have influenced power to detect correlations.

Can we relate the findings on a mild DC differentiation defect in nonLi-BD reported here to the previously reported tendency of the patients to develop islet, thyroid, and gastric autoimmunity?

Similar mild differentiation defects (i.e., a 20-30% reduced CD1a expression and a 50-70% reduced T-cell stimulatory capacity) have been

found in patients with recent onset type I diabetes and their first-degree relatives[33]. It is also known, from studies in animal models of type I diabetes and thyroiditis (i.e., studies in the NOD mouse[34] and in the BB-DP rat[35]), that mild inborn defects in the differentiation and function of DC lead to autoimmune reactivity. Restoration of such defective DC function leads to a prevention of autoimmune disease[36]. This has been taken as evidence that an optimal function of DC is not only required for an appropriate defense against foreign invaders but also for an avoidance of autoreactivity by an appropriate stimulation of T-regulatory cells[35]. Lithium treatment did restore the mildly defective DC differentiation and autologous T-cell stimulation in BD patients. Hence, it might be worthwhile to study the autoimmune preventive aspects of lithium treatment.

One can only speculate whether the here-reported data on monocyte-derived DC are of relevance for the psychiatric phenotype. There exists a theory that the macrophage system is intrinsically activated in patients with mood disorders. The corner stone of this theory is the assumption of an intrinsically enhanced production of pro-inflammatory cytokines by activated macrophages in these patients and that such enhanced production is thought to lead to a "sickness behavior", reminiscent of many mood symptoms known of depressed patients[37]. It might be that the here-described mild defect in the differentiation of DC from monocytes of nonLi-BD is due to a preferential tendency of the monocytes to develop into active macrophage-like cells. A similar imbalance, a defective DC development in favor of the macrophages, has been found in the NOD mouse model for autoimmune thyroiditis and insulitis[38]. Interestingly, NOD mice also show behavioral and hormonal abnormalities, such as a high locomotor activity in open field tests and an intrinsically high corticosterone response to repeated constraint stress, which have been taken as evidence for an altered emotional behavior of the mice[39,40].

With regard to the previously posed question of whether the here-described DC aberrancies might be of relevance for the psychiatric phenotype, it is also worth noting that DC and brain microglia are strongly related[41,42]. It has been reported that parenchymal microglia are noncommitted myeloid progenitors and that the cells can be skewed towards a DC-like profile in response to the lineage growth factor GM-CSF[43-45]. Are the defective differentiation profiles of DC from circulating monocytes found here perhaps reflected in an abnormal differentiation and function of microglia in bipolar brains? There is little information on microglia/DC/macrophages in postmortem brains of BD patients. Bayer *et al*[46] reported on an activation of frontal cortex and hippocampal microglia in one of six patients with mood disorders, yet Hamidi *et al*[47] were not able to confirm this observation and found a reduction in glial cells

(particularly of oligodendrocytes) in amygdala of BD patients not treated with lithium. Further research is indicated to study a possible relationship between monocyte-derived DC and microglia in BD patients.

We not only report here on the *in vivo* effects, but also on the *in vitro* effects of lithium on the differentiation of DC from monocytes. To our surprise, we found suppressive effects on DC generation of *in vitro* exposure to lithium, in contrast to the previously described stimulating effects on DC generation of lithium therapy *in vivo*. Lithium chloride (10 mM) *in vitro* clearly reduced the capacity of monocytes to differentiate into DC with a high potency to stimulate autologous T cells. It also resulted in a reduced differentiation of DC with a high expression of characteristic markers: the generated cells expressed lower levels of DC-SIGN and CD1a and higher levels of the residual monocyte marker CD14. This *in vitro* suppressive effect of lithium on DC differentiation can perhaps be explained by the previously found predominant production of Th2 cytokines (like IL-10 and IL-4) by cultured monocytes and lymphocytes when exposed to lithium during culture[48].

To our knowledge, there is only one report that –like we do- also compares the *in vivo* treatment effects with the *in vitro* effects of lithium on the function of immune cells in the same study[49]. Discrepant results were found in that report also: whereas lithium *in vitro* lacked any effects on the number of cytokine secreting cells, lithium treatment of patients reduced such numbers. These and our results might be explained by the difference in concentration and duration of lithium to which the immune cells are exposed in the *in vivo* and *in vitro* situation. The *in vivo* stimulatory effects found here are perhaps due to the fact that the leukocytes are exposed much longer to lithium in treatment conditions (months to years) than in the artificial *in vitro* situation (days). With regard to differences in concentration, it must be noted that we only clearly detected *in vitro* effects of LiCl with a 10 mM concentration; when we used *in vitro* 1 mM LiCl (high therapeutic serum level), only minimal effects on DC differentiation were found, although indirect effects of lithium might also play a role. Whereas the only variable *in vitro* was the exposure to lithium, *in vivo* lithium treatment must have had all sorts of effects on neuronal and other systems that, in turn, might have influenced the immune cells and their generation.

In conclusion, we found a mildly impaired differentiation of monocytes into fully active DC in BD patients. Lithium treatment resulted in a complete restoration of the impaired DC differentiation, and activated DC were obtained. In contrast, lithium *in vitro* had a suppressive effect on the generation of prototypic and fully active DC from monocytes.

Acknowledgements

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Chapter 5
Steroid Induced Effects

Chapter 5.1

A Relative Resistance of T cells to Dexamethasone in Bipolar Disorder

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Abstract

Background

A relative resistance of immune cells to steroids has been established in patients with major depression. In this study we investigated the *in vitro* responsiveness of T cells to dexamethasone (DEX) of patients with bipolar disorder.

Methods

T cells of outpatients with DSM-IV bipolar disorder (BD: n=54) and of healthy control subjects (HC: n=29) were isolated, cultured, and stimulated with phytohaemagglutinin (PHA) for 72 hours. The suppressive effect of graded concentrations of DEX ($5x10^{-9}$-10^{-5}M) on PHA-induced CD25 (IL-2R) expression was measured by fluorescence-activated cell sorting (FACS) analysis. Data were correlated to the T-cell activation status in the peripheral blood of the same patients and to their diagnosis, current mood state, ultradian cycling pattern, and current use of medication, including lithium.

Results

T cells of patients with BD were less sensitive to DEX induced suppressive effects as compared with T cells of HC. These data were particularly evident at 10^{-7}M DEX (mean % suppression ± *SEM* BD: 18.9% ± *3.5* versus HC: 35.8% ± *4.7*, p=.001). We found no correlations of this relative *in vitro* DEX resistance of T cells neither with the previously mentioned clinical characteristics nor with the actual activation status of the T cells in the BD patients.

Conclusion

A relative T-cell resistance to steroids, as has been observed in major depression previously, may be a trait phenomenon of BD, independent of mood state.

Introduction

Bipolar disorder (BD) is a severe and chronic mood disorder with a complex and an unraveled pathophysiology[1]. Several studies have reported on an activated immune system in BD (i.e., an activated T-cell system)[2-4], raised numbers of B-cells[2], an activated monocyte/macrophage system[5-11], increased serum levels of acute phase proteins[12,13], and a higher prevalence of thyroid, gastric, and islet auto-antibodies[14-17]. The outcomes of these studies have led to the hypothesis that immune activation forms part of the etiopathogenetic mechanism leading to BD. In addition, an activation of the immune system has been found in major depression (MD), another severe mood disorder with also depressive episodes but, unlike BD, without (hypo-)manic episodes[18-20]. However, it must be noted that not all reports describe an immune activation in BD and MD[21-24].

Apart from an activated immune system, there is also clear evidence for a disturbed activity of the hypothalamus-pituitary-adrenal (HPA)-axis, both in BD[25-27] and in MD[28-30]. First, cortisol levels are increased in both disorders[28,30,31], which interestingly is not accompanied by obvious signs of Cushing's Syndrome[32]. Second, the dexamethasone (DEX) suppression test[29] and the DEX/corticotrophin releasing hormone (CRH) test[27,33] are disturbed, indicating a relative steroid resistance. These findings led to the hypothesis that an impaired glucocorticoid (GC) receptor signaling may be one of the key factors in the pathogenesis of mood disorders[34]. It must, however, be noted that in other psychiatric conditions such as schizophrenia, this phenomenon of GC resistance is also present, yet less pronounced[35].

Glucocorticoids (GCs) not only play an important role in the stress response[36], but are also known for their inhibitory effects on the immune system[37-39]. Hence, it is a short step to the hypothesis that a relative steroid resistance is responsible for the immune activation in patients with mood disorders. The first publication showing an *in vitro* steroid resistance of immune cells in mood disorders was of Lowy *et al*[40]. These authors established that immune cells of nonsuppressors in the DEX-suppression test were also resistant to DEX in a mitogen-induced lymphocyte proliferation assay. Bauer *et al*[41] confirmed these data later specifically for the MD group. Patients with MD also showed less effect of orally administered DEX compared to HC on peripheral immune cell redistribution, suggesting an *in vivo* resistance to at least some effects of steroids[42]. In accordance with the view that steroid resistance is linked to immune activation, plasma cortisol and soluble serum interleukin-2 receptor (sIL-2R), a T-cell activation marker, were positively correlated

to each other in patients with MD[43]. Steroid resistance has also been found on the level of lymphocytes and macrophages in a mouse model for mood disorders[44]. Taken together, these findings point to a relative steroid resistance of immune cells in MD. However, such data are lacking for immune cells in BD.

We therefore decided to investigate to what extent T cells of patients with BD are sensitive to GCs. The phytohaemagglutinin (PHA) activated T-cell assay was used as a read-out system. T cells are easy to access and possess GR[45,46] resulting in an exquisite sensitivity of the cells to GC both *in vivo* and *in vitro*[47]. The PHA-activated T-cell assay is therefore a well-established model system to test for steroid resistance[48]. We measured as a sign of T-cell activation the PHA induced increase of CD25 (i.e., the IL-2R) on T cells. IL-2 is the growth factor for T cells and both IL-2 and its receptor are up regulated on activated T cells. We investigated whether the suppressive effect of graded concentrations of DEX on PHA-induced CD25 expression differed between T cells of bipolar patients (BD, n=54) and those of healthy control subjects (HC, n=29). We also questioned whether these *in vitro* DEX response data could be correlated to our previously described activated T-cell status in the peripheral blood of the same patients[2] and to their clinical characteristics.

Materials and Methods

Patients and Healthy Control Subjects

Patients: Heparinized blood was obtained from outpatients with DSM-IV bipolar I and II disorder (n=54) participating in the Stanley Foundation Bipolar Network (SFBN), a multi-center research program described elsewhere in detail[49,50]. All patients were recruited from the SFBN-site in The Netherlands for the here-described experiments. The Institutional Review Board of the University Medical Center Utrecht had approved the study protocol and written informed consent was obtained from all patients.

A DSM-IV[51] diagnosis of BD was made by means of the Structured Clinical Interview for DSM-IV Axis I (SCID)[52]. A detailed illness history, including past and present medication, was assessed at entry into the Network. Blood for the present study was drawn during one of the monthly follow-up visits, when a research clinician also assessed the current mood state (i.e., euthymic, depressed, (hypo)manic, or ultradian cycling) during the past week. This overall assessment of mood was based on a detailed interview[49], including the Young Mania Rating Scale[53],

the Inventory of Depressive Symptoms[54], the Global Clinical Impressions Scale – Bipolar Version[55], and the Life Chart Methodology[56].

Euthymia was defined as the absence of mood symptoms or functional impairment caused by mood symptoms for at least one week as indicated by an YMRS score of <8 and an IDS score of <14. The longitudinal

Table 1. Clinical and Demographic Characteristics.

		Bipolar Patients		Healthy Control Subjects	
Group size		54		29	
Age (years)[1]	Male	32	(59.3%)	18	(62.1%)
	Female	22	(40.7%)	11	(37.9%)
Gender		45	(21-60)	39	(22-54)
Duration of illness (years)[1]		22.2	(2-42)		
Duration of treatment (years)[1]		14.8	(1-36)		
Psychotropic medication	Carbamazepin	10	(18.5%)		
	Valproate	20	(37.0%)		
	Thyrax	14	(25.9%)		
	Antidepressives	13	(24.1%)		
	Antipsychotics	7	(13.0%)		
	Benzodiazepins	15	(27.8%)		
	Lithium	49	(90.7%)		
	Li titer in serum (mM)[2]	.77 ± *.03*			
	Cumulative period of Li treatment (months)[1]	75	(6-292)		
	No current Lithium	5	(9.3%)		
	Period without Li (months)[1]	44.6	(6-118)		
Current mood state	Euthymic	29	(53.7%)		
	Depressed	15	(27.8%)		
	Manic	9	(16.7%)		
	Cycling	1	(1.9%)		
Diagnosis (DSM-IV)	Bipolar I disorder	39	(72.2%)		
	Bipolar II disorder	15	(27.8%)		
Rapid cyclers (>4 episodes/year)		16	(29.6%)		

[1] mean (range)
[2] mean ± *SD*

methodology of the SFBN with daily self-ratings of mood and monthly follow-ups of each patient, allows a reliable association of the immune-parameter-outcomes with mood states and illness course. During the visit when the blood was drawn, patients had no serious medical somatic illness or fever. Clinical characteristics of the patients are shown in Table 1.

Blood was drawn in sodium-heparin tubes (Becton and Dickinson, Franklin Lakes, New Jersey, USA) in the morning and transported by courier to the laboratory in Rotterdam, lasting an acceptable maximum of 8-10 hours before further processing.

Healthy Control Subjects: Blood collections were obtained from healthy laboratory and hospital staff members of the Erasmus MC (The Netherlands; *n*=29) on the same days as those of the patients, with the same procedures. All HC gave written information about medication use and medical history. Informed consent was obtained. The exclusion criteria for this HC group were: any immune disorder, serious medical illness, recent infections, fever, any life-time major psychiatric disorder (including psychosis, mood disorders, anxiety disorders, or substance abuse disorders) or usage of any psychotropic or other medication (apart from anti-conceptive hormonal therapy).

T-cell Cultures

Peripheral blood mononuclear cells (PBMC) (i.e., the lymphocytes and monocytes) were prepared from the heparinized blood via Ficoll (Pharmacia, Uppsala, Sweden density 1.077 g/ml) gradient centrifugation. First, PBS (Bio Whittaker Europe, Verviers, Belgium) containing .1% BSA (Bayer, Kankakee, USA) (PBS/.1% BSA) was added to the heparinized blood sample in a dilution of 1:1. These diluted cells were distributed on the Ficoll gradient and centrifuged for 15 min at 1000 g. After centrifugation the PBMC were collected from the interface and washed with PBS/.1% BSA (centrifugation for 10 min at 760g).

Peripheral blood mononuclear cells were thereafter re-suspended in RPMI 1640 with 25 mM Hepes and L-glutamine (medium liquid, named after Roswell Park Memorial Institute, Bio Whittaker Europe, Verviers, Belgium), additionally containing 10% inactive fetal calf serum

(Bio Whittaker Europe, Verviers, Belgium; FCSi), penicillin (100 U/ml)/streptomycin (100 µg/ml; Bio Whittaker Europe, Verviers, Belgium; P/S), and extra ultraglutamine (2 mM; Bio Whittaker Europe, Verviers, Belgium; UG) (RPMI+). Samples were frozen in 10% dimethylsulfoxide and stored in liquid nitrogen. This enabled us to test T cells of BD and HC in the same series of experiments at appropriate times.

The frozen and stored PBMC suspension was quickly thawed and

washed twice in RPMI+. Viability of the population was at least 85%, using trypan blue staining. Cells were thereafter incubated with anti-CD3 microbeads (20 µl/10^7 cells; Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 minutes at 4°C (according to the manufacturer's protocols) and a magnetic cell sorting system (autoMACS; Miltenyi Biotec) was used for the selection of CD3-positive T cells.

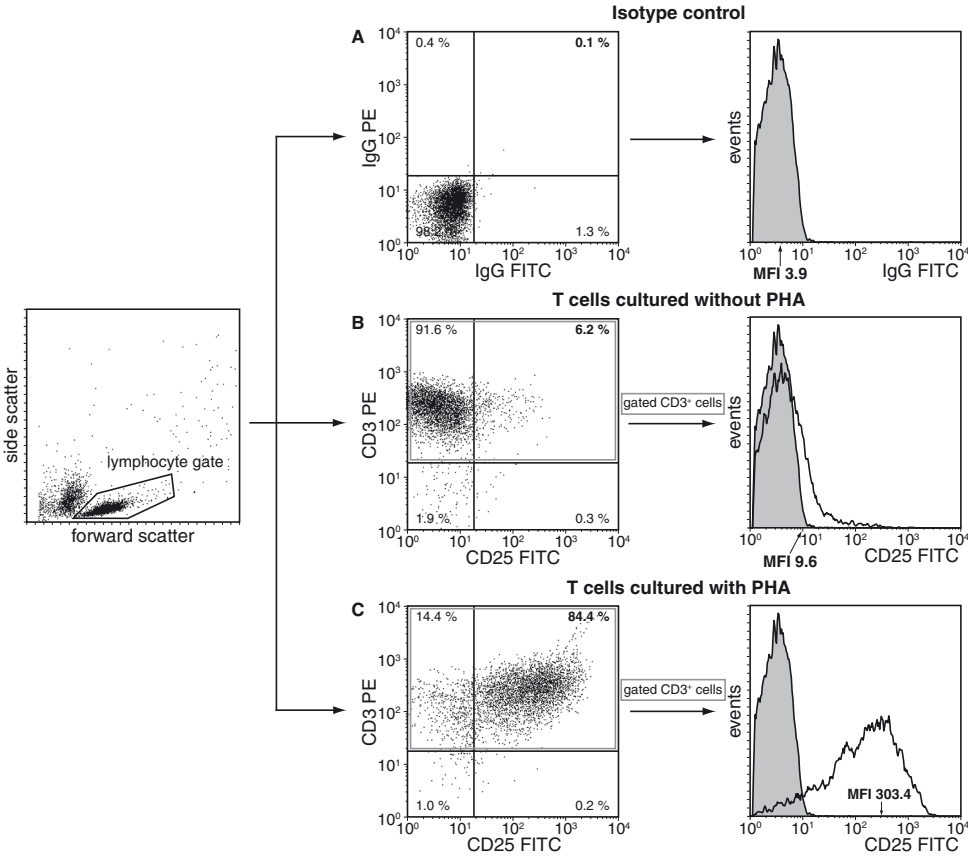
The CD3-positive T-cell preparations were washed with PBS/0.1% BSA and contained at least 85% viable cells, measured by trypan blue staining. T cells were resuspended in RPMI+ ($1.5x10^6$ cells/ml), supplemented with A+ serum (Bloodbank, The Hague, The Netherlands) and distributed over a 96-wells-plate (NUNC, Roskilde, Denmark) with or without PHA (final concentration; 100 µg/ml), a polyclonal mitogen-stimulating T-cell proliferation *in vitro*. To assess *in vitro* steroid sensitivity, graded concentrations of DEX (final concentrations; $5x10^{-9}$-10^{-5}M; 9α-fluoro-16α-methylprednisolone; SIGMA) were added to the mitogen-driven lymphocyte cultures. Each well ended with an equal volume of 200 µl and was incubated for three days (72h) at 37°C, 5% CO_2, and 95% humidity.

Fluorescence-activated Cell Sorting Analysis of T cells

Lymphocyte populations were incubated for 10 minutes in the dark at room temperature with mouse anti-human fluorescein (fluorescein isothiocyanate, FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs; Becton Dickinson (B&D), San Jose, CA, USA), and washed afterwards. The following mAbs were used: anti-IgG1 FITC, anti-IgG1 PE (both used as isotype control), anti-CD25 FITC (i.e., IL-2R α–chain, a marker for activated T cells), and anti-CD3 PE (i.e., a molecule associated with T-cell antigen receptor, a general T-cell marker).

