Immuno-neuro-endocrine networks

A study on the inflammatory state of circulating monocytes and $CD4^+$ T cells in psychiatric and endocrine autoimmune disease

Roos Drexhage

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Chapter 1

Introduction

Adapted from: The mononuclear phagocyte system and its cytokine inflammatory networks in schizophrenia and bipolar disorder.
Drexhage RC, Knijff EM, Padmos RC, Heul-Nieuwenhuijzen L, Beumer W, Versnel MA, Drexhage HA.

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General Introduction

This thesis deals with immune aspects of bipolar disorder, schizophrenia, autoimmune thyroid disease and type 1 diabetes mellitus and therefore we give a short introduction to each disease.

Bipolar disorder

Definition. Bipolar disorder is one of the major psychiatric disorders. The term "bipolar disorder" replaced the older term "manic-depressive illness" that was introduced by Emil Kraepelin (1856-1926) in the late 19th century [1].Standardized classification systems as DSM-IV [2] and ICD-10 [3] are used to describe the psychiatric disorders. According to the DSM-IV classification bipolar disorder is a chronic disorder with manic and depressive episodes and usually a full recovery between episodes. A manic episode is characterized by an elevated, expansive or irritable mood, whereas a depressed episode is characterized by a depressed mood together with the possible following symptoms: sleep disturbances, psychomotor retardation or agitation, fatigue, feelings of worthlessness or guilt, impaired thinking or concentration, change of appetite or weight and suicidal thoughts. Patients have to suffer from at least one manic episode to be diagnosed with bipolar disorder, but most if not all patients also have depressive episodes. In this aspect bipolar disorder differs from unipolar depression since in that disorder patients do not experience manic episodes but only depressive episodes.

Subtypes. Within bipolar disorder the DSM-IV distinguishes various forms based on the type and severity of mood episodes experienced. Bipolar disorder I is the classic and most severe form, characterized by one or more manic episodes or mixed episodes (in which standards are met for both depression and mania). Bipolar disorder II is characterized by one or more major depressive episodes accompanied by at least one hypomanic episode, i.e an episode with manic symptoms but without associated functional impairment. The key difference between bipolar disorder I and bipolar disorder II is thus that subtype II comprises *hypomanic* but not *manic* episodes. The third form of bipolar disorder is Cyclothymic Disorder. This disorder involves more or less chronic alternating hypomanic and minor depressive episodes. The mood cycles between highs and lows, but never reaches the full criteria of mania or major depression. Finally, the term bipolar disorder Not Otherwise Specified (NOS) is used for patients who clearly have certain symptoms of bipolar disorder but do not meet the criteria for one of the subtypes described above.

Epidemiology. Bipolar disorder is a major global health problem. The life time prevalence of bipolar disorder (classified according to DSM-III) is estimated at 1.0 -2.0% in the general population across ten European countries [4]. In the United States the life time prevalence for bipolar disorder I is 1.0% and for bipolar disorder II 1.1% [5].The Netherlands Mental

Health Survey and Incidence Study, a prospective epidemiological survey in the Dutch general population, calculated a lifetime prevalence for the total bipolar spectrum of 5.2% (CI 2.2-8.1), including 2% (CI:0.1-4.1) for bipolar disorder I [6]. Together with unipolar depression bipolar disorder belongs to the ten leading causes of disability worldwide [7]. It is associated with high suicide rates [8]. Approximately 25 percent of patients attempt suicide at some time during their lives, and around 5 percent of patients die by suicide [8] making bipolar disorder the third leading cause of death among adolescents [9].

Treatment. To achieve stabilisation of their mood episodes, bipolar patients are treated with mood stabilizers. Lithium is the oldest among these mood stabilizers and still considered (one of the first choices) because of its proven effectiveness [10] [11]. Other known mood stabilizers are the anti-epileptics valproate, carbamazepine and lamotrigine. Often other medications are needed as well, especially to treat episodes of mania (with e.g. atypical antipsychotics) or depression (with antidepressants, lamotrigine or the atypical antipsychotic quetiapine).

Schizophrenia

Definition. Schizophrenia is one of the other major psychiatric disorders. It was first described as Dementia Preacox by Emil Kraepelin. Emil Kraepelin made a differentiation between Dementia Praecox and Manic Depressive Illness based on the duration of the disease (usually longer in Dementia Praecox) and the course of the disease (intermittent in Manic Depressive Illness) [1, 12]. A few years after the original description of Dementia Praecox by Emil Kraepelin, Eugen Bleuler suggested rephrasing the disorder into schizophrenia ("fragmented mind") [13].

According to the DSM-IV schizophrenia is a mixture of positive and negative psychiatric signs and symptoms [2] Positive symptoms reflect an excess or distortion of normal perceptive function such as delusions and hallucinations. Negative symptoms reflect a diminution or loss of normal function such as affective flattening and lack of initiative.

Subtypes. The DSM-IV describes in total five subtypes of schizophrenia: paranoid type, disorganized type, catatonic type, undifferentiated type and residual type. The paranoid type has as essential feature the presence of prominent delusions or auditory hallucinations. The disorganized type has as essential feature disorganized speech or behaviour. For the catatonic type the essential feature is a marked psychomotor disturbance. In the undifferentiated type the clinical picture of the patient does not fit into one of the other subtypes. In the residual type there is no evidence for the features of the first three subtypes but negative symptoms are present.

Epidemiology. Schizophrenia is present worldwide and the prevalence is in the range of 0.5%-1.5%. There are geographic differences in prevalence, it is e.g. seen that the incidence is higher in urban areas [14]. Also migrant groups have a higher incidence: 1st and especially

2nd generation immigrants from Morocco and Surinam in the Netherlands have a 5.8 times and 2.8 higher chance respectively for the development of schizophrenia [15].
Treatment. Patients with schizophrenia are usually treated with antipsychotic medication for the positive symptoms such as psychosis and delusions [14]. There is so far no medication for the negative symptoms such as alterations in drive and lack of initiative. In general the effect of medication is not totally satisfactory and there are numerous serious side effects, such as agranulocytosis (a well-known complication of clozapine) [16].

Autoimmune thyroid disease

An archetype of organ specific endocrine autoimmune disease is autoimmune thyroid disease (AITD). AITD encompasses a diverse range of clinical entities including Hashimoto's thyroiditis, Graves's hyperthyroidism and goiter, atrophic autoimmune thyroiditis, postpartum thyroiditis, sporadic painless thyroiditis and thyroid associated opthalmopathy. The main clinical entities are Hashimoto's thyroiditis (HT) and Graves hyperthyroidism and goiter. These two forms differ in clinical phenotype (hypothyroidism versus hyperthyroidism), but resemble each other in the histological features of thyroid lymphocytic infiltration, be it that in HT this infiltration is generally more severe than in Graves hyperthyroidism and goiter. Furthermore, the natural course of Graves hyperthyroidism and goiter is without surgical or iodine ablation not uncommonly hypothyroidism [17]. Also, both HT and Graves hyperthyroidism and goiter run in the same families, implying a shared genetic background of both diseases [18].

Hashimoto's thyroiditis is the catabolic form of AITD and characterised by a gradual destruction of the thyroid gland by a T cell-mediated autoimmune process leading to low levels of thyroid hormone (hypothyroidism). HT is serologically characterized by the presence of autoantibodies against thyroid peroxidase (TPO) [19]. The other main autoantigen is thyroglobulin (Tg).

Graves' disease is the anabolic form of AITD. Here, antibodies directed against the TSH receptor of the thyroid stimulate the thyroid to grow and to produce more thyroid hormone leading to hyperthyroidism [19].

AITD is a multifactorial disease and thus the result of an inherited susceptibility and encountered environmental factors (GD: concordance rate MZ twins= 22%, DZ twins = 0% (range or CI not available) [20], HT: concordance rate MZ twins= 55% (95% CI=23%-83%), DZ twins= 0% (95%CI= 0%-25%)[20]). The low concordance rate shows that environmental factors play a role.

Type 1 diabetes

Type 1 diabetes is another typical organ specific endocrine autoimmune disease in which the insulin producing β cells of the islets of Langerhans in the pancreas are destroyed by an autoimmune process. Because of this β cell destruction, the production of insulin diminishes and finally comes to an end. This will lead to hyperglycaemia. Insulin shortage can cause keto-acidoses which is, when untreated, a fatal outcome of T1D. Chronic hyperglycemia has its own set of problems such as cardiovascular disease, chronic renal failure, and retinal and nerve damage. Several autoantigens have been reported to play a role in T1D, including insulin, GAD65 (65 kDA glutamic acid decarboxylase), the protein tyrosine phosphatase-like antigen IA2 and ICA 69 (69 kDa islet cell antigen) [21]. Of these autoantigens insulin is probably the most important one [21]. Firstly because GAD65 and ICA69 were shown to be dispensable for diabetes development in GAD65 and ICA69 knock-out mouse models [22, 23]. Secondly, because insulin deficiencies were found to abrogate the development of T1D [24, 25]. Thirdly, insulin reactive CD4 T cells and CD8 T cells isolated from the pancreas could adoptively transfer disease to syngeneic recipients [26] . Fourthly, in humans a relatively high frequency of insulin A-chain-reactive CD4+ T cells was observed in the pancreas associated lymph nodes [27]. And last but not least, a polymorphic variable number tandem repeats (VNTR) located upstream of the insulin gene leads to susceptibility or resistance to T1D in patients by influencing the level of expression of pro-insulin in the thymus [28].

Antibodies against the above mentioned autoantigens are frequently present in T1D, not only in patients but also in their 1st degree relatives [29]. However, these autoantibodies do not have an active role in the pathogenesis of diabetes but are thought to be a parallel product as a result of β cell destruction caused mainly by CD8+ T cells and macrophages [30]. They are nevertheless helpful in the prediction of progression of the disease.

The pathogenesis of T1D is again multifactorial. The involvement of genes is shown by the higher concordance rate of T1D in MZ twins (53%, 95%CI= 33%-73%) as compared to DZ twins (13%, 95%CI= 5%-21%) [31], the involvement of environmental factors is suggested by the fact that the MZ concordance rate never reaches a 100%.

AITD and T1D are frequently associated

AITD and T1D co-occur more frequently within patients and families than normal. The co-occurrence of these two organ specific autoimmune diseases is described as subtype 3A of a syndrome called Autoimmune Polyendocrine Syndrome (APS)[32]. This syndrome was initially defined as a multiple endocrine gland insufficiency associated to an autoimmune disease in a patient. These associations were noted not to be at random but in particular

combinations. Therefore, the syndrome was classified after careful clinical observations by Neufeld and Blizzard into four main subtypes [33]. With regard to the pathogenesis of both AITD and T1D several similarities can be seen. In both diseases there is a destruction of target tissue by CD8 T cells and macrophages, the presence of autoantibodies to antigens in the target gland and common genes such as MHC II, CTLA4 and PTPN22 have been suggested as actors in both diseases.

A higher prevalence of mood disorders in AITD and T1D patients

Subjects positive for TPO-antibodies were shown to have a higher risk to develop mood disorders independent from the thyroid function and this was not the consequence of eachother [34, 35]. Hypothyroidism (often a consequence of AITD) is commonly accompanied by depressive symptoms and this is in part certainly due to a lack of thyroid hormone needed for brain cells to function optimally.

In a systemic literature review of T1D patients a prevalence of depression of 12% was found in controlled studies and in the healthy controls 3.2%, whereas in the uncontrolled studies the weighted mean overall prevalence rate was 13.4% [36] and considered higher than in the general population. In a recent Dutch study there was a prevalence of 8% of patients suffering from a depressive disorder based on a CIDI interview. Furthermore, depressive effect was associated with poor glycaemic control and proliferative retinopathy in this study sample [37]. The same was shown in a study from the USA [38]. Moreover, a meta-analysis showed that depression is associated with diabetes complications [39].

With regard to studies using patients with T1D and T2D, a meta-analysis done by Gavard et al. reported a prevalence of depression of 14 % (range 9%-27%) [40]. Another meta-analysis reported an odds risk twice as high to develop depression in diabetes then in healthy controls [41]. In these meta-analyses controlled studies were used.

A higher prevalence of AITD in patients with a major mood disorder

Major depressive disorder is associated with changes in the hypothalamus-pituitary-thyroid-axis and there have been various reports on aberrancies of the thyroid in patients with a major depressive disorder [42].

In bipolar disorder we have shown in recent years that patients were found to be more prone to develop thyroid autoimmunity and this was not related to the use of lithium [43]. Moreover, bipolar patients had also a higher prevalence to the other organ-specific autoantibodies H/K adenosine triphosphatase and glutamic acid decarboxylase-65 [44].
For a further discussion on the relationship major mood disorder-endocrine autoimmunity

it is important that we previously described that also children of bipolar patients have a higher risk for endocrine autoimmunity [45]. In this research, 11 of the 126 bipolar offspring studied were found positive for TPO-antibodies (versus 4/129 healthy controls) and 3 had developed T1D (i.e. 2% versus 0.4% general population). In addition, a twin study was performed which demonstrated that healthy co-twins of bipolar index twins show a similar high incidence of TPO-antibody positivity [46]. In both TPO-antibody positive offspring and co-twins thyroid autoimmunity was raised irrespective of mood symptoms. Thus family members of bipolar patients are more vulnerable to develop thyroid autoimmunity (and possibly also T1D), but these endocrine autoimmune diseases do develop independently from the vulnerability to develop mood disorders. These findings refute the concept that mood disorders and endocrine autoimmunity are cause or consequence of each other. This is further strengthened by the observation that treatment of hypothyroid patients with thyroid hormones does not always lead to an improvement of their mood disturbances. Taken together this might imply a shared common immune pathogenesis of mood disorders, thyroid autoimmunity and possibly also T1D. For the latter it is difficult to find a relation between depression and the development of type 1 diabetes, because type 1 diabetes usually develops in younger age than depression.

A higher prevalence of autoimmune disease in patients with schizophrenia

In schizophrenia there are also reports of a higher prevalence of autoimmune disease. In a large Danish national study patients with schizophrenia had a 45% higher chance of developing an autoimmune disease, amongst which thyrotoxicosis and interstitial cystitis [47]. Moreover, these autoimmune diseases were also more prevalent in the parents of patients with schizophrenia, also showing that family members have a higher chance of developing such autoimmune disease (again indicating a putative shared immune pathogenesis of psychiatric disorder and autoimmunity). A limitation of the Danish study was that the incidence of autoimmune disease was reported until the onset of schizophrenia, autoimmune diseases developed after the diagnosis of schizophrenia were not included in the study. Schizophrenia usually develops in early adulthood while autoimmune diseases develop usually later in life, so it could be that the higher risk for autoimmunity in schizophrenia is even higher than the already higher chance of 45%.

Although thus autoimmune diseases, such as thyrotoxicosis and interstitial cystitis are described with a higher prevalence in patients with schizophrenia, there are numerous reports of a negative co-occurrence of schizophrenia and rheumatoid arthritis, and it is unknown what the underlying immune mechanism is so far [48].

A putative shared immune pathogenesis of major psychiatric disorders and autoimmune thyroid disease and autoimmune diabetes

There is mounting evidence that monocytes and their descendent cells, i.e. the macrophages and dendritic cells (collectively also called cells of the mononuclear phagocyte system, MPS), of patients with autoimmune diabetes and autoimmune thyroid disease are in a pro-inflammatory state and abnormal in their adhesion and migration (vide infra in more detail). These abnormal set points of the cells most likely play a role in breaking T cell tolerance and inducing the aberrant autoimmune response towards β cells and thyrocytes. In addition a hyperactivated state of effector T cells play a role.

Precisely such activated state of the MPS and T cell system is presently thought to underlie major mental disorders, and this concept is generally referred to as the "Macrophage-T cell Theory" of Major Mental Disorders.

Macrophage-T cell Theory of depression and schizophrenia

Although there are reports of an involvement of the immune system in major mental illnesses already in the first decennia of the 20th century, it took till the last decennia of the 20th century before detailed studies on an involvement of the immune system in major mental disorders became more numerous. These studies reported aberrant levels of pro-inflammatory cytokines in the serum, plasma and cerebrospinal fluid of patients with schizophrenia and major mood disorders [49-52]. On the basis of these reports it was hypothesized that a pro-inflammatory state of the cytokine network induces psychopathologic symptoms and is involved in the pathogenesis and pathophysiology of these major mental illnesses.

Pro-inflammatory cytokines are primarily produced by activated cells of the immune system; such as activated endothelial cells, cells of the MPS and T-cells. The realization that such cells must be involved led to the "Macrophage-T-cell theory of depression and schizophrenia", which was proposed in 1992 and adapted in 1995 [53, 54]. In this theory chronically activated macrophages (and their related cells in the brain, i.e. the microglia) and T-cells produce cytokines and inflammatory compounds, which impact brain developments and destabilize the brain functionally in such a way that other genetic and environmental influences are able to precipitate the signs and symptoms of schizophrenia and mania/depression [55]. Indeed, receptors for inflammatory cytokines are present in various brain nuclei [56] and via their triggering deregulations of important neurotransmitter and neuro-developmental systems are introduced, facilitating the development of psychiatric signs and symptoms.

In the following section we give more detail on monocytes, macrophages and dendritic cells, how these cells traffic from the tissues to the lymphoid system and behave under steady state conditions, how they interact with various T cell subsets, and how they are involved in the inflammatory immune response under "danger" conditions and in case of autoimmunity.

Cells of the mononuclear phagocyte system (mps) and their interaction with various t cell susbsets

The immune system is divided in the innate immune system and the adaptive immune system (Figure 1).

Innate immunity

The innate immune system is the first line of defence against pathogens and provides a fast response with a limited specificity. The key players are: 1) barriers, such as tears, saliva and skin; 2) defence cells, which comprise neutrophils, cell of the MPS (monocytes and macrophages), natural killer cells and mast cells; and 3) soluble factors, of which various cytokines and chemokines are examples. If the innate system fails to resolve the infection, the adaptive system will get triggered by the cells of the innate immune system. This is seen as the inter phase between innate and adaptive immunity.

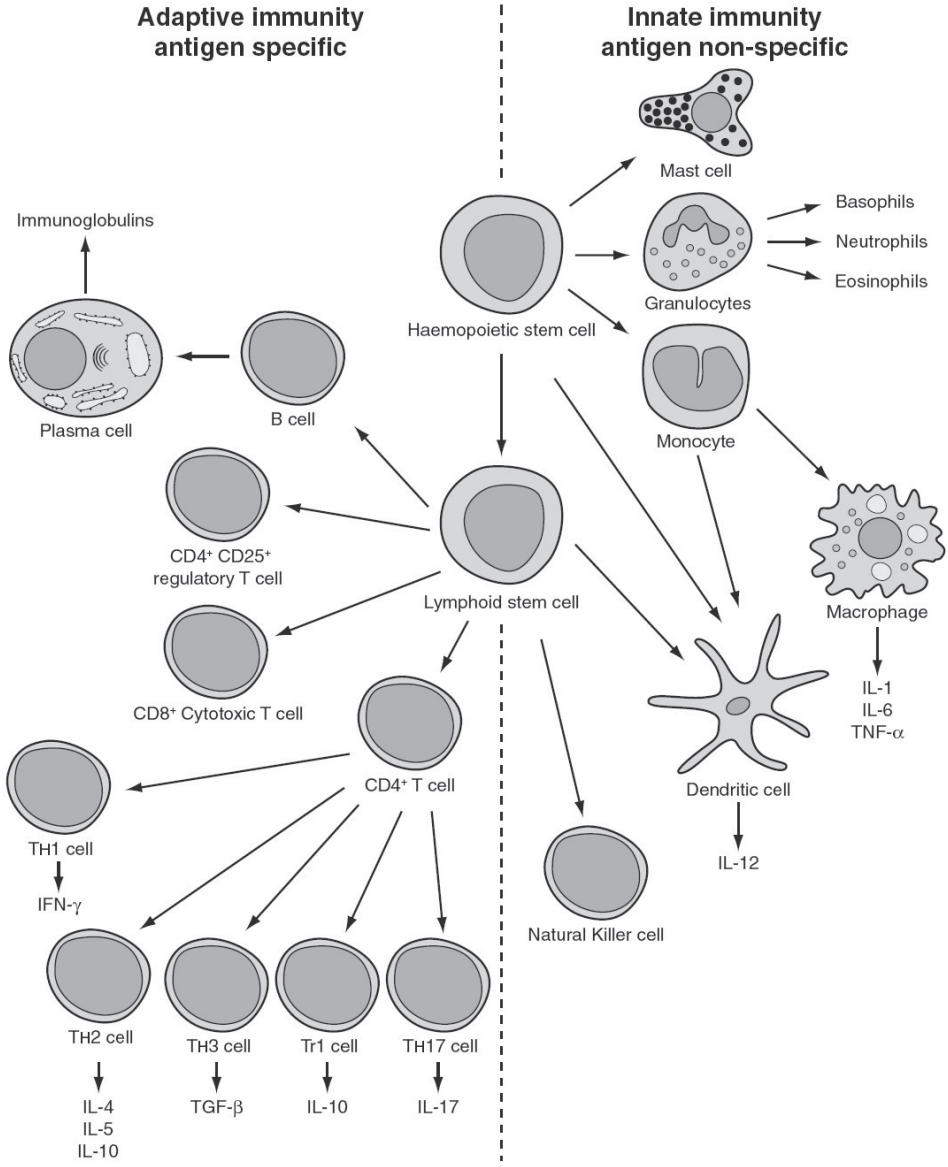
Inter phase

The cells responsible for the activation of the adaptive immune system and thus the key players of the inter phase are the various antigen presenting cells (APC). Accessory macrophages are an example of these APC but the most specialized APC are the dendritic cells (DC), which are also part of the MPS and in many instances derived from monocytes. DC pick up antigen at the site of infection, travel through lymphatics to the lymph node and present the collected antigen to cells of the adaptive immune system. With the presence of MCH class II molecules and their various co-stimulatory molecules, DC are well equipped to stimulate the naïve cells of the adaptive immune system, which will subsequently proliferate and be activated to play their role in the combat against the invading pathogens.

Adaptive immunity

The adaptive immune system is antigen specific, provides a memory and is typically activated a few days later than the innate system. The main contributors are the T cells and B cells. B cells, which transform into plasma cells, are responsible for the production

Figure 1. The innate and adaptive immune systems.



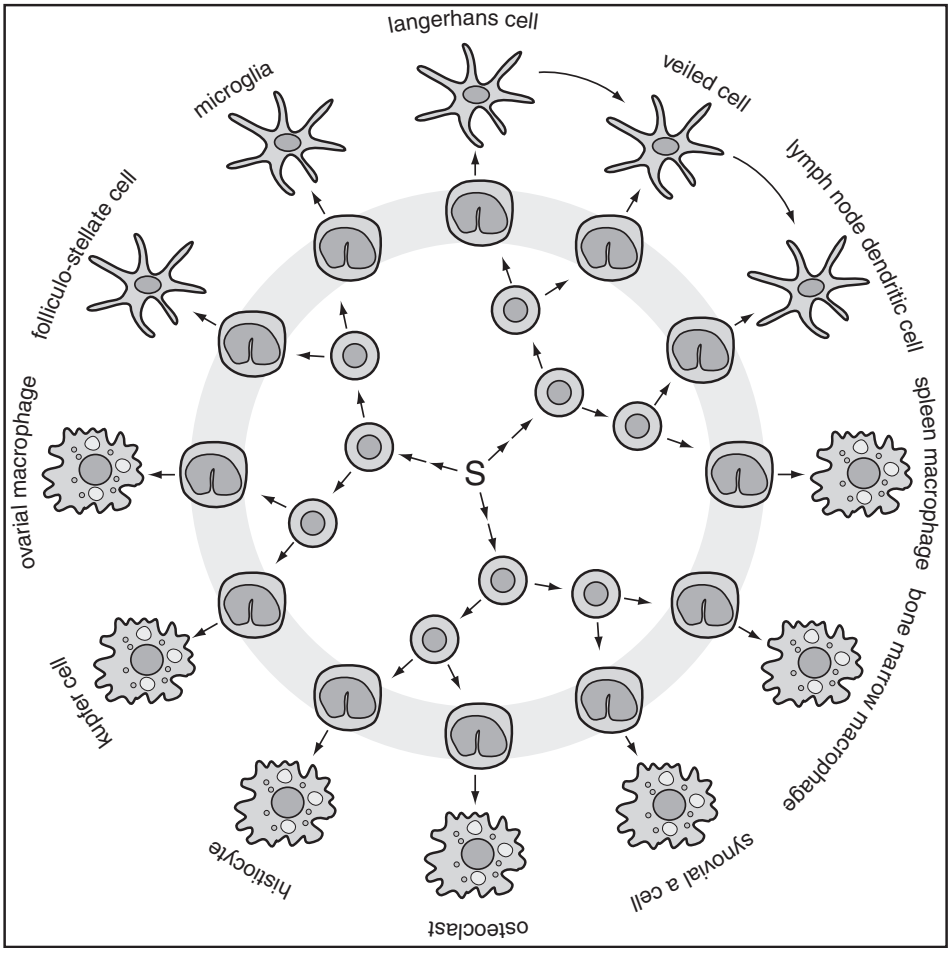
Depicted in this figure are the important players of the innate and adaptive immune system. Their roles are described in the text.

of antibodies. Antibodies neutralize extra-cellular bacteria, viruses and toxins, activate complement and facilitate pathogen phagocytosis. T cells are responsible for the so called cell-mediated immunity. T cells form a heterogeneous group of cells comprising effector cells, regulatory cells and memory cells and are typically divided into CD4+ and CD8+ T cells. The effector CD8+ T cells are cytotoxic and important in killing other body cells infected with virus, intra-cellular bacteria, or are otherwise damaged or dysfunctional. The effector CD4+ T cells are important in providing help to other immune cells and are therefore called helper T cells (Th). A naïve T helper cell (a cell which has not encountered an antigen yet in the presence of an APC) can upon stimulation by an APC differentiate into three Th subtypes, namely Th1, Th2 and Th17 cells. Th1 cells are capable of activating macrophages via Interferon γ (IFN γ), and are in this way essential in the elimination of intracellular pathogens. Th2 cells play a central role in the transformation of B cells into plasma cells. The third member of Th cells, the Th17 cell, has just recently been identified. The function of Th17 cells is not yet completely understood but is suggested to be in host defence against mainly extra-cellular pathogens which have not been cleared efficiently by the other Th cells [57]. Next to these effector and helper T cells, there are also T cells with regulatory functions as the immune system needs to be tightly regulated since it is well equipped to lethally damage the host cells if it loses control. The best known natural T regulatory cell population are the $CD4+CD25^{high}$ T cells. Naturally occurring $CD4+CD25^{high}$ Treg cells are defined by the constitutive high expression of CD25 (IL-2 is indeed required for triggering their suppressive function) and of the transcription factor FOXP3 (Forkhead Box P3). A feature of CD4+CD25+FOXP3+ T cells is that the cells themselves are "anergic" to mitogenic stimuli, but are capable to suppress the proliferation of $CD4+CD25^{neg}$ T cells when cultured together. Such suppression can be abrogated by the addition of IL-2, IL-15 or stimulation with anti-CD28 antibodies. The precise mechanisms of suppression by CD4+CD25+FOXP3+ T cells are not clarified yet, but cytokines like IL-10 and TGF-β are thought to play a role [58]. Next to CD25 and FOXP3 expression these Tregs are characterized by high surface expression of CTLA-4 and GITR. Other known regulatory T cells are the Tr1 cells (defined by a high production of the anti-inflammatory cytokine IL-10) and the Th3 cells (defined by a high production of the anti-inflammatory cytokine TGF-β) [59] .

Monocytes, macrophages and dendritic cells in more detail

Since these cells are of prime importance in this thesis, they are described in a little more detail. Monocytes are closely related to macrophages and dendritic cells and collectively these cells are often referred to as the "Mononuclear Phagocyte System (MPS)". This group of hematopoietic cells has diverse characteristics and origins. In general the cells originate in the bone marrow and migrate as monocytes through the blood to peripheral tissues.

Figure 2. The mononuclear phagocyte system.



By cell division of monoblasts (inner ring), promonocytes are formed (second ring), which form monocyte by division (shaded ring). Monocytes differentiate into the various macrophages and dendritic cells with their different names and functions (outer ring)
S: Hematopoietic stem cell.