Immediately after staining, cells were subjected to fluorescence-activated cell sorting FACS) on a FACS calibur (B&D). The researcher was blind for the identity (BD/HC) of the sample during the FACS measurement and analysis of the data. Debris and dead cells were gated out in the samples on the basis of their light scatter properties. We used FACS instrument settings to correct for auto-fluorescence and as a negative control we determined the fluorescence intensity IgG1-FITC or IgG1-PE, correcting for possible aspecific binding of these mAbs. The T-cell population was gated out by means of CD3 expression. For analysis of the T-cell activation we used CellQuestPro software (B&D Pharmingen, Alphen aan de Rijn, The Netherlands). We expressed the CD25 expression level of the CD3-positive T cells as percentages of cells staining positive for this marker or as mean fluorescence intensity (MFI) (for analysis

Figure 1. Fluorescence-activated Cell Sorting (FACS) Analysis of T cells.



Lymphocytes were gated out on the basis of forward (size) and side scatter (shape). Three stainings are shown in this figure, with two conditions: T cells cultured without PHA, which are stained with the isotype control IgG FITC/PE (a) or with CD25/CD3 (b) versus T cells cultured with PHA, which are stained with CD25/CD3 (c). The CD25 expression of the gated population is presented as both percentages of CD3/CD25 double positive cells (respectively .1% in Figure a, 6.2% in b, 84.4% in c) and histograms showing the mean fluorescence intensity (MFI) of the CD25 expression of the CD3-positive lymphocytes. For statistical analyses we used the MFI corrected for the aspecific binding of the corresponding isotype control (i.e., IgG FITC; gray histogram), which gives the following values: 5.7 (9.6 - 3.9) in the T-cell culture without PHA (b) and 299.5 (303.4 - 3.9) in the T-cell culture with PHA (c).

methods see Figure 1). In the case of DEX-suppression we used the MFI and expressed the DEX-induced effects in each assay relative to the condition without DEX. Thus, these data are expressed as "% suppression".

sIL-2R Determinations and T-cell Activation of Freshly Isolated Lymphocytes

The serum soluble IL-2 receptor levels (sIL-2R) and the number of circulating activated T cells (CD25-expressing $CD3^+$ T cells) in the periphery have been measured in the same cohort and described previously[2].

Statistical Analysis

Statistical analysis was performed with the SPSS 11.0 package for Windows (SPSS, Chicago, Illinois, USA). Data were tested for their parametric distribution with the Kolmogorov-Smirnov test. Because the majority of data showed a non-parametric distribution, we decided to use non-parametric tests. Statistical significance between groups was determined by a Mann-Whitney U test; if there were more than two groups, preceded by a Kruskal-Wallis test. To analyze the DEX-induced effects in the samples, we performed paired statistical analysis (e.g. no DEX versus 10^{-6}M DEX) with the Wilcoxon signed-rank test. Correlation coefficients of the T-cell activation with different clinical parameters were determined by the method of Spearman. All data were tested for two-tailed significance. $P<.05$ were considered to be statistically significant.

Results

CD25 Expression on Cultured T cells

We previously reported on an increased level of serum sIL-2R and on a slightly higher percentage of activated (i.e., CD25-expressing) T cells in the circulating lymphocyte fraction of patients with BD as compared with those values in HC[2]. In this sample we were able to confirm these data, both with respect to the serum sIL-2R concentrations (mean ± *SEM* BD: 512.6 IU/ml ± *25.7* versus HC: 365.7 IU/ml ± *18.7*, $p<.001$) and with respect to the percentage of CD25-expressing $CD3^+$ T cells (BD (no. available = 44): 1.5% ± *.1%* versus HC (no. available = 25): 1.1% ± *.1%*, $p<.001$).

After a 72-hours *in vitro* culture of the T-cell population, even in the

Table 2. CD25 Expression and DEX Sensitivity of T cells in Different Subgroups.

		CD25 expression after culture without addition		CD25 expression after PHA activation[1]		% suppression with 1e-7 M DEX	
		MFI ± *SEM*	*stats*	MFI ± *SEM*	*stats*	Mean(%) ± *SEM*	*stats*
HC (*n*=29)		14.3 ± *4.7*	ns[2]	318.7 ± *30.9*	ns[2]	35.8% ± *4.7*	***p*=.001**
BD (*n*=54)		14.2 ± *2.6*		349.3 ± *25.5*		18.9% ± *3.5*	
Current mood state	Euthymic (*n*=29)	13.0 ± *4.2*	ns[3]	357.9 ± *36.4*	ns[3]	19.6% ± *4.8*	ns[3]
	Symptomatic (*n*=25)	15.6 ± *2.7*		339.3 ± *36.0*		18.2% ± *5.3*	
	Depressed (*n*=15)	18.3 ± *4.0*	ns[4]	348.3 ± *57.4*	ns[4]	19.3% ± *6.4*	ns[4]
	Manic (*n*=9)	12.3 ± *3.9*		335.0 ± *32.7*		12.3% ± *9.8*	
Diagnosis	BD I (*n*=39)	15.7 ± *3.1*	ns[2]	359.7 ± *33.0*	ns[2]	20.5% ± *4.1*	ns[2]
	BD II (*n*=15)	8.9 ± *3.7*		322.4 ± *33.7*		14.9% ± *6.8*	
Ultradian cycling	RC (*n*=16)	7.6 ± *1.7*	ns[2]	296.5 ± *44.1*	ns[2]	23.9% ± *6.1*	*p*=.063
	No RC (*n*=38)	18.4 ± *3.7*		371.6 ± *30.8*		16.8% ± *4.3*	
Lithium treatment	Li-BD (*n*=49)	14.8 ± *2.9*	ns[2]	350.5 ± *27.4*	ns[2]	18.9% ± *3.7*	ns[2]
	NonLi-BD (*n*=5)	9.5 ± *6.6*		338.0 ± *73.1*		19.4% ± *11.1*	

In vitro CD25 expression (IL-2 receptor) on T cells after72 hours of culture with or without 100mg/ml of phytohaemagglutinin (PHA) expressed as mean fluorescence intensity (MFI) in samples of healthy control subjects (HC), bipolar patients (BD) and their different subgroups. The dexamethasone (DEX) responsiveness is expressed as percentage suppression of CD25 expression after addition of 10-7M DEX. All data are expressed as mean ± standard error of mean (SEM).

[1] In all cases PHA stimulated CD25 expression is significantly increased compared to spontaneous expression (Wilcoxon signed-rank test, $p<.001$)

[2] Mann-Whitney U test

[3] Mann-Whitney test (euthymic versus symptomatic group)

[4] Kruskal-Wallis test (overall comparison of the euthymic, the depressed and the manic group

BDI resp II, bipolar disorder subtype I and II; RC, rapid cycler; Li-BD, lithium user; NonLi-BD, bipolar patient without lithium treatment;ns, not statistically significant

absence of PHA, T cells became slightly activated and percentages of CD25-expressing T cells rose to 3.5% ± *2.5%* for HC and to 4.2% ± *3.3%* for BD in these non-PHA cultured samples (difference not statistically significant; ns). This observation also means that culturing of the T cells for 3 days led to the disappearance of the *ex vivo* difference between T cells of bipolar patients ("activated") in comparison to T cells of healthy controls.

PHA addition to the *in vitro* T-cell cultures resulted in a significant increase of CD25 expression on the T cells in all cases. Percentages of CD25-expressing T cells rose further to 56.6% ± *18.1%* for HC and to 51.2% ± *19.3%* for BD (ns). When data were expressed as mean fluorescence intensities (MFI), the rise of CD25 on T cells due to PHA stimulation was also clear, yet again without statistical significant differences between HC and BD (Table 2). We concluded from this set of experiments that the potential of T cells of BD patients to be activated by PHA was comparable to that of T cells of HC.

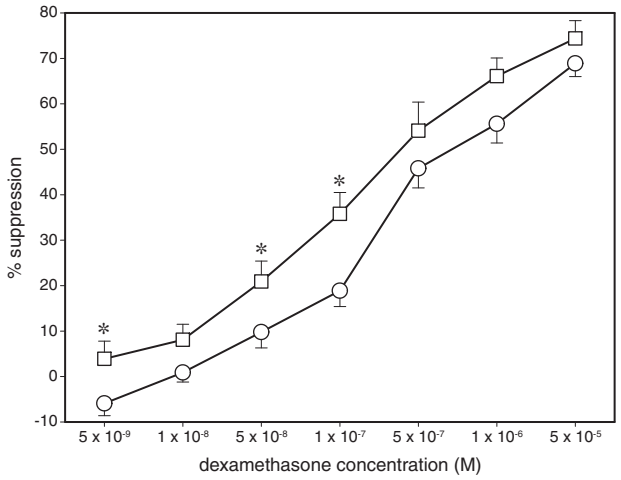
It is of note that the *in vitro* PHA-induced CD25 expression on T cells of BD patients correlated neither to the sIL-2R level in serum (r=-.109) nor to the CD25 expression on $CD3^+$ circulating T cells in freshly isolated blood (r=.033). We were also unable to find any difference with regard to the CD25 expression on *in vitro*-cultured T cells between various subgroups of BD patients. In particular, there was no effect detectable of a specific mood state, neither comparing the 4 different mood states nor comparing euthymic versus symptomatic patients (Table 2, first and second column). Clinical parameters such as duration of illness (r=-.115, p=.408) or duration of lithium treatment (r=.088, p=.554) also did not correlate with the CD25 expression on cultured T cells. The demographic parameters age (r=-.029, p=.798) and gender (mean ± *SEM* male: 328.6 ± *28.3* versus female: 349.4 ± *27.8*) did not have any effect on the outcome either, which was also the case in the HC group.

Effects of Dexamethasone Addition to Cultured T cells on CD25 Expression

Dexamethasone dose-dependently suppressed CD25 up regulation induced by PHA (Figure 2). The kinetics of the DEX induced immune suppressive effects (i.e., the shape of the curve and the maximum effect) were comparable to earlier studies using T cells of healthy control subjects to record effects of DEX[48].

In the BD group DEX induced less-pronounced suppressive effects as compared with the HC group (Figure 2). This resulted in a statistical significant smaller suppressive effect of DEX in the lower concentration

Figure 2. Dexamethasone Suppression Test with a PHA Activated T-cell Assay.



Dexamethasone (DEX) suppression test using T cells after 72 hours of culture with phytohaemagglutinin (PHA) and graded concentrations of DEX. Data are expressed as "% suppression", which means that the DEX-induced effects on CD25 expression in each assay are expressed relative to the CD25 expression in the condition without DEX. **Open squares** represent the healthy control samples (HC), the **open circles** represent that of the bipolar patients (BD), and data are expressed as mean ± standard error of mean (SEM).
* $p<.01$ Mann-Whitney U test

range ($5x10^{-9}$ through $1x10^{-7}$M, with the exception of the $1x10^{-8}$M concentration, reaching marginal significance of $p=.07$) in the BD group as compared with HC, indicating a relative resistance of T cells of BD patients to the immune suppressive effects of DEX.

We checked for possible associations between the relative DEX resistance of T cells of BD patients and various clinical parameters. We focused on the effects of 10^{-7}M DEX, because this is the DEX concentration with the clearest and most significant difference between the BD and the HC group (Figure 2). As shown in Table 2 none of the sub-groups of patients differed significantly in DEX responsiveness. With regard to age ($r=-.048$, $p=.668$), duration of illness ($r=-.123$, $p=.374$), or duration of lithium treatment ($r=.033$, $p=.822$) associations could not be found. The DEX induced effects on male (23.0 ± *4.1*) and female samples (26.7 ± *4.2*) were comparable. The same is true for the other DEX concentrations (data not shown). We also could not find any association between the percentage suppression at 10^{-7}M by DEX and the serum sIL-

2R level (r=-.226) in the patients or between this suppression and the CD25 expression on the circulating $CD3^+$ T cells in freshly isolated blood of the same samples (r=-.133).

Discussion

In this study we found – with an *in vitro* assay – that T cells of patients with BD are less sensitive to DEX induced suppressive effects as compared to T cells of HC. This DEX resistance of T cells of BD patients was only noted at the relative low *in vitro* concentration range of $5x10^{-9}$M to 10^{-7}M DEX, which indicates that T cells of BD patients are characterized by a relative, but not an absolute, DEX resistance, which disappears when high concentrations of DEX are used. It is of note that the relatively low concentrations of $5x10^{-9}$M to 10^{-7}M DEX are more physiologically relevant[57]. Previously, Modell *et al*[58] reported a shift of the DEX dose-response curve in depressed patients similar to the shift found by us, be it that these investigators studied DEX resistance *in vivo* comparing depressed patients with healthy control subjects in the DEX/CRH test using different DEX dosages (.75, 1.5, 3.0 mg).

In this study we also found that the relative resistance of T cells in BD patients was independent of the current mood state and several other clinical parameters, such as ultradian cycling pattern or duration of illness, suggesting that the DEX resistance may be a trait rather than a state phenomenon. This in *vitro* finding is also in line with earlier *in vivo* studies concerning a state-independency of a GC resistance of the HPA-axis in BD patients[27].

With regard to possible confounding factors, like age and gender, we did not find associations with the outcomes of the relative DEX resistance of the T cells. This is in accord with previous findings on a lack of association between age or gender and steroid resistance[31,59-61]. Despite this, various other confounding factors and limitations have to be taken into account regarding our current study.

First, the naturalistic set-up of this study caused a considerable diversity of currently used psychotropic medication, and groups of separate specified treatments were too small to evaluate their effects on the relative DEX resistance of the T cells. Despite this, we did analyze the possible effects of lithium treatment on the DEX resistance of T cells of bipolar patients, because this drug is in particular known for its immune modulating effect[62,63]. There are data on the *in vivo* effects of lithium treatment on the deregulation of the HPA-axis. Two studies found that lithium treatment resulted in a normalization of the abnormal

DEX responsiveness of patients with a mood disorder[41,64]. It must be noted, however, that one of these authors[61] reported in an earlier study that sufficient lithium prophylaxis did not automatically prevent the deregulation of the HPA axis. Bschor *et al*[65] even reported that the overstimulation of the HPA axis in depressed patients worsened after lithium augmentation independent from the clinical responsiveness of lithium. Taken together the clinical *in vivo* studies are thus not consistent on either a positive or negative effect of lithium treatment on the deregulation of the HPA axis and it must be noted that the greater part of these surveys do not study single treatment effects, which makes it hard to draw firm conclusions. In the here-reported study, we were unable to find an effect of lithium treatment on the DEX responsiveness of T cells, but –as is stated before- the sub-group of non-lithium treated BD patients is too small (n=5) to reach a definite conclusion and more than one medication was used in the patients. We also did not study effects of lithium medication on DEX responsiveness of T cells in healthy control subjects, further highlighting the notion that our findings on a lack of effect of lithium on the DEX responsiveness of T cells should be taken with caution.

A second important limitation of our study is that plasma cortisol levels of the tested subjects were not available, nor did we perform DEX/CRH-tests. Consequently, associations between *in vivo* and *in vitro* effects of DEX could not be calculated. Some previous studies indicated a positive correlation between *in vivo* and *in vitro* DEX effects[57,60], whereas others disputed such a link[66,67].

Previous reports on DEX induced effects on immune parameters in mood disorders solely focused on MD and/or therapy resistant depressed patients. The majority of these studies, which dealt with the *in vitro* effects of DEX, used mitogen (PHA)-induced lymphocyte proliferation assays. Already in 1988, DEX has been shown to decrease the proliferation of lymphocytes also in psychiatric patients, but with a relative resistance to DEX in the MD group when relatively low concentrations of the compound were used[57]. Later studies found comparable effects, both in MD patients[41,68] and in healthy subjects suffering from chronic stress[69]. None of the studies used CD25 expression as their readout system. One study checked the production of IL-2 in the supernatant of the lymphocyte cultures, which is the ligand for CD25. In accord with the previous studies DEX decreased the IL-2 production, but less pronounced in patients with MD compared to HC, again suggesting relative steroid resistance[41]. Regarding the *in vivo* effects of DEX on the immune system after administering the drug orally, DEX decreased the lymphocyte number and changed the expression of adhesion molecules

more prominently in healthy control subjects as compared with MD patients[42,66]. Taken together, there is thus ample evidence that immune cells of major depressed patients are relatively resistant to DEX. Our data show that T cells of patients with BD share this resistance with the immune cells of patients with MD.

What can be the mechanism underlying this relative DEX resistance in MD and BD? First, polymorphisms of the glucocorticoid receptor (GR) gene are able to cause a relative GC resistance. Several GR gene variants and single nucleotide polymorphisms (SNPs) have been coupled to GC responsiveness in the general population[70,71] but also specifically to patients with cortisol resistance[72]. Suggestive genetic associations related to the GR have been postulated in patients with mood disorders[73,74]. However, mutations in or polymorphisms of the glucocorticoid receptor alpha and beta isoforms could not be detected in PBMC of BD patients[75].

Second, stressful events or exogenous administration of DEX during fetal and early life are able to influence the structure and physiology of the brain and the stress axis in animal models[76,77] resulting in an altered set point of the adult HPA axis and an altered GR expression on adult cells. With regard to lymphocytes of MD patients, a decrease of the number of receptors was positively correlated with the inhibitory effect on mitogen-induced lymphocyte proliferation[78]. More is known about the GR and MR density in various brain areas of patients with mood disorders. Although a review states that there is only little evidence that such density is decreased in patients with MD[79], more recent papers did show a decrease in the receptor expression for both BD and MD[59,80,81].

Perturbations in the pro-inflammatory cytokine milieu, might also be involved in the relative resistance of bipolar T cells to DEX. Cytokines, such as interleukin-1β (IL-1β), IL-6, and TNFα sort their effects via various intracellular routes, which counteract the transcriptional pathways or antagonize the immune suppressive effects of GCs upon immune cells[82-84]. Because Moutsatsou *et al*[75] were unable to find GR mutations/polymorphisms in PBMC of BD patients, these authors (and others) suggested that the altered responsiveness of immune cells to GCs are caused by, for example, the raised level of pro-inflammatory cytokines in mood disorders[20].

Taken together, it thus remains unclear from these literature data whether the alterations in the GC system of bipolar patients (also found here) are simply a result of a "chronic stress environment" or are closely related to inborn neurobiological underpinnings of BD. In this context it is important to know, whether or not the here-found DEX resistance remains detectable in cell lines prepared from the T cells of the bipolar patients. B lymphoblastic cell lines of, in particular, major depressed patients

show an increase of cytosolic glucocorticoid receptors and a enhanced decrease of such receptors in response to DEX when compared to the values found in B lymphoblastic cell lines of healthy controls even after 12 weeks of culture of the cells[85]. This suggests trait-like characteristics of the abnormal DEX response in immune cells of patients with affective disorders. Similar trait-like characteristics have been found regarding altered beta-adrenergic receptor sensitivity, altered G protein-mediated cAMP signaling and altered Ca^{2+} homeostasis in B lymphoblastic cell lines of bipolar I patients[86,87]. These observations indeed urge for further studies in T lymphoblastic cellines of bipolar patients.

Our study also shows that the higher activation status of the circulating T cells in BD patients over that of circulating T cells in HC[2] does not remain after culturing the cells for 3 days. The expression of CD25 on T cells of HC and BD patients rose due to the culture conditions, but became mutually equal. In accord with this observation, Bauer *et al*[41] found the *in vitro* IL-2 production in supernatants of lymphocytes of HC and MD patients to be equal. Hence the activation of T cells found in freshly isolated lymphocyte preparations of BD patients is most likely due to a factor operative in the patients *in vivo* (perhaps the pro-inflammatory cytokine milieu eluded to earlier). Interestingly, a positive correlation between the serum level of sIL-2R and the plasma cortisol level has been found in MD[43].

In conclusion, this study found a relative glucocorticoid resistance in T cells of patients with bipolar disorder, as has been observed previously in major depression. This relative glucocorticoid resistance of T cell may be a trait phenomenon of BD since it was found independent of mood state and other clinical characteristics of the patients.

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Chapter 6
Auto-antibodies

Chapter 6.1

A High Prevalence of Organ-specific Autoimmunity in Patients with Bipolar Disorder

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Abstract

Background

In a previous study, we reported an increased prevalence of thyroperoxidase antibodies (TPO-Abs) in patients with bipolar disorder. Here, we report the prevalence of other organ-specific auto-antibodies: H^+/K^+ adenosine triphosphatase antibodies (H^+/K^+ ATPase-Abs), glutamic acid decarboxylase-65 (GAD65-Abs) and GAD-67 (GAD67-Abs).

Methods

H^+/K^+ ATPase-Abs, GAD65-Abs and GAD67-Abs were determined (via a commercially available enzyme linked immunosorbent assay for H^+/K^+ ATPase-Abs, and standardized radio immuno-assays for GAD65- and GAD67-Abs) in the sera of 239 patients with DSM-IV bipolar disorder, in 74 patients with DSM-IV schizophrenia and in 220 healthy control subjects.

Results

The positivity prevalences for H^+/K^+ ATPase-Abs and GAD65-Abs (but not that of GAD67-Abs) were raised in bipolar patients compared with those in healthy control subjects (11.7% vs. 6.1% and 11.3% vs. 2.6% respectively; $p<.05$). Schizophrenia patients did not show such statistically higher prevalence. The elevated prevalence of H^+/K^+ ATPase-Abs and GAD65-Abs in bipolar disorder was associated with neither rapid cycling nor the use of lithium. Interestingly, the presence of GAD65-Abs (and not that of TPO-Abs and H^+/K^+ ATPase-Abs) tended to be associated with the activity of bipolar disorder. The level of TPO-Abs was negatively correlated with the serum level of sIL-2R, a measure of T-cell activation.

Conclusion

Bipolar disorder is associated with organ-specific autoimmunity to the antigens TPO, H^+/K^+ ATPase and GAD65.

Introduction

Bipolar disorder is a severe, recurrent illness with a complex genetic, biological, and psychosocial etiology. There is increasing evidence that the immune system, in close interaction with the central nervous system and the endocrine system, plays a role in its pathophysiology. There is ample evidence to suggest that acute episodes of bipolar disorder (BD) are accompanied by an acute phase response and an activation of cell-mediated immune reactions, including an activation of the T cell system as evidenced by elevated levels of soluble IL-2 receptor (sIL-2R) in the serum of the patients[1-7]. An increased prevalence of thyroid auto-antibodies, that is of thyroid peroxidase auto-antibodies (TPO-Abs), in BD has been shown as well, and this higher prevalence of thyroid antibodies was associated with a higher prevalence of clinically overt thyroid failure (hypothyroidism) due to autoimmune thyroiditis[8].

Autoimmune thyroiditis represents an organ-specific or endocrine autoimmune disease. In organ-specific autoimmunity, more than one organ is often affected. The most frequent co-occurrences are between autoimmune thyroiditis and type I diabetes and between autoimmune thyroiditis and autoimmune atrophic gastritis[9,10]. These syndromes are referred to as autoimmune polyendocrine syndrome (APS) type 3a and 3b, respectively.

Autoimmune atrophic gastritis is characterized by an increased prevalence of antibodies to gastric parietal cells[11]. These auto-antibodies recognize gastric H^+/K^+ adenosine triphosphatase (H^+/K^+ ATPase), a highly specialized proton pump located in the unique intracellular membranes of gastric parietal cell membranes. The presence of these H^+/K^+ ATPase auto-antibodies (H^+/K^+ ATPase-Abs) provides a convenient diagnostic probe for chronic autoimmune (type A) atrophic gastritis[11].

Type I diabetes has several serological markers. The most frequently used markers are auto-antibodies against the 65-kDalton (65kD) isoform of glutamic acid decarboxylase (GAD65-Abs), which are positive in approximately 80% of recent-onset cases[12]. Two forms of GAD occur in mammalian tissues: a 65kD form and a 67kD form. GAD65 and GAD67 are enzymes that catalyze the reaction of L-glutamate to γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter in the central nervous system. GAD65 and GAD67 are both highly expressed in the brain, but the two forms appear to be differentially expressed in pancreatic islets. In human islets, GAD65 is abundant, but GAD67 can barely be detected (or cannot be detected at all) using various detection methods[13-16].

Because of the high prevalence of autoimmune thyroiditis in bipolar patients and the known association of autoimmune thyroiditis with

autoimmune atrophic gastritis, type I diabetes, or both, we investigated the presence of auto-antibodies to H^+/K^+ ATPase and GAD65 in the sera of a group of well-characterized bipolar patients (n=239). The same blood samples had been used in previous studies conducted by our group on the prevalence of TPO-Abs and the level of sIL-2R in bipolar patients[1,8].

As control subjects, we used a group of well-characterized schizophrenia patients (n=74) and a group of healthy individuals (n=220). Apart from auto-antibodies to H^+/K^+ ATPase and GAD65, we also determined antibodies to gastric parietal cells using the classical indirect immunofluorescence (IIF) assay and antibodies to GAD67 as a possible sign of autoimmunity against "brain" GAD.

We report a higher prevalence of auto-antibodies to H^+/K^+ ATPase and to GAD65 (but not to GAD67) in BD compared with healthy control subjects. The schizophrenia patients did not show statistically higher prevalences of these organ-specific auto-antibodies.

Materials and Methods

Patients and Healthy Control Subjects

Patients: The subjects were 239 outpatients with bipolar I, II or not otherwise specified (NOS) disorder from the Stanley Foundation Bipolar Network (SFBN), a multi-center longitudinal treatment research program performed in the United States and The Netherlands described in detail elsewhere[17,18]. A DSM-IV[19] diagnosis of bipolar disorder was made by means of the Structured Clinical Interview for DSM-IV Axis I, Patient Edition (SCID-I/P) Research Version[20]. A detailed illness history, including age of onset, previous illness course, medical history, and past and present medication, was assessed at entry into the Network. Blood for the present study was drawn during one of the monthly follow-up visits, when a research clinician also determined the present mood by means of a detailed interview including the Young Mania Rating Scale[21], the Inventory of Depressive Symptoms[22] and the Clinical Global Impressions Scale – Bipolar Version[23]. Based on the retrospective and prospective Life Chart Methodology[24] patients were defined as rapid cyclers (DSM-IV: four or more episodes of mania/hypomania, depression, or both in the previous year) or nonrapid cyclers.

Subjects were 122 (51%) women and 117 (49%) men, mean age 45 (range 23-83) years. At the time of blood collection, 124 (52%) were euthymic, 84 (35%) depressed, and 31 (13%) manic or hypomanic, all meeting syndromal DSM-IV criteria; 103 (43%) had a rapid-cycling

course. Medication use was as follows: 84% used or had used lithium, 43% used or had used carbamazepine, 70% used or had used valproate, 42% used or had used antidepressants, and 19% used or had used antipsychotics.

Healthy Control Subjects: Healthy control subjects were recruited from three groups of healthy individuals (the staff of the Altrecht Institute for Mental Health Care, Utrecht; Department of immunology, Erasmus MC, Rotterdam, The Netherlands; Department of Medicine, University of Washington Medical Center, Seattle, USA), representing 129 Dutch and 91 U.S. control subjects. All 220 healthy subjects gave written information about medication use and medical history. All were free of any psychiatric or medical illness and none used any psychotropic or other medication (apart from anticonceptive hormonal therapy). Control subjects were 145 (66%) women and 75 (34%) men, mean age 32 (range 18-59) years. The racial composition of both the patient group and the control group was comparable, being mainly of Caucasian origin.

A second control group consisted of 74 inpatients and outpatients with a DSM-IV diagnosis of schizophrenia[19], made by means of the Structured Clinical Interview for DSM-IV Axis I, Patient Edition (SCID-I/P) Research Vision[20]. A research clinician assessed current and past medication use at the time of blood sampling. Subjects were 16 (22%) women and 58 (78%) men, mean age 41 (range 19-61) years.

Blood was drawn and serum collected, which was stored immediately at –20 °C. All samples were sent frozen to the Department of Immunology of the Erasmus MC and stored there, enabling us to test patient and control material in the same series of experiments at appropriate times. The average duration of storage before testing was the same for the patient and control groups (i.e., about 3 to 4 years). It is our experience that titers of auto-antibodies do not change during such storage time when sera are not thawed and frozen several times during storage, which was not the case in this study. Blood samples had been used before in previous studies by our group[1,8].

The Institutional Review Boards of the participating centers approved the study protocol. All subjects gave informed consent.

Laboratory Methods

The H^+/K^+ ATPase antibodies in serum were determined by a specific enzyme-linked immunosorbent assay (ELISA; QUANTA Lite^{tm} GPA ELISA, INOVA Diagnostics, San Diego, USA). According to the manufacturer recommendations, a prediluted negative, a low positive, and a high positive control (supplied by manufacturer) were aliquoted in duplicate

in the wells of a preincubated 96-well ELISA plate. Patient sera were diluted to a measurable solution with horseradish peroxidase sample diluent (1:101) and afterwards added to the plate as well. The remaining part of the assay was performed according to the manufacturer's manual. Absorbance at 450-620 nm was measured with a microplate reader Multiskan RC (Thermo Life Sciences, Breda, The Netherlands). The H^+/K^+ ATPase-Abs level in the unknown samples was calculated according to the manual: ([Sample OD]/[OD of the H^+/K^+ ATPase-Abs low positive control]) x 25 units. Using the control values, the concentrations of the samples were calculated based on their optical density values. The inter-assay variation was less than 10%. Our internal control was comparable with the manufacturer's controls and stable over all the plates we used.

Antibodies to gastric parietal cells were determined via indirect immunofluorescence (IIF) according to standard routine diagnostic procedures in use at the Department of Immunology at the Erasmus MC. Briefly, serum was incubated at 1:10 dilution on cryostat sections from rat stomach (IMMCO diagnostics, New York, USA) followed by incubation with a second antibody (i.e., fluorescein isothiocyanate-conjugated sheep anti-human immunoglobulin; De Beer Medicals, Diessen, The Netherlands). In each assay, positive and negative serum controls were included. These positive and negative sera in the IIF assay were also used in the previously described ELISA for H^+/K^+ ATPase-Abs. All sections were evaluated by at least two technicians. In case of positive fluorescence, serum was screened for mitochondrial antibodies using rat kidney sections (IMMCO diagnostics, New York, USA). When positive for mitochondrial antibodies, sera were considered dubious and were not included in the analysis.

The glutamate decarboxylase-65 antibodies (GAD65-Abs) and glutamate decarboxylase-67 antibodies (GAD67-Abs) were detected by radioimmunoassay (RIA) as described by Grubin *et al*[25]. Recombinant 35S-GAD65 and GAD67 were made in an *in vitro* transcription and translation reaction (Promega, Madison, WI, USA) described elsewhere[26,27]. Samples were analyzed in triplicate. Antibody levels were expressed as a relative index to correct for inter-assay variation using the World Health Organization standard for GAD65-Abs and GAD67-Abs as positive control subjects[28]. To determine the relative index, positive and negative controls were included in all assays[25]. The threshold for positivity for both GAD antibodies was taken at the 97th percentile of the GAD index levels of the 220 healthy control subjects.

The TPO-Abs levels had been measured previously[8] with ELISA (Milenia assay, DPC, Breda, The Netherlands) according to the manufacturer's

recommended protocol. A level of 10 units/ml or higher was considered as positive for the presence of TPO-Abs.

The sIL-2R levels had been measured previously[1] by automatic Immulite chemiluminescent enzyme immunometric assay (DPC) according to manufacturer instructions.

Statistical Analysis

Data were analyzed using the SPSS statistical package for Windows (SPSS, Chicago, Illinois, USA). Logistic regression and analysis of covariance (ANCOVA) were used to determine differences between groups, corrected for age and gender.

To investigate the difference in GAD65-Abs and GAD67-Abs index between control subjects and bipolar patients, a log transformation was performed on the data to obtain Gaussian distribution. Correlations and associations between the various auto-antibodies were examined using Spearman's rho test. All tests were tested for two-tailed significance and a *p* value below .05 was considered to be statistically significant.

Results

Antibodies to H^+/K^+ ATPase

The bipolar patients had a higher prevalence of antibodies to H^+/K^+ ATPase compared with the healthy control subjects (11.7% versus 6.1%, $p=.049$, corrected for age and gender, Table 1). The percentage of rapid cyclers as well as medication use was similar in H^+/K^+ ATPase-Abs positive and negative bipolar patients (Table 2). There were also no relationships between H^+/K^+ ATPase-Abs positivity and duration of illness, present mood, or subtype of BD (Table 2).

The schizophrenia patients, our comparison group, had a prevalence of H^+/K^+ ATPase-Abs of only 6.8%, a value that was similar to that of the healthy control subjects ($p=.454$ versus healthy control subjects, Table 1).

Apart from the H^+/K^+ ATPase-Abs measured in ELISA, we also tested for antibodies to gastric parietal cells using the classical and original IIF assay. Using this assay, differences between bipolar patients and healthy control subjects could not be found (we therefore did not test the schizophrenia patients with the IFF assay). We consider this discrepancy between the two assays to be the result of the poorer sensitivity and specificity of the IIF assay compared with the newer H^+/K^+ ATPase-Abs

Table 1. Prevalence of H^+/K^+ ATPase-, GAD65- and GAD67-antibodies in Healthy Control, Bipolar and Schizophrenia Subjects and the Mean GAD65-Abs and GAD67-Abs Index.

	HC (*n*=220)	**BD** (*n*=239)	*p* value vs. HC	**SCH** (*n*=74)	*p* value vs. HC
H^+/K^+ ATPase-Abs positivity[1] (%)	6.10%	11.70%	.049	6.80%	.454
GAD65-Abs positivity[1] (%)	2.60%	11.30%	.002	4.10%	.488
GAD65-Abs index[2]	.018 ± *.035*	.025 ± *.019*	.038	.014 ± *.071*	.702
GAD65-Abs index[3]	.702 ± *.016*	.706 ± *.009*	.016	.699 ± *.031*	.566
GAD67-Abs positivity[4] (%)	3.00%	6.80%	.159	9.50%	.166
GAD67-Abs index[5]	-.032 ± *.167*	-.006 ± *.400*	.158	-.005 ± *.389*	.295
GAD67-Abs index[6]	.673 ± *.078*	.678 ± *.137*	.247	.677 ± *.149*	.466

[1] Logistic regression, adjusted for age, gender, and GAD67-Abs index
[2] Mean ± *SD*; ANCOVA on index value, adjusted for age, gender, and GAD67-Abs index
[3] Mean log-transformed ± *SD*; ANCOVA on log index, adjusted for age, gender, and GAD67-Abs log index
[4] Logistic regression, adjusted for age, gender, and GAD65-Abs index
[5] Mean ± *SD*; ANCOVA on index value, adjusted for age, gender, and GAD65-Abs index
[6] Mean log-transformed ± *SD*; ANCOVA on log-index, adjusted for age, gender, and GAD65-Abs log-index

ANCOVA, analysis of covariance; H^+/K^+ ATPase-Abs, H^+/K^+ adenosine triphosphatase antibodies; GAD65-Abs, glutamic acid decarboxylase-65-antibodies; GAD67-Abs, glutamic acid decarboxylase-67-antibodies; vs., versus; HC, healthy control subject; BD, bipolar disorder; SCH, schizophrenia.

ELISA. Reading of the IIF requires subjective interpretation and is subject to variability in the quality and reproducibility of the tissue sections used to manufacture the slides (indeed, the negative standard used for IIF gave weak positive results in the H^+/K^+ ATPase-Abs ELISA).

Antibodies to GAD65 and GAD67

The prevalence of GAD65-Abs positivity (defined as a level higher than the 97th percentile of levels found in the healthy control subjects) was significantly higher in the group of bipolar patients compared with the control subjects. A prevalence of 11.3% GAD65-Abs positivity was found in bipolar patients compared with the 2.6% positivity in the control subjects (p=.002, corrected for age and gender, Table 1). This was also reflected in the calculated mean GAD65-Abs index that was significantly higher in bipolar patients compared with the control subjects. Both the normal mean value and the log transformed mean value (to obtain a

Table 2. Characteristics of Bipolar Patients Positive and Negative for, respectively, H^+/K^+ ATPase Antibodies (H^+/K^+ATPase-Abs) and GAD65 Antibodies (GAD65-Abs).

	H^+/K^+ ATPase-Abs positive		H^+/K^+ ATPase-Abs negative			GAD65-Abs positive		GAD65-Abs negative		
Characteristics	*n*[1]	%	*n*[2]	%	*p* value[3]	*n*[1]	%	*n*[2]	%	*p* value[3]
Rapid cycling	13/25	52%	78/193	40%	.581	12/27	44%	80/191	42%	.520
Medication use										
lithium	22/28	79%	181/212	85%	.999	22/27	82%	181/212	85%	.830
carbamazepine	10/27	37%	95/211	45%	.861	11/27	41%	93/210	44%	.660
valproate	23/28	82%	144/212	68%	.502	18/27	67%	148/212	70%	.920
antidepressives	13/28	46%	83/204	41%	.364	14/26	54%	82/206	40%	.750
antipsychotics	6/28	21%	39/204	19%	.851	4/26	15%	41/206	20%	.230
Years since first medication	12.04 ± *9.37* (*n*=25)		14.80 ± *9.93* (*n*=193)		.702	13.65 ± *8.37* (*n*=23)		14.51 ± *10.06* (*n*=194)		.878
Years since first symptoms	24.35 ± *14.08* (*n*=26)		22.98 ± *12.06* (*n*=201)		.482	25.54 ± *13.00* (*n*=24)		22.96 ± *12.19* (*n*=194)		.538
Present mood										
euthymic	12/24	50%	98/188	52%	.225	11/26	42%	100/186	54%	.018
depressive + manic	12/24	50%	90/188	48%	.741	15/26	58%	86/186	46%	.065
depressive	9/24	37%	65/188	35%	.109	10/26	39%	63/186	34%	.147
manic	3/24	13%	25/188	13%	.111	5/26	19%	23/186	12%	.062
Bipolar subtypes										
Bipolar I	19/28	68%	169/210	81%	.575	20/26	77%	167/211	79%	.930
Bipolar II	9/28	32%	34/210	16%	.947	6/26	23%	37/211	18%	.918
Bipolar NOS	0/28	0%	7/210	3%	.938	0/26	0%	7/211	3%	.922
BMI	28.55 ± *6.31* (*n*=26)		27.56 ± *6.25* (*n*=197)		.455	27.91 ± *6.66* (*n*=24)		27.64 ± *6.23* (*n*=198)		.152
sIL-2R expression[4]	366.14 ± *117.27* (*n*=14)		429.09 ± *203.04* (*n*=131)		.156	422.42 ± *128.79* (*n*=12)		421.81 ± *203.17* (*n*=132)		.850

Data are represented as number, percentages, or mean ± *SD*

[1] Number of bipolar patients with indicated characteristic present per number H^+/K^+ ATPase-Abs positive bipolar patients (or respectively per number GAD65-Abs-positive bipolar patients)

[2] Number of bipolar patients with indicated characteristic present per number H^+/K^+ ATPase-Abs negative bipolar patients (or respectively per number GAD65-Abs-negative bipolar patients)

[3] Logistic regression adjusted for age and gender

[4] Determined in sera, IU/ml

n, numbers; H^+/K^+ ATPase-Abs, H^+/K^+ adenosine triphosphatase antibodies; GAD65-Abs, glutamic acid decarboxylase-65-antibodies; BMI, body mass index weight/(length)^2; sIL-2R, soluble interleukin-2 receptor.