The main descendents of these circulating monocytes, the macrophages and DC, occur in virtually all organs (Figure 2). Each organ contains multiple different macrophage and DC subpopulations. Local myeloid precursors for macrophages and DC are also present in various organs, including the brain [60].

The best-known functions ascribed to macrophages – clearance by phagocytosis and digestion - and to DC – primary activation of the adaptive immune system – have long been the leading principle in distinguishing these cell types. However, the biologic reality

is much more complex. The cells of the MPS comprise a large family of functionally and/or developmentally related cells that form a continuum in which the cells recognized as prototypical 'macrophages' and 'DC' form the extremes of a spectrum (Figure 2). Both DC and macrophages are involved in the regulation of the immune response and both DC and macrophages are capable of up and downregulating immune responses. In 'danger' situations the cells of the MPS become pro-inflammatory by perceiving danger signals, and then guide an effective T cell response to eliminate the danger-evoking signal. In normal situations without any danger the MPS cells stay in their "tolerogenic steady state", yet are active and do interact with T cells, but in such a way that tolerance to auto-antigens is induced and upheld.

In sum, the MPS comprises a large family of functionally and/or developmentally related cells essential for both inducing tolerance towards auto-antigens and for inducing an effective immune response towards dangerous intruders.

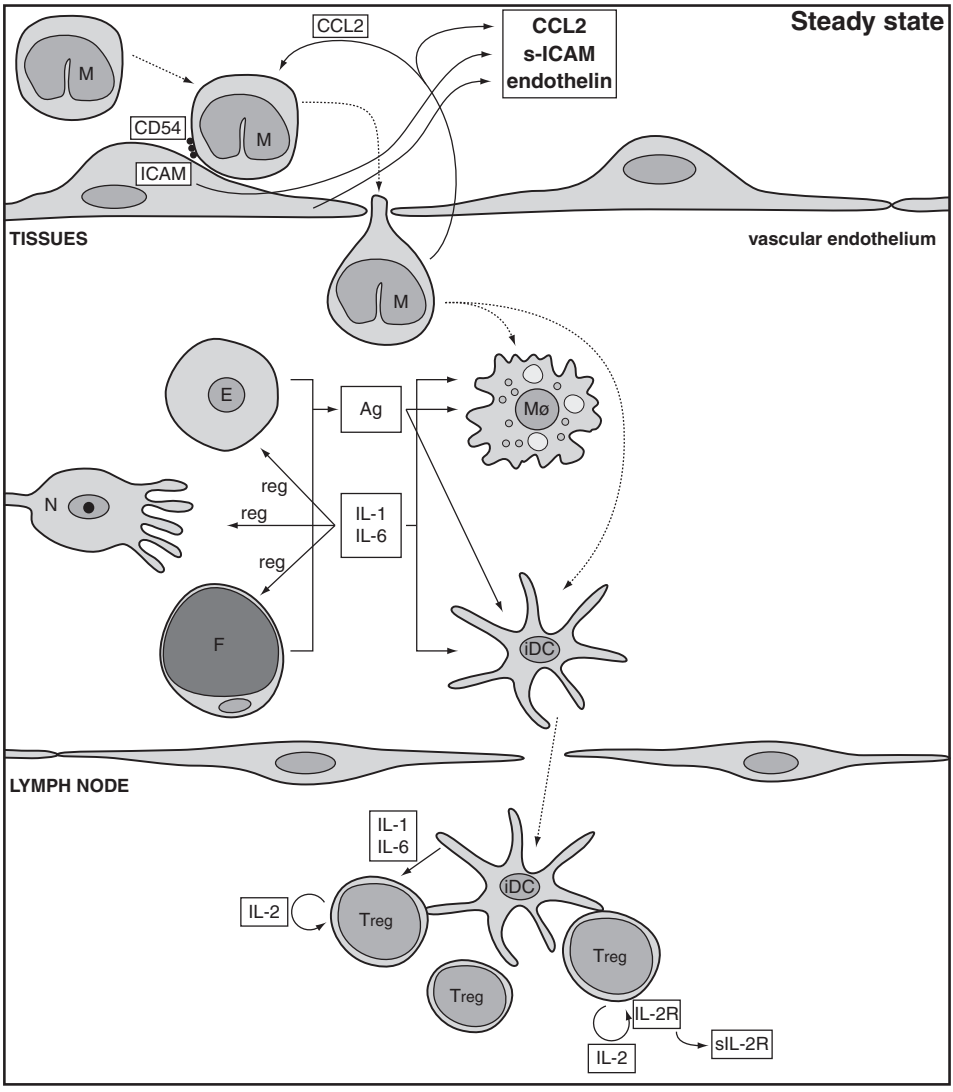
Trafficking and role of cells of the MPS and T cells under steady state conditions

Under normal physiological conditions there is a low but continuous trafficking of immune cells, in particular of monocytes and the monocyte-derived macrophages and dendritic cells (DC), from the peripheral tissues, such as from the skin (the DC is known here as Langerhans cell), the mucosa, the endocrine organs and the brain to the draining lymph nodes (known there as veiled cells [61]). Part of the cells enter the tissues from the bloodstream transversing the endothelial cells, although there are also indications that a proportion stems from local organ-dwelled precursors.

Influx of monocytes under steady state conditions.

Intercellular adhesion molecule (ICAM) is one of the adhesion molecules involved in the transmigration of leukocytes through endothelium (Figure 3) and is constitutively present on the surface of endothelial cells [62]. Monocytes in peripheral blood carrying the LFA-1 receptor (CD54) bind to ICAM-1 on endothelial cells to transmigrate into the tissues. The presence of ICAM on the surface of endothelial cells is increased by pro-inflammatory cytokines, causing more monocytes to migrate into the tissue. Soluble (s) ICAM is the soluble form of ICAM and shed by endothelium. In normal conditions it is present in human serum in concentrations of 100-450 ng/ml, and when endothelium is activated levels are increased. Then also levels of endothelin-1 are increased [63]. Early tissue-infiltrating monocytes produce the chemokine (C-C motif) ligand 2 (CCL2). CCL2 promotes further migration of monocytes into the tissue. The cell surface receptors on monocytes that bind CCL2 are CCR2 and CCR4. Normal serum levels of CCL2 are 100-225 pg/ml [49, 64].

Figure 3. Traffic: Steady state situation.



Under normal conditions, some circulating monocytes diapedese through the vascular endothelium entering the tissues. ICAM, CD45 and CCL2 are instrumental in this diapedesis. In the tissues the monocyte-descendent cells, the macrophages and immature DCs play a role in the physiological regulation of the growth and functioning of neighbouring parenchymal cells, for example, endocrine cells, neurons and fat cells; in this regulation IL-1 and IL-6 play a role. The macrophages and iDC also take up autoantigens of these cells and travel with these to the draining lymph nodes, where they stimulate, in particular, natural T regulator cells. In the proliferation of T regulator cells IL-2 and the shed IL-2R are produced. E: Endocrine cell; F: Fat cell; iDC: Immature dendritic cell; M: Monocyte; MØ: Macrophage; N: Neuron.

Neuro-endocrine regulatory role of tissue macrophages and DC under steady state conditions.

Infiltrated monocytes or local precursors differentiate under steady state conditions into tissue macrophages (the histiocytes) and immature DC (iDC). Such cells are primarily involved in functions of tissue homoeostasis and growth- and function-regulation of neighboring parenchyma and not in functions of defense (since there are no dangerous microbes or compounds around). To give a few examples: iDC in the anterior pituitary are known as folliculo-stellate cells and involved in the architecture of the anterior pituitary and the regulation of the secretion of e.g. FSH, LH, GH and TSH by gonadotrophs, somatotrophs and thyrotrophs [65]. iDC and macrophages in the thyroid, the islets of Langerhans and the ovaries are involved in the regulation of endocrine cell proliferation (e.g. follicle and islet formation) and in the dampening of the secretion of thyroid hormones and insulin [66-68]. Interleukin-1 (IL-1) and IL-6 produced by these steady state iDC and tissue macrophages play important roles in these neuro-endocrine regulatory processes.

Tissue macrophages and iDC in the brain are known as microglia and have the same regulatory functions as in other peripheral tissues. Animal studies have shown that microglia arise from primitive myeloid progenitors and not from circulating monocytes and are thus a distinct population in the MPS [60]. Microglia are in particular important for synaptogenesis, synaptic functioning and neuronal sprouting [55]. Under steady state conditions microglia promotes neurogenesis by the production of neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) that support the survival of neurons [69]. Also during brain development microglia expresses thrombospondins [70], a family of extracellular matrix proteins, able to induce synaptogenesis [71]. Microglial dysfunction impacts synaptogenesis, synaptic functioning and neuronal sprouting: The absence of thrombospondins induces a dramatic reduction in the number of synapses formed during postnatal stages [71], whereas a mutation in KARAP/DAP12, which switches microglia from a steady state to a pro-inflammatory set point, impacts synaptic functions and neuronal sprouting in the hippocampus [72].

Efflux of iDC from tissues under steady state conditions and their role in tolerance induction preventing autoimmunity.

Under steady state conditions iDC also take up – due to their strong endocytic capability - the compounds present in their vicinity, such as insulin when in the islets, thyroglobulin when in the thyroid, etc. etc., thus in fact "auto-antigens". The iDC, maturing partly under the influence of local cytokines (such as TNF) to semi-mature DC, thereafter travel via the lymphatics (known there as veiled cells, [61] to the draining lymph nodes (known as interdigitating cells) carrying the auto-antigens along. In the draining lymph nodes the superb antigen-presenting cell (APC) capability of the DC becomes evident and they start to trigger and expand in particular subsets of naturally occurring T regulatory cells[58].

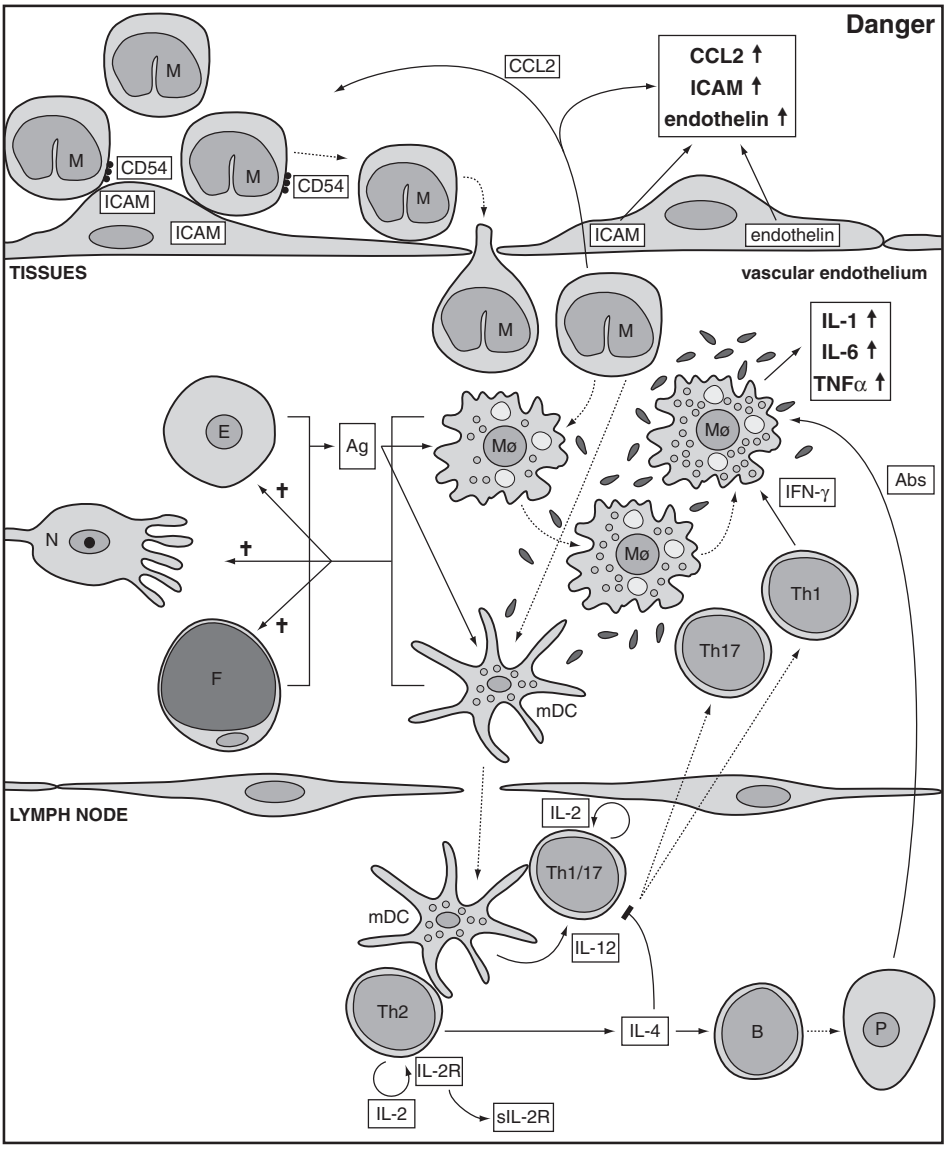
Apart from playing a role in positive and negative selection shaping the T cell repertoire, the thymus also creates naturally occurring T regulatory cells preventing autoimmunity. This is essential since negative selection is imperfect and autoreactive T effector cells have escaped to the periphery.

In triggering this population of T cells DC build up a strong non-reactivity (=tolerance) towards self under steady state conditions. The expansion and proliferation of the T regulator cells is heavily dependent on the activity of Interleukin-2 (IL-2), a cytokine expressed and produced by Treg cells when triggered by the iDC. Secreted IL-2 activates the Treg cells to proliferate and differentiate via the IL-2 receptor (IL-2R, CD25) in an autocrine fashion. Activated Treg cells shed the IL-2R (CD25) from their cell surface and the natural activity of such shed sIL-2R is to bind IL-2 inactivating it and so fine-tuning the immune response. sIL-2R (sCD25) produced by activated Treg cells is spilled over in the peripheral blood and circulating levels of sIL2R are under normal conditions 300-550 U/ml. *In sum, under steady state conditions the normal low level of trafficking of cells of the MPS (monocytes, macrophages and iDC) serves a neuro-endocrine regulatory role and promotes tolerance via the stimulation of natural T regulatory cells preventing autoimmunity towards neuro-endocrine tissues.*

Trafficking and role of cells of the MPS and T cells under inflammatory ("danger") conditions

When microbes infiltrate the tissues or when the tissue is necrotic (due to e.g. ischemia) the disturbance in tissue homoeostasis is perceived as a "danger" signal, putting a program of inflammation into motion. To perceive such danger signals the tissue macrophages and iDC are equipped with pathogen sensors, recognizing signals such as lipo-polysaccharides (LPS), lipotichoic acid (LTA), poly-IC, oxidative radicals and necrotic debris. There are 3 families of pathogen sensors: Toll-like receptors (recognize bacteria, fungi, viruses and protozoa); NOD-like receptors (recognize bacteria); and RIG-I-like receptors (recognize viruses) [73]. When triggered via such receptors the tissue macrophages and iDC change into inflammatory macrophages and mature (m) DC (Figure 4).

Figure 4. Traffic: Danger situation.



Under situations of danger (here a microbial infection) there is an increased influx of monocytes, which develop in the tissues into stimulated macrophages and mature DCs. Stimulated macrophages have increased phagocytic and lytic potential to kill the microbes, but are often also more toxic to the parenchyma. Mature DCs have an altered regulatory function for neighboring parenchymal cells and upon arrival in the draining lymph node do not stimulate Treg cells, but effector T cells to build up an adaptive immune response. †: *Requiescat in pace* – macrophages and dendritic cells kill neighbouring cells; Ab: Antibody; Ag: Antigen; B: B cell; DC: Dendritic cell; E: Endocrine cell; F: Fat cell; iDC: Immature dendritic cell; M: Monocyte; mDC: Mature dendritic cell; MØ: Macrophage; N: Neuron; P: Plasma cell.

Inflammatory macrophages.

Inflammatory macrophages produce large quantities of the pro-inflammatory cytokines IL-1β, TNFα and IL-6 and of CCL2. CCL2 attracts more monocytes and macrophages. The function of IL-1β is to activate the macrophages to an enhanced phagocytosis and killing, to increase the expression of adhesion molecules, including ICAM, on the surface of endothelial cells to attract more immune cells and to increase inflammatory compound production. The receptor for IL-1 is the IL-1R, which can be shed. IL-1 receptor antagonist (IL-1RA) – also produced by inflammatory macrophages - is opposing the effects of IL-1. The shed IL-1R as well as IL-1RA fine-tune the IL-1 inflammatory cascade. The functions of TNFα and IL-6 are similar and they also have similar regulatory cascades of soluble receptors and inactivating compounds.

Mature DC and the stimulation of the effector T cell response.

Mature (m) DC are in particular meant to and equipped to stimulate the adaptive immune response towards the dangerous microbes or noxes, i.e. the antigen-specific effector T cell response and B cell response. They have upregulated particular cytokines (IL-1, IL-6, IL-12, IL-4 and IL-23) and co-stimulatory molecules (CD80/86) and travel to the draining lymph node carrying along the microbial or otherwise "dangerous" antigens. In the lymph node the function of IL-1 and IL-6 produced by the mDC is the stimulation of the T effector lymphocytes to proliferate, whereas IL-6 has an additional function in the differentiation of B-lymphocytes. IL-2 produced by the DC-triggered effector T cells again acts here (under "danger" conditions) as the autocrine proliferation factor for the T effector cells. Thus: High levels of serum IL-2 or sIL-2R indicate T cell proliferation of either the Treg or T effector compartment.

The IL-12 produced by the mDC drives naive T cells in the direction of a Th1 development. Th1 cells are capable of secreting IFN-γ, which activates inflammatory macrophages to an even higher phagocytic and killing capacity, it also strongly activates cytotoxic T cells and natural killer cells, thus hyper-activating the inflammatory response towards the dangerous noxe.

The IL-23 produced by the mDC drives naïve T cells in the direction of a Th17 development, i.e. T cells capable of secreting IL-17, of which the function is not entirely known, but probably has similar functions as IFN-γ.

IL-4 produced by the mDC drives naïve T cells in the direction of a Th2 development. Th2 cells secrete the cytokine IL-4 too, which primes B cells and stimulates class switching, IL-4 is thus instrumental in the production of IgG antibodies towards the dangerous noxe.

IL-4 secretion by T cells provides a positive feedback by encouraging the growth of more Th2 cells, however IL-4 also inhibits through a negative feedback the development of Th1 cells. On its turn IFN-γ inhibits the Th2 response through a negative feedback, and thus TH1 and Th2 development are reciprocal phenomena.

In sum, under conditions of danger tissue macrophages develop a program of hyper-secretion of pro-inflammatory compounds promoting local inflammation, while matured DC are capable of stimulating Th1, Th2 and Th17 effector mechanisms, of which IFN-γ, IL-4 and Il-17 are indicators.

Abnormalities of monocytes, dendritic cells, t cells and macrophages in endocrine autoimmunity

Any abnormality of monocytes and DC switching the cells into a "danger" mode and leading to malfunctioning of the tolerogenic steady state processes could in principle lead to a loss of tolerance by an escape of autoreactive T cells from control and/or an imbalance (either in number or in function) between T effector and T regulatory cells [74-76]. Such a signal which switches steady state APC towards a danger mode when carrying along autoantigens to the draining lymph nodes is e.g. the introduction of a strong danger signal during immunization with e.g. thyroglobulin by adding heat-killed M tuberculosis together with oil (i.e. "Freund's complete adjuvans"). This protocol is followed in the induction of Experimental Allergic Thyroiditis (EAT) and also in other Experimental Allergic models using the appropriate autoantigens, such as Basic Myelin Protein to induce Experimental Allergic Encephalitis (EAE).

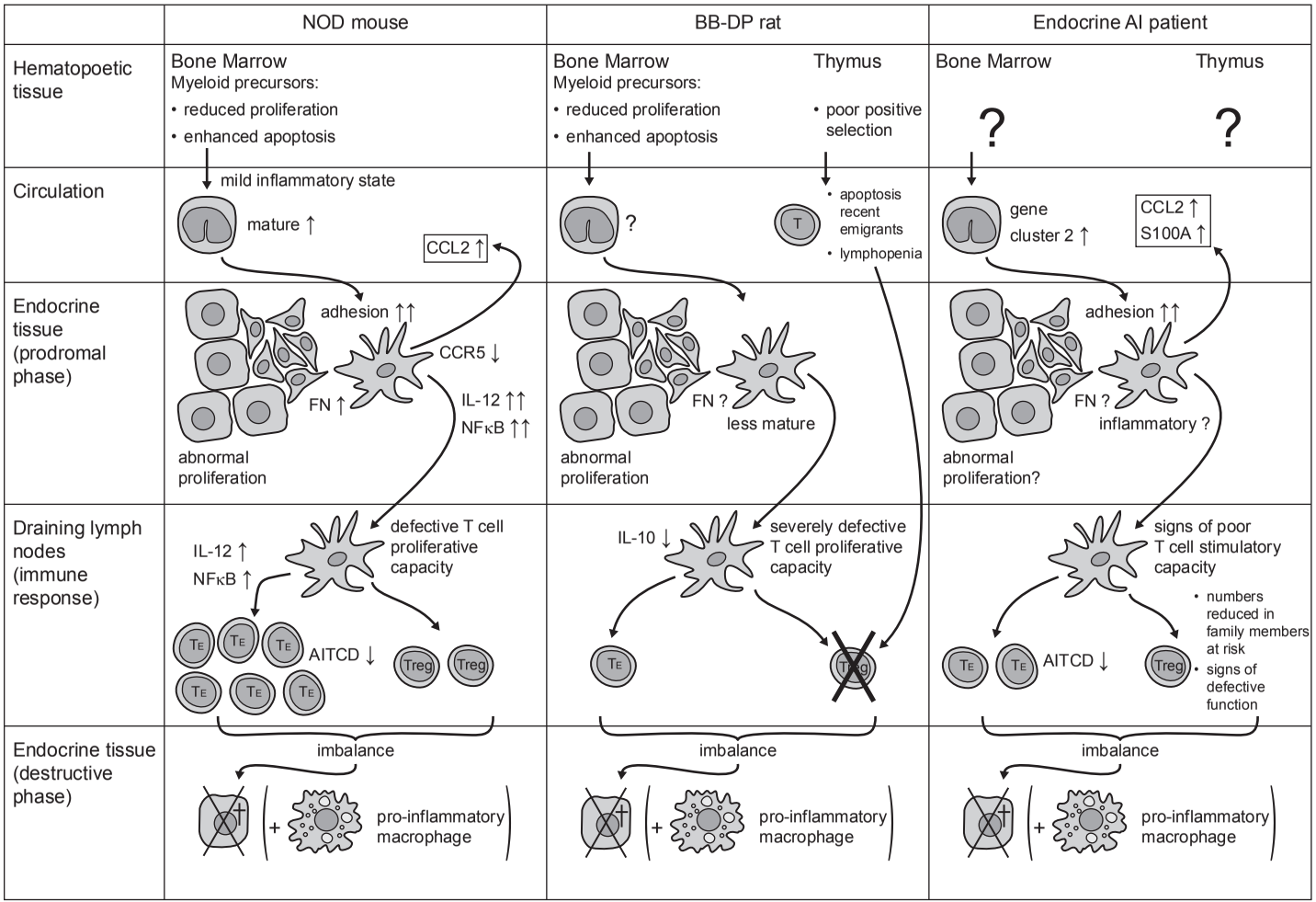
However, an aberrant "danger" set point of steady state monocytes and DC can also be due to an "inborn" aberrant set point of the cells leading to spontaneous organ specific autoimmunity. Indeed, such an inborn aberrancy has extensively been described in the NOD mouse and the BB-DP rat, both excellent animal models of thyroid and islet autoimmunity [32, 77-81]. In these animal models an aberrant "danger" set point of steady state monocytes and DC is already detectable before the actual autoimmunization phase, i.e. the enhanced DC accumulation in the target gland-to-be at around 5-8 weeks of age and the immune reaction in the draining lymph node and the production of autoantibodies from week 8-10 onwards. It is thus tempting to speculate that these "inborn" aberrancies of the steady state monocytes and DC are causal in the break of tolerance and indeed corrections of the abnormal monocytes and DC have led to prevention of disease in these models [82, 83].

It can also be envisaged that, next to aberrancies of the steady state monocytes and DC, intrinsic defects in the function of T cells are a reason for a poor tolerance to autoantigens. Again these defects have extensively been described in the NOD mouse and the BB-DP rat preceding the autoimmune process and corrections have prevented or even cured the starting disease.

Interestingly the defects in monocytes, dendritic cells and T cells are similar, but not identical in the NOD and BB-DP rat model and are also linked to different genes in both models. This notion points to a concept that different molecular immune pathogenic mechanisms lead to a comparable functional defect in DC-T cell interaction resulting in a similar endpoint, i.e. autoimmunity and a final destruction of target cells. It is possible, that similar diverse immune molecular pathogenic pathways exist for human T1D and AITD, and that these diseases are in fact composed of various syndromes with diverse molecular mechanisms.

Based on extensive research in our group over the last 10-15 years and based on the literature the aberrancies of the monocyte, macrophage, DC and T cell compartments in the NOD mouse, the BB-DP rat and in T1D and AITD patients can be summarized as follows (Figure 5):

Figure 5. Traffic: Autoimmune conditions in the NOD mouse, BB-DP rat and the endocrine autoimmune patient.



Collectively the various aberrancies in endocrine cell, monocyte, DC and T cell functions in both the NOD mouse and the BB-DP rat (models of spontaneous endocrine autoimmunity) are highly suggestive for a basic underlying inborn defect in growth and development of myeloid and lymphoid precursors in the bone marrow and thymus and of epithelial and mesenchymal cell precursors in the target glands.

A. The myeloid precursors of the bone marrow show a reduced proliferation and a reduced apoptosis resistance, but an accelerated and deviant maturation. This results in

1) A mature (partly inflammatory) set point of circulating monocytes with an enhanced adhesiveness to ECM (as shown in the NOD mouse)

2) An accelerated maturation of DC from these monocytes/precursors with an intrinsic high inflammatory set point, but with a poor T cell stimulatory T cell capacity (depending on the availability of growth factors). Such "inflammatory" DC are poor stimulators of AITCD and natural T regulator cells (both shown in the NOD and BB-DP rat)

B. The growth disturbances of the parenchyma and connective tissue in the target gland prior to the autoimmune process lead to an abnormal structure of the extra-cellular matrix (ECM) with a high fibronectin (FN) content (as shown in the NOD mouse). The "inflammatory" DC generated in the tissues from infiltrated sticky monocytes will acquire an even higher pro-inflammatory state due to an abnormal interaction with the abnormal ECM (high FN content). Indeed the DC in the tissues are pro-inflammatory (high NFκB) and have a disturbed IL-12/IL-10 ratio (as shown both in the NOD mouse and the BB-DP rat) and they will thus primarily stimulate Th1 pathways, tipping the already disturbed T cell balance from tolerance to auto-immunization.

C. The disturbances in the growth and development of lymphoid precursors in the thymus lead to

1. A lymphopenia and in particular a strongly reduced number of natural T regulator cells due to an enhanced apoptosis of recent thymic emigrants (RTE) as shown in the BB-DP rat,
2. Various T cell apoptosis defects leading to abnormal positive and negative selection in the thymus (BB-DP rat) and a defective AITCD (NOD mouse)
3. A fitness defect of T regulator cells in the NOD mouse (due to a lack of growth stimuli, e.g. IL-2)

These imbalances between the various T cell tolerogenic and autoimmunization differentiation routes will aggravate the antigen presenting disturbances of the autoimmune DC.

D. In endocrine autoimmune patients similar aberrancies as in the NOD mouse and BB-DP rat have been found, i.e. there are signs of

1. An abnormal adhesive state of circulating monocytes (gene cluster 2 upregulated, more adhesiveness to FN and endothelial cells) leading to an over production of pro-inflammatory factors (CCL2 and S100A)
2. A defective generation of veiled cells and dendritic antigen presenting cells (APC) from monocytes resulting in APC with a poor capability of stimulating tolerogenic T cells or to induce AITCD
3. A resistance of T cells to undergo apoptosis in AITCD
4. A defective function of natural T regulator cells, and
5. A reduced number of T regulator cells in individuals at risk for endocrine autoimmunity.