Gaussian distribution) were significantly different, also after adjustments for age and gender (p=.038 and p=.016 respectively; Table 1).

In contrast to the prevalence of GAD65-Abs positivity, that of GAD67-Abs positivity was not significantly higher in the group of bipolar patients and a prevalence value of 6.8% versus 3% (p=.159) was found (Table 1).

The data in Table 2 show that there was no correlation between GAD65-Abs positivity and rapid cycling or medication use in the bipolar group or between GAD65-Abs positivity and years since first symptoms or duration of treatment. In the GAD65-Abs positive and negative groups, body mass index (BMI) did also not differ. In line with this observation there was also no correlation between BMI and GAD65-Abs positivity (r=.01, p=.881). Interestingly, bipolar patients with GAD65-Abs tended to have more active forms of the illness (either mania/hypomania or depression) compared with the GAD65-Abs negative bipolar patients. Table 2 shows symptomatic BD in 58% of GAD65-Abs positive patients and 46% in GAD65-Abs negative patients, although this difference did not reach statistical significance (adjusted for age and gender, p=.065). When we compared GAD65-Abs positivity in patients with symptomatic BD with values found in euthymic patients, this trend was also visible: values of 15% versus 9.9% (data not shown) were found respectively, but again this difference was not statistically significant (adjusted for age and gender p=.085).

With regard to our comparison group, the schizophrenia patients showed a normal prevalence and index value of GAD65-Abs, which was comparable to that of the control group (Table 1). The prevalence of GAD67-Abs positivity was relatively high in the schizophrenia group, that is 9.5%, yet the difference with the control group was not statistically significant (Table 1).

Comparisons of Antibodies to H^+/K^+ ATPase and GAD65/GAD67 to Antibodies to TPO and to Serum Levels of Soluble IL-2 Receptor (sIL-2R)

In a previous study, we reported on a higher prevalence of antibodies to TPO and autoimmune hypothyroidism in the same cohort of bipolar patients as studied here[8]. We also reported on a higher level of sIL-2R in the patients of this bipolar cohort and took this as a sign of T-cell activation[1].

In this study, we correlated the positivity for antibodies to H^+/K^+ ATPase and to GAD65 with each other and to positivity for antibodies to TPO in our test groups. We were unable to find any significant correlations.

We also studied the relationship between the serum levels of sIL-2R and the three organ-specific auto-antibodies. Tables 2 and 3 show that the levels of sIL-2R did not correlate with the level of antibodies to GAD65 or

Table 3. Correlations of Auto-antibodies with sIL-2R.

	sIL-2R[1]
H^+/K^+ ATPase-Abs	*r*=-.103
	(*p*=.130)
GAD65-Abs	*r*=-.104
	(*p*=.127)
GAD67-Abs	*r*=-.087
	(*p*=.202)
TPO-Abs	*r*=-.286
	(*p*<.001)

[1] Spearman's rho test
H^+/K^+ ATPase-Abs, H^+/K^+ adenosine triphosphatase antibodies; GAD65-Abs, glutamic acid decarboxylase-65-antibodies; GAD67-Abs, glutamic acid decarboxylase-67-antibodies; TPO-Abs, thyroperoxidase antibodies; sIL-2R, soluble IL-2 receptor.

with the level of antibodies to H^+/K^+ ATPase. Tables 3, however, also shows that the levels of sIL-2R did significantly and negatively correlate to the titer of TPO-Abs (interestingly, for all three auto-antibodies, correlation coefficients were negative). Also, TPO-Abs positive patients had a lower sIL-2R level compared with TPO-Abs negative patients (i.e., 336 versus 435 pg/ml [mean]), but this did not reach statistical significance.

Discussion

In this cross-sectional study, we found that bipolar patients not only have a higher prevalence of antibodies to TPO as reported previously[8], but also a higher prevalence of antibodies to H^+/K^+ ATPase and GAD65 compared with healthy control subjects. These higher prevalences of H^+/K^+ ATPase-Abs and GAD65-Abs were not found in our comparison group of schizophrenia patients (this also applied for a normal 5% prevalence of TPO-Abs in the schizophrenia patients; unpublished results). The prevalence of GAD67-Abs was not increased in bipolar patients.

The tendency to develop organ-specific autoimmunity did not correlate with a rapid-cycling course of BD or the use of medications. Although it is known that lithium in particular has immune-modulating effects[29], we did not find a correlation between past or present lithium exposure and TPO- Abs[8], H^+/K^+ ATPase-Abs, or GAD65-Abs. Likewise, Vestegaard and Schou[30] found that lithium did not induce diabetes mellitus. To the best

of our knowledge, there are no data on the prevalence of autoimmune atrophic gastritis in lithium-treated subjects.

Although our sample of bipolar patients is one of the largest studied, the sample size still limits the power of statistical analysis. Another limitation of the study is that the healthy control group and the comparison schizophrenia group are not entirely comparable with the bipolar patient group because they differed slightly in the female:male ratio and in age. Gender and age do affect the prevalence of TPO-Abs and H^+/K^+ ATPase-Abs (higher in women at older age[11,31]), yet it is questionable whether this also applies for GAD65-Abs[32]. To rule out a possible confounding effect of age and gender, we adjusted for these variables in our statistical models. Hence, we are confident that the higher prevalence of TPO-Abs, H^+/K^+ ATPase-Abs and GAD65-Abs in BD reported here, is not the result of such confounding effects. In addition, the values found in this study for positivity of the organ-specific auto-antibodies in the healthy control group and the comparison schizophrenia group are in the range of values found in population-based control groups in the literature (i.e., around 4-7%)[33,34].

There are, however, putative confounding factors that we could not correct for because exact and reliable information was not available for a substantial proportion of our controls. One of these is the smoking behavior (we assume that almost all of our control subjects did not smoke, whereas most of the patients did). Smoking is known to activate the cell-mediated immune system, yet effects on the prevalence of organ-specific auto-antibodies range from unknown (H^+/K^+ ATPase-Abs, GAD65-Abs) to nonexistent, to protective (TPO-Abs)[31].

It is also of interest that the GAD65-Abs index has been reported to relate to the BMI; both positive[35] and negative correlations[36-39] have been found. In our patients, a correlation did not exist between the BMI and GAD65-Abs (see Table 2). A correlation of BMI to GAD65-Abs was not possible in our control group because we lacked reliable information on the weight of our healthy subjects. It must be noted in this regard that our patient group was slightly overweight (i.e., mean BMI of 28, normal 20-25).

The impact of mood state on the functioning of the immune system of bipolar patients is controversial. Hornig *et al*[2], Maes *et al*[4], and Rapaport *et al*[6] showed that the activation of the cell-mediated immune system and of the acute phase response did not normalize with patients' remission. These authors concluded that the immune arousal of patients with a mood disorder was a trait phenomenon; however, Tsai *et al*[7] showed a normalization of cell-mediated immune system activation and acute phase response in patients in remission and concluded that there is a state dependency of the immune activation.

We previously reported on the activation of the T cell system in BD and used as one of the parameters the sIL-2R levels. We showed higher levels of sIL-2R in the sera of depressed, manic/hypomanic and euthymic patients compared with healthy control subjects, yet the levels of sIL-2R in manic/hypomanic patients were the highest. We concluded that the cellular immune activation is both a trait and a state phenomenon of the disorder[1].

We show here some influence of the illness on positivity for GAD65-Abs, albeit a borderline statistically significant effect (p=.065). The trend detected was that there were more euthymic subjects among bipolar GAD65-Abs negative than bipolar GAD65-Abs positive patients. The latter had a higher prevalence of both depression and mania compared with euthymic patients. In our previous study on the prevalence of TPO-Abs in BD, we found no association with current mood[8].

Interestingly, we found negative correlations between the serum level of sIL-2R and the titer of the three organ-specific auto-antibodies, and this reached statistical significance for TPO-Abs. A possible explanation for the discrepancy might be found in the actual function of the raised sIL-2R in the serum of the bipolar patients. The function of this shed receptor, although indicating T cell activation, is to capture circulating IL-2. Raised levels of the shed receptor thus result in the actual down regulation of T cell activation, representing a negative feedback system[40]. This down regulation might also affect the activity of autoreactive B cells and the production of auto-antibodies.

GAD65-Abs is an important marker of type I diabetes. The risk to develop type I diabetes is, however, not only dependent on the positivity for GAD65-Abs, but is also, and in particular, influenced by the titer of the GAD65-Abs and the co-occurrence of other islet-related auto-antibodies such as antibodies to insulin, insulinoma antibody 2 (IA-2), or islet cell antibody (ICA)[12,41]. The prevalence of diabetes is elevated in bipolar patients[42,43]; however, most reports on the subject did not distinguish between type I and II diabetes. Regenold *et al*[44], limiting themselves to type II diabetes, showed a higher prevalence of diabetes mellitus in bipolar patients. We are not aware of any study that systematically investigated the prevalence of type I diabetes in bipolar patients or the co-occurrence of GAD65-Abs, ICA, IA-2, or insulin auto-antibodies. In the entire Stanley Foundation Bipolar Network cohort, 27 out of 967 patients (i.e., 2.8%) self-reported that they had diabetes mellitus, and 6 of those 27 were insulin dependent (R.W. Kupka, personal communication). Hence, at least 6 out 967 bipolar patients (i.e., .62%) had insulin-dependent diabetes, a figure that is slightly higher than the prevalence figures for type I diabetes in the literature for community-based population groups in the United Kingdom of the same age as our SFBN cohort

(i.e., .4%)[45]. Values range in such community-based population groups from .2% at 20 years of age to .8% at 80 years of age, however[45]. Moreover, prevalence also depends on geography (e.g., low in European Mediterranean countries, high in Nordic countries). We therefore cannot conclude that there is a putative-raised prevalence of type I diabetes in BD because we lack solid prevalence figures for type I diabetes in both the Stanley Foundation group and the matched healthy control group.

The same arguments can be used in a discussion of whether the raised prevalence of H^+/K^+ ATPase-Abs in BD is a sign of a higher risk for autoimmune atrophic gastritis because gastric auto-antibodies are characteristic of (type A) autoimmune gastritis[11]. Controlled epidemiological studies need to be performed to answer this question.

Because our findings suggest that BD is associated with organ-specific autoimmunity, the question of whether bipolar mood disorder is itself an autoimmune disease can be raised. GAD65 expression is not restricted to the pancreas, but is also present in nervous tissue. The specific GAD65-Abs in bipolar patients may thus be viewed as not directed against pancreatic tissue alone, but against nervous tissue as well. Would our observation of a raised prevalence of GAD65-Abs in bipolar patients mean that BD belongs to the spectrum of brain-specific autoimmune diseases, with GAD65-Abs in fact directed against GAD65-positive nervous tissue in the brain? It has been shown that the expression of GAD65 and GAD67 is reduced in the brains of bipolar patients[46-48], whereas auto-antibodies to brain structures have also been detected in mood disorders and schizophrenia[49-52]. GAD67-Abs were not elevated in our bipolar patients (this report), however, whereas GAD67 is expressed in nervous tissue. A high titer of GAD67-Abs (together with a high titer of GAD65-Abs) is a hallmark of an uncommon neurological autoimmune disease Stiff Man Syndrome, which is clinically associated with insulin-dependent diabetes mellitus[53].

Collectively, although the low prevalence of GAD67-Abs suggests that an autoimmune reaction to nervous tissue is not likely in BD, we cannot rule out with certainty that the disorder itself is an organ-specific autoimmune disease. More types of antibodies to other autoantigens of the brain need to be determined.

In conclusion, our data are compatible with the view that patients with bipolar disorder show various signs of an enhanced organ-specific autoimmune reaction to TPO, H^+/K^+ ATPase and GAD65. The positivity of TPO-Abs indeed led to a greater prevalence of autoimmune thyroid failure in our bipolar patients[8]. It remains unclear whether the higher prevalence of antibodies to H^+/K^+ ATPase and GAD65 are linked to a higher risk for developing autoimmune atrophic gastritis and type I diabetes.

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Chapter 6.2

Antinuclear Antibodies in Bipolar Disorder: a Cross-sectional Study and a Review of the Literature

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Abstract

Background

The association between mood disorders and organ specific autoimmune diseases has previously been studied in a large cohort of well-characterized bipolar patients of the Stanley Foundation Bipolar Network (SFBN). With regard to antibodies against thyroid peroxidase, glutamic acid decarboxylase subtype 65, and H^+/K^+-adenosine triphosphatase prevalences were found increased. The purpose of the present study was to determine the seroprevalence of antinuclear antibodies (ANA) in the SFBN cohort of bipolar patients.

Methods

Screening for ANA positivity was performed, using indirect immunofluorescence (IIF) with HEp2000-cells in 290 bipolar samples. Data were compared to 149 healthy control subjects and 74 schizophrenic patients. The fine specificity of ANA was determined in ANA positive samples focusing on antibodies against extractable nuclear antigens (ENA; such as SS-A/Ro and SS-B/La) and dsDNA (using standard techniques).

Results

The prevalence ANA was significantly increased in bipolar patients (8.6%) compared to healthy controls (3.4%) and also increased, although not significantly, compared to schizophrenic patients (2.7%). Regarding the specificity of these ANA, 3 bipolar patients had detectable serum levels of SS-A/Ro and/or SS-B/La Abs and 4 patients produced dsDNA-Abs. In the samples of the healthy control subjects and schizophrenic patients, ENA-Abs or dsDNA-Abs were lacking.

Conclusion

In conclusion, we found a significant (factor 2.5) increase of the prevalence of ANA in bipolar disorder. Together with our data on thyroid, pancreatic and gastric autoimmunity in bipolar disorder, it is suggestive that bipolar disorder is characterized by another set point of the immune system leading to vulnerability for autoimmune processes.

Introduction

The association between mood disorders and autoimmune diseases has frequently been studied within the research field of immuno-psychiatry[1-3]. The majority of these studies have focused on autoimmune thyroiditis. Various groups determined the prevalence of auto-antibodies (Abs) directed against thyroid antigens in patients with mood disorders. The state of the art is that autoAbs associated with autoimmune thyroiditis are more prevalent in patients with both unipolar[4-7] and bipolar disorder[8-12] compared with the prevalence found in the general population. It must be noted, however, that some consider thyroid autoimmunity to be caused by lithium, which is frequently used in the treatment of mood disorders[13,14].

We studied in a large cohort of well-characterized bipolar patients collected within the Stanley Foundation Bipolar Network (SFBN; a multi-center longitudinal research program), the prevalence of auto-antibodies against thyroid peroxidase (TPO-Abs; associated with autoimmune thyroiditis), glutamic acid decarboxylase subtype 65 (GAD65-Abs; associated with diabetes mellitus type I), and hydrogen-potassium adenosine triphosphatase (H^+/K^+ ATPase-Abs; associated with atrophic gastritis and pernicious anemia). TPO-Abs (28% versus 13% in a healthy population) and autoimmune thyroiditis (17% versus 1%) were highly prevalent in this cohort [12]. Moreover, the prevalence figures of autoAbs against GAD65 and H^+/K^+ ATPase were increased (11% versus 3%, and 12% versus 6%, respectively)[15].

Systemic autoimmune diseases like systemic lupus erythematosus (SLE)[16,17], rheumatoid arthritis[18], and Sjögren's syndrome[19,20], have been reported to possess a high comorbidity with psychiatric disorders. Severe mood disturbances co-occur in 40-70% of patients with these systemic autoimmune diseases[18,21,22]. Specifically for SLE, the association with psychiatric disorders is very tight because psychosis is one of the diagnostic criteria for this disease[16].

Antinuclear antibodies (ANA) are commonly found in the serum of patients with systemic autoimmune disorders. Various authors have addressed the question whether ANA are also present with a higher frequency in serum of patients with mood disorders[23-36]. The results and prevalence figures are rather variable in these studies and conclusions are thus difficult to draw. The variability is probably due to various factors, including the fact that studies were performed with a variety of techniques, with different criteria for ANA positivity, and with relatively small or heterogeneous populations.

The purpose of the present study was to determine the seroprevalence

of ANA in a well-characterized cohort of 290 bipolar patients collected within the SFBN. We compared the bipolar samples with healthy control subjects and schizophrenic patients. Subsequently, the fine specificity of ANA was determined in all the positive samples focusing on antibodies against extractable nuclear antigens (ENA) such as antibodies against SS-A/Ro and SS-B/La (Sjögren's Syndrome antigen A and B, also called Ro and La; associated with Sjögren's Syndrome) and double-stranded DNA (dsDNA; a hallmark of SLE), using well established and state of the art standard techniques.

Materials and Methods

Subjects

Bipolar Patients: Blood samples were obtained from 290 outpatients with bipolar I, II, or not otherwise specified (NOS) disorder participating in a multi-center longitudinal treatment research program, the SFBN. This study was performed in the United States and The Netherlands and is described elsewhere in detail[37,38]. A DSM-IV[39] diagnosis of bipolar disorder was made by means of the Structured Clinical Interview for DSM-IV Axis I (SCID)[40]. A detailed illness history, including age of onset, previous illness course, medical history and medication, was assessed at entry into the Network. Blood for the present study was drawn during one of the monthly follow-up visits, when a research clinician also assessed the current mood state, by means of a detailed interview including the Young Mania Rating Scale[41], the Inventory of Depressive Symptoms[42], and the Clinical Global Impressions Scale – Bipolar Version[43]. [40,44]

Subjects were 160 (55%) females and 130 (45%) males, mean age 45 years (range 22-83). At the time of blood collection, 165 (57%) were euthymic, 74 (26%) depressed, 27 (9%) manic/hypomanic, 17 (6%) cycling, and of 7 (2%) patients the mood state was unknown. Current treatment was as follows; 146 (50%) used lithium, 64 (22%) used carbamazepine, 118 (41%) used valproate, 111 (38%) used antidepressants, and 63 (22%) used antipsychotics. 236 (81%) Patients had used lithium in the past. The mean duration of illness was 21.8 years (range 1-67), however the main duration of treatment was 14.9 years (range 0-40).

Healthy Control Subjects: Blood was also collected from 149 healthy control subjects (i.e., the staff of the Altrecht Institute for Mental Health Care, Utrecht and of the Department of Immunology, Erasmus MC, Rotterdam, The Netherlands). Each one gave written information about

medication use and medical history and was free of any psychiatric or medical illness. Nobody used psychotropic medication. Control subjects were 99 (66%) females and 50 (34%) males, mean age 32 years (range 19-62). The racial composition of both the patient group and the control group was comparable, being mainly of Caucasian origin.

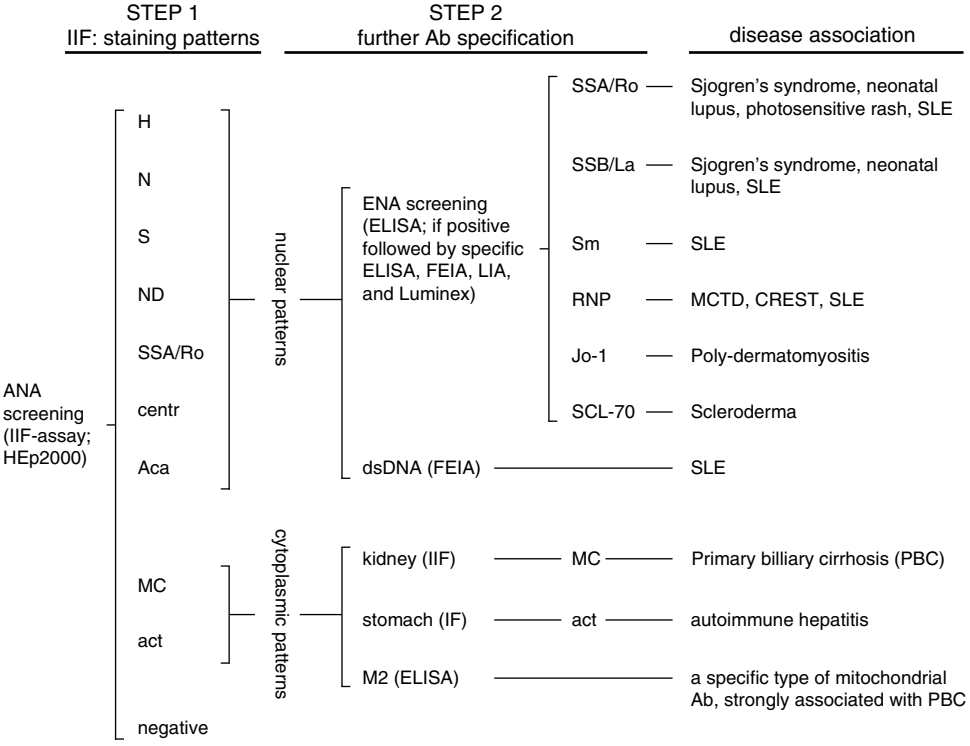
Schizophrenic Patients: A second control group, used as disease contrast group, consisted of 74 in- and outpatients with a DSM-IV diagnosis of schizophrenic[39], made by means of the SCID[40]. A research clinician assessed current and past medication use at the time of blood sampling. Subjects were 16 (22%) females and 58 (78%) males, mean age 41 years (range 19-61).

Blood was drawn and serum collected which was divided in aliquots and stored immediately at minus 20°C. All samples were sent frozen to the Department of Immunology of the Erasmus MC and finally stored there at minus 80°C, enabling us to test patient and control material in the same series of experiments at appropriate times. The average duration of storage before testing was the same for the patient and control groups, i.e. about 4 to 5 years. It is our experience that titers of auto-antibodies do not change during such storage time when sera are not repeatedly thawed and frozen during storage, which was not the case. Our group used aliquots of the blood and serum samples for previously published studies[12,15], see also below. The Institutional Review Boards (IRBs) of the participating centers approved the study protocol. All 513 subjects gave informed consent.

Antibody Assays

Antinuclear Antibody (ANA) Screening with Indirect Immunofluorescence (IIF) (STEP 1): Indirect immunofluorescence was performed to screen for antibodies against nuclear antigens (i.e., antinuclear antibodies or ANA), according to standard routine diagnostic procedures as used at the Department of Immunology (Diagnostic unit; Erasmus MC, Rotterdam, The Netherlands). HEp-2000 cells were used as substrate for the IIF assay (Immuno Concepts, Sacramento, CA, USA). Briefly, serum was incubated at 1:80 dilutions on glass slides with HEp-2000 cells and further diluted if necessary. Subsequently, incubation with a second antibody, i.e. fluorescein isothiocyanate-conjugated sheep anti-human immunoglobulin G, took place. In each assay, blancs, positive and negative serum controls were included. All slides were evaluated by at least two independent and experienced technicians. In all cases, the technicians were blinded for the diagnosis. Sera with titer >80 were considered as being positive samples. In case of positive fluorescence,

Figure 1. Test Algorithm of the Antinuclear Antibody Screening.



Test algorithm of this study; after the first ANA screening, all positive IF samples with a nuclear pattern were tested for specific antibodies, either one of the extractable nuclear antigen antibodies (ENA-Abs) or dsDNA-Abs. These specific antibodies are associated with various systemic autoimmune diseases, depicted in the last column.
ANA, antinuclear antibody; IIF, indirect immune fluorescence; HEp, human epithelial cell line; H, homogenous; N, nucleolar; S, speckled; ND, nuclear dots; centr, centriole; Aca, anti-centromere pattern; MC, mitochondrium; act, actin; Ab, antibody; ENA, extractable nuclear antigen, ELISA, enzyme linked immunosorbent assay; FEIA, fluorescent-enzyme immunoassay; LIA, line-blot immunoassay; dsDNA, double stranded DNA; M2, antibodies against a specific target within mitochondria; SS-A/Ro, Sjögren's Syndrome antigen A (also called Ro); SS-B/La, Sjögren's Syndrome antigen B (also called La); Sm, Smith; RNP, ribonuclear proteins; Jo-1, Histidyl-tRNA sythetase; SCL-70, scleroderma Abs directed against topoisomerase-1; SLE, systemic lupus erythematosus; CREST, autoimmune disease with symptoms of calcinosis, Raynaud's phenomenon, esophagus spasm, scleroderma, and teleangiectasy; MCTD, mixed connective tissue disease; PBC, primary billiary cirrhosis.

the staining pattern was also described, distinguishing the various nuclear patterns and cytoplasmic patterns (Figure 1). While ANA testing is an excellent screening test, it is by no means a test to determine the specificity of the ANA. Therefore we continued with further specification for antibodies against extractable nuclear antigens (ENA-Abs) or double stranded DNA (dsDNA-Abs).

The samples with a positive cytoplasmic pattern in HEp-2000 cells were further tested on rat kidney and stomach slides (IIF) and with a specific M2-ELISA (INOVA Diagnostics, San Diego, CA, USA) to confirm antibody specificity against mitochondria or actin (Figure 1).

ENA-Abs and dsDNA-Abs Specification (STEP 2): According to the test algorithm of this study (Figure 1), all positive IF samples with a nuclear pattern were tested with sensitive techniques for the presence of specific antinuclear antibodies, either one of the ENA-Abs (i.e., SS-A/Ro, SS-B/La, Smith [Sm], ribonuclear protein [RNP], Histidyl-tRNA sythetase [Jo-1], and scleroderma Abs [SCL-70]) or dsDNA-Abs.

In order to screen for ENA-Abs, enzyme-linked immunosorbent assays (Quanta Lite^{tm} ENA 6) were used according to the manufacturer's manual (INOVA Diagnostics, San Diego, CA, USA). If screening revealed positivity for one of the ENA-Abs, an Ab-specific ELISA (SCL-70 ELISA: Varelisa, Pharmacia Diagnostics AB, Freiburg Germany/other ENA ELISA's: INOVA Diagnostics, San Diego, CA, USA), a fluorescent-enzyme immunoassay (FEIA; ImmunoCAP-FEIA system, Pharmacia Diagnostics AB, Uppsala, Sweden), a line-blot immunoassay (LIA; INNO-LIA ANA update, Innogenetics, Gent, Belgium), and Luminex technology (FIDIS™ connective 10, BioMedical Diagnostics, Marne la Vallée, France), were performed to confirm this finding. Ultimately, a serum sample was labeled as positive for a specific ENA when at least two tests had a positive result.

Anti-dsDNA antibodies were determined with a FEIA (ImmunoCAP-FEIA system [Pharmacia Diagnostics AB, Uppsala, Sweden]). All assays were carried out in the diagnostic unit of the Department of Immunology, Erasmus MC Rotterdam, The Netherlands.

Antibody Assays against TPO and GAD65: In the majority of subjects used for the present study, endocrine auto-antibodies were determined previously. The presence of antibodies against TPO had been measured with an enzyme linked immunosorbent assay (ELISA; Milenia assay, DPC, Breda, The Netherlands) according to the method indicated by the manufacturer[12]. Second, Abs against H^+/K^+ ATPase and GAD65 were determined, respectively by an ELISA (QUANTA Lite^{tm} GPA ELISA, INOVA Diagnostics, Inc., San Diego USA) and by radioimmunoassay (RIA; FIRM[45]), as previously published[15].

Statistical Analysis

Statistical analysis was performed with the SPSS 11.0 package for Windows (SPSS, Chicago, Illinois, USA). All comparisons of categorized variables with 2x2 tables were tested for statistical significance using the Fisher exact test. We choose for this strict statistical approach, instead of Chi square tests, because figures in the 2x2 tables are often less than 5. Statistical significance of continuous variables between (sub-)groups was determined by Mann-Whitney U tests. All tests were tested for two-tailed significance and *p* values below .05 were considered to be statistically significant.

Results

Antibodies against Nuclear Antigens are increased in Bipolar Patients

As depicted in Table 1, 25 bipolar patients showed a positive nuclear IIF staining. The prevalence of such ANA positivity in bipolar disorder (8.6%) is statistically significant increased compared to the prevalence in the healthy control subject group (3.4%, p=.04). However, significance was not reached compared to the prevalence among schizophrenic patients (2.7%, p=.13), probably due to low numbers. The patterns of nuclear staining patterns were variable and not significantly different between patient groups and controls (Table 1).

Antibodies against Antigens in Cytoplasm in HEp2000-Cells are not increased in Bipolar Patients

Although in the IIF assays, the majority of positive samples showed a nuclear staining pattern, some samples showed Ab-reactivity to antigens within the cytoplasm, such as to actin (5) and to mitochondria (2; Table 1). However, numbers of positive samples are low and differences between patient groups and controls were not significant.

Further Typing of Antibodies against Specific Nuclear Antigens

The second step of our test algorithm (Figure 1) was to further identify the ANA specificity. We investigated antibodies against ENA and dsDNA in all the samples with a positive nuclear IIF staining. Of all the positive

Table 1. Results of STEP 1: ANA Screening.

	Bipolar Disorder (*n*=290)	**Healthy Control Subjects** (*n*=149)	*Stats HC vs. BD*[1]	**Schizophrenic Patients** (*n*=74)	*Stats SCH vs. BD*[1]
ANA screening: IIF assay					
1) IIF staining					
Positive	8.6% (25)	3.4% (5)	$p<.05$	2.7% (2)	ns
Negative	91.4% (265)	96.6% (144)	($\chi^2=4.29$)	97.3% (72)	($\chi^2=3.01$)
2) IIF patterns					
N	1.4% (4)	.7% (1)		1.4% (1)	
H	4.5% (13)	0		0	
Combination N/H	.3% (1)	0		0	
S	.3% (1)	.7% (1)		1.4% (1)	
ND	.7% (2)	1.3% (2)		0	
SS-A/Ro	.7% (2)	0		0	
centr	.3% (1)	.7% (1)		0	
Aca	.3% (1)	0		0	
Cytoplasmic staining					
MC	.7% (2)	0	ns	0	ns
Act	1.4% (4)	.7% (1)	ns	0	ns

[1] Statistics is performed by using Fisher exact test. The Pearson Chi-Square value (χ^2) is depicted between brackets after the *p* value

ANA, antinuclear antibodies; IIF, indirect immunofluorescence; N, nucleolar; H, homogenous; S, speckled; ND, nuclear dots; SS-A/Ro, Sjögren's Syndrome antigen A; centr, centriole; Aca, anti-centromere pattern; MC, mitochondrium; act, actin; Abs, antibodies; BD, bipolar disorder; HC, healthy control subjects; SCH, schizophrenic patients; ns, not statistically significant.

nuclear IIF serum samples tested in the bipolar group, two samples had Abs against SS-A/Ro, and one sample had Abs both against SS-A/Ro and SS-B/La (Table 2). Other ENA-Abs (i.e., Sm, RNP, Jo-1, and SCL-70) could not be detected. None of the healthy control samples or of the schizophrenic samples had Abs against one of the ENA. Regarding Abs against dsDNA, 4 bipolar samples were positive, although one of these samples had a low titer (13.7 IU/ml). This finding contrasted to the absence of dsDNA-Abs in the two other groups tested (i.e., healthy control subjects and schizophrenic patients). All in all numbers of positivity are too low to evaluate statistically.

Table 2. Results of STEP 2: Further ANA Specification.

	Bipolar Disorder (*n*=25)	**Healthy Control Subjects** (*n*=5)	*Stats HC vs. BD*[1]	**Schizophrenic Patients** (*n*=2)
Specific nuclear Abs				
Total of positive samples after typing	7	0	*p=.101 (χ^2=3.66)*	0
ENA-Abs	3			
SS-A/Ro	*2*			
SS-A/Ro & SS-B/La	*1*			
Sm	*0*			
RNP	*0*			
Jo-1	*0*			
SCL-70	*0*			
dsDNA-Abs	4	0		0

[1] Statistics is performed by using Fisher exact test. The Pearson Chi-Square value (χ^2) is depicted between brackets after the *p* value

ENA, extractable nuclear antigen; SS-A/Ro, Sjögren's Syndrome antigen A (also called Ro); SS-B/La, Sjögren's Syndrome antigen B (also called La); Sm, Smith; RNP, ribonuclear proteins; Jo-1, Histidyl-tRNA sythetase; SCL-70, scleroderma Abs directed against topoisomerase-1; dsDNA-Abs, antibodies against double stranded DNA; BD, bipolar disorder; HC, healthy control subjects; SCH, schizophrenic patients; ns, not statistically significant.

Association of ANA, ENA-Abs, and dsDNA-Abs with Demographic or Clinical Characteristics

Age neither influenced the presence of ANA, nor the presence of ENA-Abs or dsDNA-Abs. Yet, Abs against ENA or dsDNA are only present in 7 samples and these numbers are too low to draw valid statistical conclusions. The same applies to the cycling pattern, the duration of illness, and the duration of treatment (Table 3). Nevertheless, for some demographic and clinical features we found interesting trends, although none of them statistically significant. In the entire bipolar patient sample 55% were female patients, while in the patients with detectable ANA or Abs against ENA or dsDNA the percentage of female patients was increased (respectively 68% and 86%). In the patients with detectable ANA, ENA-Abs, or dsDNA-Abs, slightly more patients were manic or depressed (60% for ANA and 71% for ENA- and dsDNA-Abs) compared with the bipolar cohort as a whole (41%). Separating the patients in the

Table 3. Association of some Demographic and Clinical Characteristics.

	Entire Bipolar Cohort (*n*=290)	ANA Screening (STEP 1) Positive (*n*=25)	*Stats*	ENA- or dsDNA-Abs (STEP 2) Positive (*n*=7)	*Stats*
Age[1]	44.9 ± *10.9*	45.2 ± *10.8*	*ns*	50.7 ± *5.7*	*ns*
Female[2]	55% (160/290)	68% (17/25)	*ns* (χ^2=1.06)	86% (6/7)	*ns* (χ^2=1.50)
Euthymic mood state[2] *(7 missing values)*	57% (165/283)	40% (10/25)	*ns* (χ^2=2.452)	29% (2/7)	*ns* (χ^2=1.40)
Duration of illness (years)[1]	21.8 ± *12.2*	24.7 ± *10.8*	*ns*	24.6 ± *13.7*	*ns*
Treatment					
Duration of treatment (years)[1]	14.9 ± *9.6*	15.8 ± *9.1*	*ns*	16.0 ± *8.7*	*ns*
Duration of lithium treatment (months)[1]	77 ± *62*	98 ± *58*	*ns*	57 ± *14*	*ns*
Lithium (current)[2]	50% (146/290)	32% (8/25)	*ns* (χ^2=2.41)	71% (5/7)	*ns* (χ^2=.52)
Carbamazepine (current)[2]	22% (64/290)	28% (7/25)	*ns* (χ^2=.19)	29% (2/7)	*ns* (χ^2=.00)
Valproate (current)[2]	41% (118/290)	60% (15/25)	*ns* (χ^2=2.77)	29% (2/7)	*ns* (χ^2=.07)
AutoAbs status					
TPO-Abs positive[2] *(1 missing value)*	27% (77/289)	32% (8/25)	*ns* (χ^2=.00)	57% (4/7)	*ns* (χ^2=.09)
H^+/K^+ ATPase-Abs positive[2] *(4 missing values)*	11% (31/286)	20% (5/25)	*ns* (χ^2=1.10)	14% (1/7)	*ns* (χ^2=.11)
GAD65-Abs positive[2] *(7 missing values)*	10% (29/283)	4% (1/25)	*ns* (χ^2=.43)	14% (1/7)	*ns* (χ^2=.15)

Association of some demographic and clinical characteristics of the bipolar patients with:
1) Results of antinuclear antibody (ANA) screening (first step of test algorithm)
2) Results of further ANA specification (second step), either for antibodies against extractable nuclear antigens (ENA-Abs) or double stranded DNA (dsDNA-Abs)

[1] Statistics is performed by using Mann-Whitney U test. Data are expressed as mean ± *SD*

[2] Statistics is performed by using Fisher exact test. The Pearson Chi-Square value (χ^2) is depicted between brackets after the p value. Data are expressed as percentage of the different subgroups followed by the exact number between brackets

IIF, indirect immunofluorescence; ns, not statistically significant; Abs, antibodies; TPO, thyroid peroxidase; GAD65, glutamic acid decarboxylase subtype 65; H^+/K^+ ATPase, hydrogen-potassium adenosine triphosphatase.

four different mood states did not reveal extra information.

Because it is well known that different types of medication can affect the production of ANA, special attention was paid to the different types of treatment. Only 32% of the ANA-positive group used lithium compared with 50% in the entire bipolar cohort. However, in the group of patients with Abs against ENA or dsDNA, 5 out of 7 (71%) patients used lithium, which can not exclude a possible association between current lithium use and the production of specific ANA. The opposite is found with regard to valproate, which was used more frequently (60%) in the positive ANA group than in the total group (38%). However, few patients (29%) with Abs against ENA or dsDNA used valproate. No clear effects of carbamazepine or other treatment approaches could be found.

We also checked in our database for associations of ANA, ENA-Abs or dsDNA-Abs with the previous determined autoAbs against TPO, H^+/K^+ ATPase and GAD65. No associations were found (Table 3). However, concerning the samples positive for Abs against ENA or dsDNA, 4 out of 7 samples had also at least one organ-specific autoAb.

Discussion

In this study we found a significantly increased prevalence of antinuclear antibodies (ANA) in bipolar patients (8.6%) compared to healthy controls (3.4%) and also, although not significantly, to schizophrenic patients (2.7%). Regarding the specificity of these ANA for well-known nuclear antigens, 3 patients had detectable serum levels of Abs against SS-A/Ro and/or SS-B/La and four patients produced dsDNA-Abs. Although it is rather remarkable that 7 bipolar patients had specific Abs against nuclear targets, while healthy control subjects and schizophrenic patients did not, group sizes were too small to reach statistical significance with regard to Abs against ENA or dsDNA.

It must be noted that specific ANA against SS-A/Ro, SS-B/La and dsDNA are associated with systemic autoimmune diseases, respectively Sjögren's Syndrome and SLE. Questionnaires, obtained during inclusion of the study, of the 7 patients with specific ANA were viewed retrospectively, focusing on physical complaints. Although the information is rather sparse, none of them had clear indications for either SLE or Sjögren's syndrome. In two other studies among psychiatric patients with various diagnoses a similar approach was followed, but these studies also did not reveal any comorbid systemic autoimmune diseases[26,32].

As mentioned in the introduction, the current study is not the first on the seroprevalence of ANA in patients with mood disorders. An

Table 4. Survey on Studies investigating Prevalence of Antibodies directed against Nuclear Targets in Patients with Mood Disorders.