1. A reduced proliferation and enhanced apoptosis of myeloid precursors

Serreze et al. [84] were the first to describe that hematopoietic precursors in the bone marrow of NOD mice proliferated poorly to CSF-1 (M-CSF) stimulation resulting in a quantitative inability to generate typical and functional macrophages. They proposed that this defect in macrophage differentiation yielded antigen presenting cells that were unable to activate tolerogenic mechanisms, but remained capable of activating low level effector responses.

From our group Nikolic et al. [85] later also found a model that in the NOD mouse in a GM-CSF-driven bone marrow culture (to yield DC) the precursor cells had a reduced proliferation and a higher rate of apoptosis in comparison to 3 control strains; this resulted in a reduced yield of dendritic cells at the end of the culture.
Sommandas et al. [86] did a similar observation in the BB-DP rat system: In a GM-CSF/IL-4 driven bone marrow culture the rate of apoptosis of precursors was higher in the BB-DP rat as compared to the BB-DR rat, a line of BB rats without islet and thyroid autoimmunity because this line lacks the mutation in an important IDD gene, the *lymphopenia* (*lyp)* gene. This gene is responsible for the severe lymphopenia of the rat (see later); apparently this gene is also directly or indirectly (via lymphocyte cytokines) important for a normal myeloid precursor growth and apoptosis.

2. An activated state of circulating monocytes

The abnormal differentiation of myeloid precursors is not without consequences for the final maturation stage reached by the progeny.
NOD mouse. Nikolic et al. [85] showed in the GM-CSF-driven NOD mouse bone marrow culture system that the precursors for DC did not follow the sequence of normal development to DC via three successive stages (i.e. earliest precursors – pro-monocytes - monocytes to finally yield immature DC), but showed an accelerated maturation, characterized by an almost immediate transition of the earliest precursors into DC, which had not an immature, but a mature, macrophage-like phenotype. A reflection of such similar maturation acceleration and deviation can be observed in the circulation of the NOD mouse, where there is an overrepresentation of mature (Ly-6C^{low}) monocytes over that of immature (Ly-6C^{high}) monocytes. Interestingly, recent not yet published experiments of our group (Beumer et al., to be published) show an upregulation of a number of pro-inflammatory genes, amongst which IL-1β, PTGS2, THBS and PAI-2, in the expanded mature Ly-6C^{low} circulating monocytes in the NOD mouse.
Also the macrophages of the NOD mouse have a pro-inflammatory phenotype. A high PTGS2 expression level has been reported in NOD peritoneal and bone-marrow derived macrophages [87] as well as a high production of the pro-inflammatory cytokines IL-1β and TNF-α [88, 89] and of oxidative radicals [90].
BB-DP rat. Unfortunately studies on the pro-inflammatory state of circulating monocytes are lacking in the BB-DP rat model. However there are reports on a pro-inflammatory state of the peritoneal macrophages in the BB-DP rat producing high quantities of TNF-α [91] and oxidative radicals [90].
Patients: In AITD and T1D patients pro-inflammatory abnormalities of circulating monocytes (e.g. increased IL-1β, ROS and PTGS2 production) [92-94] similar to those observed in the NOD mouse have been reported, although these findings have also been

doubted [95, 96]. In a recently reported study we found a pro-inflammatory monocyte gene expression pattern consisting of a coherent set of inflammation related genes (a.o. IL-1 β, IL-6, TNF, PTGS2, so-called cluster 1 genes) and a coherent set of motility-adhesion-chemotaxis genes (a.o. CDC42, EMP1, CCL2, so-called cluster 2 genes) in the majority of LADA patients (60%) and to a limited extent in adult-onset T1D patients (28%). In monocytes of childhood-onset T1D cases only the motility-adhesion-chemotaxis cluster 2 gene set was upregulated in 43% of patients [97]. These data firstly suggest heterogeneity of the pro-inflammatory activation set points of circulating monocytes in different forms of T1D. Secondly they show that an upregulation of a set of motility-adhesion-chemotaxis genes is characteristic for at least proportions of LADA, adult and childhood onset T1D patients.
Monocyte gene expression data for AITD patients are lacking; Chapter 6 addresses this issue.

3. An increased fibronectin adhesiveness of monocytes and dendritic cells

This increased adhesiveness leads to a pro-inflammatory state of the cells and there is a link with a pre-existent abnormal morphogenesis of islets and thyroid tissue.
NOD mouse. From our group Bouma et al.. [98] showed an increased adhesiveness of NOD monocytes (in particular of the expanded mature (=Ly6C$_{low}$) population) to fibronectin (FN). These mature monocytes are in particular the precursors of the steady state macrophages and DC in the tissues [99] and indeed Wildenberg et al.. [100] showed that the circulating mature NOD monocytes primarily produce the DC in the peripheral tissues of the NOD mouse. Wildenberg et al.. [101] were also able to show that such tissue DC also lacked the expression of CCR5 in the NOD mouse model and that this reduced CCR5 expression leads to an over production of IL-12, a cytokine capable of skewing the T cell response in a damaging Th1 direction.
Acharya et al.. [102] took these observations further and showed that adhesion of NOD DC to adhesive substrates, such as FN, collagen and vitronectin, indeed modulate the activation and stimulatory capacity of the cells; specifically NOD-DC cultured on FN acquired a pro-inflammatory state, i.e. they were Th1 skewers and induced the highest expression of IFN-γ in T cells.
The increased adherence of NOD monocytes and DC to FN and its consequences for the inflammatory state is relevant, since increased amounts of FN are present in the NOD pancreas particularly at the islet edges in the early postnatal period, where it is associated with an enhanced accumulation of monocytes and DC at these sites [103]. The increased amounts of FN at the islet edges is part of a general abnormal morphogenesis of the NOD islets prior to the onset of the autoimmune reaction: The islets are irregularly shaped, later becoming mega-islets, which are in particular the target of the early infiltration of DC and

later infiltration with macrophages and T cells [104]. The islets also show an increased ratio of α over β cells [105]. It is relevant to recall here that DC have the capacity to influence the growth and function of islet cells and perhaps their early enhanced infiltration is linked to this function.

BB-DP rat. Studies on the adhesive capacity of monocytes and DC to FN are lacking in the BB-DP rat model. However an abnormal morphogenesis of the BB rat thyroid prior to the autoimmune process has been described: There is a reduced thyroid growth in the stage preceding thyroid infiltration with DC, macrophages and T cells and isolated thyroid follicles isolated at that time (before 9 weeks of age) show a reduced spontaneous and TSH-Induced proliferation [67]. We also noted in these tissue digestion experiments that the thyroid tissue of BB-DP rats has other digestion kinetics as compared to control strains, suggesting a defiant ECM composition (not published results). Strikingly similar observations have been done in another model of spontaneous autoimmune thyroiditis, the Obese Strain (OS) chicken: A decreased in vitro growth rate of thyroid cells in the pre-autoimmune stage, which was suggested to be an inborn abnormality since it could already be detected in OS fetuses [106].

Patients. An interesting parallel exists between the NOD mouse and T1D and AITD patients regarding the aberrant adhesiveness of monocytes to FN. In general it is known that human monocytes are "activated" after adherence to FN (so-called P-monocytes) and have acquired an enhanced capability to produce inflammatory cytokines and chemokines [107, 108].

Monocytes of T1D patients display an increased adherence to FN (and to endothelial cells) and express higher surface levels of the integrin CD11b/CD18 as compared to monocytes of healthy controls and T2D patients [109, 110]. Interestingly, adherence to FN of monocytes of T1D patients induced an even higher pro-inflammatory state over that of healthy controls with a stronger increased production of MRP8/14 (a pro-inflammatory compound of the S100 family), of the pro-inflammatory chemokines CCL2 and CCL3 [111], but with a reduced production of IL-10 [110].

Monocytes of AITD patients also show an altered interaction with FN. The cells have, after adherence to FN, a strongly reduced motility response to chemo-attractants [108]. Recent not yet published experiments (Burgerhout et al.., to be published) show that this reduced motility response is linked to a pro-inflammatory state of monocytes (in particular to an overexpression of the genes for PTGS2, IL-1β, PTX3), suggesting that also in AITD monocytes might be strongly pro-inflammatory activated after FN adherence.

4. A defective capability of dendritic cells to support tolerogenic mechanisms

NOD mouse. As stated before Nikolic et al.. [85] showed a poor generation of DC from bone marrow precursors. The generated DC are not only macrophage-like, but also had a reduced capability to stimulate T cells. Peng et al.. [112] showed that the maturation defect of NOD DC was linked to the IDD10/17/18 region on chromosome 3 of the NOD mouse. Also in other studies *in vitro* generation of DC from NOD BM precursors appeared to be defective [112, 113], and there are indications that this reduced generation and deviant differentiation of NOD DC contributes significantly to imbalances in the T cell system in such a way that autoimmunity prevails. First of all, transfers of properly terminally differentiated DC prevent diabetes development in the NOD mouse [82] and Dahlen et al.. [83] suggested that such transfers worked due to the correction of the low level of co-stimulation given by defective NOD DC: The transferred maturated DC would give a full activation of auto-reactive T cells and would consequently induce Activated Immune T cell Death (AITCD) and/or the upregulation of CTLA-4, an important switch-off signal for activated T cells. Other mechanisms might play a role as well: properly terminally differentiated DC are also better equipped to expand CD4+CD25+ regulatory T cells [114]. Interestingly, correcting the IDD10/17/18 locus in NOD mice results in significantly more tolerogenic DC in the pancreas draining lymph nodes [112].

It is important to again highlight that we found that the "proliferation defective" DC had a macrophage-like inflammatory phenotype, despite their defect in proliferation capability for T cells [85]. Other investigators have also found the pro-inflammatory state of NOD DC cultured from bone-marrow precursors: DC over expressed various IFN-induced genes and this abnormality was linked to the IDD5 region [115]. In another report NOD DC showed high levels of NFκB and an increased IL-12p70 production; not further identified non-MHC IDD genes of the NOD were involved in this pro-inflammatory set point [116]. In contrast to our results these latter authors did not find a reduced generation of DC and a reduced capability of these DC to stimulate T cells to proliferate, but found an increased generation of NOD DC and a superb T cell proliferative capability of the cells. It must be noted that in their experiments NOD BM precursors were cultured in GM-SCF *in combination* with IL-4, in contrast to our studies where we used GM-CSF *alone*. When we cultured BM precursors of the NOD mouse also in GM-SCF *in combination* with IL-4 (instead of *in the absence* of IL-4, see before) we found that the DC differentiation defects had largely disappeared (Nikolic et al.., unpublished results). We interpret these findings that NOD BM precursors need specific growth factors, such as IL-4, to develop into fully T cell stimulatory DC, which is not necessary for other mouse strains.

Thus the defective DC generation and poor T cell stimulatory capacity of DC can only be seen in the NOD mouse system under sub-optimal culture conditions of precursor cells. These suboptimal conditions probably do not normally occur in the living animal,

since we were not able to trace back the above-described *in vitro* poor T cell stimulatory capacities of NOD DC in *ex vivo* isolated DC populations. DC isolated from the NOD spleen and lymph nodes were excellent T cell stimulatory DC [117]. It is however important to note that the *ex vivo* and *in vivo* spleen, lymph node and tissue NOD are – despite their normal proliferative capacity - nevertheless abnormal in their inflammatory state: The cells are in vivo CCR5 defective and high IL-12 producers [101].

BB-DP rat. In the BB rat model the DC developmental defects are much clearer as compared to the situation in the NOD mouse. With regard to the BB rat model we already reported in the 1990's that *ex vivo* preparations of thyroid DC and of spleen DC displayed abnormalities. Firstly, the DC preparation of the thyroid gland of the BB-DP rat contained more monocyte-like precursors and fewer cells with the typical characteristics of DC [66]. Secondly, the *ex vivo* spleen DC of the BB-DP rat showed a lower MHC class II expression, had a lower capability to form homotypic and T cell aggregations and had a reduced capability to stimulate T cells in syngeneic (syn)-MLR [118], in particular the ART2+ T regulatory cell population of the BB-rat was poorly stimulated by the BB-DP DC. We took these abnormalities in both the thyroid and spleen DC population as signs of defects in the differentiation and maturation of BB-DP DC [86, 119], which had in particular an impact on the development of natural regulatory T cells.

In later studies we found similar differentiation defects with regard to DC cultured from bone-marrow precursors (see also above): i.e. a reduced MHC class II cell surface expression and a poor capability to differentiate into fully mature DC, capable of fully stimulating T cells [86, 119]. Various genes of the BB-DP rat are involved in these functional defects, including genes of the MHC complex (IDD1) and the *lyp* gene (IDD2) [119]. Similar to the NOD mouse model, bone marrow precursor derived DC of the BB-DP rat are in a pro-inflammatory state before the onset of autoimmune disease: The DC produce however not more of the pro-inflammatory IL-12 (as in the NOD mouse model), but reduced quantities of the anti-inflammatory cytokine IL-10 [119].

Patients. In AITD, T1D patients and high-risk first-degree relatives there are conflicting data with regard to the defects in DC differentiation from precursors, DC function and the pro-inflammatory state of DC. This might be related to the existence of heterogeneous pathogenic mechanisms for different forms of T1D (e.g. for LADA, juvenile onset and adult onset T1D, see before), however other factors (technical differences between the assays, influence of environmental factors) cannot be ruled out.

We [120] were the first (1995) to describe that in a group of established T1D individuals (of all ages) a veiled/dendritic APC population generated from monocytes had a poor capability of clustering with T cells and a poor capability of stimulating T cells. We hypothesized that such defect would impact tolerance induction more than the induction of effector immune forces (as is the case in the NOD mouse and BB-DP rat). Veiled/dendritic APC can

be generated from monocytes when cultured for 24 hrs. under non-adherent conditions and in the presence of anti-oxidants [61]. These veiled APC are strongly positive for MHC-II, share some markers with monocyte-derived DC (e.g. CD1a, CD83) and are excellent stimulators of T cells, yet lack markers known of classical GM-CSF/IL-4 induced monocyte-derived DC (e.g. DC-SIGN). Veiled APC are stronger IL-10 producers than IL-12 producers (certainly in comparison to classical DC) and skew T cell immune responses generated in their presence away from Th1 responses [61]. The defect in veiled/dendritic APC found in 1995 could thus indeed be instrumental in the break of T cell tolerance.

T cell stimulatory functions of monocyte-derived classical DC. Takahashi et al.. [121] extended our early observations of 1995 and showed that also GM-CSF/IL-4 induced monocyte-derived classical DC were like veiled/dendritic APC defective and had a reduced expression of CD1a, MHC-II, CD80 and CD86 and a reduced T cell stimulatory function in at risk first degree relatives of juvenile onset T1D patients as well as in the patients themselves. It must be noted that these authors cultured their DC on an ECM matrix, namely collagen (a not-common procedure).

Also Skarsvik et al.. [122] were able to show monocyte-derived DC differentiation defects in a juvenile onset T1D cohort as well as in healthy children with an HLA-risk genotype for T1D. In addition they found a decreased production of IL-12 and TNF-α from these defective DC.

We also studied in the period 2000 to 2003 the generation and function of classical monocyte-derived GM-CSF/IL4 induced DC in relatively large series of T1D and AITD patients [110]. Although we did find a lower expression of maturity markers on patient DC, differences in expression levels did barely reach statistical significance (and only in T1D patients and not in AITD patients). In these studies we did however find a reduced CD54 expression on T1D monocytes. In addition the studies showed that CD54 stimulation of monocytes of healthy individuals resulted in an accelerated differentiation of DC from these monocytes. It can thus be envisaged that DC differentiation might be defective in T1D when cells are cultured in the presence of ligands for CD54, such as an ECM matrix, as done by Takahashi et al.. (see before), but this needs further experimentation. The observations nevertheless highlight again the importance of the ECM matrix and its proteins, like FN, in the unveiling of myeloid defects in endocrine autoimmune diseases.

Vuckovic and Hart [123] did not study in vitro differentiation defects of DC from monocytes, but measured the number of myeloid DC (mDC) in the circulation and found reduced numbers particularly in young patients with juvenile onset T1D; they found however the expression of MHC-II, CD40 and CD86 normal on these circulating mDC.

In support of a view that mDC in T1D patients have an altered inflammatory set point Mollah et al.. [124] recently showed that monocytes and monocyte-derived DC of T1D patients (all ages) had an abnormal inflammatory signaling response, i.e. an altered NFκB

metabolism set point (high levels of Rel-A, Rel-B and SHP-1), making their response to LPS abnormal. A similar finding was done by Meyers et al.. [125] who found that TLR4 activation of PBMC of newly diagnosed T1D patients induced a higher frequency of IL-1β expressing monocytes, but a reduction in the percentage of IL-6 expressing monocyte-derived mDC. These reports which all found a defective generation and/or (T cell stimulatory) function of VC/DC are in contrast with a report finding a normal DC generation/function in T1D individuals: Zacher et al.. [96] found no gross differences in the generation of DC from monocytes (apart from a slightly and transient downregulation of CD1a and a slight reduction of CD80 and CD83 marker expression on mature DCs); the T cell stimulatory capability of these DC was normal.
Peng et al.. [126] found an increase in the number of circulating MHC-II positive DC in T1D patients, but this appeared due to an increase in plasmacytoid DC (pDC) and not to an increase in mDC. In accord with these findings the mixed mDC/pDC population of the T1D patients was found to secrete higher amounts of IFN-α.

5. Defects in natural regulatory T cells

The interest in $CD4^+CD25^+FOXP3^+$ T cells as a specific sub-population of naturally occurring thymus-derived regulatory T cells (Treg cells) has a historical association with the day-3-mouse thymectomy model. Day 3 neonatal thymectomy-induced organ-specific autoimmune disease (including T1D and AITD) is due to a lack of $CD4^+CD25^+FOXP3^+$ T cell migration into the periphery. These regulatory T cells typically migrate out of the thymus in this early period. Injection of purified $CD4^+CD25^+FOXP3^+$ T cells into neonatally thymectomized mice prevents the development of endocrine autoimmunity [127, 128].
NOD mouse. Several lines of evidence show that Treg cells are functionally active in pre-diabetic NOD mice. Although NOD mice harbour Treg cells capable of preventing disease progression early in life pathogenic effector T cells (Teff cells) proceed to immune mediated destruction of the β-islet cells. Controversial data exist as to whether a progressive resistance of autoreactive Teff cells to Treg cell-mediated suppression is due to defective Treg cell functions or to an accelerated decline in Treg cell frequency. Taken together present data suggest that Treg cells of NOD mice increasingly acquire a generalized fitness defect due to a lack of DC support (see before) and/or a defect in the availability of IL-2 which is required for sustaining homoiostasis and competitive fitness of Treg cells in vivo at inflammatory sites, including the peri-insulitis of the NOD. This relative IL-2 deficiency occurs at the time when the initial non-destructive peri-insulitis changes into an aggressive insulitis and is thought to be linked to a reduced production of IL-2 by the Teff cells in the insulitis. Consistently, low dose administration of IL-2 in pre-diabetic female mice resulted in enhanced frequency of Treg cells and this regimen resulted in T1D protection [129].

BB-DP rat. ART2+ T cells (formerly known as RT6+ T cells) are known for quite some time as an important thymus-derived Treg cell population in the rat. ART2+ Treg cells are important in the prevention of autoimmune diabetes/thyroiditis in the BB rat model, and BB-DP rats lack these ART2+ cells. The ART2+Treg cell deficiency in the BB-DP rat is due to a mutation in the *lyp* (Ian-5) gene [119]. There exists a sub-line of BB rats without diabetes, lacking the mutated *lyp* gene and having normal levels of ART2+ cells: The BB-Diabetes Resistant (DR) rats. ART2 depletion induces diabetes in BB-Diabetes Resistant (DR) rats, while an adoptive transfer of ART2+ cells from BB-DR rats to BB-DP rats prevents diabetes. Hillebrands et al.. [130] showed that there are two distinct potent suppressive ART2+ Treg cell populations: the CD4+ART2+CD25+FOXP3+-CD45RC-PD-1+ and the CD4+ART2+CD25-FOXP3-CD45RC-PD-1+ population. The former overlaps with the natural occurring CD4+CD25+FOXP3+ Treg cells of the mouse. Although this population is normal in number in the BB-DP rat, the cells were incapable of homeostatic expansion and survival upon transfer to nude BB rats [131]. The *lyp* gene has a strong impact on longevity of BB-DP T cells.

Patients: In humans in total 20 mutations in the FOXP3 gene have been described, which lead to the absence or malfunctioning of regulatory T cells. Patients lacking these regulatory T cells suffer from an overwhelming autoimmune syndrome IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy X-linked syndrome) [132]. This syndrome presents during the first months of life and is usually lethal within the first two years [133]. Data on Treg cell numbers and function in regular T1D and AITD patients are limited. In general normal numbers of Treg cells have been reported, but there are some indications that Treg cell function might be impaired. Co-cultures of Treg with CD4+CD25- T cells showed that diabetic Treg where 2-fold less able to inhibit proliferation of CD4+CD25- T cells as compared to Tregs from HLA-identical controls, while the cytokine profile in the co-cultures of T1D patients showed a pro-inflammatory profile (more IFN-gamma, less IL-10) [134]. Interestingly it has also been shown that there exists an enhanced apoptosis of Treg cells in recent-onset T1D, individuals at risk to develop T1D [135] and in AITD patients [136]. This enhanced apoptosis was partly due to IL-2 deprivation, which was shown to exist in the patients (see the analogy with the NOD mouse). We reported in a study on family members of AITD patients at risk to develop AITD a reduced number of CD4+ CD25high cells and a reduced level of sCD25 in serum and we took these data as suggestive for a reduction in the number of natural Treg cells [137]. In the discussion the question on the number of CD4+CD25+ FOXP3+ T regulatory cells in AITD will come back.

6. Defects in T cell apoptosis (AITCD)

T cell apoptosis is an important phenomenon in both central and peripheral tolerance induction. In the thymus the vast majority of double positive thymocytes are deleted by thymus APC (dendritic cells and epithelial cells) in the process of negative selection to get rid of erroneously developed high-affinity autoreactive T cells. In the periphery excessive immune responses are terminated by activated immune T cell death (AITCD); this process needs highly potent APC and recently activated T cells. In both apoptotic processes Fas-FasL interactions play an important role. Cross-linking of Fas by its natural ligand (FasL) leads to the recruitment of the adapter molecule, FADD and subsequently caspase-8 to the membrane. The association between FADD and caspase-8 (the death-inducing signaling complex, DISC) leads to the activation of a downstream protease cascade (caspase 10 and 7) with final activation of caspase-3 as the last step leading to apoptosis. There also exists another route of apoptosis activation involving caspase -9.

NOD mouse. In a set of experiments in which we tested the stimulatory capacity of ex vivo isolated NOD spleen and lymph node DC to stimulate syngeneic T cells we noted a prolonged response, which was accompanied by an increase in the proportion of activated memory T cells and a reduced rate of apoptosis in the cultures [117]. We interpreted these findings that AITCD was disturbed in the NOD mouse due to an intrinsic defect in T cell apoptosis. This decreased apoptosis of NOD T cells was similar to the then earlier observed resistance of NOD thymocytes and peripheral lymphocytes to the induction of apoptosis by other means, such as dexamethasone, γ-irradiation and cyclophosphamide [138], and is in accord with the observation that in vivo immune responses are enhanced and prolonged in NOD mice [139]. The defect in apoptosis segregates with various genetic loci in the NOD mouse, a.o. with genes in the region of IDD6 [140]. With regard to Fas expression a study described an impaired Fas expression and a reduced expression of caspase-8 in peripheral NOD lymphocytes displaying AITCD resistance [141]. Interestingly treatment with 1-25 $(OH)_2$ Vitamin D3 and galectin restores the apoptosis resistance defect and prevent and even cures starting diabetes in the NOD mouse.

BB-DP rat. Recent thymus lymphocyte emigrants (RTE: Thy1-ART2- lymphocytes) disappear within 7 days of export from spleen and lymph nodes due to apoptosis [142, 143], this leads to the severe peripheral lymphopenia of the BB-DP rat, particularly impacting the Treg cell compartment (ART2+ cells) with only very few cells finally expressing the mature T cell phenotype (Thy1-ART2+) [144]. The life span of RTE is extremely shortened due to the mutation in the *lyp* gene.

We have reported that thymus branched cortical macrophages of BB-DP rats were poor rescuers of developing thymocytes from apoptosis including the regulatory $ART2^+$ T cells [81]. Previous work has shown that transfers of thymus APC of BB-DR rats to BB-DP rats are partially able to restore the defects in $ART2^+$ T cells in BB-DP rats and to prevent insulitis and

diabetes [145]. Thus, it is possible that the defects at the level of the thymus of the branched cortical macrophage APC population contribute to the faulty tolerance induction of the BB-DP rat due to a heightened apoptosis of primarily Treg cells.

Patients: In T1D patients there are reports on a defective Fas expression and AITCD in peripheral T lymphocytes [146, 147]. Later it was shown [148], that a defective expression and function of caspase 3 contributed to the AITCD defect. Interestingly De Franco et al.. showed that there were differences between juvenile onset T1D and adult onset T1D regarding the defects in intracellular apoptotic pathways (either via caspase 8 or 9).

With regard to AITD a proportion of pediatric patients with Hashimoto's thyroiditis (HT) and Graves' disease (GD) display a reduced apoptosis and a decreased function of Fas on peripheral T cells, the defect involved both caspase 8 and 9 [149]. This functional defect perhaps exists in the presence of a higher Fas expression on T cells, Maruoka et al.. [150] showed that the intensity of Fas expression on peripheral T cells was disease stage dependent and was not decreased but increased in severe forms of AITD. Genetic studies support the T cell apoptosis defect in AITD, since GWAS identified ZFAT as a candidate gene for AITD: ZFAT is a critical molecule in cell survival [151]. Interestingly treatment with statins corrects the T cell apoptosis defect in HT also improving thyroid function [152].

The group of Smith [153] has opened another interesting approach to the problem: They found increased numbers of IGF-1 R positive T cells in the circulation and orbit of patients with GD. IGF-1 enhanced proliferation and inhibited Fas-mediated apoptosis of the T cells, thus implicating a role for growth factors in aggravating the T cell apoptosis defect .

Concluding hypothesis

Collectively the various aberrancies in monocyte, DC, T cell, macrophage and endocrine cell functions in animal models of spontaneous endocrine autoimmunity are highly suggestive for a basic underlying inborn defect in growth and development of myeloid, lymphoid, epithelial and mesenchymal cell precursors with a reduced proliferation, reduced apoptosis resistance, but accelerated and deviant maturation. These growth and developmental defects would in such view result in

1. Imbalances between various T cell tolerogenic and autoimmunization differentiation routes, i.e.
    a) T cell apoptosis defects leading to abnormal negative selection in the thymus,
    b) A defective generation of natural Treg cells in the thymus (e.g. due to an enhanced apoptosis of RTE),
    c) A fitness defect of Treg cells (due to a lack of growth stimuli, e.g. IL-2) and
    d) A defective AITCD (apoptosis defect)

2. An enhanced, but deviant differentiation and maturation of myeloid cells.
This results in
   e) A mature (partly inflammatory) set point of circulating monocytes with an enhanced adhesiveness to ECM,
   f) An accelerated maturation of DC, leading to an intrinsic high inflammatory set point, but with a poor T cell stimulatory capacity (depending on the availability of growth factors). Such DC are poor stimulators of AITCD and natural Treg cells.
3. An abnormal parenchyma and extracellular matrix (ECM) compartment in the target gland prior to the autoimmune process. DC generated in the tissues from infiltrated monocytes will acquire an even higher pro-inflammatory state due to an abnormal interaction with the abnormal ECM (high FN content).
Such pro-inflammatory DC (with a disturbed IL-12/IL10 ratio) primarily stimulate Th1 pathways, tipping the already disturbed T cell balance to overt autosensitisation.

Abnormalities of monocytes, macrophages, dendritic cells and t cells in schizophrenia and bipolar disorder

There are limited data on abnormalities of monocytes, macrophages, dendritic cells and T cells in schizophrenia and bipolar disorder.