Reference	Patients	#	Ab-target	Technique	Prevalence
von Brauchitsch 1972	Unipolar depressive disorders	37	ANA[1]	Immunofluorescent tumor imprint technique	41% (UP)*[1]
	Manic depressive disorder (depr.)	9			22% (BD)*[1]
	Nondepressed psychiatric disorders	124			10% (PD)=
	General population	nd			3% (HC)
Shopsin *et al* 1973	Psychiatric patients	100	ANA[1]	FCT test	3% (PP)=
	From which 25 mood disorder				4% (MD)=
Gottfries and Gottfries 1974	Psychiatric patients	627	ANA[1]	IIF[1]	14% (PP)*[2]
	From which 19 mood disorder				16% (MD)*[2]
Johnstone and Whaley 1975	Psychiatric patients	100	ANA[2]	IIF[1]	25% (PP)*[2]
	From which 45 mood disorder				27% (MD)*[2]
	Osteoarthritis patients (as control)	112			14% (Co)
			dsDNA	ACP technique	0% (in all)
Deberdt *et al* 1976	Unipolar depressive disorders	26	ANA[1]	IIF (guinea pig hepatocyte)	12% (UP)*[1]
	Manic depressive disorder (depr.)	27			29% (BD)*[1]
Ghose *et al* 1977	Mood disorders	90	ANA[3]	nd	8% (MD)=
	General population	nd			9% (HC)
Plantey 1978	Unipolar depressive disorder	19	ANA[3]	IIF[1]	0% (UP)=
	Manic depressive disorder (depr.)	6			0% (BD)
Gastpar and Müller 1981	Mood disorders	100	ANA[3]	IIF (rat liver)	2% (MD)=
	From which 11 BD		basalmembrane	IIF (rat kidney)	1% (MD)
Myers *et al* 1985	Mood disorders	57	ANA[3]	nd	.02% (MD)=

Table 4. Survey on Studies investigating Prevalence of Antibodies directed against Nuclear Targets in Patients with Mood Disorders (continued).

Reference	Patients	#	Ab-target	Technique	Prevalence
Villemain *et al* 1988	Mood disorders	30	ANA[2]	IIF (mouse liver)	13% (MD)*[1]
	From which 11 BD and 19 UP				0% (HC)
	Healthy control subjects	20	ds/ssDNA	RIA	0% (in all)
			histone	ELISA	30% (MD)*[1]
					0% (HC)
			centromere	IIF (Hep2)	0% (in all)
			ENA-Abs	immunodiffusion	0% (in all)
Yannitsi *et al* 1990	Psychiatric patients	307	ANA[2]	IIF (HEp2)	40% (PP)*[2]
	From which 14 mood disorders				7% (HC)
	Healthy control subjects	150	SS-A/Ro, SS-B/La	immunodiffusion	<1% (in all)
			RNP, Sm	immunodiffusion	0% (in all)
			dsDNA	ELISA	<1% (in all)
Maes *et al* 1991	Unipolar depressive disorder	36	ANA[2]	IIF (HEp2)	72% (UP)*[1]
	Healthy control subjects	14			0% (HC)
			cardiolipin	ELISA	11% (UP)
					7% (HC)
De Vries *et al* 1994	Psychiatric patients	100	ANA[2]	IIF (human granulo's)	7% (PP) =
	From which 29 BD				4% (HC)
	Healthy control subjects	859	dsDNA	IIF (Crithidia luciliae)	0% (in all)
Hornig *et al* 1999	Bipolar disorder	103	ANA[2]	IIF (HEp2)	65% (BD)=
	Major depression	46			70% (UP)=
	Healthy control subjects	22			68% (HC)
			dsDNA	ELISA	No diff

Table 4. Survey on Studies investigating Prevalence of Antibodies directed against Nuclear Targets in Patients with Mood Disorders.

[1] *low titers (i.e., titer ≤ 80)/weak staining: excluded*
[2] *low titers (i.e., titer ≤ 80)/weak staining: included*
[3] *in study: nothing mentioned about borderline findings*
[4] *no substrate mentioned in methods section*
= prevalence not significantly different versus HC
**[1] prevalence of patients with mood disorder significantly increased versus HC*
**[2] prevalence of psychiatric patients significantly increased versus HC, but not specific for mood disorders*

#, number of patients in the various (sub)groups; Ab, antibody; ANA, antinuclear antibodies; ENA, extractable nuclear antigens; dsDNA, double stranded DNA; ss, single stranded; FCT test, fluorescent calf thymus test; IIF, indirect immunofluorescence technique (between brackets are the used substrates); HEp2, human epithelial cell line; granulo's, granulocytes; ACP technique, ammonium sulphate precipitation technique; ELISA, enzyme linked immunoassay; Immunodiff. Test, immunodiffusion screenings test; nd, not described in paper; UP, unipolar depressed patients; BD, bipolar patients; PD, nondepressed psychiatric patients; HC, healthy control subjects; PP, psychiatric patients; MD, patients with a mood disorder; Co, control group; depr., depressed mood.

overview of 14 studies investigating the prevalence of ANA in patients either with unipolar or bipolar disorder is summarized in Table 4. In these studies, different techniques for ANA detection were used. Although 9 out of 14 used IIF techniques, the antigenic substrates vary. Our current investigation is the first to use the HEp2000-cell line as an antigenic substrate, which is presently used in many of the immunodiagnostic centres. Table 4 shows that prevalences range from 0%-72%. Next to the variability in technique or the heterogeneity of subject groups, this could also be caused by the different criteria used as cut off for (ANA) positivity, like including or excluding non-specific (borderline) findings. Therefore, a general conclusion cannot be drawn from these studies. Seven studies[24,28-31,35,36] found no difference between patients and control groups, but 7 other studies[23,25-27,32-34] found an increased seroprevalence of ANA. Our study seems to fit with this increased prevalence of ANA, be it under strict conditions by excluding non-specific findings (i.e., low titers and weak nucleolar stainings). Further typing of the positive ANA samples revealed indications that the prevalence of specific ANA, like ENA-Abs and dsDNA-Abs, is increased in bipolar disorder, as well. Although our bipolar cohort is, to the best of our knowledge, the largest ever studied (Table 4), statistical significance could not be reached for these low prevalent Abs against ENA and dsDNA. We calculated[46] that with the differences we have found, group sizes should have been at least 450 individuals each to accomplish this goal. This is in accordance with previous data both with regard to the few psychiatric studies determining ENA-Abs[32,33] or dsDNA-Abs[26,32,33,35,36].

The two confounding factors stated frequently in literature regarding the seroprevalence of ANA, are treatment and gender. Regarding psychotropic treatment, chlorpromazine is often mentioned as a possible ANA inducer[25,47,48]. None of our patients used this antipsychotic. Other studies[26,49] brought up lithium as possible ANA inducer, but other studies did not confirm this[28,30,31]. Our data even tend to contradict an ANA-inducing effect of lithium, as fewer ANA positive patients were treated with lithium compared with the total cohort, but the difference is not statistically significant. Regarding results of the second step (i.e., specifically determining ENA-Abs or dsDNA-Abs), 5 out of 7 patients used lithium, which in contrast does suggest an effect of lithium, but again the difference is not statistically significant. With the possible exception of valproate, which was (also not significantly) more prevalent in the group of bipolar patients positive for ANA screening, other psychotropic treatments were not associated with the presence of ANA. Regarding these associations it should be noted that our study is naturalistic, which could have led to bias by indication for the various treatments. Moreover, most patients received more than one type of medication. This problem was already encountered by various other groups, nevertheless the majority of them confirmed the lack of significant association between the presence of ANA and the various psychotropic treatments[23,32,34,36].

Concerning gender, we found in our sample a higher prevalence of females in the ANA positive groups, although not statistically significant. Regarding previous studies, some did find a significant association between ANA and female gender[23,25,35,36], whereas others did not[27,34]. Within the results of the ANA screening, logistic regression correcting for age and gender did not alter the overall conclusions (data not shown). Unfortunately, the number of positive samples was too small to legitimally perform logistic regression correcting for all the possible confounding factors.

A common topic of discussion in immunologic research in mood disorders is the state or trait relationships of the immune alterations. We found a non-significant trend towards increased ANA positivity in patients who were in a manic or depressive episode, as was previously found by Hornig *et al*[36]. With regard to the disease specificity, we did not find increased ANA positivity in the schizophrenic patients, which confirms other studies[23,24]. However, other studies reject that the presence of ANA can be disease specific, either by concluding that ANA prevalence is increased in psychiatric patients regardless of their diagnosis[25,33], or by concluding that psychiatric patients in general do not have an increased risk on ANA[24,35].

In conclusion, we found an increased prevalence of ANA in bipolar disorder, compared with healthy control subjects. Larger groups need

to be investigated to establish whether this also results in a significant increased prevalence of specific ANA, associated with systemic autoimmune diseases such as Sjögren's Syndrome or SLE. Nevertheless, together with our previously published data on thyroid, pancreatic and gastric autoimmunity in bipolar disorder, it is suggestive that bipolar disorder is characterized by an altered set point of the immune system leading to vulnerability for autoimmune processes in general.

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Chapter 7
Conclusions and General Discussion

7.1 Conclusions

Although the exact pathophysiology of bipolar disorder has not been unraveled, this thesis adds to the increasing evidence that aberrancies in the immune system of patients with bipolar disorder represent one of the jigsaw pieces in it's complex pathophysiology[1-4]. The main results of this thesis work are summarized in Figure 1 and show:

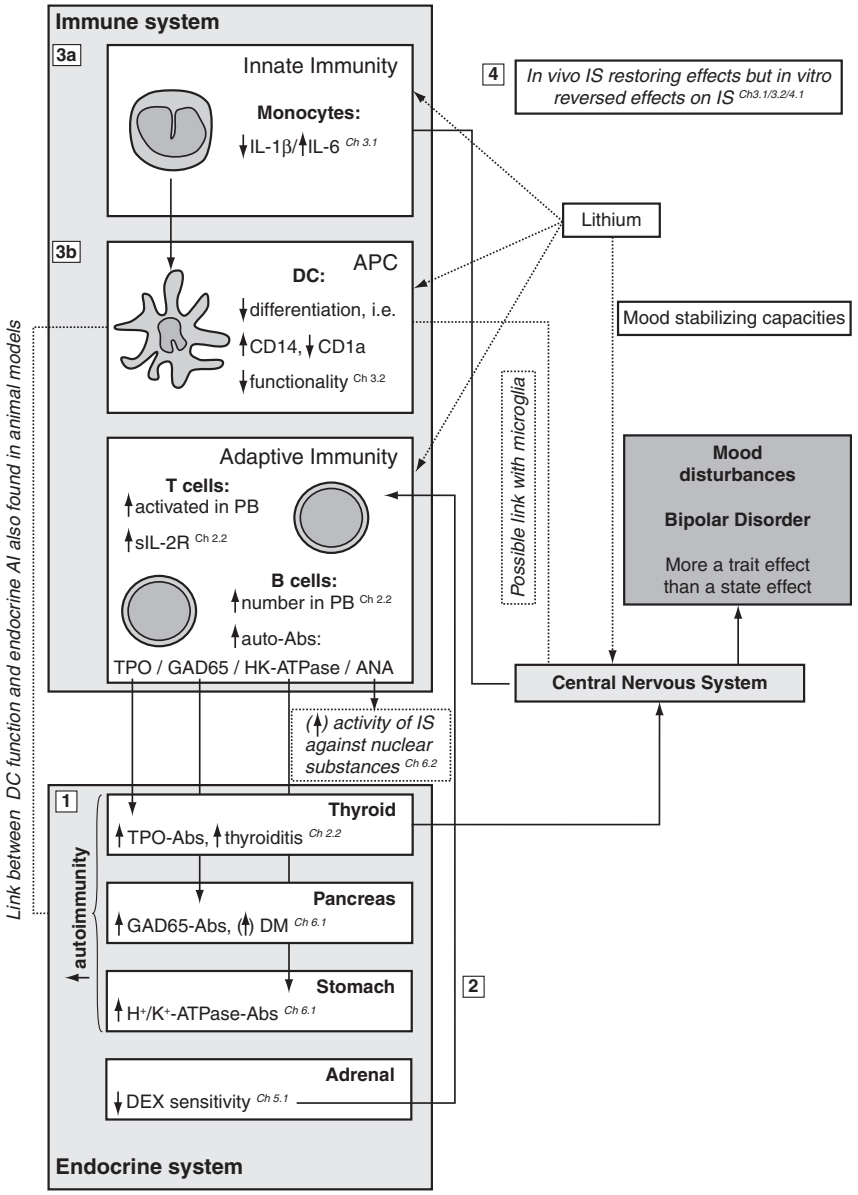
1. that the increased sensitivity of bipolar patients for autoimmune phenomena is not restricted to the thyroid[5] and that the prevalences of auto-antibodies against GAD65 (associated with diabetes mellitus type I), H^+/K^+ ATPase (associated with autoimmune gastritis), and ANA (associated with various systemic autoimmune diseases) in the serum of bipolar patients were also increased[Chapter 6.1, 6.2].
2. that T cells of bipolar patients were less sensitive to the suppressive effect of dexamethasone (DEX), a synthetic steroid[Chapter 5.1].
3. that monocytes of bipolar patients were aberrant:
   a. LPS-stimulated monocytes of non-lithium treated bipolar patients were characterized by an abnormal IL-1β/IL-6 production ratio (i.e., a relatively low IL-1β in comparison to a relatively high IL-6 production)[Chapter 3.1].
   b. monocytes of non-lithium treated bipolar patients had a reduced capacity to differentiate into fully active DC compared to healthy control subjects [Chapter 4.1].
4. that lithium treatment restored the monocyte aberrancies[Chapter 3.1, 4.1].

In summary, our results show clear indications for various B cell, T cell, and monocyte/macrophage aberrancies in patients with bipolar disorder compared to healthy control subjects. Also taking the literature into consideration, it is for us obvious that the immune system is affected in bipolar disorder. Various hypotheses have been proposed regarding the involvement of the immune system in mood disorders. In the following section we will discuss briefly some of the immune hypotheses in light of our current findings.

Autoimmune Theory

From a clinical point of view, the link between psychiatric disorders and autoimmune diseases is very intriguing. In the early 1960s Burch proposed an autoimmune hypothesis for schizophrenia, based on the age of onset, the sex differences and the relapsing remitting course of this psychiatric disorder[6]. A recent review by Jones *et al* once again puts emphasis on this

Figure 1. Main Results of the Thesis.



IL, interleukin; DC, dendritic cell; APC, antigen presenting cell; PB, peripheral blood; TPO, thyroid peroxidase; GAD, glutamic acid decarboxylase; HK-ATPase, enzyme in protonpump; ANA, antinuclear antibodies;Abs, antibodies; DM, diabetes mellitus;DEX, dexamethasone; IS, immune system; BD, bipolar disorder.

hypothesis in schizophrenia by putting forth several levels of evidence[7]. Comparable models have also been formed for other psychiatric disorders, including mood disorders. However, from an immunological point of view the conclusion that some psychiatric disorders have an autoimmune basis is too simple based on the current state of research.

In our studies we found a strong association between bipolar disorder and organ specific and systemic autoimmune diseases[Chapter 6.1 and 6.2] (i.e., we found an increased prevalence of TPO-antibodies (Abs), GAD65-Abs, H^+/K^+ ATPase-Abs, and ANA in bipolar disorder). These auto-antibodies are often markers of underlying more pathogenic events, such as the presence of auto reactive $CD4^+$ Th1 cells and $CD8^+$ cytotoxic T cells, leading to organ destruction and failure. Indeed in a previous study[5] we showed that bipolar patients have a higher risk of developing autoimmune thyroiditis, linked to the presence of TPO-Abs. Concerning the other auto-antibodies under investigation, the results are more difficult to interpret regarding their clinical consequences. GAD65-Abs are a marker for Diabetes Mellitus (DM) type I. Although the rate of diabetes was slightly increased in our SFBN cohort, the distinction between autoimmune induced pancreas destruction (DM type I) and DM due to insulin resistance (DM type II) was difficult to make in the bipolar group[Chapter 6.1]. Also, exact clinical data on atrophic gastritis or pernicious anemia, regularly associated with Abs against H^+/K^+ ATPase, were not present in our cohort and are also not present in the literature[Chapter 6.1]. Furthermore, there were no clear indications that in the bipolar group more patients suffered from systemic autoimmune diseases, like SLE or Sjögrens syndrome[Chapter 6.2].

We also found circumstantial evidence that bipolar disorder is associated with autoimmunity. Namely we showed that monocytes of bipolar patients are mildly hampered in their differentiation into fully active DC, both on the level of membrane markers (phenotype) and on the level of their decreased potency to stimulate autologous T cells. DC are an important determinant in autoimmunity as similar mild differentiation defects have been found in DC of patients with recent onset type I diabetes and their first-degree relatives[8]. In animal models, such as the NOD mice[9] or the BB-DP rat[10] aberrancies of DC are found consistently, similar to our findings in bipolar disorder. Restoring the DC disturbances in these animal models does prevent the development of autoimmunity[11]. Moreover, the typical reduced glucocorticoid sensitivity found in patients with mood disorders has also been found in animal models for both organ specific autoimmune disorders, such as the Obese strain of chickens (animal model with a spontaneously occurring Hashimoto-like autoimmune thyroiditis) and systemic autoimmune

diseases, such as murine lupus erythematosus[12].

Despite these findings, we do not think it is likely that bipolar disorder is an autoimmune disease, since specific brain antibodies have not been found in this disorder. We consider it more likely that the association between bipolar disorder and autoimmunity is caused by a common defect in the immune system, like in the monocyte/macrophage system forming the basis for, on the one hand the mood symptoms, and on the other hand the autoimmune phenomena.

Glucocorticoid Resistance Hypothesis

A consistent finding in mood disorders is a disturbance of the HPA axis[13]. The main feature of this disturbance can be summarized as an increased relative resistance of the sensing GC receptors in the body, such as the brain, for steroids, causing a disturbed feedback mechanism and increased circulating cortisol levels. The awareness of this aberrancy resulted in the glucocorticoid resistance hypothesis for mood disorders[14-17]. Our data, regarding the diminished suppressive effect of DEX on T cells[Chapter 5.1], supports this hypothesis on the level of circulating T cells. Moreover, the resistance to GC on the T cell level could explain the previously found T-cell activation in bipolar patients, indicated by a higher serum level of sIL-2R and increased numbers of $CD3^+CD25^+$ T cells[18].

Apart from possible intrinsic molecular defects in the downstream intracellular chain from the GC receptor, which causes the glucocorticoid resistance, there is also evidence that an increased level of products of activated macrophages, like the pro-inflammatory cytokine IL-1β, leads to a residual sensitivity to GC[19-21]. This finding is interesting since it links the glucocorticoid resistance hypothesis to the macrophage theory of depression.

Macrophage Theory of Depression

The basis of the macrophage theory of depression[22-24] is the finding that pro-inflammatory cytokines, produced by monocytes/macrophages[25] like IL-1β and IL-6, are able to induce behavioral changes[26] and that the production of these cytokines is increased in patients with major depression.

In order to expand this theory to bipolar disorder, we showed that monocytes of non-lithium treated bipolar patients were characterized by an abnormal IL-1β/IL-6 production ratio (i.e., a relatively low IL-1β production in comparison to a relatively high IL-6 production[Chapter 3.1]). Although we were able to confirm that the pro-inflammatory set point of

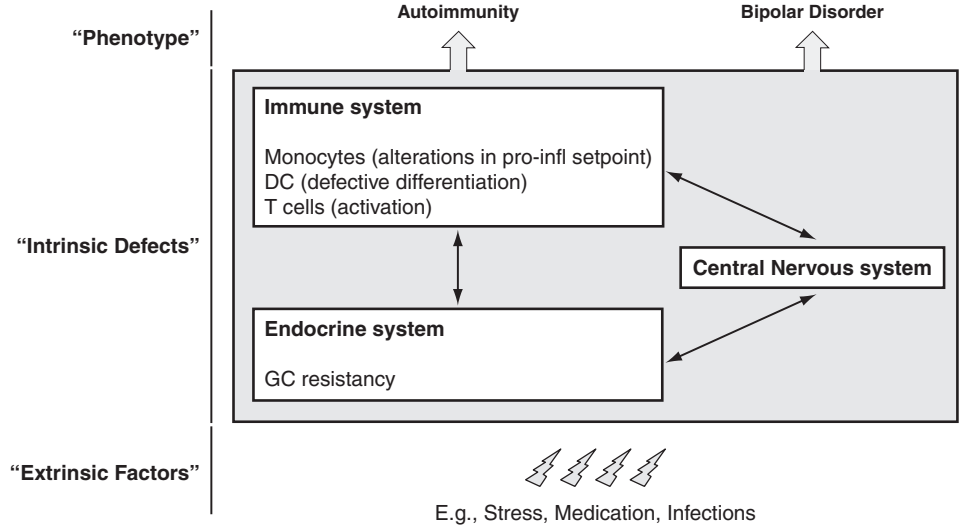
the monocytes of bipolar patients is altered, we were not able to confirm that there is simply an increased production of these pro-inflammatory cytokines. The implications of the disbalanced production of IL-1β and IL-6 can be on the level of the regulation of neurotransmitters in the brain, where both cytokines sort differential effects[27,28].