Numbers of peripheral monocytes and expression of surface markers

There are early reports showing that the number of circulating monocytes is aberrant in schizophrenia. Rothermundt et al.. [154] reported a slight increase in the mean absolute and relative monocyte counts. Zorrilla et al.. [155] and Falcone et al.. [156] supported these observations showing a monocytosis and a higher number of CD14+ cells in respectively non-medicated schizophrenia patients and in children with new-onset psychosis (psychosis NOS, schizophreniform disorder and schizo-affective disorder). Another study however refuted this monocytosis, be it on a small sample of patients [157]. In the cerebrospinal fluid of patients with schizophrenia, monocytes and macrophages show an accumulation during acute psychotic episodes [158]. With regard to adhesion molecule expression Theodoropoulou et al.. [159] showed that there is an increased percentage of circulating peripheral blood mononuclear cells (PBMC) expressing ICAM-1 in patients with schizophrenia, but the authors did not make a distinction between circulating lymphocytes and monocytes. Nevertheless their observation supports an activated state of immune cells in the circulation of patients with schizophrenia, facilitating endothelial transmigration of the cells.

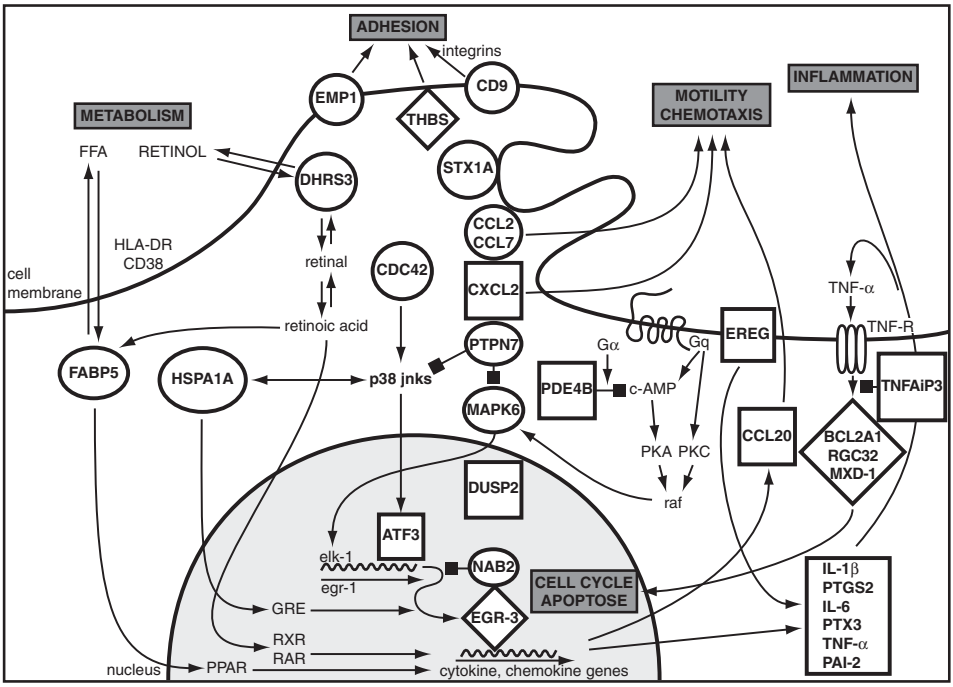
For bipolar disorder there are no reports on aberrant absolute or relative monocyte counts. We did measure CCR2 expression on monocytes of patients with bipolar disorder [50]. CCR2 is mainly expressed on the mature $CD14^{+}CD16^{+}$ set of monocytes (the counterpart of the mature Ly-$6C^{low}$ monocytes of the mouse). Expression of CCR2 was not significantly different between monocytes of BD patients and healthy controls, nor were there differences between the number of mature ($CD14^{+}CD16^{+}$) and immature ($CD14^{+}CD16^{neg}$) circulating monocytes in BD patients.

Abnormal inflammatory gene expression

We [50] previously reported an aberrant expression of pro-inflammatory genes in monocytes of bipolar patients and of offspring of bipolar patients, at risk to develop the disease. To find the genes we performed Affymetrix analyses on pools of purified monocytes of bipolar patients and matched healthy controls to search for aberrantly expressed genes. We validated the identified molecules using quantitative real time PCR (RQ-PCR) on monocytes of 42 individual bipolar patients and 54 children of a bipolar parent, of whom 13 had and 3 developed a mood disorder within 2-3 years after blood collection. We detected an aberrant coherent layout of 21 genes for inflammatory (PDE4B, IL-1β, IL-6, TNF-α, PTGS2, PTX3, FcαR), chemokinesis/motility (CCL2, CCL7, CCL20, CXCL2, CCR2, CDC42), cell survival/apoptosis (A10 or TNFAIP3, BCL2A1, EMP1) and MAP kinases pathway (MAPK6, Pac-1 / or DUSP2, NAB2, ATF3, HePTP) molecules in the circulating monocytes of bipolar patients and children of a bipolar patient with or later developing a major mood disorder. These 21 molecules were all mutually strongly correlating forming a monocyte "mRNA signature". However, careful analysis detected within this signature 2 sub-sets of genes which in particular mutually correlated: an inflammatory subset 1 of 13 genes (ATF3, DUSP2, PDE4B, IL-1β, IL-6, TNF-α, PTGS2, PTX3, FCαR, CCL20, CXCL2, TNFAIP3, BCL2A1) and a adhesion/motility/ chemotaxis subset 2 of 9 genes (EMP1, CCL2, CCL7, CDC42, MAPK6, NAB2, HePTP (Figure 6).

We also determined the contribution of genetic and environmental influences on the association monocyte pro-inflammatory state – bipolar disorder and carried out a signature study on monozygotic and dizygotic concordant and discordant twin pairs [160] From this study we concluded that the association of the monocyte pro-inflammatory state with BD is primarily the result of a common shared environment, yet for some of the sub-cluster 2 genes a genetic influence could predominate.

Figure 6. A hypothetical scheme of the interaction between the various fingerprint genes.



Squared genes: cluster 1a genes; genes in diamonds: cluster 1b genes; encircled genes: cluster 2 genes.

Numbers of peripheral T cells and T cell subsets

Numerous studies have been performed on the number of $CD3^+$ T cells, the $CD4^+$ and $CD8^+$ subsets, the B cells and the NK cells in schizophrenia and bipolar disorder (Table I). In essence it has been found that these lymphocyte subsets are normal in number [50, 154, 155, 157, 159, 161-171]; however there might be some indication that $CD8^+$ T cells, particularly CD45RA cells are increased in schizophrenia [172].

The limited studies carried out on T cells positive for CD25 (low, intermediate and high positive; these populations are generally considered as a sign of T cell activation/proliferation) show that these cells are probably increased in the circulation of SCZ and BD patients (in the latter particularly in the manic phase). However it is not known whether we are dealing here with an activation/proliferation of the T effector or the T regulatory population (Figure 7). For that, intracellular FACS staining of $CD4^+$T cells for IFN-γ (Th1), IL-4 (Th2) and FOXP3 (natural T regulatory cells) should be undertaken, and such studies are reported in Chapters 5 and 7.

Table I

		SCHIZOPHRENIA		BIPOLAR DISORDER	
Monocytes	- number - (CD14)	(↓) ↑↑ [154, 155, 161]	↑	↓ = [50, 161]	n.c.
T cells	CD3	↓↓ = = = = = ↑ [154, 159, 162-166, 168]	=	= = [167, 169]	(=)
	CD4	↓↓ = = = ↑↑ [159, 161, 162, 164-166, 168]	=	= ↑ [161, 169]	n.c.
	CD8	= = = ↑(↑) [159, 164, 165, 168, 171]	=↑	= [169]	(=)
	CD45 RA	↑↑ [165, 168]	↑		
	CD45 RO	(↓) [168]	n.c.		
	CD25	= ↑↑ [154, 159, 168]	n.c.		
B cells	CD19	↓ = = [161, 163, 165]	n.c.	= ↑ [161, 169]	n.c.
NK cells	CD56	↓ = [161, 164]	n.c.	= = [161, 169]	=

Synopsis of the literature regarding the percentages of circulating immune cells in schizophrenia and bipolar disorder. References are indicated between brackets. ↓: reference indicates a downregulation, ↑: reference indicates an upregulation, =: reference indicates a normal level, (↓); downregulated but low number of included patients. The summary columns reflect our conclusions based on this literature; we have concluded to raised numbers of monocytes in schizophrenia because the cumulative index of 3 studies shows upregulation (admitted there are too few studies), we have concluded to normal-to-putatively raised numbers of CD8+ T cells in schizophrenia studies because the cumulative index of 5 studies shows some weak upregulation and to normal numbers of CD3 and CD4 T cell, because the cumulative index of 8 studies shows normal numbers of cells. **n.c.** means not conclusive due to low number of studies and/or clearly conflicting outcomes.

Cytokines of the MPS and T cell system

Most of the available literature in psychiatric patients does not concentrate on the cells of the MPS or the T cells, but on levels of MPS- and T cell-derived cytokines. Table II gives a synopsis of the present literature on the serum levels of cytokines and chemokines related to the MPS and T cell system in SCZ and BD patients. It is however important to note that cytokine levels in serum or plasma are strongly confounded by a number of conditions, such as age, gender, lifestyle, metabolic syndrome, visceral obesity, diet, smoking and medication. These factors have to be taken into account when studying the effect of disease states, here psychiatric conditions. In doing so, the present literature indicates

1) That s-ICAM and endothelin-1 are reduced in 2 out of 2 studies in SCZ, suggesting a reduced activation of the endothelial system [173, 174]. However preliminary data of our group show an increased level of s-ICAM in SCZ patients (Beumer et al.. to be published), while there are also unpublished data on raised levels of endothelin-1 in BD patients (S Bahn, personal communication). Therefore further studies on endothelial factors are needed before firm conclusions on the state of endothelial activation in SCZ and BD can be drawn.
2) That studies on CCL2 levels are too few (n=1) and involve too few patients to draw any conclusion [49, 50, 175]. We will present in this thesis (Chapter 4), an additional CCL2 study on SCZ patients (n = 145).
3) That the IL-1, IL-6 and TNF cytokine networks are activated in SCZ and BD, be it inconsistently since not all studies were able to find the activation [49, 50, 159, 176-200]. Activation was found in general (taking SCZ and BD together) in 28 out of 52 studies (none showed reduced levels), pointing to an inconsistent pro-inflammatory activity of the MPS and/or the endothelial system in SCZ and BD patients. In many of the positive studies various confounding factors such as medication, age, gender and life-style have been taken into account, which makes these observations reasonably solid.
4) That the IL-2 system is probably activated in SCZ. Increased serum levels for the IL-2 system were found in 4 out of 10 studies (note 2 studies showed reduced levels) [159, 179-182, 186-188, 191, 193]. Medication did not influence levels of IL-2. It is also our experience that the IL-2 system is activated in SCZ, but independently from the IL-1, IL-6 and TNF cytokine networks, suggesting that the MPS/endothelial system is activated by separate mechanisms than the T cell system (see Chapter 3).
In BD there are fewer reports (n=3) on the IL-2 system, but 2 indicate an activated T cell system particularly in periods of mania, including our study [183, 195, 197]. In our study sIL-2R levels were raised in particular during a manic episode, but also (be it less) during euthymic and depressive episodes [169].
5) That there are too few reports and too many with contradictory results on an activation or non-balance of the IL-12, IFN-γ and IL-4 systems and thus insufficient indications for an activation of either the Th1 and Th2 effector systems in SCZ and BD [183, 186, 188, 192, 197, 201-204]. Studies on the Th17 system are completely lacking. Chapters 5 and 7 will address these issues.

Table II

		SCHIZOPHRENIA		BIPOLAR DISORDER	
Endothelium	ICAM	↓ ↓ ↑ [173, 174]	**n.c.**		
	Endothelin			↑	**n.c.**
Monocytes	CCL2	= ↑ [49, 175]	**n.c.**	= [50]	**n.c.**
	IL-1	= = = = = ↑ ↑ ↑ ↑ [49, 159, 176-182]	↑	= ↑ [50, 183]	**(↑)**
	IL-6	= = = = = = = = = ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ [49, 177-180, 182, 184-194]	↑	= = ↑ ↑ ↑ ↑ [50, 183, 195-198]	↑
	TNF	= = = = = = ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ [49, 159, 176, 178, 180, 182, 186, 188, 191, 192, 194, 199, 200]	↑	= ↑ ↑ ↑ [183, 196-198]	↑
T cells	IL-2	↓ ↓ = = = = ↑ ↑ ↑ ↑ [159, 179-182, 186-188, 191, 193]	**(↑)**	↓ ↑ ↑ [183, 195, 197]	**n.c.**
	Th1: IL-12	= ↑ [203, 204]	**n.c.**	= [204]	**n.c.**
	Th1: IFNγ	= ↑ [186, 202]	**n.c.**	= ↑ [197, 201]	**n.c.**
	Th2: IL-4	↓ ↓ = = [186, 188, 192, 202]	**n.c.**	= ↑ ↑ [183, 197, 201]	**n.c.**

Synopsis of the literature regarding the serum levels of indicated cytokines and their prime sources in schizophrenia and bipolar disorder. References are indicated between brackets. ↓: reference indicates a downregulation, ↑: reference indicates an upregulation, =: reference indicates a normal level. The summary columns reflect our conclusions based on this literature; we have concluded to upregulation of IL-1, Il-6 and TNF on the basis of sufficient studies of which about half show upregulation, whereas none showed a downregulation, we have concluded to IL-2 as putatively upregulated in schizophrenia because there are sufficient studies of which the cumulative index shows some upregulation, **n.c.** means not conclusive due to low number of studies and/or clearly conflicting outcomes

Conclusion

Studies on monocytes, macrophages, T cells and cytokine networks, taking into consideration the various confounding factors as described, indicate an activated pro-inflammatory state of the immune system in both SCZ and BD, particularly of the MPS and the T cell system. Measurements of serum cytokines of the MPS and T cells system are not consistent and precise enough to detect this activation in an individual patient or in small groups of patients. Gene expression studies in immune cells and numeric determinations of the various sub-types of T cells hold a promise here.

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Chapter 2

Experimental Questions

Chapter II. Experimental questions

Do patients with schizophrenia and AITD have a pro-inflammatory monocyte gene expression signature and is there a difference or overlap with the previously found monocyte pro-inflammatory gene expression signature in patients with bipolar disorder?

In a previous study Padmos et al found a pro-inflammatory gene expression signature in the monocytes of patients with bipolar disorder [1] and in the monocytes of T1D patients (in particular LADA patients). We extended these studies to patients with schizophrenia and AITD and first searched for "schizophrenia-related and AITD-related monocyte genes" via Affymetrix analysis on pools of monocytes of patients with schizophrenia and AITD. Genes which were more than three times higher or lower expressed and had a known function in inflammation or inflammation-related processes were chosen for further verification using Q-PCR together with the genes already found aberrant in patients with bipolar disorder and T1D. This verification was performed on a set of patients with bipolar disorder, a set of patients with an acute form of recent onset schizophrenia and on a set of patients with AITD. The expression levels of the different genes were compared between the various disorders and a cluster analysis was performed to group the various genes (See Chapter 3 and 6).

Do monozygotic discordant co-twins of an index twin with juvenile-onset T1D have the same pro-inflammatory monocyte gene expression signature?

Padmos et al previously described that monocytes of 43% of patients with juvenile onset T1D have monocytes lacking the gene overexpression of part of the signature (i.e. lacking cluster 1 containing most of the pro-inflammatory cytokines and compounds), but with the remaining part intact (i.e. cluster 2 containing predominantly adhesion and motility factors and chemokines, like CCL2 and CCL7) [2]. In the here given study we investigated the presence of the pro-inflammatory mRNA gene expression signature in the monocytes in monozygotic co-twins of index twins with a juvenile onset T1D, who were discordant for T1D for already for many years. We aimed at detecting putative differences in inflammatory gene expression between the twins explaining the discordance despite monozygosity (Chapter 8).

Do patients with bipolar disorder and schizophrenia have aberrancies in their Th1, Th2, Th17 and natural T regulator cell compartment?

As stated in the introduction, there is evidence for the involvement of the T cell system in the pathogenesis of schizophrenia, bipolar disorder and AITD. Therefore, we investigated the frequency of Th1, Th2, Th17 and natural T regulator cells in the circulation of patients

with schizophrenia, bipolar disorder and AITD using detailed FACS analysis. We related T cell outcomes to the pro-inflammatory state of the monocytes (i.e. the presence of the pro-inflammatory mRNA gene expression signature) and in the bipolar patients with the presence of TPO-antibodies in the same patients (Chapter 5 and 7).

Is an altered inflammatory setpoint of the monocytes and T cells in patients with bipolar disorder, schizophrenia and AITD reflected in higher serum levels of pro-inflammatory and T cell cytokines/chemokines and how are these levels related in patients with chronic forms of schizophrenia to the presence of the metabolic syndrome, a common co-morbidity of chronic schizophrenia?

Cytokine levels were investigated in the serum of patients with bipolar disorder, recent-onset acute schizophrenia and AITD using a cytometric bead array and serum levels of cytokines were correlated with the monocyte gene expression signatures and the T cell state in the same patients (Chapters 4, 5, 6, 7).

The incidence of the metabolic syndrome is higher in patients with chronic schizophrenia and the question is whether this is due to the disease itself or due to the use of atypical antipsychotic medication. One of the traits of the metabolic syndrome is visceral obesity and the adipocytes in the white adipose tissue are able to produce several pro-inflammatory cytokines and chemokines which play a role in insulin resistance. Moreover low HDL (another trait of the metabolic syndrome) negatively impacts the inflammatory state of the MPS. In the study reported in Chapter 4 we measured serum levels of pro-inflammatory cytokines and chemokines in a group of 145 patients with naturalistically treated chronic schizophrenia and correlated the levels to the presence and traits of the metabolic syndrome.

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Chapter 3

Inflammatory gene expression in monocytes of patients with schizophrenia: Overlap and difference with bipolar disorder.

A study in naturalistically treated patients

Short title: Monocyte fingerprint in psychiatric disorders

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Abstract

Accumulating evidence indicates an activated inflammatory response system as a vulnerability factor for schizophrenia (SZ) and bipolar disorder (BD).

We aimed to detect a specific inflammatory monocyte gene expression signature in SZ and compare such signature with our recently described inflammatory monocyte gene signature in BD.

A quantitative-PCR case-control gene expression study was carried out on monocytes of 27 SZ patients and compared to outcomes collected in 56 BD patients (all patients naturalistically treated). For Q-PCR we used 9 "SZ specific genes" (found in whole genome analysis), the 19 BD signature genes (previously found by us) and 6 recently described autoimmune diabetes inflammatory monocyte genes.

Monocytes of SZ patients had (similar to those of BD patients) a high inflammatory set point composed of three sub-sets of strongly correlating genes characterized by different sets of transcription / MAPK regulating factors:

1) Sub-set 1A, characterized by ATF3 and DUSP2, and sub-set 1B, characterized by EGR3 and MXD1, were shared between BD and SZ patients (up-regulated in 67 and 51% and 34 and 41% respectively).
2) Sub-set 2, characterized by PTPN7 and NAB2 was *up-regulated* in the monocytes of 62% BD, but *down-regulated* in the monocytes of 48% of SZ patients.

Our approach shows that monocytes of SZ and BD patients overlap, but also differ in inflammatory gene expression. Our approach opens new avenues for nosological classifications of psychoses based on the inflammatory state of patients, enabling selection of those patients who might benefit from an anti-inflammatory treatment.

Key words: schizophrenia, bipolar disorder, inflammation, monocytes, Kraepelinian dichotomy.

Introduction

We [1] recently described a sensitive quantitative PCR (Q-PCR) assay system to detect the pro-inflammatory state of circulating monocytes of naturastically treated patients with bipolar disorder (BD patients) and detected in the monocytes a coherent, mutually-correlating set of 19 aberrantly expressed inflammatory genes ("a gene signature or fingerprint"), supporting the concept of an activated inflammatory response system (IRS) in mood disorders [2].

Since the concept of an activated IRS also extends to schizophrenia [2], we hypothesized that the same or a similar abnormal inflammatory gene fingerprint could also be found in monocytes of patients with schizophrenia (SZ patients) and we decided to test for the 19 aberrantly expressed "bipolar signature genes" in the circulating monocytes of naturalistically treated SZ patients. In addition, we searched for new "schizophrenia inflammatory genes" using Affymetrix whole genome expression profiling on monocytes of SZ patients and selected those genes which were markedly aberrantly expressed and clearly involved in inflammation (yielding 15 "new" genes, for details see Results).

Autoimmune diabetes, thyroiditis and gastritis are about three times more prevalent in BD [3], whereas autoimmune thyrotoxicosis and Sjogren's disease are more prevalent in SZ [4]. Given our recently reported overlap in monocyte gene expression signatures between BD and autoimmune diabetes, we additionally included in our analysis 6 "specific autoimmune diabetes signature monocyte genes [5]" to be complete.

Thus, using Q-PCR, we validated for this report the abnormal expression of in total 34 monocyte activation genes in 27 patients with schizophrenia (compared to monocytes of 32 age/gender matched healthy controls) and 56 patients with bipolar disorder (42 patients of the reported series [1] *plus* 14 new cases, altogether compared to monocytes of 48 age/gender matched healthy controls).

Patients and Methods

Patients with Schizophrenia:

Patients with schizophrenia were diagnosed according to the DSM-IV criteria and recruited at the department of Psychiatry of the Erasmus Medical Center in Rotterdam. All patients were in-patients. Patients were diagnosed with schizophrenia according to the DSM-IV criteria after a Comprehensive Assesment of Symptoms and History (CASH) interview [6] and by consensus between two senior psychiatrists who were blinded to the results. For patients with symptoms for less than 6 months, a final diagnosis was made after 6 months to comply with the DSM-IV criterium. All patients were acutely psychotic.

The SZ patients were virtually all recent onset cases and had a median duration of illness of only 0.3 yrs (median, range 0-3 yrs). All cases did not suffer from another severe medical illness (including infections and allergies), verified with a medical history assessment and routinely laboratory testing (Hb, Ht, leukocyte count, blood smear and kindney/liver function) on admission.

For Affymetrix micro array analysis (searching for aberrantly expressed genes in monocytes) two monocyte pools of schizophrenia patients were used. Each pool was compared to a monocyte pool of age- and gender-matched HC (pools were used for minimizing inter-individual differences in mRNA expression and to reduce costs for this expensive methodology): Patient pool 1 consisted of 4 male cases, ages 22, 26, 27 and 20 yrs, patient pool 2 consisted of three male cases, ages 17, 19 and 27 yrs. Pool 1 and 2 were compared to 2 healthy control pools of 2X2 males, ages between 22 and 26 yrs.

For Q-PCR (verifying the found genes) 27 additional SZ patients diagnosed according to the DSM-IV criteria were recruited at the department of Psychiatry of the Erasmus Medical Center in Rotterdam.

All but one of the patients received antipsychotic medication at the time of blood withdrawal; none of the patients were drug naïve. The demographics, duration of illness and drug usage, of the patients used in Q-PCR are summarized in Table I.

For the Q-PCR on SZ patients we used a control group of 32 healthy individuals (HC), who were age/gender matched to the SZ patients. These were recruited from enrolling laboratory staff, medical staff and medical students (Table I). The inclusion criteria for the HC were an absence of any psychiatric and autoimmune disorder and an absent history of these disorders in first-degree family members. HC had to be in self-proclaimed good health and free of any obvious medical illness for at least two weeks prior to the blood withdrawal, including acute infections and allergic reactions.

Table I. Characteristics of schizophrenia and bipolar patients and their respective healthy controls used for Q-PCR.

	Schizophrenia		Matched Healthy Controls
Group size	27		32
Age (years)[1]	27	(17-59)	27 (21-47)
Gender			
Male	22	(81%)	25 (78%)
Female	5	(19%)	7 (22%)
Duration illness (years)[1]	0.3	(0-3)	
Age of onset[1]	26	(17-58)	
Medication			
Typical antipsychotics	10	(37%)	
Atypical antipsychotics	15	(56%)	
Other	1	(4%)	
None	1	(4%)	

	Bipolar Disorder [2,3]		Matched Healthy Controls
Group Size	56		48
Dutch twin study	37		
Dutch site SFBN	19		
Age (years)[1]	42	(26-61)	42 (23-57)
Gender			
Male	22	(39%)	20 (42%)
Female	34	(61%)	28 (58%)
Duration illness[1]	16	(3.5-40)	
Age of onset[1]	26	(6-49)	
Medication			
Lithium	32		
Antipsychotics	8		
Antipsychotics and lithium	5		
Other	11		

[1]Mean (range)
[2] The bipolar patients did not have a history of drug or alcohol dependency for at least six months; this was not known for the schizophrenic patients.
[3] Data on 42 of these 56 patients have been given before in Padmos et al (Padmos et al. 2008a)

Patients with Bipolar Disorder:

In total, 56 outpatients with DSM-IV bipolar I or II disorder were recruited from two studies, i.e. the Dutch site of the former Stanley Foundation Bipolar Network (SFBN), an international multi-center research program described elsewhere in detail [7] (n=19 patients) and from an ongoing Dutch twin study on bipolar disorder described in detail in Vonk et al [8] (n=37). Characteristics of BD patients are given in Table I. Diagnosis was also made by means of the SCID. Present mood state was evaluated via the Young Mania Rating Scale (YMRS) and the Inventory for Depressive Symptomatology (IDS). The BD patients did not have another severe medical illness, verified with a medical history assessment.

Since age and gender differed between our BD and SZ patients (Table I) we compared outcomes of the bipolar group to those of an extra group of 48 HC, who were age/gender matched to the BD patients (Table I). For inclusion criteria of the HC see above.

The Medical Ethical Review Committee of the University Medical Center Utrecht (bipolar patients) and the Medical Ethical Review Committee of the ErasmusMC Rotterdam (schizophrenia patients) had approved the studies. Written informed consent was obtained from all participants after a complete description of the study had been given.

Laboratory methods

Blood collection and preparation

Blood (drawn in the morning hours) was collected in clotting tubes for serum preparation (stored at -80°C) and in sodium-heparin tubes for immune cell preparation. From the heparinized blood, peripheral blood mononuclear cell (PBMC) suspensions were prepared in the afternoon by low-density gradient centrifugation, as described in detail before [9], within 8 hours to avoid activation of the monocytes (erythrophagy). PBMCs were frozen in 10% dimethylsulfoxide and stored in liquid nitrogen. This enabled us to test patient and control immune cells in the same series of experiments later.

Isolation of monocytes

CD14 positive monocytes were isolated from frozen PBMCs by magnetic cell sorting system (Miltenyi Biotec) The purity of monocytes was > 95% (determined by morphological screening after trypan blue staining and FACS). As reported elsewhere positive versus negative selection of immune cells did not influence gene expression profiles [10].

Affymetrix whole genome gene expression profiling

RNA was isolated from purified monocytes using RNAeasy columns as described by the manufacturer (Qiagen) and previously [11]. Fragmented cRNA was hybridized to U95Av2 microarrays (Affymetrix, manufacturer's protocol). For all experiments, the 5' / 3' ratios of GAPDH were 2 or less (usually 0.9 – 1.1).

Quantitative –PCR (Q-PCR)

RNA was isolated from monocytes as described above. To obtain cDNA for Q-PCR, one μg RNA was reversed-transcribed using the cDNA high capacity cDNA reverse Transcription kit (Applied Biosystems).Q-PCR was performed as previously described in detail [11] and in the legend of Table II.

Statistics

Scanned micro array images were analyzed using Affymetrix Microarray Suite 4.2 software. Further analysis was performed using RMA software, modification de Ridder [12] and Ingenuity Systems (www.ingenuity.com) software. Statistical analysis was performed using the SPSS 15.0 package for Windows. Data were tested for normal distribution using the Kolmogorov-Smirnov test. Depending on the distribution pattern and the total number of subjects, parametric (normal distribution and ≥ 50 subjects) or non-parametric tests (skewed distribution or <50 subjects) were used. Bonferroni correction for multiple testing was used for the Affymetrix data (since this was a non-hypothesis driven approach). Correction for multiple testing was not used for the analysis of the Q-PCR data, because we focused in on the effect of specific genes found in Affymetrix analysis.. The specific tests used are mentioned under tables and in legends.

Table II. Q-PCR analysis of monocytes of bipolar patients (n=56, 42 from a previous study (Padmos et al. 2008a) plus 14 new cases) and schizophrenia patients (n=27) as compared to healthy control values (HC SCZ: n=32; HC BD n=48), set at 1 fold.

	Schizophrenia		Bipolar Disorder	
Genes selected by whole genome screening in this study in schizophrenia				
EGR3	**5,36**[1]	**< 0,01**[3]	2,52	0,16
MXD1	**1,49**	**< 0,01**	1,43	0,06
MAFF	**5,10**	**< 0,01**	**2,95**	**0,01**
F3	**5,56**	**< 0,01**	**1,87**	**0,02**
SERPINB2	**1,84**	**< 0,01**	1,10	0,06
THBS	**4,31**	**< 0,01**	**2,02**	**0,05**
EREG	**7,36**	**< 0,01**	**2,31**	**< 0,01**
CXCL3	**3,99**	**< 0,01**	**3,33**	**< 0,01**
RGC32	0,85[2]	0,07	**2,61**	**< 0,01**
Genes selected in a previous study on bipolar patients (Padmos et al. 2008a)				
DUSP2	**5,36**	**< 0,01**	**4,96**	**< 0,01**
ATF3	**3,50**	**< 0,01**	**3,55**	**< 0,01**
MAPK6	**1,21**	**< 0,01**	**1,80**	**< 0,01**
PDE4B	**3,91**	**< 0,01**	**3,00**	**< 0,01**

	Schizophrenia		Bipolar Disorder	
IL6	**7,89**	**< 0,01**	**5,39**	**< 0,01**
IL1B	**9,20**	**< 0,01**	**6,45**	**< 0,01**
TNF	**3,91**	**< 0,01**	**1,87**	**< 0,01**
TNFAIP3	**3,22**	**< 0,01**	**2,31**	**< 0,01**
BCL2A1	**2,39**	**< 0,01**	**3,30**	**< 0,01**
PTX3	**2,51**	**< 0,01**	**2,63**	**< 0,01**
PTGS2	**4,34**	**< 0,01**	**3,20**	**< 0,01**
CCL7	**1,12**	**< 0,01**	**8,47**	**< 0,01**
CDC42	**1,49**	**< 0,01**	**1,99**	**< 0,01**
CCL20	**23,53**	**< 0,01**	**10,63**	**< 0,01**
CXCL2	**3,76**	**< 0,01**	**5,31**	**< 0,01**
CCL2	**1,60**	**< 0,01**	**3,83**	**< 0,01**
CCR2	0,85	0,53	0,62	0,10
NAB2	0,76	0,21	**2,58**	**< 0,01**
EMP1	0,97	0,88	**2,19**	**< 0,01**
Genes selected in previous study on autoimmune diabetes (Padmos et al. 2008b)				
PTPN7	**0,78**	**0,04**	**2,04**	**< 0,01**
CD9	1,49	0,59	**2,16**	**< 0,01**
STX-1A	0,64	0,22	**3,04**	**< 0,01**
DHRS3	1,08	0,51	1,87	0,08
FABP5	1,08	0,75	1,21	0,09
HSPA1A	1,06	0,93	0,79	0,40

Q-PCR was performed with Taqman Universal PCR mastermix (Applied Biosystems, Foster City, CA). All Taqman probes and consensus primers were pre-formulated and designed by Applied Biosystems (Assays on Demand, see supplementary Table 1). PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and finally 1 min at 60°C. PCR amplification of the reference gene *ABL* was performed for each sample to allow normalization between the samples. *ABL* was chosen as a reference gene because it was previously shown that *ABL* was the most consistently expressed reference gene in haematopoietic cells (Beillard et al. 2003). The quantitative value obtained from Q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from normalized CT values (CT gene- CT housekeeping gene), by the ΔΔCT method ($2^{-\Delta\Delta CT}$, User Bulletin 2, Applied Biosystems, Foster City, California). To correct for inter-assay variance, we set the mean of the studied genes found in the healthy control groups in the same assay for each gene to 1 ($\sum\Delta CT$ HC= 0, 2^{-0}=1). The fold change values of the genes in patient monocytes were expressed relative to this set mean of 1.

Data are expressed relative to this HC value. HC SCZ: n = 32; HC BD: n = 48

[1] Values >1: patients have a higher expression than control group.

[2] Values<1: patients have a lower expression than control group.

[3] P tested by univariate ANCOVA vs. control subjects; age and gender are included in this model.

Purity of monocytes was over 90% (as determined by morphology on each sample) and over 92% as determined by FACS analysis. Yield of monocytes was 28% (±10%) in the patient groups group and 21% (±8%) for the HC group (n.s.) of the Ficoll-isolated peripheral blood mononuclear cells.

The ΔCT values are available in the Supplementary Table I

Results

Whole genome expression profiling of potential inflammatory biomarker genes in monocytes of schizophrenia patients

Affymetrix micro array analysis was performed to search for aberrantly expressed genes in monocytes on two monocyte pools of naturalistically treated SZ patients. Each pool was compared to a monocyte pool of age- and gender-matched HC (pools were used for minimizing inter-individual differences in mRNA expression and to reduce costs for this expensive methodology): All raw data obtained by Affymetrix analysis are available as MIAMExpress submission (http://www.ebi.ac.uk/miamexpress/).

We analyzed the data using a modified RMA analysis [12] and considered for Ingenuity analysis genes which were more than 2 fold statistically differentially expressed ($p < 0.01$, corrected for multiple testing) between SZ patients and HC. This resulted in 298 discriminating genes (185 up regulated and 113 down regulated). Major pathways found in Ingenuity analysis were pathways involved in inflammatory and immune mediated disease. To select for genes which could serve as potential biomarkers for the "schizophrenia inflammatory condition", we took the top genes from the up and down list, which were statistically more than 3.5 fold significantly differentially expressed between SZ and HC with the purpose of only identifying strongly discriminating genes. This resulted in 22 over expressed genes. None of the genes was more than 3.5 fold lower expressed (the first gene of the list of the lower expressed genes was CCR2, which was 2.9 fold lower expressed, but this gene had already been selected in our previous bipolar study [1]). Because we were searching for regulators and biomarkers of inflammation, out of these 22 aberrantly expressed genes we selected only genes clearly involved in inflammation. This resulted in 14 selected aberrantly expressed genes for SZ, and it is of note that five of these genes had previously been found over expressed in bipolar patients, i.e. PDE4B, IL1, PTGS2/COX2, CCL20 and CXCL2 [1], pointing to a strong overlap of inflammatory set points between monocytes of bipolar disorder and schizophrenia. In sum, 9 new "schizophrenia specific" up-regulated genes (MXD1, F3, MAFF, EGR3, THBS, SERPINB2/PAI-2, RGC32, EREG and CXCL3) were finally selected and we included these 9 new genes together with the 19 aberrantly expressed "bipolar signature genes" and the 6 "autoimmune diabetes signature genes" in the validating Q-PCR analysis of the 27 SZ patients, 56 BD patients (42 patients of the previous reported study of Padmos [1] plus 14 new cases) and their 32 and 48 matched healthy controls respectively.

RQ-PCR analysis of monocytes of schizophrenia and bipolar patients.

Table II (and Supplementary Table I) show that of the 34 genes tested, the mRNA expression levels of 25 genes were significantly different ($p < 0.05$ by ANCOVA, corrected for age and gender) in the monocytes of the 27 SZ patients as compared to HC, while in the monocytes of the 56 BD patients 27 genes were significantly different expressed. Data obtained in RQ-PCR on the mRNA expression levels of the various genes in the patient groups correlated very strongly to those obtained in the above-described Affymetrix analysis (SZ: $r = 0.708$, BD: $r = 0.663$, Spearman's Rho).

There were no differences in mRNA gene expression for the different groups of healthy controls (Supplementary Table I).

The aberrantly expressed genes were mostly shared between the 2 disorders but also in part not shared. Of the 9 "schizophrenia specific" genes, 8 were confirmed in RQ-PCR in schizophrenia (RGC32 appeared not to be higher expressed); while in bipolar patients 5 of these genes were significantly higher expressed (MAFF, F3, EREG, CXCL3 and RGC32).

Of the 19 "bipolar signature genes" 16 were statistically significant up-regulated in schizophrenia, while for 3 genes statistical significance was not reached (CCR2, NAB2 and EMP1). Confirming our previous data, we found almost all 19 "bipolar signature genes" (apart from CCR2) statistically significant over expressed in this extended set of bipolar patients.

Of the 6 "autoimmune diabetes signature genes", 5 were not aberrantly expressed in schizophrenia, while PTPN7 was statistically significantly down-regulated. Interestingly, in the bipolar sample 3 of the 6 genes were up regulated, including PTPN7.

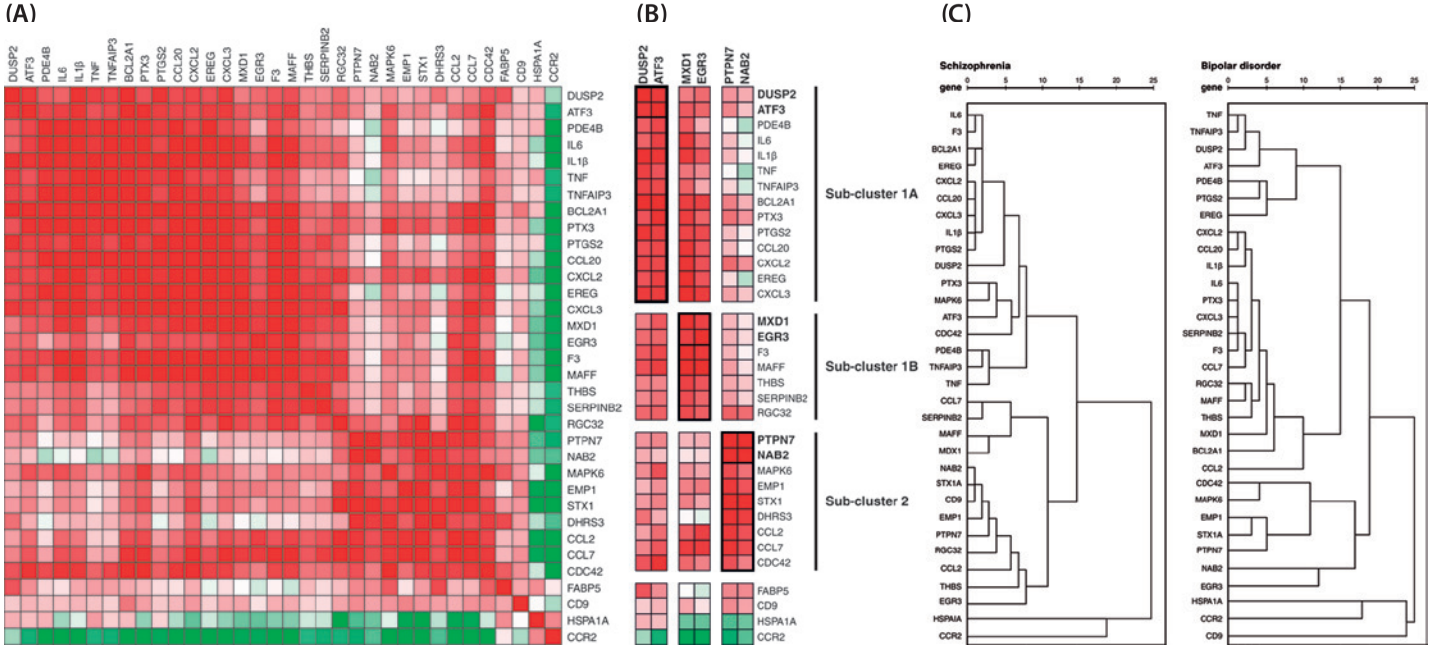
Cluster analysis and identification of sub-clusters in the pro-inflammatory signature

To study their mutually interdependent state in expression, we subsequently performed a cluster analysis on the RQ-PCR data. The heat map and dendrograms of this analysis are given in Fig 1a. In sum, expression levels of virtually all genes correlated to each other, yet two sub-clusters of mutually very strongly correlating genes (correlation coefficient higher than 0.60) could clearly be identified (two major red blocks in the figure), each predominantly correlating to a different set of transcription/MAPK regulating factors, i.e. ATF3/DUSP2 and PTPN7/NAB2 respectively. These two sets of transcription factors were mutually, but not strongly, correlating (Fig 1b).

The first sub-cluster correlating to ATF3 and DUSP2 consisted predominantly of various well-known inflammatory compounds, such as the pro-inflammatory cytokines IL-1, IL-6 and TNF, the inflammatory compounds PTGS2/COX2 and PTX3, various inflammatory chemokines (CCL20, CXCL2 and CXCL3) and PDE4B (Fig 1b).

The second sub-cluster (further indicated as sub-cluster 2) correlating to PTPN7 and NAB2 consisted predominantly of various adhesion/motility/chemotactic factors, such as EMP1, CDC42, CCL2 and CCL7 (Fig 1b).

Figure 1: Heat map of gene correlation.



Correlation of expression of the various genes; data represent Spearman's correlation coefficients, tested on the relative mRNA expression of the genes in 83 individuals: 56 bipolar patients, 27 schizophrenic patients. Significant positive correlations ($p<0.05$) are given in a red scale (darkest red are correlations over 0.60), significant negative correlations are given in a green scale. White fields are not significant. Figure 1a: The correlations of all tested genes to each other are shown.

Figure 1b: Three sets of MAPK regulators/transcription factors have been extracted from Figure 1a, namely DUSP2/ATF3, MXD1/EGR3 and PTPN7/NAB2 and correlations to the other gens are shown. Note that 1) DUSP2/ATF3 correlate the strongest to sub-cluster 1A genes (and weaker to the other subsets of genes), 2) MXD1/EGR3 correlate the strongest to sub-cluster 1B genes and many of the sub-cluster 1A genes (but weaker to DUSP2/ATF3) and 3) that PTPN7/NAB2 correlate the strongest to sub-cluster 2 genes.

Table III

		SCZ		BD	
CLUSTER 1A					
DUSP2		**5.36**	**< 0,01**	**4.96**	**< 0,01**
ATF3		**3.50**	**< 0,01**	**3.55**	**< 0,01**
	PDE4B	3.91	< 0,01	3.00	< 0,01
	IL6	7.89	< 0,01	5.39	< 0,01
	IL1B	9.20	< 0,01	6.45	< 0,01
	TNF	3.91	< 0,01	1.87	< 0,01
	TNFAIP3	3.22	< 0,01	2.31	< 0,01
	BCL2A1	2.39	< 0,01	3.30	< 0,01
	PTX3	2.51	< 0,01	2.63	< 0,01
	PTGS2	4.34	< 0,01	3.20	< 0,01
	CCL20	23.53	< 0,01	10.63	< 0,01
	CXCL2	3.76	< 0,01	5.31	< 0,01
	EREG	7.36	< 0,01	2.31	< 0,01
	CXCL3	3.99	< 0,01	3.33	< 0,01
CLUSTER 1B					
MXD		**1.49**	**< 0,01**	1.43	0.06
F3		**5.56**	**< 0,01**	**1.87**	**0.02**
MAFF		**5.10**	**< 0,01**	**2.95**	**0.01**
EGR 3		**5.36**	**< 0,01**	2.52	0.16
	THBS	4.31	< 0,01	2.02	0.05
	PAI-2	1.84	< 0,01	1.10	0.06
	RGC32	0.85	0.07	2.61	< 0,01
CLUSTER 2					
HEPTP		**0.78**	**0.04**	**2.04**	**< 0,01**
NAB2		**0.76**	**0.21**	**2.58**	**< 0,01**
MAPK6		**1.21**	**< 0,01**	**1.80**	**< 0,01**
	EMP1	0.97	0.88	2.19	< 0,01
	STX-1A	0.64	0.22	3.04	< 0,01
	DHRS3	1.08	0.51	1.87	0.08
	CCL2	1.60	< 0,01	3.83	< 0,01
	CCL7	1.12	< 0,01	8.47	< 0,01
	CDC42	1.49	< 0,01	1.99	< 0,01
	FABP5	1.08	0.75	1.21	0.09
	CD9	1.49	0.59	2.16	< 0,01
	HSP 70	1.06	0.93	0.79	0.40
	CCR2	0.85	0.53	0.62	0.10

The quantitative value obtained from Q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from the normalized CT values (CT gene- CT housekeeping gene), via the ΔΔCT method (User Bulletin, Applied Biosystems). The fold change of the HC was set to 1.
Data are expressed relative to this HC value. HC SCZ: n = 32; HC BD: n = 48
Values >1: patients have a higher expression than control group. Red indicates significantly up regulated. Values<1: patients have a lower expression than control group. Green indicates significantly down regulated. P tested by univariate ANCOVA vs. control subjects; age and gender are included in this model.

In careful analysis (Fig1b and Supplementary Fig 1), sub-cluster 1 contained a further sub-cluster consisting of the transcription factors EGR3 and MXD1, which were mutually strongly correlating and strongly to the transcription factors MAFF and F3, but more weakly to ATF3/DUSP2 and PTPN7/NAB2 (Fig 1b, this sub-sub cluster is further indicated as sub-cluster 1B, the other remaining set being sub-cluster 1A).

The expression of the three sub-clusters in schizophrenia and bipolar disorder

Table III and IV show that sub-cluster 1A is expressed in the monocytes of both SZ and BD patients. If one defines sub-cluster 1A positivity as positive for ATF3 and/or DUSP 2 (i.e. an expression level higher than the mean + one standard deviation of the healthy control values) 67% of BD and 52% of SZ patients are positive versus 24 and 24% of their matched healthy controls respectively (Table IV).

With regard to sub-cluster 1B, Table III and IV show that SZ patients and BD patients are positive, but in lower proportions. Using the same type of definition as for sub-cluster 1A (but now for EGR3 and/or MXD1), it appeared that SZ patients were significantly positive for 41 % versus 34% of BD patients (versus 13 and 22 % of their HC, Table IV).

With regard to sub-cluster 2, Table III and IV show that only BD patients show an up regulation of these genes. Interestingly, SZ patients show a significant down regulation of two transcription factors belonging to this sub-cluster, i.e. PTPN7 and NAB2 (significant for PTPN7), while MAPK6 is up regulated (yet not to the same extent as in BD). It is noteworthy that the adhesion/motility factors EMP1 and STX1A are down-regulated too. If one defines sub-cluster 2 positivity as positive for PTPN7 and/or NAB2 (see above for definition), even a significantly reduced expression in SZ versus HC can be seen (7 % versus 21%), while in BD there is a significant increased expression (62% versus 32%) (Table IV). Vice versa, if sub-cluster 2 is defined as a "negative" signature (Table IV, last column) a higher proportion of SZ patients is positive for such reduced expression (48%).

Table IV. The prevalence of sub-clusters in bipolar patients, schizophrenia patients and healthy controls

	Cluster 1a		**Cluster 1 b**		**Cluster 2**		**Cluster 2**	
	Dusp2 and/or ATF3 positivity		MXD1 and/or EGR3 positivity		PTPN7 and/or NAB2 positivity		PTPN7 and/or NAB2 negative	
Schizophrenia	52%	(14/27)[a]	41%	(11/27)[a]	7%	(2/27)[b]	48%	(13/27)[ab]
Healthy Controls	24%	(7/29)	13%	(4/30)	21%	(6/29)	21%	(6/29)
Bipolar Disorder	67%	(26/39)[a]	34%	(12/36)	62%	(24/39)[a]	5%	(2/39)[a]
Healthy Controls	24%	(10/41)	22%	(8/36)	32%	(13/41)	25%	(10/40)

[a] $p < 0.05$ vs. healthy controls
[b] $p < 0.05$ vs. bipolar disorder

Positive is defined as an mRNA expression more than one SD away of the mean level found in the healthy controls. Signature is defined on the transcription factors positive. P Values are obtained from Chi-squared test

Relation of monocyte inflammatory gene expression to medication use, disease duration and disease activity.

Medication use. To test for the influence of lithium and antipsychotics, we turned to the group of BD patients, since virtually all patients with SZ were on anti-psychotics; only one was not, but had used an anti-psychotic in the past. Of the 56 BD patients, 32 were on lithium, 8 were on anti-psychotics, 5 used both and 11 were at the time of blood extraction not on lithium or an anti-psychotic, but had used this medication in the past (more than 6 months ago). Table Va shows the effects of the medications in this BD group. Use of lithium and antipsychotics either alone or in combination resulted in a significant decrease of PDE4B, but not of other genes (though there was a near significant trend for a decreasing effect of lithium and antipsychotics on other important signature 1A genes such as IL1 and TNF).

Table V. Correlations of aberrant gene expression

A. With medication use in bipolar patients.

Medication	Genes	p		B	95% Confidence Interval
Lithium	PDE4B	0.002	down	-12.302	-4.736 to -19.867
Antipsychotics	PDE4B	0.002	down	-16.613	-6.555 to -26.671
Both	PDE4B	0.005	down	-17.142	-5.468 to -28.817

Linear regression with lithium, antipsychotics and both medications were included in the model. The values of patients on the indicated drug are set to 1 in the model. B=regression coefficient.

B. With the mood status of bipolar patients

Genes	Depressive (n=9) vs Euthymic (n=40)			Manic (n=7) vs Euthymic (n=40)		
	B	95% CI	p	B	95% CI	p
CCL2	105,24	37,49 – 172,98	0,003	22,93	-44,81 – 90,68	0,500
STX1A	36,59	13,52 – 59,69	0,002	8,77	-14,30 – 31,83	0,449
DHRS	9,91	3,00 – 16,83	0,006	-0,28	-6,78 – 6,22	0,930
PTPN7	3,82	1,08 – 6,56	0,007	2,25	-0,49 – 4,99	0,106
MAPK6	2,76	0,83 – 4,69	0,006	2,29	0,36 – 4,22	0,021
EMP1	12,72	7,31 – 18,12	0,001	3,83	-1,58 – 9,24	0,161

Determination of the influence of mood on mRNA expression of molecules via ANCOVA analysis. The values of patients with an euthymic mood are set to 1. B = regression coefficient.

With regard to sub-cluster 2 genes there was a near significant increasing trend of the use of antipsychotics in BD patients on genes such as PTPN7, NAB2 and STX1A (data not shown). This increasing effect of anti-psychotics on sub-cluster 2 genes was also reflected in the "lifetime cumulative antipsychotic drug usage in haloperidol equivalents" which positively and significantly correlated to important sub-cluster 2 genes as PTPN7 (r=0,55), HSPA1A (r=0,61) and EMP1 (r=0,62).

Duration of illness. The patients with schizophrenia were in general recent onset cases and had a median duration of illness of only a couple of months (Table I). Effects of duration of illness were not noticeable.

The BD patients had a median duration of illness of 16 years (range 3.5 – 40 years, Table I); in this latter group there was a weak, though significant correlation between disease duration and the expression of some of the signature genes: we found a weak positive correlation for IL6, PTX3, CCL2 and EMP1 (r=+ 0.30 to 0.40) and a weak negative correlation for HSPA1A (r= - 0.30), indicating a slightly stronger expression of part of the inflammatory fingerprint over years.

Disease quality and severity. In the SZ patients disease quality and severity (as expressed in the various PANSS scales) did not correlate to any of the various gene expression levels.

With regard to BD we previously reported [1] that the actual mood status of the patients tested was to some extent related to the inflammatory gene expression: During a manic episode, the mRNA expression of 2/19 genes were significantly increased in monocytes of manic versus euthymic BD patients; during depressive episodes 6/19 genes. In the presently extended series of 56 bipolar patients we largely confirmed this observation and now found 1/34 genes during mania (n = 7 patients) and 6/34 genes during depression (n = 9 patients) raised (in comparison to euthymic patients (n = 40 patients), Table Vb). Interestingly these were all cluster 2 genes. Although active disease thus is related to a higher expression of many of the cluster 2 signature genes (in depressive phases more than in manic phases), it must be noted that virtually all of the cluster 2 genes were still significantly higher in euthymic bipolar patients as compared to healthy controls.

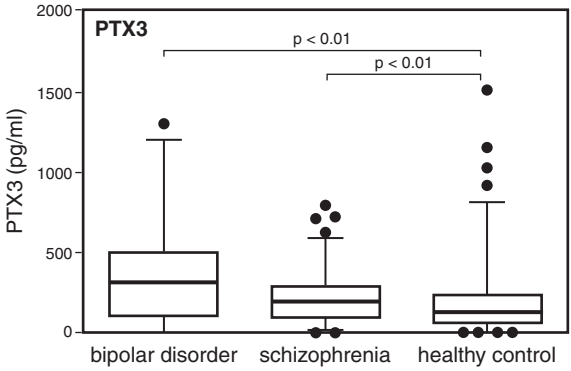
Inflammatory gene expression at the protein level.

We previously reported on the IL1β, IL6, TNFα, CCL7 and CCL2 levels in the serum of these sets of BD and SZ patients [1, 13], and found that only IL1β was increased in BD (as compared to HC), while IL1β, IL6, TNFα and CCL2 were all increased in SZ patients (as compared to HC).

With regard to PTX3 we were able to measure in this study serum levels and found these increased in BD and SZ patients (Figure 2). The figure shows that in BD and SZ PTX3 serum levels are significantly raised over HC levels, i.e. about 2.5 fold in BD and about 1.5 fold in

SZ. When we correlated serum PTX3 protein levels to monocyte gene expression levels we found a significant positive correlation, though weakly (r=0,184, p=0.05, Spearman's correlation), indicating that factors other than monocyte gene expression also determine serum PTX3 levels.

Figure 2: Serum PTX3 levels in bipolar (BP) patients, schizophrenia (SCZ) patients and healthy controls (HC).



Box plots of PTX3 are given. The serum PTX3 level was determined via an in-house ELISA (M7M) on the serum of 58 bipolar patients, 181 schizophrenia patients and 188 healthy controls, some of whom were also used for Q-PCR used according to the manufacturer's protocol (for patient details see [13]). The box indicates the lower and upper quartiles. The line within the box represents the median. The whiskers extend to the 2.5 and 97.5 percentiles. The outliers are characterized by the filled dots. ANCOVA was used for statistical evaluation. Age and gender were included in the statistical model, other confounding factors such as adiposity could however not be investigated, since we were not informed on the BMI in the majority of the cases tested here. The figure shows that in bipolar disorder and schizophrenia, PTX3 serum levels are significantly raised over healthy control levels, i.e. about 2.5 fold in bipolar disorder and about 1.5 fold in schizophrenia. Correlating serum PTX3 protein levels to monocyte gene expression levels we found a significant positive correlation, although it was only weakly so (r=0,184, p=0.05, Spearman's correlation).

Discussion

The outcomes of our study show that circulating monocytes are set at a high inflammatory gene expression set point in both schizophrenia and bipolar disorder. On the protein level these high gene expression set points were (weakly, but significantly) reflected in high serum levels of pro-inflammatory cytokines and compounds.

Though monocytes of BD and SZ patients clearly overlapped in signature gene expression sets 1A and B, they also differed with regard to signature gene expression set 2: We found the MAPkinase regulating factors PTPN7 and NAB2 *up-regulated* in monocytes of BD patients, but *down-regulated* in monocytes of SZ patients. Our immune biomarker

approach thus made a distinction between BD and SZ possible. Though thus supporting the dichotomy between BD and SZ as introduced in 1899 by Kraepelin [14], our immune data also lend support to the recently expressed view by geneticists [15-18] that BD and SZ are strongly overlapping entities sharing the same vulnerability genes, since we found monocytes of considerable proportions of BD and SZ patients to share an up regulated pro-inflammatory gene sub-cluster 1A, composed mainly of a network of well known pro-inflammatory cytokines and compounds such as IL1, IL6, TNF, PTGS2/COX2 and PTX3, many of which have previously been found up-regulated mainly at the protein level in mood disorders and schizophrenia [1, 13].

In addition, we found monocytes of particularly SZ patients to be set at a further and higher inflammatory set point, due to an extra up regulation of in particular the transcription factors/regulators EGR3, MXD1, MAFF and F3. These transcription factors/regulators are mainly involved in proliferation and differentiation of monocytes, but also play a role in the regulation of the inflammatory set point of monocytes/macrophages [19-24]. Our data, which show a strong correlation of EGR3, MXD1, MAFF and F3 to the inflammatory cytokines and compounds to which ATF3 and DUSP2 were also correlating, supports such view of involvement of these transcription factors in inflammation. Interestingly, the expression of EGR3, MXD1, MAFF and F3 were in particular correlated to the up regulation of the adipogenic and vascular pathology factors THBS and SERPINB2/PAI-2, and it is here of note that the incidence of the metabolic syndrome is increased in BD and SZ [25].