Ongoing research on immune activation in mood disorders also made the link to indoleamine 2,3-dioxygenase (IDO). This enzyme is, amongst others, present in macrophages and DC can be activated by immune activation[29]. Indoleamine 2,3-dioxygenase catabolizes tryptophan, one of the building stones of serotonin, and an increased activity of IDO can thus lead to a deficiency of serotonergic (5HT) neurotransmission[30]. Furthermore, one of the breakdown products of tryptophan, quinolinate and kynurenate, are respectively neurodegenerative and neuroprotective. In the "neurodegeneration hypothesis of depression" it is postulated that IDO is activated via immune activation, which results in an alteration of these neurodegenerative and neuroprotective compounds. This dysbalance can contribute to the development of mood disorders[31]. In our studies we did not measure the IDO activity.

Modified Hypothesis

The studies performed in this thesis enable us to adjust our current hypotheses (Figure 2). We found in our studies indications for intrinsic defects (i.e., not related to the state of bipolar disorder): an alteration in the pro-inflammatory set point of bipolar monocytes, a defective generation of DC from bipolar monocytes, and a resistance of bipolar T cells for glucocorticoids. These aberrancies have in earlier research either been implicated in the induction of mood disturbances (i.e., aberrant pro-inflammatory set point of monocytes in the macrophage theory of depression) or in the induction of autoimmunity (i.e., defective DC generation[8-11], or GC resistance[12]). To join these findings into one comprehensive model (Figure 2), the multidirectional interplay between the immune system, the endocrine system, and the central nervous system is crucial (i.e., neuro-immuno-endocrine axis). We hypothesize that the equilibrium of the neuro-immuno-endocrine axis is altered in patients with bipolar disorder. Such disequilibrium can eventually develop into a mood disorder, when other predisposing factors/vulnerabilities for mood disorders are also present, such as polymorphisms (acquired or congenital). On the other hand, this disequilibrium of the neuro-immuno-endocrine axis can also lead to clinically overt autoimmune diseases, explaining the high association between bipolar disorder and autoimmunity. In these cases other disturbances in the immune system should be additionally

Figure 2. Modified Hypothesis on the Pathophysiology of Bipolar Disorder.



DC, dendritic cell; GC, glucocorticoids.

present, such as a decreased function of regulatory T cells, tolerogenic DC, or a cross reaction with a similar antigen. Probably, multiple factors play a role in the adjustment of the neuro-immuno-endocrine axis, such as genetically determined polymorphisms of important molecules in this axis (cytokines, chemokines, cytokine-/GC-receptors) or environmental factors (earlier infections, stress factors during pregnancy or early life).

In conclusion, our current hypothesis is that intrinsic aberrancies of the immune system, particularly in the monocyte/macrophage system, lead to both mood disturbances as well as to autoimmunity depending on other elicitating and genetically determined intrinsic factors in the endocrine or central nervous system and/or extrinsic factors such as stress and infections, which we currently did not study.

7.2 Methodological Considerations: Strengths and Limitations of our Studies

The Classification of Bipolar Disorder

The Diagnostic and Statistical Manual of Mental Disorders (DSM) is a classification method originally introduced to categorize the variety of

psychiatric symptoms for research purposes. However, the diagnostic boundaries in classification methods, such as DSM-IV, remain hazy and the individual phenotypes within one diagnosis diverge. Bipolar disorder, as a diagnostic entity, is in this respect no exception, especially when you take the whole bipolar spectrum into account. In the present study we used the fourth edition[32] and only included patients, who clearly fulfilled the diagnostic criteria for bipolar disorder I or II (narrow phenotype) to make the study group as uniform as possible. We therefore did not include patients with cyclothymic or dysthymic disorder.

A strong point in this study is the registration of a large variety of demographic and clinical characteristics of the bipolar patients in this SFBN cohort into a central database. In this way possible confounders, such as age, gender, or mood state[33,34], could be taken into consideration in our statistical analyses.

Treatment

With regard to the psychotropic treatment, which is an important co-variable in our database, the following has to be mentioned. Patients were treated "as usual" and on average 90% of patients used 2 or more different types of psychotropic medications at the time of drawing blood, depending on their clinical features. This "polytreatment" makes it hard to draw firm conclusions on the effects of a single treatment on the different immune parameters. Because lithium is a clear immune modulator[35,36], as mentioned previously, we decided to focus mainly in our data analyses on the effects of this particular drug.

Duration of Illness

As mentioned in the introduction of this thesis, an accurate diagnosis during the first mood episode of patients with bipolar disorder is very difficult[37,38]. The time-gap between the disease onset and the starting point of treatment (on average 10 years) in our SFBN patient sample is in this respect very illustrative. For a biological study, as described in this thesis, it would be an advantage if one could catch patients early in their disease development, when they are still free of treatment. However, this is a general problem in the immunopsychiatric research field and it underlines the importance of finding early biological markers for bipolar disorder. Possibly these markers can be found in an aberrant function or set point of the immune system.

Sample Size

Compared to other studies on bipolar patients, the sample sizes of the immune studies within the SFBN cohort are among the largest ever studied. In the majority of our studies, sample sizes were large enough to control for single confounding factors, which were included in our database. However, to legitimately take all the possible confounders within our study into account and to build one statistical model, group sizes need to be larger. In the auto-antibody studies, which included over 200 bipolar patients, we were able to perform more elaborative statistical analyses. In these studies we did not find clear indications for important confounders.

Healthy Control Subjects: Age, Gender, and Psychiatric Interview

A general point of concern in our studies is the age of the subject groups. The mean age of the included healthy control subjects was in general not significantly lower as compared to the bipolar patients. Although on a single study level the role of age introduces differences in outcomes[33,39-41], a recent meta-analysis[3] on immune assays in mood disorders showed that age did not demonstrate a significant moderating effect with any of the immune variables. Statistical analysis in our studies did not reveal age as a confounding factor either.

In the meta-analysis mentioned above[3], female gender was associated with increases in B- and T-cell numbers. In particular, we paid attention to this possible confounding factor and matched the bipolar and the healthy control group as good as possible for gender. Performing statistical analyses in this respect, we were not able to find differences between the male and female group with the exception of the production of auto-antibodies, as was expected[42].

Another point of concern in the healthy control subject group is the fact that they did not undergo a (semi-)structured psychiatric interview. We did ask them in a small questionnaire if they were healthy (both physically and mentally) and if they took any kind of medication at the time of drawing blood. They were only allowed to take oral anticonceptives, otherwise they were excluded. We also excluded two individuals with a positive family history for psychiatric disorders (schizophrenia and major depression). Virtually all healthy volunteers were co-workers or medical students and with virtual certainty it can be states that our healthy control group was free of major psychiatric disorders. However, we could not control for any potential low level stress or mood symptoms.

Smoking Behavior

Finally, smoking habits influence the immune system[33,43], although one study disputes this[39]. Unfortunately we could not take smoking into consideration in our evaluations, because details on the smoking behavior of individual patients are lacking in the SFBN cohort. As a general rule, the majority of the bipolar and schizophrenia patients are most likely smokers, in contrast to our healthy controls.

Technical Flaws of the Assays

Some of the experimental designs, particularly the bio-assays such as the DC generation, used for the immune measurements in this thesis are sensitive to various external factors, such as the batch of FCS or the types of plastics used, stressing the importance of taking internal controls along in the experiments. The studies performed in this thesis were performed with this awareness and paired control samples were taken along in the same set of experiments. For future research it would be advantageous to develop experimental designs, which are more standardized and less variable. This would also be of benefit to decrease the variability of results between different research groups and reports in the literature.

Lithium

As mentioned previously, we also studied the effects of lithium in the immune studies reported in this thesis. The main reason being the previously reported immune modulating capacity of this potent mood stabilizing drug[35,36]. In general our data shows that lithium treatment clearly influences the immune status of our patients. Lithium treatment restored the aberrant IL-1β/IL-6 ratio and the aberrant capacity of monocytes to differentiate into potent DC. Despite that, the production of auto-antibodies or the relative DEX resistancy of T cells was not significantly affected by lithium treatment.

Besides the *in vivo* treatment effect of lithium, we also studied the *in vitro* acute effects of lithium by adding lithium chloride (LiCl) to our various immune assays. As a general rule, *in vitro* addition of LiCl did not have the same effects as *in vivo* lithium treatment. In the monocyte-derived DC cultures the suppressed DC generation and activation after *in vitro* exposure to LiCl (10mM) contrasted with the restored, activated phenotype due to lithium treatment *in vivo*[Chapter 4.1]. This phenomenon was also observed with regard to the capacity of monocytes to produce pro-

inflammatory cytokines. *In vitro* addition of LiCl resulted in a decreased IL-1β production and a minimal effect on the IL-6 production[Chapter 3.1], yet *in vivo* treatment restored the disturbed balance between these cytokines in bipolar patients. This clear difference between *in vivo* and *ex vivo* lithium exposure was also observed by Boufidou *et al*[44]. As argued by him and us in chapters 3.1 and 4.1, this discrepancy could be caused by factors such as differences in duration of lithium exposure, concentration of lithium, or factors which play a role *in vivo* but are excluded in an *in vitro* system, such as the interaction between different immune cells.

7.3 Possible Implications of our Findings

7.3.1 Implications for the Clinics

Because the exact role of the immune system in mood disorders has not yet been unraveled, the practical implications are rather limited. However, our data show a clear association between bipolar disorder and immune disorders, such as thyroid autoimmunity. As autoimmune thyroiditis has a high prevalence in bipolar patients and is relatively easy to treat, our data thus support the practice to evaluate the thyroid function of patients with a mood disorder. In particular when risk factors for thyroid pathology, such as treatment with lithium, female gender, or age above 44 years, are present along with the mood disorder, it is more than reasonable to measure the thyroid status of these patients thereby facilitating the early detection of thyroiditis, which means suppletion with thyroid hormone can start as soon as possible[45,46]. If somatic complaints or diagnostics are suggestive of an autoimmune disease a consult with a medical immunologist should be performed as well.

7.3.2 Implications for the Development of new Treatment Strategies

One of the main conclusions of this thesis is that the immune system is by and large activated (more activated T cells[18], aberrant pro-inflammatory cytokine set-point of monocytes, increased prevalence of autoimmunity) in patients with bipolar disorder, in line with the general opinion for mood disorders. In this respect, it would be logical to incorporate the involvement of the immune system in treatment strategies. Although the studies described in this thesis did not study possible mechanisms for new treatment strategies, it is from a clinical point of view, interesting to

review the findings in the literature in this respect. Some experimental treatment strategies aiming at immune suppression are listed in the following section.

Omega-3 Polyunsaturated Fatty Acids

Although the exact working mechanism of omega-3 polyunsaturated fatty acids, mainly of fish oil, is not known, there is clear evidence that omega-3 fatty acids have anti-inflammatory properties as reviewed several times[47,48]. This dietary compound was shown to be associated with unipolar depression, both in epidemiological studies[49,50] and on the cell membrane level[51]. Efforts to show therapeutic effects of suppletion of omega-3 fatty acids were successful in depressed patients[52,53]. This experimental treatment strategy also appeared to be effective in treating bipolar disorder, either for short-term effects[54], improving lithium-induced psoriasis [55], reducing depressive symptoms[56], or ameliorating signs of irritability[57].

PDE-IV Inhibitor

Another experimental approach is the treatment with phosphodiesterases IV (PDE-IV) inhibitors. Phosphodiesterases are responsible for hydrolysis of the cyclic nucleotides cAMP and cGMP, particularly in nerve and immune cells. Rolipram, a selective PDE-IV inhibitor, suppresses the expression of pro-inflammatory cytokines and other components of the immune system[58,59]. In animal models, treatment with rolipram indicates antidepressive effects[60,61]. Although nausea is a severe side effect of rolipram, a positive effect on symptoms of depression is shown in some human trials as well[58,62]. However, some studies showed that some already existing antidepressants, like imipramine or amitriptyline, remain superior compared to rolipram[63,64]. The search is now shifted to PDE4-treatment regimens with fewer side effects.

Interference with HPA-axis

One of the best-known immune suppressants is the corticosteroid-treatment approach. In patients with mood disorders, the levels of endogenous produced cortisol are increased. However, the simultaneous resistance of the glucocorticoid receptor makes this story complicated[16,65]. Treatment with cortisol ameliorates the mood state in some patients, but is also able to induce psychiatric symptoms[66,67], including manic episodes[68]. Drugs designed specifically to restore the feedback function within the

HPA axis may be integral for future treatment of mood disorders[16,69]. In this respect, limited clinical trials have been designed treating patients with depression with CRH antagonists[70], GR antagonists[71], and with steroid-synthesis inhibitors like metyrapone [72,73]. The first results are, at least for short-term effects, promising.

DHEA

Regarding hormones with immunomodulating capacity, dehydroepiandosterone (DHEA) has to be mentioned as well[74]. DHEA is an inactive androgen, transformed into active sex steroids in peripheral tissues. Briefly, the plasma concentrations decrease throughout adult life[75]. DHEA administration to patients with SLE[76,77] or diabetes mellitus[78,79] showed beneficial effects for their mood state. Moreover, DHEA treatment may have significant antidepressant effects, mainly in patients with major depression[80,81].

NSAID

Non-Steroidal Anti-Inflammatory Drugs (NSAID), such as ibuprofen and aspirin, act both as anti-inflammatory and analgesic agents via the inhibition of prostaglandin syntase. Trials in patients with mood disorders with these well-known NSAID have not yet been published. However, another prostaglandin syntase inhibitor (reboxetine), specifically targeting cyclooxygenase-2 (COX-2), has shown to have beneficial therapeutic effects in major depression[82].

Monoclonal Abs

Monoclonal antibodies to pro-inflammatory cytokines, such as IL-6 or TNFα, are novel treatment strategies in severe inflammatory diseases like Crohn's disease and rheumatoid arthritis. To the best of my knowledge, trials with these kinds of treatment regimens have not been performed in patients with severe mood disorders. This is probably due to the high costs, the intensive treatment protocols and the ethical considerations concerning serious side effects, including pneumonia, tuberculosis, lymphoma, and hepatotoxicity. It must be remembered here that major depression is the most common psychiatric diagnosis in Crohn's disease. Therapy with monoclonal anti-TNFα antibodies, such as infliximab, improved the quality of life in patients with active Crohn[83] and decreased the frequency of major depression in these patients[83,84]. Recently, a study with another monoclonal anti-TNFα antibody, Etanercept, in

psoriasis patients, showed a significant relieve of fatigue and symptoms of depression associated with this chronic disease[85].

7.3.3 Implications for Future Research

The results obtained in the studies published in this thesis open doors for future research regarding the role of the immune system in the pathophysiology of bipolar disorder. Although some of them have already been mentioned previously in this chapter, we want to approach these aspects more systematically in the following section.

1. The majority of techniques used in this study, particularly in the bioassays, are time consuming and sensitive to many factors, such as methodological approaches. New techniques have to be developed to solve these kinds of problems. Presently, techniques like genomics and proteomics are considered to have great potential for the future.
2. Although the implementation of DSM-IV criteria improved the uniformity of patient groups in psychiatric research, problems with heterogeneity within diagnostic entities remain. In the future, the use of potential biomarkers would be of benefit to approach a psychiatric diagnosis in a novel way. Ongoing research on the relationship between mood disorders and the immune system will most likely provide such potential diagnostic markers.
3. As mentioned previously it is difficult to collect bipolar patients with a recent onset of bipolar disorder or without therapeutic interventions. It is very important to perform studies in this early stage of disease development, to be able to investigate state or trait effects, the effects of being chronically ill, and/or the effects of treatment. For this reason one has to search for other approaches, e.g. by studying in follow-up studies high risk groups such as offspring or siblings of bipolar patients.
4. The studies presented in this thesis are restricted to aberrancies in single isolated components of the immune system, like monocyte/macrophages or T cells *in vitro*. Clearly, there is a need to integrate the findings in such isolated systems to a more holistic approach, which include the nervous system and the endocrine system as well as taking into consideration genetic and environmental determinants. This could be achieved by using sophisticated animal models or developing new animal models, which show the behavioral or the biological aspects of mood disorders. Various animal models have already shown their value. However, most animal models, such as animal models of sleep deprivation, olfactory bulbectomy, or chronic stress, at best highlight only part

of the clinical symptoms[86]. It would be challenging to develop an animal model for all aspects of bipolar disorder, mimicking behavioral components of both mood poles, which can be used for neurobiological studies.

7.4 Final Remark

The studies in this thesis confirm that aberrancies in the immune system are integral parts of bipolar disorder. From a clinical point of view, the association of bipolar disorder with autoimmune disease is of great interest.

The links between behaviour, the brain, the immune system and the endocrine system are intriguing and complex. It is expected that through scientific research insight will grow into the interactions between these systems. The ultimate goal would be to integrate all the findings, as small pieces of the big jigsaw, into one comprehensive model. Such a model will lead to new diagnostic tools and therapies. The journey of finding the crucial steps in the pathophysiological mechanism leading to bipolar disorder is not finished yet. It remains an interesting yet difficult challenge.

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Abbreviations

Abs	antibodies
ACTH	adreno-corticotrophin hormone
APC	antigen-presenting cells
AVP	vasopressin
BB-DP	biobreeding diabetic prone rat
BD	bipolar disorder
BMI	body mass index
BSA	bovine serum albumine
CD	cluster of differentiation
cpm	counts per minute
CTLA	cytotoxic T-lymphocyte associated antigen
CRH	corticotrophin-releasing factor
DC	dendritic cells
DM	diabetes mellitus
DEX	dexamethasone
DST	dexamethasone suppression test
DEX/CRH test	combined dexamethasone/corticotrophin-releasing hormone test
DM	diabetes mellitus
DSM	diagnostic and statistical manual of mental disorders
ESR	erythrocyte sedimentation rate
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein
GAD65-Abs	glutamic acid decarboxylase isotype 65
GC	glucocorticoid
GM-CSF	granulocyte-macrophpage colony-stimulating factor
HC	healthy control subjects
H^+/K^+ ATPase-Abs	H^+/K^+ adenosine triphosphatase antibodies
HPA axis	hypothalamus pituitary adrenal axis
ICAM	intercellular adhesion molecule
IIF	indirect immune fluorescence
Ig	immuneglobulin
IL	interleukin
LiCl	lithium chloride
mAbs	monoclonal antibodies
MD	major depression
MFI	mean fluorescence intensity

MHC	major histocompatibility complex
MLR	mixed lymphocyte reactions
M	mol/liter
NOD	non-obese diabetic mouse
PBMC	Peripheral blood mononuclear cells
PBS	phosphate buffered saline
PHA	phytohaemagglutinin
PE	phycoerythrin
P/S	penicillin/streptomycin
SCID	structured clinical interview for DSM-IV axis I
sIL-2R	serum-interleukin-2 receptor
SLE	systemic lupus erythematodes
SFBN	Stanley Foundation Bipolar Network
Th	T helper cell
TNF	tumor necrosis factor
TPO-Abs	thyreoperoxidase-antibodies
UG	ultraglutamine
VLA	very late antigen

Summary

The main objective of this thesis was to obtain more insight in the role of the immune system in the pathogenesis of bipolar disorder by investigating various aberrancies in the immune system of patients with bipolar disorder. In **Chapter 1** some general concepts, important for the perception of this thesis, are presented. Background information on bipolar disorder, the immune system, and the hypothalamus pituitary adrenal axis is given. Bipolar disorder is a severe and chronic mood disorder with a prevalence ranging from .3-3.7% (generally 1.3-1.6%) and a complex etiology. There is increasing evidence that the immune system plays a significant role in the pathophysiology of mood disorders, but research in this field particularly focussed on major depression. In this chapter an overview of earlier published research on immune abnormalities in mood disorders, especially bipolar disorder, is given. It is clear that not all immune findings point in the same direction, mainly due to diverse methodological approaches. Consequently, much of the involved mechanisms need further clarification. Also the role of lithium is mentioned, a potent mood stabilizer with immune modulating capacity.