Our study has limitations. Firstly, patients with schizophrenia were predominantly male and young in the age group of 20 years and virtually all had recent onset disease and were on antipsychotic medication; the BD patients were predominantly female in the age group of 40 years, had mainly longstanding disease and many used lithium and/or antipsychotics. All patients were naturalistically treated and none of our patients was "treatment naïve". It could be argued that age and gender simply explained the differences between SZ and BD and that medication is the causal factor for the high inflammatory gene expression level in monocytes of psychiatric patients.

With regard to age and gender, it must be noted that we compared the patient findings with those of age and gender matched control groups and that the monocyte inflammatory state in these control groups of different age and gender did not differ (Supplementary Table I).

With regard to the effects of medication it must be noted that effects of lithium and anti-psychotics are generally anti-inflammatory in character [26-28] and the data presented here, as well as our previous data [1], support such an immune suppressive action and show that, if any, these medications do not induce but correct the abnormal inflammatory set point of patient monocytes (see Table Va). However we can not totally rule out an

important effect of elicit drugs on the induction of the specific characteristics of the schizophrenic monocyte signature, since our study group of SZ patients was not controlled for this variable (the BD patients were, see Table I).

Another limitation is that we have made in our experimental design a selection of aberrantly expressed genes by selecting in whole genome analysis only highly over and under expressed genes (more or less than 3.5 fold), which were clearly involved in inflammation and inflammatory processes. Although this approach proved to be fruitful in detecting the three fingerprint patterns as described here (which also made a distinction between BD and SZ possible), we may have missed important causal genes for the inflammatory set points, since our assumption that the sheer expression level of genes is important for the inflammatory state, is naïve. Clearly, further studies of additional genes that are less aberrantly expressed but are critically involved in inflammation and/or have previously been described as aberrant in psychiatric disorders are clearly needed to see whether they are essential components of the inflammatory monocyte gene fingerprints.

The criteria used in psychiatry for validating nosological categories have usually been restricted to clinical features, outcome and family history [29]. Kraepelin used these tools in formulating his ideas, leading to his dichotomous classification between schizophrenia and bipolar disorder. Given that the main goal of modern psychiatry is to provide effective treatment, the view has been expressed that the ultimate validator for a diagnostic system must be treatment response based on a detailed knowledge of pathogenesis [18].
Our study, using powerful new research genomic tools, precisely provides such new immune biological validators, which probably not only play a role in the immune pathogenesis of schizophrenia and bipolar disorder but are also potential treatment targets. There are several reports indicating that pharmacological interferences with some of the found up-regulated inflammatory signature genes, i.e. interference with PTGS2/COX-2, PDE4B and TNF, may alleviate signs and symptoms of schizophrenia and depression [30-37] and it can thus be envisaged that in particular patients positive for monocyte cluster 1 genes would benefit from a treatment with COX-2, PDE4 and TNF inhibitors.

In conclusion, we here describe the first steps in an immune molecular dissecting approach on inflammatory monocytes, which have already led to the identification of 3 coherent sets of putative immune biomarker genes, opening new avenues for nosological distinctions in psychiatric disease based on inflammation. Our approach could also lead to putative sub-classification of patients with psychotic or bipolar disorder, who could possibly benefit from adjunctive anti-inflammatory treatment targeting important fingerprint genes.

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Supplementary table I. ΔCT values (mean, SD) of Q-PCR Analysis of Monocytes of Bipolar Patients, Schizophrenia Patients and Healthy Controls.

Gene	BD		HC-BD		SCZ		HC-SCZ		p-value
PDE4B	-3.32	1.40	-1.72	1.90	-2.59	1.87	-0.81	1.70	0.901
MAPK6	-3.13	1.26	-1.98	1.79	-1.13	0.98	-1.01	0.75	0.515
DUSP2	-2.55	1.65	0.08	2.25	-2.10	1.90	0.39	2.39	0.824
NAB2	0.37	2.07	2.76	1.21	2.43	1.06	1.94	1.05	0.011
PTPN7	-0.47	1.69	1.77	0.77	2.11	0.75	1.83	0.59	0.774
ATF3	-3.14	1.50	-0.70	1.78	-1.12	1.34	0.41	1.48	0.703
TNF	-3.03	1.63	-1.62	1.60	-4.15	2.70	-2.05	2.39	0.938
IL1B	-7.37	2.55	-3.28	2.80	-6.57	3.09	-3.45	2.95	0.561
IL-6	2.19	4.42	5.14	2.77	1.47	4.51	5.51	3.34	0.775
CCL2	-1.69	3.54	2.72	1.74	1.61	1.63	2.73	1.72	0.621
CCL7	-0.18	4.73	6.75	3.19	6.26	3.28	7.25	2.63	0.319
CXCL2	-5.60	2.65	-1.43	2.99	-3.04	2.70	-0.78	2.63	0.936
CCL20	1.39	4.28	4.55	3.77	1.32	5.08	6.05	4.64	0.428
PTX3	-1.83	2.22	-0.15	1.49	-0.75	1.69	0.44	1.26	0.856
PTGS2	-3.60	1.87	-2.13	2.01	-3.74	2.00	-1.35	2.03	0.342
TNFAIP3	-4.36	2.00	-2.78	1.87	-3.88	1.99	-2.78	1.50	0.484
BCL2A1	-5.64	2.75	-2.38	1.87	-5.19	2.20	-3.27	1.70	0.450
EMP1	-2.19	2.12	0.74	1.60	1.94	1.30	1.68	1.23	0.687
CD9	-2.20	1.67	-0.85	1.53	-0.99	1.69	-0.82	1.60	0.313
CDC42	-1.48	2.07	0.72	1.25	-0.11	1.46	1.01	1.28	0.224
STX-1A	2.11	2.94	5.77	1.48	6.53	1.29	6.16	1.11	0.902
DHRS3	-1.54	1.70	1.81	0.86	3.18	1.27	4.16	3.00	0.160
FABP5	-0.75	1.56	-1.45	0.39	-1.94	0.71	-1.96	0.26	0.195
HSPA1A	-3.30	1.86	-4.71	1.58	-3.43	1.25	-4.02	1.26	0.197
RGC32	-0.05	2.14	1.74	2.02	1.41	1.69	2.13	1.59	0.908
EREG	-0.94	2.50	0.17	1.96	-1.26	2.64	1.61	2.20	0.366
CXCL3	-1.62	3.45	0.91	2.82	-0.95	3.58	2.35	3.31	0.659
F3	1.08	2.20	2.05	2.25	0.99	3.13	3.85	2.61	0.317
THBS	-5.69	1.80	-4.65	2.10	-5.37	2.42	-3.36	2.02	0.322
EGR3	-2.34	1.82	-2.09	2.32	-2.43	0.96	-0.36	2.77	0.287
SERPINB2	1.32	2.78	2.61	1.53	1.77	1.80	3.24	1.59	0.101
MAFF	-0.38	1.55	1.01	2.11	-0.70	1.33	1.81	2.53	0.699
MXD1	-3.67	0.97	-3.65	0.97	-3.93	0.92	-3.14	0.84	0.666

Delta CT values of HC-BD (n = 48) vs HC-SCZ (n = 32). P values were tested by univariate ANCOVA HC-BD vs. HC-SZ; age and gender are included in this model.

Supplementary Table II. Function of genes.

Gene	Function
DUSP2	Transcription factor in the MAPKinase pathway
ATF3	Transcription factor in the MAPKinase pathway
NAB2	Transcription factor involved in repressing the MAPK-pathway
EGR3	Transcription factor involved in early growth response,
	Proliferation/differentiation monocytes
PDE4B	Regulation of signal transduction
PAI-2	Adipogenesis, Vascular pathology, Inflammation
	Proliferation and differentiation of monocytes/macrophages
IL6	Inflammation
IL1B	Inflammation
TNF	Inflammation
PTX3	Inflammation
PTGS2	Inflammation
TNFAIP3	Apoptosis, Inflammation
BCL2A1	Apoptosis, Cell cycle
CCL2	Chemotaxis, Motility
CCL7	Chemotaxis, Motility
CCL20	Chemotaxis, Motility
CXCL2	Chemotaxis, Motility
EREG	Proliferation
CXCL3	Chemotaxis, Inflammation
MXD1	Transcriptional repressor, Proliferation/differentiation monocytes, Cell cycle, Apoptosis
F3	Transcription factor involved in blood coagulation, Proliferation/differentiation monocytes
MAFF	Transcription factor involved in cellular stress response, Proliferation/differentiation monocytes
THBS	Adipogenesis, Vascular pathology, Adhesion
EMP1	Cell-cell adhesion, Interactions with the extracellular membrane
CD9	Cell adhesion, Migration and Motility
STX-1A	Membrane fusion
RGC32	Cell cycle progression, Apoptosis
PTPN7	Transcription factor involved in MAPKinase regulation, T and B cell development
MAPK6	Part of the MAPKinase pathway, Inflammation
CDC42	Signaling in the MAPK-pathway, Motility, Cytoskeletal organization of the cell, Chemotaxis
FABP5	Fatty acid uptake, Transport and Metabolism
DHRS3	Vitamin A metabolism, Metabolism
HSPA1A	Protein folding, Cellular stress response
CCR2	Receptor for CCL2, Chemotaxis

Chalfont & Latimer
Watford
Croxley
Chorleywood
Rickmansworth
Moor Park
Northwood
Northwood Hills
Pinner
Ruislip Manor
Ruislip
West Ruislip
Eastcote
North Harrow
Harrow & Wealdstone
Harrow-on-the-Hill
Ruislip Gardens
Rayners Lane
West Harrow
Northwick Park
Preston Road
Kenton
Stanmore
Canons Park
Queensbury
Kingsbury
Edgware
Burnt Oak
Colindale
Hendon Central
Brent Cross
Golders Green
Hampstead
Neasden
Wembley Park
Dollis Hill
Willesden Green
Kilburn
West Hampstead
Finchley Road & Frognal
Belsize Park
Chalk Farm
South Ruislip
Northolt
Greenford
South Harrow
Sudbury Hill
Sudbury Town
South Kenton
North Wembley
Wembley Central
Stonebridge Park
Harlesden
Willesden Junction
Kensal Rise
Brondesbury Park
Brondesbury
Kensal Green
Queen's Park
Finchley Road
Swiss Cottage
St. John's Wood
Alperton
Perivale
Hanger Lane
Park Royal
North Ealing
Ealing Broadway
North Acton
West Acton
East Acton
White City
Shepherd's Bush
Holland Park
Kilburn Park
Maida Vale
Warwick Avenue
Paddington
Edgware Road
Royal Oak
Westbourne Park
Ladbroke Grove
Latimer Road
Bayswater
Queensway
Lancaster Gate
Notting Hill Gate
Baker Street
Great Portland Street
Euston
Warren Street
Marylebone
Regent's Park
Bond Street
Oxford Circus
Marble Arch
Tottenham Court Road
Acton Central
Shepherd's Bush
Kensington (Olympia)
High Street Kensington
Hyde Park Corner
Green Park
Piccadilly Circus
Ealing Common
South Acton
Goldhawk Road
Knightsbridge
Acton Town
South Ealing
Hammersmith
Barons Court
Gloucester Road
Sloane Square
St. James's Park
Northfields
Boston Manor
Chiswick Park
Turnham Green
Stamford Brook
Ravenscourt Park
West Kensington
Earl's Court
South Kensington
Victoria
Westminster
Hounslow East
Osterley
Hounslow West
Hounslow Central
Hatton Cross
for Heathrow Terminal 4
Heathrow Terminal 4
Gunnersbury
Kew Gardens
Richmond
West Brompton
Fulham Broadway
Parsons Green
Putney Bridge
River Thames
East Putney
Southfields
Wimbledon Park
Wimbledon
Pimlico
Vauxhall
Waterloo
Oval
Stockwell
Clapham North
Clapham Common
Clapham South
Balham
Tooting Bec
Tooting Broadway
Colliers Wood
South Wimbledon
Morden

Chapter 4

Patients with schizophrenia show raised serum levels of the pro-inflammatory chemokine CCL2. Association with the metabolic syndrome in patients?

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Dear Editors,

Serum levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α are raised in schizophrenia [1]. However, apart from these pro-inflammatory cytokines, chemokines play an important role in modulating brain function [2]. The system of chemokines and their receptors has been described as a major regulating system of the brain and the receptor for CCL2, CCR2, is expressed in the brain by astrocytes, microglia and neurons [3]. CCL2 (Chemokine (C-C motif) ligand 2) is an important pro-inflammatory chemokine, playing a key role in the recruitment of monocytes to inflammatory foci [4, 5]. Interestingly, a genotypic association was found between the A-2518G polymorphism of the CCL2 gene and resistance to anti-psychotic medication [6] and a predominance of negative symptoms over positive symptoms [7]. The A-2518G polymorphism affects the production of CCL2 [6]. However, it is unclear if CCL2 serum levels are changed in patients with schizophrenia. One study reported a normal level of CCL2 in the serum of a small group of institutionalized male patients [8], while another study reported an elevation of CCL2 in the cerebrospinal fluid of a small group of patients with psychosis serving as controls for a study on neuro-psychiatric lupus [9].

Here we present a study on the CCL2 levels in the serum of 145 patients with chronic schizophrenia. Patients participated in a study on the prevalence of abnormalities in glucose metabolism in patients with schizophrenia or schizo-affective disorder treated with anti-psychotics; the demographic data, study design, hyperglycemia measurements, diabetes and metabolic syndrome definition and outcomes have been reported in detail before [10]. Adult healthy controls (HC, $n = 105$) were laboratory, medical staff and students, with blood collection at the same time as the patients. The medical ethics review board METIGG (Utrecht, The Netherlands) approved the study.

Serum CCL2 levels were measured using the Cytometric Bead Array kit (CBA, BD Biosciences, San Diego, USA) according to the manufacturer's protocol. Serum levels of CCL2 were significantly higher in the 145 patients (Fig 1a, median 196.38 pg/ml, range up to 958.45 pg/ml) compared to the 105 HC (median 141.41 pg/ml, range up to 310.35 pg/ml, $p < 0.001$). Because patients and HC differed in age and had different male/female ratios, data were also analyzed as randomly selected nested case-controls of 65 cases with schizophrenia and 65 age and gender matched HC. This nested study showed that CCL2 levels were also significantly higher in patients (Fig 1a, median 184.67 pg/ml, range up to 673.23 pg/ml) than in HC (median 151.27 pg/ml, range up to 310.35 pg/ml, $p < 0.009$).

Increased CCL2 levels were independent of the use of anti-psychotic medication, both regarding the class of drug (typical versus atypical) as well as regarding the individual drug. Eleven patients did not use any anti-psychotic drug for at least three months. The CCL2 level of this "drug-free" chronic schizophrenic population was higher than that of the HC (yet numbers were too small to reach significance). To verify whether the raised CCL2

level was indeed not due to treatment we retested the 11 drug-free patients together with 13 newly collected drug-naïve patients (duration of schizophrenia less than 1 year) and 36 age and gender matched HC for their CCL2 levels in a separate assay. CCL2 levels were statistically significantly higher than the HC (patients: median 302.20 pg/ml, range up to 595.50 pg/ml; HC: median 154.90 pg/ml, range up to 534.30 pg/ml, $p < 0.001$).

Serum levels of CCL2 correlated positively ($r = 0.152$, $p < 0.05$) to the prevalence of the metabolic syndrome (MS, prevalence in our study population 51/145), defined according to a modified definition of the National Cholesterol Education Program's Adult Treatment panel III. And, also it correlated in particular and negatively ($r = -0.108$, $p < 0.052$) to the HDL levels. As a consequence, the levels of CCL2 were significantly higher in patients with MS (Fig 1a, median 221.91 pg/ml, range up to 955.60 pg/ml) compared to those without (median 179.08 pg/ml, range up to 711.82 pg/ml, $p < 0.026$), though it must be noted that those without MS still had higher serum CCL2 levels compared to the HC population (Fig 1a, median 141.41 pg/ml, range up to 310.35 pg/ml, $p < 0.001$). It is tempting to speculate that the extra increased CCL2 levels in schizophrenia patients with the MS reflect a higher load of atherosclerotic plaques in these patients, since CCL2 plays a critical role in fatty streak development, e.g. hypercholesterolemic CCL2 deficient mice have less arterial lipid deposition [3].

The CBA allows the simultaneous quantification of IL-1β, TNF-α and IL-6 in the same test and we found all three cytokines elevated in the patients (Fig 1b). These cytokines did not correlate to the MS and there was no difference between cytokine levels in patients with or without the MS. The levels of CCL2 showed a strong positive correlation to the levels of IL-6, IL-1β and TNF-α.

Limitation of our study is that outcomes are largely based on patients with chronic stable schizophrenia with the vast majority of patients on anti-psychotic medication while drug-free/naive patients have hardly been studied. Moreover we were not informed on the hyperglycemia and the MS in our HC. So, our finding that CCL2 levels are not due to medication and correlate at least in part to the presence of MS (and predominantly to a low HDL-cholesterol) should be taken cautiously and needs confirmation in a confirmative study using patients before and after treatment characterized for the MS and dito HC.

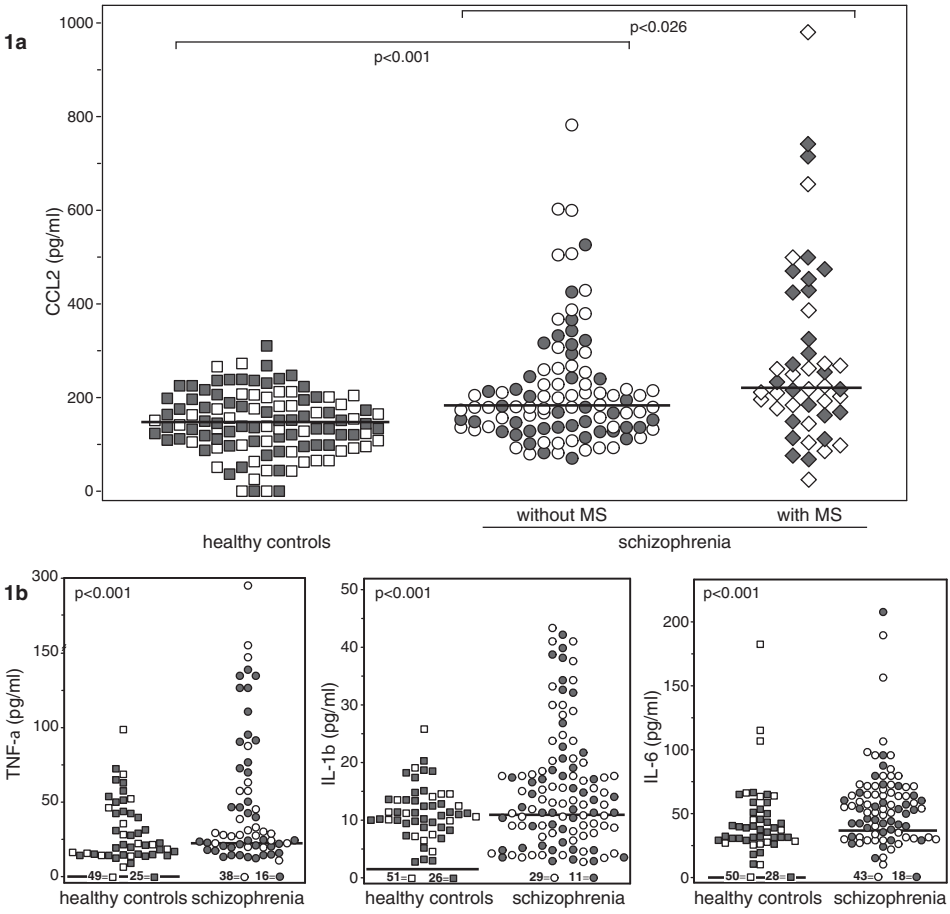

Figure 1a:

CCL2 levels are significantly ($p < 0.026$) higher in patients with schizophrenia with the metabolic syndrome (open diamond: n = 52, median 221.91 pg/ml, range up to 955.60 pg/ml) compared to patients without the metabolic syndrome (open circle: n = 93, median 179.08 pg/ml, range up to 711.82 pg/ml), while the CCL2 levels of the latter group are significantly higher ($p < 0.001$) compared to the levels of healthy controls (open quadrangle: n = 105, median 141.41 pg/ml, range up to 310.35 pg/ml).

CCL2 levels are also significantly ($p < 0.02$) higher in the nested age and gender matched patients with schizophrenia with the metabolic syndrome (closed diamond: n = 23, median 261.61 pg/ml, range up to 673.23 pg/ml) compared to patients without the metabolic syndrome (closed circle: n = 42, median 156.63 pg/ml, range up to 435.58 pg/ml), while the CCL2 levels of the latter group are significantly higher ($p < 0.04$) compared to the levels of healthy controls (closed quadrangle: n = 65, median 151.12 pg/ml, range up to 310.35 pg/ml).

Fig 1b:

a) TNF-α levels are significantly ($p < 0.001$) higher in patients with schizophrenia (open circle: n = 145, median 22.15 pg/ml, range up to 271.06 pg/ml) compared to healthy controls (open quadrangle: n = 105, median 0 pg/ml, range up to 98.68 pg/ml). Note that 49 healthy controls have non-detectable

TNF-α levels compared to 38 patients with schizophrenia (indicated by 49 = ❒ and 38 = O).
TNF-α levels are also significantly ($p < 0.035$) higher in the nested age and gender-matched patients with schizophrenia (closed circle: n = 53, median 20.02 pg/ml, range up to 146.69 pg/ml) compared to healthy controls (closed quadrangle: n = 58, median 13.93 pg/ml, range up to 72.35 pg/ml) Note that 25 healthy controls have non-detectable TNF-α levels compared to 16 patients with schizophrenia (indicated by 25 = ▪ and 16 = •).
IL-1β levels are significantly ($p < 0.001$) higher in patients with schizophrenia (open circle: n = 145, median 9.09 pg/ml, range up to 43.34 pg/ml) compared to healthy controls (open quadrangle: n = 105, median 1.37 pg/ml, range up to 25.80 pg/ml). Note that 51 healthy controls have non-detectable IL-1β levels compared to 29 patients with schizophrenia (indicated by 51 = ❒ and 29 = O).
IL-1β levels are also significantly ($p < 0.007$) higher in the nested age and gender-matched patients with schizophrenia (closed circle: n = 57, median 10.61 pg/ml, range up to 43.34 pg/ml) compared to healthy controls (closed quadrangle: n = 62, median 6.89 pg/ml, range up to 20.28 pg/ml). Note that 26 healthy controls have non-detectable IL-1β levels compared to 11 patients with schizophrenia (indicated by 26 = ▪ and 11 = •).
IL-6 levels are significantly ($p < 0.001$) higher in patients with schizophrenia (open circle: n = 145, median 31.95 pg/ml, range up to 617.10 pg/ml) compared to healthy controls (open quadrangle: n = 105, median 0 pg/ml, range up to 182.46 pg/ml). Note that 50 healthy controls have non-detectable IL-6 levels compared to 43 patients with schizophrenia (indicated by 50 = ❒ and 43 = O).
IL-6 levels are also significantly ($p < 0.032$) higher in the nested age and gender-matched patients with schizophrenia (closed circle: n = 52, median 36.49 pg/ml, range up to 208.47 pg/ml) compared to healthy controls (closed quadrangle: n = 57, median 26.20 pg/ml, range up to 115.10 pg/ml). Note that 28 healthy controls have non-detectable IL-6 levels compared to 18 patients with schizophrenia (indicated by 28 = ▪ and 18 = •).

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Chalfont & Latimer
Watford
Croxley
Chorleywood
Rickmansworth
Moor Park
Northwood
Northwood Hills
Pinner
Ruislip Manor
Ruislip
West Ruislip
Eastcote
North Harrow
Harrow & Wealdstone
Harrow-on-the-Hill
Ruislip Gardens
Rayners Lane
West Harrow
Northwick Park
Preston Road
Kenton
Stanmore
Canons Park
Queensbury
Kingsbury
Edgware
Burnt Oak
Colindale
Hendon Central
Brent Cross
Golders Green
Hampstead
Neasden
Wembley Park
Dollis Hill
Willesden Green
Kilburn
West Hampstead
Finchley Road & Frognal
Belsize Park
Chalk Farm
South Ruislip
Northolt
Greenford
South Harrow
Sudbury Hill
Sudbury Town
South Kenton
North Wembley
Wembley Central
Stonebridge Park
Harlesden
Willesden Junction
Kensal Rise
Brondesbury Park
Brondesbury
Kensal Green
Queen's Park
Finchley Road
Swiss Cottage
St. John's Wood
Alperton
Perivale
Hanger Lane
Park Royal
North Ealing
Ealing Broadway
North Acton
West Acton
East Acton
White City
Shepherd's Bush
Holland Park
Kilburn Park
Maida Vale
Warwick Avenue
Paddington
Edgware Road
Royal Oak
Westbourne Park
Ladbroke Grove
Latimer Road
Bayswater
Queensway
Lancaster Gate
Notting Hill Gate
Baker Street
Great Portland Street
Euston
Warren Street
Marylebone
Regent's Park
Bond Street
Oxford Circus
Marble Arch
Tottenham Court Road
Acton Central
Shepherd's Bush
Kensington (Olympia)
High Street Kensington
Hyde Park Corner
Green Park
Piccadilly Circus
Ealing Common
South Acton
Goldhawk Road
Knightsbridge
Acton Town
South Ealing
Hammersmith
Barons Court
Gloucester Road
Sloane Square
St. James's Park
Northfields
Boston Manor
Chiswick Park
Turnham Green
Stamford Brook
Ravenscourt Park
West Kensington
Earl's Court
South Kensington
Victoria
Westminster
Hounslow East
Osterley
Hounslow West
Hounslow Central
Hatton Cross
for Heathrow Terminal 4
Heathrow Terminal 4
Gunnersbury
Kew Gardens
Richmond
West Brompton
Fulham Broadway
Parsons Green
Putney Bridge
River Thames
East Putney
Southfields
Wimbledon Park
Wimbledon
Pimlico
Vauxhall
Waterloo
Oval
Stockwell
Clapham North
Clapham Common
Clapham South
Balham
Tooting Bec
Tooting Broadway
Colliers Wood
South Wimbledon
Morden

Chapter 5

An activated set point of T cell and monocyte inflammatory networks in recent onset schizophrenia patients involves both pro- and anti-inflammatory forces.

Short title: T cells in schizophrenia

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Abstract

We recently described a pro-inflammatory gene expression signature in the monocytes of 60% of patients with recent onset schizophrenia (SCZ). We here investigated whether the T cell system is also in a pro-inflammatory state.

A detailed FACS analysis, e.g. of $CD3^+CD25^+$ T cells, IFN-γ^+, IL-4^+, IL-$17A^+$ ($CD4^+$) lymphocytes and $CD4^+CD25^{high}FoxP3^+$ regulatory T cells, was carried out on peripheral blood of 26 patients with recent onset schizophrenia (of whom in 19 the monocyte inflammatory gene expression signature had been determined) and in age/gender matched healthy controls. Various relevant T cell cytokines, e.g. sCD25, IFN-γ, IL-17A and IL-4, were measured in serum by a multiplex assay.

We detected:

a) Not only higher percentages of pro-inflammatory prone monocytes, activated $CD3^+CD25^+$ T cells and pro-inflammatory Th17 cells in patients, but also higher percentages of anti-inflammatory $CD4^+CD25^{high}FoxP3^+$ regulatory T cells and IL-4^+ lymphocytes.

b) That this activated T cell set point was reflected in a significantly raised serum level of sCD25.

c) That the up regulation of IL-4^+ containing lymphocytes was predominantly found in patients characterized by a monocyte pro-inflammatory set point.

d) That regulatory T cell and Th17 cell numbers were higher in patients irrespective of the monocyte pro-inflammatory state.

Our data do not support the concept that the T cell system is in a simple pro-inflammatory state in recent onset schizophrenia, but do show that the monocyte and T cell networks are activated and involve both pro- and anti-inflammatory forces. This suggests control within an activated inflammatory system.

Keywords: Schizophrenia, activated inflammatory response system, Th17, regulatory T cells, cytokines

Introduction

There is accumulating evidence that an activation of the inflammatory response system plays an important role in the pathogenesis of schizophrenia. In support of this view we recently described an inflammatory gene expression signature, i.e. a coherent aberrant expression of a set of 34 pro-inflammatory genes, in the circulating monocytes of around 60% of patients with recent onset schizophrenia [1].

Apart from cells of the monocyte lineage T cells are important contributors to the inflammatory response, but literature on T cell numbers and cytokines is scarce and conjectural in schizophrenia. Although it has been shown [2] that infusion with IL-2, the well-known growth factor for T cells, is capable of inducing psychosis-related symptoms in psychiatrically healthy individuals, there are at present inconclusive results on the levels of IL-2 or its soluble receptor (sIL-2R, synonymous to sCD25) in the circulation of patients with schizophrenia [3]. The same holds true for the blood levels of IFN-γ and IL-4, the hallmark cytokines of the $CD4^+$Th1 and Th2cell subsets respectively; higher [4-5], lower [6] or unaltered levels [7] of these cytokines and T cell subsets have been described in patients with schizophrenia.

In recent years a new pro-inflammatory T cell cytokine IL-17, has been discovered predominantly produced by so-called $CD4^+$ "Th17 cells" [8]. Th17 cells protect the host against bacteria and fungi by activating macrophages via the production of IL-17 (but also IL-21 and IL-22). In contrast, they also play a role in the pathogenesis of autoimmune diseases such as psoriasis and rheumatoid arthritis [8]. Functions of Th17 cells are thus similar to those of Th1 cells.

The inflammation inducing effects of Th17, Th1 cells and of monocytes/macrophages are controlled by a special class of T cells, the regulatory $CD4^+CD25^{high}FoxP3^+$ T cells [9-10]. The main function of these inborn, thymus-derived regulatory T cells is tempering the inflammatory response thereby maintaining homeostasis and tolerance to self-antigens. Cytokines involved in this anti-inflammatory action are thought to be IL-10 and TGF-β. Patients lacking these regulatory T cells due to a genetic mutation in the gene coding for FoxP3 suffer from a severe and rapidly lethal auto-immune syndrome (Immune dysregulation, Polyendocrinopathy, Enteropathy X-linked syndrome)[11].

The aim of this study was to evaluate T cell related inflammatory networks in schizophrenia following the hypothesis that not only the monocyte system, but also the T cells system would be in a pro-inflammatory state. We therefore determined the percentages of IL-2r ($CD25^+$) T cells and of IFN-γ, IL-4 and IL-17A containing blood lymphocytes in the circulation of 26 recent onset patients, using intracellular staining and FACS analysis. In addition we determined via FACS the percentage of the anti-inflammatory regulatory

$CD4^{+}CD25^{high}FoxP3^{+}$ T cells.
Next to these FACS analyses we evaluated the serum levels of the catabolic pro-inflammatory T cell cytokines IFN-γ, IL-17A, IL-22 (but also of the monocyte/macrophage cytokines CCL2, TNF-α, IL-1β and PTX3) and of the anabolic or anti-inflammatory cytokines sCD25, TGF-β, IL-10, IL-6, IL-4 and IL-5.
In 19 of the tested patients the monocyte inflammatory gene profiles had been determined in the above mentioned previously reported study [1]. This enabled us to not only relate the T cell pro-inflammatory and anti-inflammatory state to various clinical variables which we measured in the patients (for example the GAF-score, medication, smoking), but also to the pro-inflammatory state of their monocytes.

Methods

Patients with schizophrenia: 26 acutely psychotic inpatients with schizophrenia diagnosed according to the DSM-IV criteria were recruited at the department of Psychiatry of the Erasmus Medical Center in Rotterdam. Schizophrenia was diagnosed according to the DSM-IV criteria after a Comprehensive Assessment of Symptoms and History (CASH) interview [12] and by consensus between two senior psychiatrists who were blinded to the results based on all clinical available evidence. In patients with symptoms for less than 6 months, a final diagnosis was made after 6 months to comply with the DSM-IV criterium. All patients were acutely psychotic on admission. Their level of functioning on admission and at discharge was determined by the Global Assessment of Functioning-score on the fifth axis of the DSM-IV.
The patients with schizophrenia were virtually all (except one) recent onset cases and had a median duration of illness of only 0.3 yrs (median, range 0-3 yrs). Absence of severe medical illness (including infections and allergies) was established by medical history assessment, physical examination and routine laboratory testing (Hb, Ht, leukocyte count, blood smear and kidney/liver function) on admission. Almost all patients received antipsychotic medication at the time of blood collection, however with a low lifetime cumulative haloperidol equivalent (median: 42 mg hal.eq.) [13] ; none of the patients were drug naïve.
The demographics of the patients are summarized in Table I.

Healthy Controls: Age- and gender-matched healthy controls (HC, Table I) were recruited from enrolling laboratory staff, medical staff and medical students. The inclusion criteria for the HC were an absence of any psychiatric and autoimmune disorder in themselves and in first-degree family members. HC had to be in self-proclaimed good health and

free of any obvious medical illness for at least two weeks prior to the blood withdrawal, including acute infections and allergic reactions.
The Medical Ethical Review Committee of the Erasmus MC Rotterdam had approved the study. Written informed consent was obtained from all participants after a complete description of the study had been given.

Table I. Characteristics of patients with schizophrenia and healthy controls.

		Schizophrenia		Healthy Controls	
Group size		26		26	
Age (years)1		24	(17-39)	25	(21-39)
Gender	Male	23	88%	23	88%
	Female	3	12%	3	12%
Smoking		18	69%	9	35%
GAF score1	On admission	21-30	(1-10 – 41-50)		
	At discharge	51-60	(21-30 – 71-80)		
Medication	None	1			
	Typical antipsychotics	13			
	Atypical antipsychotics	12			

[1] Median with range. GAF = global assessment of functioning. GAF range from 0 to 100, higher scores indicate better functioning.

Laboratory methods

Blood collection and preparation

Blood (drawn in the morning hours) was collected in clotting tubes for serum preparation (stored at -80°C) and in sodium-heparin tubes for immune cell preparation, on average 20 ml blood was collected from each patient. From the heparinised blood, peripheral blood mononuclear cell (PBMC) suspensions were prepared by low-density gradient centrifugation, as described in detail before [14], within 8 hours to avoid activation of the monocytes (erythrophagy). PBMCs were frozen in 10% dimethylsulfoxide and stored in liquid nitrogen. This enabled us to test patient and control immune cells in the same series of experiments later.

Flow cytometric analyses

FACS (fluorescence-activated cell sorting) analysis was used to measure intracellular cytokine content in PBMC of patients and age and gender matched HC. As hallmark intracellular cytokines we used: IFN-γ, IL-4 and IL-17A. To enable the enumeration of

regulatory T cells we intracellularly stained for FoxP3. Membrane-staining was done for CD3, CD4, CD25 and CD45RO. This enabled us to assign the cytokine staining to the enigmatic Th1, Th2 and Th17 cells in either the total population of $CD4^+$ cells or the memory population. It also enabled us to enumerate the enigmatic regulatory $CD4^+CD25^{high}FoxP3^+$ T cell population.

For the analysis PBMCs were suspended in complete culture medium. Cell suspensions were then stimulated with PMA (Sigma Aldrich, Missouri, USA), ionomycin (Sigma Aldrich, Missouri, USA) in the presence of Golgistop (Becton Dickinson, New Jersey, USA) for 4 hours in 37 C under a 5% CO2 environment for T effector cells, regulatory T cells were not stimulated.

Cells were harvested and stained extracellularly with anti-CD4 (PerCP-Cy5.5; Becton Dickinson, New Jersey, USA) and anti-CD45RO FITC (DAKO, Glostrup, Denmark). For determination of regulatory T cells, non-stimulated PBMC samples were stained with anti-CD3 (PerCP; BD Biosciences, California, USA), anti-CD4 (APC; BD Biosciences, California, USA) and anti-CD25 (FITC; BD Biosciences, California, USA) via standard protocol.

Following extracellular staining, the cells were fixed and permeabilized according to the manufacturers instructions (eBioscience, California, USA) and then stained for FoxP3 (PE; Becton Dickinson, New Jersey, USA), IL-4 (PE; Becton Dickonson, New Jersey, USA), IFN-γ (APC; Becton Dickinson, New Jersey, USA), IL-17A (PE; eBioscience, California, USA).

Isotype antibody controls were used to confirm antibody specificity for CD25. Stained cells were analyzed by four colour flowcytometry (FACSCalibur, BD Biosciences, California, USA) as described previously and analyzed using FlowJo (Tree Star Inc. Ashland, Oregon, USA) research software. Supplementary Figure 1 gives the dot plots of the stainings and definition of cell populations.

mRNA gene expression in monocytes

The definition and determination of the mRNA gene expression fingerprints in monocytes has been previously described in detail [1].

Serum cytokine determinations

Serum cytokines (IFN-γ, IL-17, IL-10, IL-6, IL-4, TNF-α, CCL2, IL-5, IL-1β and PTX3) were measured using the Cytometric Bead Array kits (BenderMedSystems, California, USA) according to the manufacturer's protocol. Bead flow cytometry allows the simultaneous quantification of various proteins in the same test (http://www.bendermedsystems.com/bm_files/47/BM_Factsheet_3_A4_SCREEN.pdf). Twenty-five µls of serum per test were used. Samples were analyzed in a FACSCanto flow cytometer (BD Biosciences, California, USA) using the FlowCytomix Pro 2.3 Software (BenderMedSystems, California, USA). Results are expressed as picograms per milliliter.

For TGF-β (Diaclone, France) and sIL-2r (R & D Systems, Minneapolis, USA) commercially available ELISAs were used according to the manufacturer's protocol.

Statistics

Statistical analysis was performed using the SPSS 15.0 package for Windows. Data were tested for normal distribution using the Kolmogorov-Smirnov test. Parametric tests were used when the data had a normal distribution. When normal distribution was not reached, data were log-transformed. If the data were still skewed after log-transformation non-parametric tests were used. In the text, tables and figures the original data have been used. Levels of significance were set at p = 0.05 (2-tailed). The specific tests used are mentioned under tables and in legends.

Results

Percentages of monocytes and lymphocytes

FACS analysis of the samples showed that the percentage of monocytes was higher in the schizophrenia samples as compared to those of the gender and age matched HC (SCZ: 24.33 +- 7.91, HC: 20.64 +- 4.52, $p = 0.049$, Mann-Whitney test). Although the percentage of lymphocytes was smaller in the schizophrenia samples as compared to the HC, values did not reach statistical significance (SCZ: 70.97 +- 7.90, HC: 75.08 +- 5.02, $p = 0.077$, Mann-Whitney test). Blood leukocyte counts were within the normal range on admission for all patients (routine clinical determination, mean 6.4 +- 1.5 x 10.9/L, reference range for our center: 3.5 -10.0 x 10.9/ L).

IFN-γ, IL-4 and IL-17A containing lymphocytes

We first analyzed via FACS the capability of the total lymphocyte fraction to produce IFN-γ, IL-4 and IL-17A. After stimulation with PMA and ionomycin for 4hrs (a procedure to re-activate lymphocytes), the percentages of IFN-γ and IL-17A containing lymphocytes were not different between schizophrenia samples and HC samples (IFN-γ: SCZ: 24.47 +- 7.60, HC: 27.63 +- 9.45, $p = 0.199$; IL-17A: SCZ: 0.39 +- 0.29, HC: 0.27 +- 0.12, $p = 0.137$). However lymphocytes capable of producing IL-4 were more numerous in patients with schizophrenia as compared to lymphocytes of HC (Fig 1a). Differences could not be detected with regard to the Mean Fluorescence Intensity (MFI, which reflects the actual content of the cytokine per lymphocyte).

CD3+CD25+ cells

Activation of T cells is classically determined by CD25 (IL-2r) expression, illustrating the higher capability of the T cells to react to the growth factor IL-2. Percentages of $CD3^+CD25^+$ T cells were higher in patients with schizophrenia as compared to HC (Fig 1c). Moreover, serum levels of the shed receptor, sCD25 (=sIL-2r), were also higher in patients with schizophrenia and correlated positively to $CD3^+CD25^+$ T cells ($r = 0.394$, $p = 0.007$) and IL-4 containing lymphocytes ($r = 0.462$, $p = 0.001$).

Classical Th1 (CD4+IFN-γ+), Th2 (CD4+IL-4+), Th17 (CD4+IL-17A+) and regulatory CD4+CD25highFoxP3+ T cells

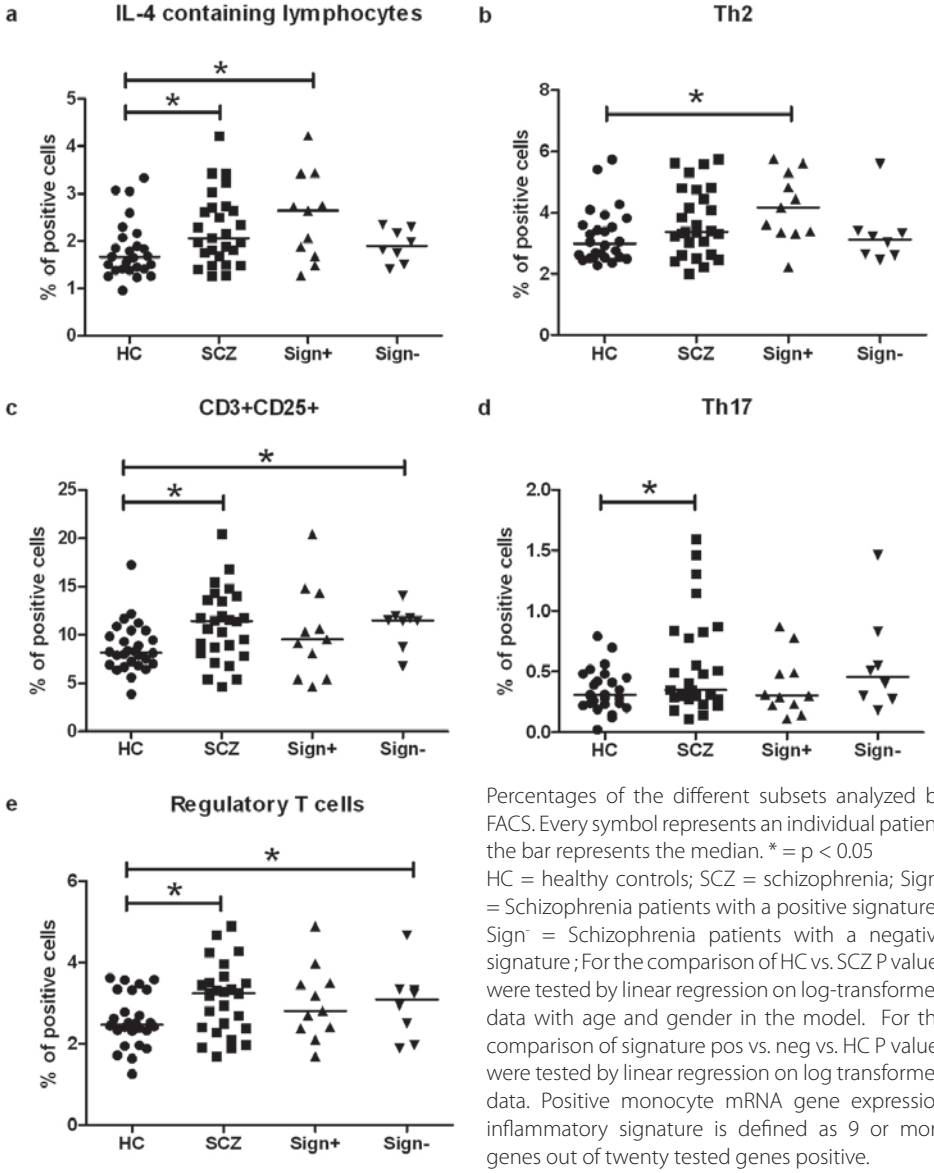
We subsequently studied the percentages of classical Th1 cells ($CD4^+$ T cells capable to produce IFN-γ), classical Th2 cells ($CD4^+$ T cells capable to produce IL-4) or classical Th17 cells ($CD4^+$ T cells capable to produce IL-17A).

The percentage of classical Th1 cells was not different in patients with schizophrenia (SCZ: 7.55 +- 4.19, HC: 7.30 +- 3.50, $p = 0.905$), while that of classical Th2 cells showed a trend to be higher in line with the finding that IL-4 producing lymphocytes were higher (Fig 1b).

Statistically higher percentages of classical Th17 cells were found in the circulation of patients with schizophrenia as compared to that of HC (Fig 1d). Virtually all these Th17 cells were of memory type, i.e. they were $CD45RO^+$.

Figure 1e also shows that $CD4^+CD25^{high}FoxP3^+$ T cell (regulatory T cells) percentages were higher in patients with schizophrenia as compared to HC (Fig 1e). There was a strong positive correlation between the percentage of regulatory T cells and the serum levels of sIL-2R in the patients ($r = 0.447$, $p = 0.002$).

Fig 1 FACS analysis data of the different subsets tested



Percentages of the different subsets analyzed by FACS. Every symbol represents an individual patient, the bar represents the median. * = $p < 0.05$
HC = healthy controls; SCZ = schizophrenia; $Sign^+$ = Schizophrenia patients with a positive signature $Sign^-$ = Schizophrenia patients with a negative signature ; For the comparison of HC vs. SCZ P values were tested by linear regression on log-transformed data with age and gender in the model. For the comparison of signature pos vs. neg vs. HC P values were tested by linear regression on log transformed data. Positive monocyte mRNA gene expression inflammatory signature is defined as 9 or more genes out of twenty tested genes positive.

Positivity of a gene is defined as more than 1 standard deviation higher expressed than the mean expression of the tested gene in healthy controls.

Percentages expressed as mean +- standard deviation; IL-4 containing lymphocytes: SCZ: 2.27 +- 0.76, HC: 1.82 +- 0.61, $p = 0.015$, $Sign^+$: 2.51 +- 0.93, $Sign^-$: 1.91 +- 0.35, p = 0.01; 3Th2: SCZ: 3.65 +- 1.14, HC: 3.23 +- 0.90, $p = 0.124$, $Sign^+$: 4.18 +- 1.12, $Sign^-$: 3.29 +- 1.00, p = 0.006

$CD3^+CD25^+$ T cells: SCZ: 11.01 +- 3.79, HC: 8.32 +- 2.01, $p = 0.002$, $Sign^+$: 10.25 +- 4.75, $Sign^-$: 10.95 +- 2.25, p = 0.029

Th17: SCZ: 0.56 +- 0.42, HC: 0.34 +- 0.18, $p = 0.042$, $Sign^+$: 0.38 +- 0.25, $Sign^-$: 0.56 +- 0.41

$CD4^+CD25^{high}FoxP3^+$ T cell: SCZ: 3.01 +- 0.84, HC: 2.57 +- 0.65, $p = 0.032$, $Sign^+$: 3.01 +- 3.30 +- 0.85, p = 0.043

Table II Correlations between the different T cell subsets

Th17	lymphocytes	monocytes	CD3CD25+	IL4 containing lymphocytes	Th2	Regulatory T cells		
,411**	.202	-.205	,460**	.170	-.218	.052	*r*	Th1
.002	.155	.149	.001	.223	.116	.712	p	
	.081	-.089	,441**	.092	-.020	.089	*r*	Th17
	.571	.534	.001	.512	.889	.526	p	
		-,961**	-.255	-.097	-.223	-.075	*r*	lymphocytes
		.000	.071	.499	.116	.600	p	
			.200	.063	.189	.054	*r*	monocytes
			.160	.662	.184	.705	p	
				,548**	.232	,516**	*r*	CD3CD25+
				.000	.094	.000	p	
					,653**	,397**	*r*	IL4 containing lymphocytes
					.000	.003	p	
						.123	*r*	Th2
						.382	p	

r = correlationcoefficient
Correlations of the different subsets.
P values were tested by Pearsons correlation on log transformed data.

Correlations between the different T cell subsets (Table II)

The percentages of $CD3^+CD25^+$ cells correlated significantly to the percentages of regulatory T cells, classical Th1 cells and Th17 cells, but not to Th2 cells . Percentages of Th17 cells correlated with those of Th1 cells, but not to the Th2 cells. The percentages of regulatory T cells did not correlate to those of Th17 cells. There was also no correlation for regulatory T cells and Th1 or Th2 cells. Although the correlation coefficient was negative for the correlation between Th1 and Th2 cells (as could be expected from the reciprocal character of the subsets), values did not reach statistical significance.

Table III Cytokine levels in serum

		Schizophrenia	Healthy Controls	p-value
Monocyte activation	CCL2	33.39 (12.46 -60.30)	25.74 (14.10 -60.90)	0.495
	IL-1β	0.02 (0 - 0.55)	0.05 (0 - 2.55)	0.447
	PTX3	66.50 (7.36 - 381.49)	73.07 (13.90 - 368.80)	0.493
	IL-6	nd	nd	
	TNFα	nd	nd	
T cell proliferation activity	sIL-2R	1960 +- 593	1496 +- 473	0.009
Th1	IFNγ	nd	nd	
Th2	IL-5	0.05 (0 - 0.41)	0.01 (0 - 2.69)	0.281
	IL-4	nd	nd	
Th17	IL-22	47.43 (41.17 - 94.38)	45.34 (41.17 - 59.98)	0.265
	IL17a	nd	nd	
Regulatory T cells	TGFβ	13872 (7602 - 68981)	12655 (4761 - 23198)	0.204
	IL-10	nd	nd	

nd = not detected

Levels of cytokines are expressed as median with range. All values are in picograms per milliliter. Normal distribution was not obtained after log-transformation. The P values were calculated thus by Mann-Whitney tests.

sIL-2r had a normal distribution and the level is thus expressed as mean +- standard deviation in IU/ml. The P value was calculated by ANOVA with age and gender in the model.

Pro-inflammatory and anti-inflammatory cytokines in serum

Levels of IFN-γ, IL-17, IL-10, IL-6, IL-4 and TNF-α were lower than the detection limit in the serum of patients and controls (Table III).

Levels of CCL2, IL-5, IL-1β and PTX3 were detectable, but levels were not different in the serum of patients with schizophrenia as compared to those of HC. Levels of the Th17 related cytokine IL-22 and of the regulatory T cell related cytokine TGF-β were higher in the serum of patients with schizophrenia, however levels did not reach statistical significance (Table III).

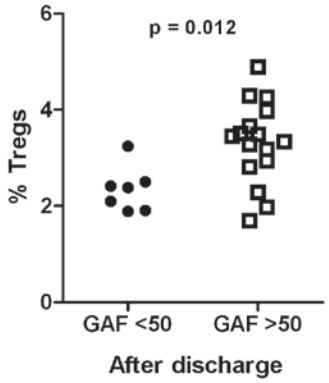
We were unable to find correlations between the cytokines listed in this section and the percentages of the various T cell subsets.

Relation of T cell inflammatory parameters to clinical variables

We were unable to find any correlation between the T cell variables reported here and antipsychotic drug, smoking, positive symptoms, negative symptoms or GAF score on admission.

A positive correlation was found between the level of functioning of the patients with schizophrenia at discharge and their regulatory T cell percentages on admission (Fig 2): Patients with regulatory T cell percentages above 3,25% (n = 16) on admission all had a GAF score of 50 or higher at discharge, whereas all patients (n = 7) with regulatory T cell percentages lower than 3,25% had a GAF score of 49 or lower at discharge (of 3 patients GAF score at discharge were not available). This led to a positive correlation between regulatory T cell percentage on admission and GAF-score at discharge ($r = 0.442$, $p = 0.035$, with Bonferroni correction for 6 tests statistical significance was not reached).

Figure 2 Relation of regulatory T cell percentage on admission and GAF score at discharge



GAF = global assessment of functioning.
% Tregs = percentage of $CD4^{+}CD25^{high}FoxP3^{+}$ cells on admission.
P values were tested by linear regression on log transformed values. $p = 0.012$ $df = 21$

Relation of T cell inflammatory parameters to the pro-inflammatory state of patient monocytes

Samples of 19 of the monocyte pro-inflammatory signature-tested patients could also be used in the present study and in 11/19 of these patients the signature was present as compared to 3/23 of the HC (Legend of supplementary Figure 2 for definition of a positive signature).

The higher percentages of IL-4 containing lymphocytes and of classical Th2 cells found in our patients with schizophrenia were largely confined to patients with schizophrenia with a positive monocyte pro-inflammatory signature (Fig 1a-b, Suppl Fig 2).
The higher percentages of Th17 cells, regulatory T cells and $CD3^+CD25^+$ cells were found in both monocyte signature positive and negative patients with schizophrenia. In the signature negative patients significantly higher percentages of regulatory T cells and $CD3^+CD25^+$ cells were found (Fig 1c-e).

Discussion

This study shows a strongly activated set point of the T cell and monocyte inflammatory network in patients with recent onset schizophrenia. On the one hand an activation of pro-inflammatory forces was found, i.e. higher percentages of circulating monocytes with a pro-inflammatory gene expression signature, activated ($CD3^+CD25^+$) T cells and pro-inflammatory Th17 cells, on the other hand there was also an activation of anti-inflammatory forces, i.e. increases in $CD4^+CD25^{high}FoxP3^+$ T cells and IL-4 containing lymphocytes.

The up regulation of IL-4 containing lymphocytes and of classical Th2 cells, i.e. the $CD4^+$ population of IL-4 containing lymphocytes, was predominantly found in patients with schizophrenia characterized by a monocyte pro-inflammatory gene expression set point. This constellation of the co-occurrence of pro-inflammatory prone monocytes with higher percentages of IL-4 containing lymphocytes is counterintuitive, since IL-4 dampens the inflammatory state of monocytes and macrophages, turning the cells in so-called M2 cells [15]. We like to interpret our data by hypothesizing that the higher percentage of IL-4 containing lymphocytes in these monocyte gene expression signature positive patients serves as a control mechanism to counteract the monocyte proneness for inflammation keeping an actual higher production of inflammatory monocyte cytokines under control. This interpretation might then explain the normal serum levels of the pro-inflammatory monocyte/macrophage cytokines CCL2, IL-1β and PTX3 we found in our patients. On the other hand, preliminary data of our group (to be published) in a cohort of older (on average 40 yrs) patients with chronic naturalistically treated schizophrenia showed a much lower inflammatory monocyte gene expression than reported here for acute schizophrenia, while serum levels of inflammatory cytokines were clearly raised in the group of older patients, but – apart from disease- also linked to obesity and serum lipid levels. This points towards a clear and not congruent role for disease duration, treatment, activity and/or diet/obesity in the inflammatory profiles of circulating cells and cytokine levels. This

impacts further studies for biomarker development needing to control for these variables, unfortunately in this study we were not informed on metabolic parameters of patients and controls and could not adjust for this.

The increases in both the pro-inflammatory subset of Th17 cells and in the anti-inflammatory subset of regulatory T cells in SCZ patients were found irrespective of the monocyte pro-inflammatory state (be it a little more outspoken in the signature negatives). Increases in the regulatory T cells and Th17 cells also correlated to increased numbers of $CD3^{+}CD25^{+}$ T cells and higher levels of serum sCD25. Although we like to interpret these CD25 data as reflecting a higher tendency for T cell proliferation, which might then underlie the higher percentages of Th17 and regulatory T cells, there are data in the literature which show that T cell proliferation is in fact reduced in acute forms of schizophrenia when T cells were stimulated with anti-CD3 [16].This study also found a normal expression of CD25 on CD4+T cells in patients with schizophrenia. Further studies are needed to clarify this issue.

There is strong evidence that Th17 are inflammation-promoting cells in chronic inflammatory conditions such as rheumatoid arthritis, asthma, psoriasis and multiple sclerosis and the number of Th17 cells as well as the levels of the Th17 derived cytokines IL-17, IL-21 and IL-22 are higher in affected tissues [17]. Although patients with schizophrenia do have molecular alterations in their brain vasculature [18], it is unknown whether Th17 cells or the Th17 cytokines pass the blood-brain-barrier to exert an effect. In a mouse model of multiple sclerosis Th17 cells do migrate into the brain [19]. Studies on post mortem brains of schizophrenia patients and animal models of schizophrenia-like disease could be instrumental to solve this question of Th17 influence on behavior.
There is ample evidence that regulatory T cells are capable of dampening the effect of Th17 cells [17] , Th1 cells [10] and pro-inflammatory monocytes [20] in immune reactions. It is therefore tempting to hypothesize that the increase in anti-inflammatory regulatory T cells is meant (like that proposed of the IL-4 containing lymphocytes) to keep the pro-inflammatory immune forces in patients with schizophrenia under control.