In **Chapter 2** the Stanley Medical Research Institute, formerly known as Stanley Foundation Bipolar Network (SFBN), is introduced. From this multi-center research program, we included 81 bipolar patients from the SFBN site in Utrecht. Blood was drawn, demographical and clinical data were collected and standardised psychiatric interviews were assessed. Previous immune studies in this SFBN cohort revealed an increased prevalence of thyroid autoimmunity in bipolar disorder and a raised activation state of T cells and elevated numbers of B cells in peripheral blood. These studies contributed to our research questions, which are presented in this chapter. In summary, the studies described in this thesis were performed to gain more insight into different parts of the immune system of patients with bipolar disorder.

With regard to the innate immune system, represented by monocytes, we found indications for an altered pro-inflammatory cytokine balance, as described in **Chapter 3**. LPS-stimulated monocytes of non-lithium treated bipolar patients were characterized by an abnormal IL-1β/IL-6 production ratio (i.e., a relatively low IL-1β in comparison to a relatively high IL-6 production). Lithium treatment restored this status. In addition we studied the role of these cytokines in lithium-induced psoriasis, one of the major side effects of this mood stabilizing drug. The production profile of pro-inflammatory cytokines of monocytes of patients with lithium-induced psoriasis is different from that of regular psoriasis, indicating differences in their pathogenesis.

In the next chapter, **Chapter 4**, we focussed on the interphase between the innate and the adaptive (T and B cells) immune system, represented by monocyte-derived dendritic cells (DC). Monocytes of non-lithium treated bipolar patients had a reduced capacity to differentiate into fully active DC compared to healthy control subjects. The disturbances in the phenotype of monocyte-derived DC, both the marker expression on the cell membrane (CD1a and CD14) and the capacity to stimulate autologous T cells, were restored after lithium treatment.

Chapter 5 focussed on a part of the adaptive immune system (i.e. T cells) and more specific at their responsiveness to glucocorticoids (GC). In earlier studies, we found T cells of bipolar patients to be activated and we questioned whether this could be linked to the concept of GC-resistancy. *In vitro* experiments with T cells of bipolar patients showed evidence for a lesser responsiveness of T cells to steroids compared to healthy controls.

The first immune study in the SFBN cohort found a clearly increased prevalence of thyroperoxidase antibodies (TPO, auto-antibodies against the thyroid) in bipolar patients. Also the prevalence of the associated autoimmune thyroiditis was increased. We questioned whether this was also the case for other auto-antibodies, both organ-specific Abs (**Chapter 6.1**) and more generalized Abs (**Chapter 6.2**). Regarding organ-specific auto-antibodies, the prevalence of auto-antibodies against H^+/K^+ ATPase (against proton pumps in the parietal cells in the stomach) and glutamic acid decarboxylase-65 (against the beta cells in the pancreas) was also elevated. With regard to generalized Abs, we also provided evidence for an increased prevalence of antinuclear antibodies (ANA), associated with various forms of systemic autoimmune diseases.

In all our studies, special attention was paid on the effects of lithium, a potent mood stabilizer with immunomodulatory effects. In general, lithium treatment normalized the immune aberrancies in bipolar disorder. For example it restored the aberrant pro-inflammatory cytokine profile and recovered the potency to develop monocyte-derived DC. We also studied the *in vitro* effects of lithium chloride in various experimental set-ups, such as pro-inflammatory cytokine production in cell cultures and monocyte-derived DC cultures. As a general rule, *in vitro* addition of LiCl did not have the same effects as *in vivo* lithium treatment. This discrepancy could be caused by factors such as differences in duration of lithium exposure, concentration of lithium, or factors which play a role *in vivo* but are excluded in an *in vitro* system, such as the interaction between different immune cells.

Finally in **Chapter 7** the main findings and conclusions of this thesis

are summarized and discussed. The present study shows clear indications for various B cell, T cell, and monocyte/macrophage aberrancies in patients with bipolar disorder compared to healthy control subjects. Also taking the literature into consideration, it seems obvious that the immune system is affected in bipolar disorder and plays a role in the pathogenesis. Various hypotheses have been proposed regarding the involvement of the immune system in mood disorders, such as the autoimmune theory, the glucocorticoid resistance hypothesis, and the macrophage theory of depression. In the light of the immune findings in the SFBN cohort, we formulated an **adapted hypothesis** in which the neuro-immuno-endocrine axis plays an important role. Intrinsic aberrancies of the immune system, particularly in the monocyte/macrophage system, could lead to both mood disturbances as well as to autoimmunity. Whether it will lead to mood disturbances, autoimmunity, or both depends on other elicitating and genetically determined intrinsic factors in the endocrine or central nervous system and/or extrinsic factors such as stress and infections.

The findings in this thesis have some **implications for the clinic**. First, the association of bipolar disorder with autoimmune diseases, mainly autoimmune thyroiditis, is striking. Because the exact role of the immune system in mood disorders has not yet been unraveled, the practical implications are rather limited. As autoimmune thyroiditis has a high prevalence in bipolar patients and is relatively easy to treat, diagnostic tests of the thyroid function should be implicated in the good clinical practice of the treatment of patients with a mood disorder, in particular when risk factors for thyroid pathology co-exist. Second, this thesis confirms the involvement of the immune system in the pathophysiology of bipolar disorder, which can be of help in the development of future treatment strategies.

Finally, the pathophysiology of bipolar disorder and the links between behaviour, the brain, the immune system and the endocrine system are intriguing and complex. The ultimate goal would be to integrate all the findings into one comprehensive model. Such a model will lead to new diagnostic tools and therapies. The journey of finding the crucial steps in the pathophysiological mechanism leading to bipolar disorder is not finished yet. It remains an interesting yet difficult challenge.

Samenvatting

Bipolaire stoornis, vroeger manisch-depressieve stoornis genoemd, wordt gekenmerkt door het met intervallen optreden van heftige en extreme stemmingsschommelingen, waarbij de stemming zeer laag (depressief) of juist zeer uitgelaten (manisch) kan zijn. Soms komen in zo'n periode zowel kenmerken van een depressie als van een manie voor (gemengde episode). Kenmerkend is dat in deze periodes het normale leven (bijvoorbeeld werken, gezinsleven of sociale relaties) van een patiënt ernstig wordt verstoord. De episoden komen meestal geïsoleerd voor en worden afgewisseld door periodes met een gezonde (euthyme) stemming, waarin patiënten normaal kunnen functioneren. Echter, soms gaan de periodes direct in elkaar over.

Bipolaire stoornis komt bij 1.3-1.6% van de algemene bevolking voor, evenveel bij vrouwen als bij mannen. Bipolaire patiënten worden over het algemeen behandeld met een stemmingsstabilisator, zoals lithium, carbamazepine of valproaat. Echter, in veel gevallen gebruiken patiënten meer dan 1 type medicijn. De behandeling kan er voor bedoeld zijn om een depressie te verlichten, symptomen van een manie onder controle te brengen, of ernstige stemmingswisselingen te voorkomen. Over het algemeen gebruiken bipolaire patiënten ook medicatie in de periode dat ze een normale stemming hebben, om terugval te voorkomen.

De oorzaak van de bipolaire stoornis is niet precies bekend, maar uit onderzoek blijkt dat er verschillende factoren bij betrokken zijn. Onder andere speelt erfelijkheid een rol. Zo is de kans voor een broer of zus van een bipolaire patiënt om ook deze ziekte te ontwikkelen 40-70% voor een eeneiige tweeling en 5-10% voor een "gewone" broer of zus (dit ten opzichte van 1.3-1.6% voor de algemene bevolking). Omgevingsfactoren, zoals ingrijpende gebeurtenissen en sociale factoren, kunnen evenzeer een rol spelen bij het uitlokken van een manie of een depressie. Andere biologische factoren, zoals hormonale veranderingen en kleine structurele veranderingen in het brein, spelen ook mee. Recent onderzoek heeft aangetoond dat bij patiënten met een depressieve stoornis, dat wil zeggen bij patiënten die nog nooit een manische periode hebben doorgemaakt, tevens het immuunsysteem (ook wel afweersysteem genoemd) een rol speelt. Wetenschappelijk onderzoek naar dergelijke immunologische veranderingen bij bipolaire patiënten is in veel mindere mate verricht.

Het onderzoek, dat in dit proefschrift beschreven staat, had als belangrijkste doel om meer inzicht te krijgen in het functioneren van het immuunsysteem bij bipolaire patiënten. Voordat de resultaten van dit onderzoek beschreven worden, zullen hieronder eerst kort enkele immunologische basisprincipes besproken worden.

Het immuunsysteem is een verdedigingssysteem met als doel indringers (zoals bacteriën en virussen) of veranderde eigen cellen (zoals kankercellen) te bestrijden. Het immuunsysteem is een erg vernuftig en complex systeem, waarin verschillende stoffen en cellen met elkaar samenwerken en communiceren. Het is onder te verdelen in een "aspecifiek" en een "specifiek" deel. Met de cellen uit het aspecifieke immuunsysteem worden binnendringers snel maar vaak weinig effectief te lijf gegaan. Het in gang zetten van het specifieke deel kost meer tijd maar resulteert meestal in een sterke afweer, waarbij tevens langdurige bescherming tegen een specifieke indringer verzorgd wordt. Witte bloedcellen zijn cellen van het immuunsysteem, die zich in het bloed, in de huid en verspreid over het lichaam bevinden. Er zijn verschillende soorten witte bloedcellen, die afhankelijk van het type voornamelijk een rol spelen in het aspecifieke of specifieke immuunsysteem. Monocyten en macrofagen zijn bijvoorbeeld cellen uit het aspecifieke immuunsysteem en lymfocyten (B en T cellen) zijn cellen uit het specifieke immuunsysteem (zie ook figuur 1, hoofdstuk 1). Als het immuunsysteem niet goed is "afgesteld" kan dit tot verschillende ziektes leiden. Indien het immuunsysteem in ernstige mate te kort schiet, bijvoorbeeld door het wegvallen van bepaalde witte bloedcellen, spreken we van een immuundeficiëntie. Bij een overgevoelig immuunsysteem zal het lichaam overdreven reageren op onschuldige prikkels, zoals bij een allergie tegen huisstofmijt. Een overactief immuunsysteem kan een deel van het eigen lichaam gaan aanvallen. Dit noemen we een auto-immuunreactie, die kan leiden tot een auto-immuunziekte. Ziektes als diabetes mellitus type I, reumatoïde artritis en schildklier auto-immuniteit zijn hier voorbeelden van. Soms zijn verstoringen in het immuunsysteem echter subtieler.

Het immuunsysteem werkt niet volledig op zichzelf; zo kunnen stoffen uit het immuunsysteem in het brein effect sorteren en kunnen hormonen (zoals stresshormonen) het immuunsysteem beïnvloeden. Een voorbeeld om dit te illustreren is het bekende fenomeen, dat mensen met een flinke griep en koorts vaak ook een beetje somber zijn en minder eetlust hebben. Onder andere om deze reden zijn onderzoekers de relatie tussen het immuunsysteem, het hormonale systeem en het brein (waaronder ook gedrag en psychiatrische ziektebeelden) gaan onderzoeken.

Om het immuunsysteem van bipolaire patiënten te kunnen onderzoeken werd voor onze studie van 81 bipolaire patiënten bloed afgenomen. Met speciale technieken werden de verschillende witte bloedcellen uit het bloed geïsoleerd. Deze patiënten namen deel aan een breder onderzoek vanuit een stichting die het "Stanley Medical Research Institute" heet, voorheen ook het "Stanley Foundation Bipolar Network" (SFBN) genoemd. Naast de afname van bloed, werden over deze

patiënten uitgebreide, grotendeels gestandaardiseerde, vragenlijsten ingevuld met klinische en demografische gegevens. Tevens werd er een groep gezonde controles verzameld, die qua leeftijd en geslacht overeen kwamen met de patiëntengroep. Ook van deze groep mensen werd bloed afgenomen volgens dezelfde procedures. Eerder onderzoek in deze patiëntenpopulatie wees uit dat er voor bipolaire patiënten een groter risico was op het ontwikkelen van schildklierauto-immuniteit. Verder bleek ook dat bepaalde cellen uit het specifieke immuunsysteem (T cellen) meer geactiveerd waren, dan die van gezonde controles. Het doel van ons vervolg onderzoek, beschreven in dit proefschrift, was om andere facetten van het immuunsysteem in meer detail te bestuderen.

Wat betreft het aspecifieke immuunsysteem, vonden wij dat de monocyten van bipolaire patiënten een subtiele verandering in hun "status" hadden. De verhouding van bepaalde boodschapperstoffen, betrokken bij het aanzetten van ontstekingsreacties (pro-inflammatoire cytokinen; IL-1β en IL-6), was anders ingesteld bij bipolaire patiënten ten opzichte van gezonde controles. Lithiumbehandeling, waarvan al eerder bekend was dat het invloed heeft op het immuunsysteem, herstelde deze balans voor een groot deel. Aangezien één van de bijwerkingen van lithium psoriasis is (een huidziekte, waarbij het immuunsysteem ook verstoord is), hebben we ook naar de productie van dezelfde pro-inflammatoire cytokinen in de patiënten met "gewone" psoriasis en met lithiumgeïnduceerde psoriasis gekeken. Het productieprofiel verschilde tussen deze twee vormen van psoriasis, wat een verschil in de ontstaansmechanismen doet vermoeden.

Monocyten kunnen zich ontwikkelen tot dendritische cellen, die op hun beurt het specifieke immuunsysteem kunnen activeren. Wij vonden dat deze dendritische cellen van bipolaire patiënten minder ontwikkeld waren, dan die van gezonde controles. Behandeling met lithium herstelde deze ontwikkeling voor een deel.

In een andere studie keken we naar het effect van glucocorticoïden (remmers van het immuunsysteem, die ook een rol spelen in de stressreactie van het lichaam). Het bleek dat T cellen van bipolaire patiënten minder gevoelig voor deze glucocorticoïden zijn.

Zoals eerder vermeld, hebben patiënten met een bipolaire stoornis een groter risico op het ontwikkelen van schildklierauto-immuniteit. Wij wilden weten of dit ook het geval was voor andere vormen van auto-immuniteit. Wij deden dit door markers te meten in het bloed, zogenaamde autoantistoffen, die zich richten tegen een bepaald orgaan of andere structuur in het lichaam. In het geval van schildlierauto-immuniteit heet deze autoantistof "TPO-antistof". Deze bleek vaker voor te komen bij bipolaire patiënten (28% t.o.v. 13% in gezonde controles).

De markers, geassocieerd met auto-immuniteit tegen de maag (H^+/K^+ ATPase antistoffen) en tegen eilandjes van Langerhans in de alvleesklier (GAD-65 antistoffen), kwamen ook meer frequent voor bij bipolaire patiënten (resp. 11.7% t.o.v. 6.1% en 11.3% t.o.v. 2.6%). Ook autoantistoffen geassocieerd met een breder pallet van auto-immuunziekten, zoals het Syndroom van Sjögren en SLE, kwamen vaker voor bij bipolaire patiënten (antinucleaire antistoffen; 8.6% t.o.v. 3.4%). Echter, statistisch konden we in onze bipolaire patiëntengroep niet bewijzen dat ook de geassocieerde auto-immuunziekten vaker voorkwamen. Een te traag werkende schildklier ten gevolge van auto-immuniteit vormt hier een uitzondering op. Deze ziekte komt duidelijk meer voor bij bipolaire patiënten.

Een algemene conclusie, die uit dit proefschrift getrokken kan worden, is dat er op diverse niveaus van het immuunsysteem van bipolaire patiënten verschillen worden gevonden vergeleken met gezonde controles. Het immuunsysteem lijkt dan ook een rol te spelen bij het ontstaan van een bipolaire stoornis. Echter, het immuunsysteem is slechts een puzzelstuk in de complete verklaring van de oorsprong van de bipolaire stoornis. Verschillende theorieën zijn hier al over geformuleerd, maar geen enkele beslaat alles. Het zal nog een lange, maar uitdagende weg zijn om alle puzzelstukken te vinden die de complete ontstaanswijze van de bipolaire stoornis verklaren.

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Lieve Marije, ik ben trots dat jij straks naast me wil staan, want behalve mijn zusje ben je veel meer voor me. Ik hoop dat je op alle fronten binnenkort meer rust krijgt en dat de toekomst jullie samen toelacht, want dat verdien je!

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Esther

Curriculum Vitae

Esther Marianne Knijff, the author of this thesis, was born on October 28th 1975 in Gouda. She grew up in Waddinxveen and attended secondary school at the Coornhert Gymnasium in Gouda. In 1994, she started Medicine School in Leuven (België). After obtaining her first candidature, she continued Medical School from 1996 onwards in Rotterdam.

She matriculated in 2000 and obtained the medical degree in 2003 (cum laude). She followed her research course, entitled "Lithium induced psoriasis in bipolar patients", under supervision of Prof.Dr. H.A. Drexhage at the department of Immunology, Erasmus MC Rotterdam. During her internships and after obtaining her medical degree, she extended research at this department focusing on the immune system in bipolar patients in close collaboration with the Utrecht site of the "Stanley Medical Research Institute" (Prof.Dr. W.A. Nolen and Dr. R.W. Kupka), leading to this thesis. Since January 1st 2006, she is resident in training to become a psychiatrist at the Erasmus MC Rotterdam (educational head Prof.Dr. M.W. Hengeveld).

She is married with Albert Tertoolen and lives in Rotterdam.

Esther Marianne Knijff, de auteur van dit proefschrift, werd op 28 oktober 1975 geboren in Gouda. Zij groeide op in Waddinxveen en volgde het VWO-gymnasium aan het Coornhert Gymnasium in Gouda. In 1994 ving zij de studie Geneeskunde aan in Leuven (België). Na het behalen van haar eerste kandidatuur vervolgde zij vanaf 1996 dezelfde studie in Rotterdam. In 2000 behaalde zij haar doctoraal examen en in 2003 haar artsexamen (cum laude). Haar afstudeer onderzoek deed zij op de afdeling immunologie van het Erasmus MC Rotterdam onder supervisie van prof. dr. H.A. Drexhage met als onderwerp: "Lithium geïnduceerde psoriasis bij bipolaire patiënten". Gedurende haar co-schappen en in aansluiting op het artsexamen breidde zij het onderzoek op deze afdeling naar het immuun systeem van bipolaire patiënten uit in nauwe samenwerking met het "Stanley Medical Research Institute", locatie Utrecht (prof. dr. W.A. Nolen en dr. R.W. Kupka). Dit onderzoek heeft uiteindelijk tot dit proefschrift geleid. Sinds 1 januari 2006 is zij in opleiding tot psychiater in het Erasmus MC Rotterdam (opleider prof. dr. M.W. Hengeveld).

Zij is getrouwd met Albert Tertoolen en woont in Rotterdam.

List of Publications

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