Another point of interest with regard to both the rise in Th17 and regulatory T cells is that the development of regulatory T cells has been reported to be tightly linked to that of Th17 cells. Both cell lineages share common developmental pathways [17, 21]. Also Foxp3-lineage-commited regulatory T cells were directly converted into IL-17-expressing cells by CpG treatment and interestingly IDO acts as a pivotal molecular switch in this process: Only in the absence of IDO this conversion occurs, whereas this was blocked when IDO was active [22]. Although IDO activation and abnormal tryptophan break-down is thought to play a role in schizophrenia, we were not able to find in this study a correlation between percentages of regulatory T cells and those of Th17 cells.

With regard to clinical characteristics we found that a high regulatory T cell percentage on admission correlated with a better clinical outcome (GAF score) at discharge. Interestingly in this respect is that IL-10, one of the key cytokines produced by regulatory T cells, has direct effects on brain function and is able to dampen down sickness behavior [23]. However, effects of the regulatory T cells could also be mediated by a direct suppression of the pro-inflammatory immune forces in the brain e.g. those exerted by pro-inflammatory microglia.
However and with regard to this clinical effect, a limitation of our study is that it was not designed to investigate parameters of prediction and p values did not reach significance with Bonferroni correction. Suffice to say that a study on the predictive value of regulatory T cells for outcome in psychosis in a larger group of patients is now imperative.

Our study has also other limitations.
We only used peripheral blood samples and we were not able to investigate the cerebrospinal fluid (CSF). Pathologic abnormalities of the brain are probably directly reflected in the CSF, and indeed in schizophrenia there are reports on cell aberrancies in CSF. A recent paper investigated T cell subsets in CSF and peripheral blood of patients with a major psychiatric disorder and found an overlapping low grade inflammation in CSF as well as in peripheral blood [24] . Unfortunately, we were unable to obtain CSF samples from our patients.
Furthermore, the study has a small sample size and outcomes should be replicated on a larger and independent sample size. The study subjects were the same in which the monocyte signature was determined, and it could be that this sample of patients with schizophrenia has an extreme activation of the immune system. However, we have found a similar strong inflammatory monocyte signature in patients with mania [1, 25] and in post partum psychosis (to be published). In the latter conditions the T cell system was not activated (to be published).
Our study is naturalistic and all patients (except one) were on –very recently started- antipsychotic medication. As antipsychotic medication exerts anti-inflammatory effects [26-28], this effect on the outcome cannot be ruled out [1, 25]. Moreover, there were more male participants than female participants in this study. Although healthy controls were age and gender matched, we cannot completely rule out a general effect of gender on the activation state of monocytes and/or T cells. Moreover, we cannot rule out a general effect of smoking in this study sample because there was a difference in the smoking status of the patients (69%) versus our healthy controls (35%). We did however not find a correlation for smoking with one of the other immune parameters.
A further limitation is that our FACS data represent percentages, not the number, of cells within the lymphocyte population. We were unable to give absolute cell numbers, since

we had not performed a leukocyte count in the same blood sample as used for FACS, although leukocyte counts routinely performed in the patient at the same time did not show abnormalities.

Taken together, our data do not support the concept that the T cell system is in a simple pro-inflammatory state (our hypothesis), but do show that the monocyte and T cell networks are activated in recent onset schizophrenia and involve both pro- and anti-inflammatory forces.
The question remains when and why these activated set points are installed and what the consequences are for the pathology of schizophrenia. Here the animal models of the disease might become important tools for further study. In rodents chronic immune activation in the peri-natal period by reagents mimicking infections with commensal bacteria and viruses (such as LPS and GpC) do lead to altered set points of the inflammatory system which cause abnormalities in brain development and consequent psychosis-related behaviour [29-30]. Further studies in these animal models into aberrant set points of their macrophage/T cell system are indicated.

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All other authors declare that they have no conflicts of interest.

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Chapter 6

An inflammatory gene-expression fingerprint in monocytes of autoimmune thyroid disease patients

Short title: Monocytes in AITD

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Abstract

Context: In monocytes of patients with autoimmune diabetes we recently identified a gene expression fingerprint of 2 partly overlapping gene clusters, a "PDE4B associated" cluster (consisting of 12 core pro-inflammatory cytokine/compound genes), a "FABP5 associated" cluster (3 core genes) and a set of 9 overlapping chemotaxis, adhesion and cell assembly genes correlating to both PDE4B and FABP5.

Objective: To study whether a similar monocyte inflammatory fingerprint as found in autoimmune diabetes is present in autoimmune thyroid disease (AITD).

Design and patients: Q-PCR analysis for 28 genes in monocytes of 67 AITD patients and 70 healthy controls (HC). The tested 28 genes were the 24 genes previously found abnormally expressed in monocytes of autoimmune diabetes patients plus 4 "extra" genes found in whole genome analysis of monocytes of AITD patients reported here.

Results: Monocytes of 24% of AITD and 50 % of LADA patients shared an inflammatory finger print consisting of the set of 24 genes of the PDE4B, FABP5 and overlapping gene sets. This study in addition revealed that FCAR, the gene for the Fcα receptor I and PPBP, the gene for CXCL7 were part of this pro-inflammatory monocyte fingerprint.

Conclusions: Our study provides an important tool to determine a shared, specific pro-inflammatory state of monocytes in AITD and LADA patients, enabling further research into the role of such pro-inflammatory cells in the failure to preserve tolerance in these conditions and of key fingerprint genes involved.

Introduction

Autoimmune thyroiditis is an autoimmune disease, in which T cells and antibodies specific for thyroid antigens target thyrocytes, resulting in the destruction of the gland leading to hypothyroidism.

There is a large body of literature showing that in the animal models for spontaneously developing autoimmune thyroiditis (the BB-DP rat and the NOD mouse) cells of the monuclear phagocyte series, i.e. monocytes, macrophages (MØ) and dendritic cells (DC), are aberrant in function and involved in the induction of autoantigen specific T cells and antibodies (1-3). Under normal, steady state conditions DC and MØ take up auto-antigens, travel with these to the draining lymph node and activate T regulatory cells specific for these autoantigens, inducing a state of tolerance. However, in the BB-DP rat and the NOD mouse DC and MØ are in a pro-inflammatory state, resulting in a predominant stimulation of autoimmune effector T and B cells. In the BB-DP rat steady state DC are defective in IL-10 production and have a reduced capability to stimulate a sub-population of T regulatory cells in the rat (the ART-2+ T cells, 1), while in the NOD mouse steady state DC express high levels of NFκB and IL-12 and have a macrophage-like appearance (2, 3). Correction of the aberrant DC in the NOD mouse via transfers of normal DC led to the prevention and/or the delay of autoimmunity (4,5), supporting the view that an aberrant pro-inflammatory set point of steady state DC is one of the cornerstones in the induction of autoimmunity in this model. Not only the DC, but also their immediate pre-cursors in the peripheral blood i.e. the monocytes, are in an aberrant pro-inflammatory state in the NOD mouse, which is shown by an overrepresentation of the fraction of Ly-6C^{low} monocytes (6).

With regard to thyroid autoimmune patients, monocyte integrin- and chemokine-mediated functions are hampered and we previously hypothesized that – similar to the NOD mouse and BB-DP rat - these abnormalities might underlie misbalances in monocyte inflammatory set points important for the loss of tolerance (7,8).

Autoimmune thyroiditis has many characteristics in common with autoimmune diabetes and the diseases often co-occur in patients and their families (9). Also the BB-DP rat and NOD mouse are characterized by such co-occurrence (10). In monocytes of patients with autoimmune diabetes we (11) recently identified at the molecular, i.e. at the gene expression level, a fingerprint of 2 partly overlapping gene expression clusters, reflecting the abnormal inflammatory set point of the cells in this disease. One cluster, an "inflammatory compound" cluster, consisted of 12 core pro-inflammatory cytokine/compound genes strongly correlating to the expression of PDE4B (the "PDE4B associated"

cluster) and was detected in 60% of adult onset LADA, but in only 10% of juvenile onset type 1 diabetic patients. A second cluster consisted of 3 core genes strongly correlating to the expression of FABP5 (the "FABP5 associated" cluster), and was detected in 43% of juvenile-onset type 1 diabetic and 33% of adult onset LADA patients. Apart from these 15 core genes there were 9 other overlapping chemotaxis, adhesion and cell assembly genes correlating positively but weaker to the expression of PDE4B as well as that of FABP5.

A gene expression study into an abnormal inflammatory set point of monocytes in autoimmune thyroiditis is lacking.

We here report outcomes of a Q-PCR analysis, to investigate whether the in total 24 genes found aberrantly expressed in monocytes of autoimmune diabetic patients were also aberrantly expressed in the monocytes of patients with autoimmune thyroiditis. We in addition focused on finding new and discriminating genes abnormally expressed in monocytes of autoimmune thyroiditis patients. We used whole genome expression profiling (in the same way as we did for the autoimmune diabetes study) to identify aberrantly expressed genes in the monocytes of patients with autoimmune thyroiditis and found 4 new and extra "thyroid-autoimmune-specific" genes. The extra found 4 genes were also validated via Q-PCR analysis in the monocytes of the thyroid autoimmune patients.

Material and Methods

Patients

Autoimmune hypothyroid and hyperthyroid patients: Newly diagnosed hypothyroid Hashimoto's thyroiditis (HT, n=34) and hyperthyroid Graves' patients (n=33, together indicated as autoimmune thyroid disease (AITD) patients, n=67), who visited the outpatient clinic of the Department of Internal Medicine, Maasstad Ziekenhuis, Rotterdam, the Netherlands, were recruited. All patients with HT were clinically hypothyroid, had a lowered serum free thyroxin level (fT4, cut off levels: 10.0- 24.0 pmol/L) and a raised thyroid stimulating hormone level (TSH, cut off levels: 0.4-4.0 mU/L), and were positive for thyroid peroxidase antibodies (TPO-abs, cut off level: 35 IU/ml, Immulite, DPC, Breda, The Netherlands). TSH-receptor antibodies were negative (cut off level: 1.5 IU/L Brahms Diagnostics, Berlin, Germany). The hyperthyroid Graves' patients all had a raised fT4 level and a decreased TSH level. They also had a diffuse non-nodular appearance of thyroid on palpation, scan or ultrasound. In addition, patients were positive for TSH-receptor antibodies. None of the newly diagnosed HT and Graves' patients received medication

(also no thyroid medication) at the time of blood collection.

Healthy Controls: Age and gender matched healthy controls (HC, n=70) were recruited via enrolling laboratory staff, medical staff and medical students. The inclusion criteria for the HC were an absence of any thyroid and autoimmune disorder and an absent history of these disorders in first-degree family members. HC had to be in self-proclaimed good health and free of any obvious medical illness for at least two weeks prior to the blood withdrawal, including acute infections and allergic reactions.

The Medical Ethical Review Committee of the Erasmus MC Rotterdam approved the studies. Written informed consent was obtained from all participants after a complete description of the study was given.

Characteristics of both patients and HC are given in Table 1.

Table I. Characteristics of AITD patients amd healthy controls used for RQ-PCR, and IL1β, IL6 ELISAs.

		AITD patients	Healthy controls
Group size		34 HT	70
		33 GD	
Age (years)1		41 (18-88)	40 (21-54)
Gender	Male	8 (23%)	5 (18%)
	Female	27 (77%)	23 (82%)

1Mean (range)

HT= Hashimoto's thyroiditis, GD= Graves' disease, AITD= autoimmune thyroid disease

Laboratory methods

Blood collection and preparation

Blood was collected in clotting tubes for serum preparation (stored at -80°C) and in sodium-heparin tubes for immune cell preparation. From the heparinized blood peripheral blood mononuclear cell (PBMC) suspensions were prepared by low-density gradient centrifugation within 8 hours to avoid activation of the monocytes. PBMCs were frozen in 10% dimethylsulfoxide and stored in liquid nitrogen. This enabled us to test patient and control immune cells in the same series of experiments.

Isolation of monocytes

CD14 positive monocytes were isolated from frozen PBMCs from selected subjects (Table 1) by magnetic cell sorting system (Miltenyi Biotec) The purity of monocytes was > 95% (determined by morphological screening after trypan blue staining and FACS). As reported elsewhere positive versus negative selection of immune cells did not influence gene expression profiles (11,12).

ELISAs

The CCL2, CCL7, IL-6 and IL1-β levels in sera of the various patient groups were determined via commercially available ELISAs (R&D Systems, Minneapolis MN, USA.) used according to the manufacturers protocol.

Affymetrix whole genome gene expression profiling

RNA was isolated from purified monocytes using RNAeasy columns as described by the manufacturer (Qiagen, Hilden, Germany). Fragmented cRNA was hybridized to U95Av2 microarrays (Affymetrix, manufacturers protocol). For all experiments, the 5' / 3' ratios of GAPDH were 2 or less (usually 0.9 – 1.1). 2 pools of autoimmune thyroiditis (AITD) patients, each pool was compared to a monocyte pool of age and gender-matched healthy controls (HC), (pools were used for minimizing inter-individual differences in mRNA expression): Pool 1 consisted of 2 females with HT (Hashimoto's thyroiditis) , ages 34 and 40 yrs, pool 2 consisted of 2 females, ages 49 and 54 yrs with HT, pool 1 and 2 were compared to a pool of 4 healthy control females, ages 33-46 yrs.

Quantitative –PCR (Q-PCR)

RNA was isolated from monocytes of the AITD patients and matched healthy controls. To obtain cDNA for Q-PCR, we used the extensively described BIOMED-1 protocol (13). One μg RNA was reversed transcribed using SuperscriptII (Invitrogen) and random hexamers (Amersham Biosciences, Roosendaal, The Netherlands) for 50 min at 42°C.

Q-PCR was performed with Taqman Universal PCR mastermix (Applied Biosystems, Foster City, CA). All Taqman probes and consensus primers were pre-formulated and designed by Applied Biosystems (Assays on Demand, Appendix 1). PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and finally 1 min at 60°C. PCR amplification of housekeeping gene *ABL* and *RPL32* were performed for each sample to allow normalization between the samples. *ABL* and *RPL32* were chosen as housekeeping gene because it was previously shown that they were the most consistently expressed housekeeping gene in haematopoietic cells (14). The quantitative value obtained from Q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from normalized CT values (CT gene- CT housekeeping genes (avg)), by the ΔΔCT method ($2^{-\Delta\Delta CT}$, User Bulletin 2, Applied Biosystems, Foster City, California, see Table 2). To correct for inter-assay variance we set the mean of the studied genes found in the healthy control groups in the same assay for each gene to 1 ($\sum\Delta CT\ HC= 0$, $2^{-0}=1$). The fold change values of the genes in patient monocytes were expressed relative to this set mean of 1 (Table 2).

Statistics

Scanned microarray images were analyzed using Affymetrix Microarray Suite 4.2 software. Further analysis was performed using Rosetta Resolver (www.rosettabio.com) software and Ingenuity Systems (www.ingenuity.com) software. Statistical analysis was performed using the SPSS 15.0 package for Windows. Data were tested for normal distribution using the Kolmogorov-Smirnov test. Depending on the distribution pattern and the total number of subjects, parametric (normal distribution and ≥ 50 subjects) or non-parametric tests (skewed distribution or <50 subjects) were used. Levels of significance were set at p=0.05 (2-tailed). The specific tests used are mentioned under tables and in legends.

Results

Whole genome expression profiling for inflammatory genes in monocytes of autoimmune thyroiditis patients

Affymetrix microarray analysis to search for aberrantly expressed genes in monocytes was performed on 2 pools of autoimmune thyroiditis (AITD) patients. All raw data obtained by Affymetrix are available as MIAMExpress submission E-MEXP- (http://www.ebi.ac.uk/miamexpress/).

In Rosetta Resolver analysis of the expression data we took only molecules into consideration, which were more than 4 fold statistically significantly differentially expressed between patients and HC, with the purpose to identify strongly discriminating molecules. This resulted in 32 genes for AITD patients (27 over and 5 under expressed). These genes were analyzed via Ingenuity pathway analysis software. Major functional networks/pathways found to be involved were cell growth/differentiation, cell-to-cell interaction, and signaling and cell movement.

To select for genes which could serve as biomarkers for the monocyte inflammatory condition we took the genes in the upper or lower 33 percentile of the list, but with at least a 5 fold abnormal expression and of which the function is known and involved in inflammation or related processes such as cell movement, cell-to-cell interaction or signaling. This selection resulted in 4 genes *FCAR, EIF2S3, ADAM17* and *PPBP* (all up-regulated, there were no discriminating negative genes fulfilling the criteria). These 4 up-regulated genes were validated in Q-PCR together (see below) with the 24 genes previously found abnormally expressed in autoimmune diabetes patients (11).

Q-PCR analysis of monocytes of thyroid autoimmune patients

Table II shows that in Q-PCR 5 of the in total 28 studied genes were significantly aberrantly expressed in the 34 (hypothyroid) Hashimoto's thyroiditis (HT) patients tested.

We decided to also include in Q-PCR patients with (hyperthyroid) Graves' disease (GD), the other major autoimmune condition of the thyroid. This enabled us to investigate whether the thyroxine/TSH status influenced the pro-inflammatory monocyte gene expression or whether the aberrant gene expression was mainly due to the autoimmune condition per se. Therefore outcomes in the HT patients were compared to those of 33 GD patients. Table II shows that virtually all genes were similarly aberrantly expressed in the monocytes of HT and GD patients and it appeared that only *EMP1* and *EIF2S3* showed a clear correlation (Spearman) with fT4 and T3 serum levels (fT4: *EMP1* r=0.41, *EIF2S3* r=0.27; T3: *EMP1* r= 0.35, *EIF2S3* r=0.41, $p<0.05$ for all correlations). Thus only these genes were clearly influenced by the thyroid hormonal state.

Because of the similar aberrant expression levels of the various genes in monocytes of HT and GD patients and the closely related immune-pathology of both autoimmune disorders, we analyzed the two clinical conditions together considering them as representing one autoimmune condition, i.e. autoimmune thyroid disease (AITD, Table II). Only 6 of the 28 genes were statistically significant higher expressed in monocytes of the entire group of 67 AITD patients (Table II). These aberrantly expressed genes were 3 of the 9 chemotaxis/adhesion/cell assembly set genes and 3 of the 12 PDE4B associated genes.

Of the new "thyroid-autoimmune-specific" genes none could be verified in Q-PCR as a gene significantly abnormally expressed in monocytes of the entire group of 67 AITD patients.

It is also of note that we were not able to find a clinical variable (age, gender, age of onset, and titer of TPO and TSH-R abs) linked to the expression level of any of the fingerprint genes, apart from the genes linked to the thyroid hormonal state (*EMP1* and *EIF2S3,* see before).

Table II. Q-PCR analysis of monocytes of autoimmune thyroid disease (AITD) (n=67) as compared to healthy control values (HC AIT: n=70), set at 1 fold. AITD split up in Hashimoto's autoimmune thyroiditis (HT) (n=34) and Graves'disease (GD) (n=33).

	HT		GD		AITD	
Genes selected by whole genome screening in this study in autoimmune thyroid disease.						
FCAR	1.85	0.57	1.57	0.84	1.72	0.82
EIF2S3	1.13	0.54	1.44	0.05	1.28	0.15
ADAM17	1.53	0.28	1.75	0.06	1.64	0.09
PPBP	1.31	0.83	2.59	0.16	1.93	0.51

	HT		GD		AITD	
PDE4B correlating set: genes selected in a previous study on autoimmune diabetes (Padmos et al)						
PDE4B	4.62	0.04	4.54	0.22	4.58	0.04
CCL20	26.3	0.64	37.84	0.24	31.98	0.67
DUSP2	4.83	0.22	5.23	0.72	5.02	0.32
IL1B	9.39	0.99	12.47	0.72	10.9	0.82
PTGS2	9.31	0.15	11.61	0.07	10.44	0.05
IL6	38.7	0.17	43.08	<0.01	40.86	0.01
BCL2A1	4.13	0.28	4.67	0.04	4.39	0.06
PTX3	6.47	0.27	4.54	0.21	5.52	0.94
ATF3	4.45	0.08	4.71	0.06	4.58	0.84
TNFAIP3	4.99	0.71	5.09	0.96	3.4	0.37
NAB2	3.11	0.1	4.15	0.42	3.62	0.13
TNF	3.73	0.67	4.04	0.29	3.88	0.36
FABP5 correlating set: genes selected in a previous study on autoimmune diabetes (Padmos et al)						
CD9	3.03	0.55	6.41	0.42	4.69	0.39
HSPA1A	1.21	0.48	1.78	0.1	1.49	0.15
FABP5	2.87	0.21	5.7	0.99	4.26	0.43
Overlapping set of chemotaxis/adhesion/cell assembly genes selected in a previous study on autoimmune diabetes (Padmos et al)						
PTPN7	1.96	0.04	2.06	0.24	2.01	0.05
MAPK6	1.89	0.52	1.61	0.2	1.75	0.24
DHRS3	2.69	< 0.01	2.68	0.02	2.69	< 0.01
CCL7	21.66	0.83	20.21	0.05	20.95	0.2
STX1A	5.93	0.24	3.98	0.48	4.97	0.24
EMP1	5.13	0.03	2.14	0.98	3.66	0.17
CDC42	4.31	< 0.01	6.26	< 0.01	5.27	< 0.01
CXCL2	10.14	0.79	13.46	0.43	11.78	0.52
CCL2	8.75	0.34	7.65	0.59	8.21	0.36

The quantitative value obtained from Q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from normalized CT values (CT gene- CT reference gene ABL), by the ΔΔCT methods ($2^{-\Delta\Delta CT}$, user Bulletin 2, Applied Biosystems, Foster City, California). Data were standardized to the HC (thus the HC were used as the calibrator). The fold change of the HC is therefore 1. Groups were compared using the Mann-Whitney test. HT, Hashimoto's thyroiditis; GD, Graves'disease; AITD, autoimmune thyroid disease

Cluster analysis of Q-PCR data of monocytes of thyroid autoimmune patients

In a next step we studied the mutually interdependent state of expression of the 28 genes and therefore performed a cluster analysis on the Q-PCR data. The heat map of this correlation analysis is given in Fig 1.

In sum, although expression levels of the large majority of the 28 genes correlated to each other, two clusters of strongly correlating genes could clearly be identified: One cluster (darkest red, Fig 1., correlation coefficient > 0.70) was formed by a large part of the previously identified PDE4B associated cluster (genes as PTX3, ATF3, IL-6, IL1B, CCL20, PDE4B and PTGS2), the second cluster (also dark red, Fig 1., correlation coefficient > 0.70) was formed by a large part of the previously identified chemotaxis/adhesion/cell assembly gene set (genes as CXCL2, CCL2, CCL7, MAPK6, PTPN7 and STX1A).

With regard to the FABP5 correlating set previously identified in autoimmune diabetes (FABP5, CD9 and HSPA1A), there was not a clear indication of such strongly correlating set in AITD, genes correlated to each other at correlation coefficients of only 0.12 to 0.26.

Special in this study on AITD patients is that the 4 genes newly identified in Affymetrix (FCAR, ADAM, PPBP and EIF2S3) also correlated to each other at reasonable level with correlation coefficients of 0.21 to 0.48. Special is that of these 4 genes FCAR in particular correlated in expression level strongly to both the PDE4B associated genes and the genes of the chemotaxis/adhesion/cell assembly set, with correlation coefficients > 0.5 (see Fig. 1).

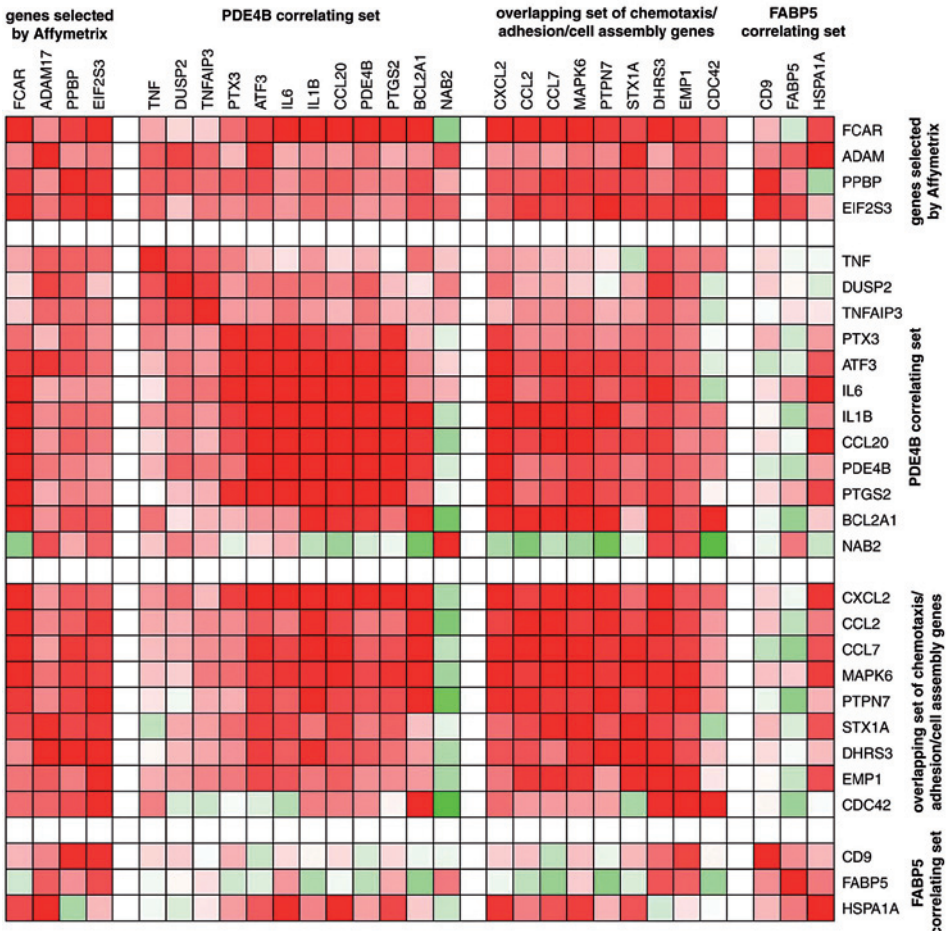
A comparison of the prevalences of the monocyte gene expression profiles in autoimmune thyroid disease patients with those in autoimmune diabetes patients published previously

On the basis of the cluster analysis we decided to express the data as a heat map of the individual genes as expressed in monocytes of individual AITD patients (see Figure 2, in this figure the various reds mean over expression as compared to the mean expression of a particular gene in monocytes of HC, the various greens mean a reduced expression as compared to the mean expression of a particular gene in monocytes of HC).

As can be seen from these heat maps about one quarter of the AITD patients (i.e16/67 patients, 7 HT patients and 9 GD patients) showed a pro-inflammatory monocyte set point of particular the PDE4B associated genes set (gene set B) and the chemotaxis/ adhesion/ cell assembly gene set (gene set C) (both columns for about a quarter strongly red), while one third had absolutely no signs of an high pro-inflammatory monocyte set point (predominantly strongly green).

Figure 2 also shows the heat maps of the monocyte gene expression levels of the AITD patients in comparison to those of LADA patients and childhood onset T1D children previously reported (see 11, for the actual raw gene expression data of monocytes of LADA and childhood onset type 1 diabetes patients we refer to these previously published

Figure 1. Expression levels of monocyte gene clusters in AITD patients



Correlation analysis of expression levels of the 28 genes identified in AITD patients. Data represent Spearman's correlation coefficients illustrated as heatmap by Treeview. Red indicates strong correlation, green represents anti-correlation. The 28 genes are divided in 4 groups; Genes selected by Affymetrix; PDE4B correlating genes; overlapping set of chemotaxis/adhesion and cell assembly genes and FABP5 correlating genes.

data). As can be seen from these heat maps about half of the LADA patients showed a pro-inflammatory monocyte set point similar to the pro-inflammatory set point found in the one quarter of AITD patients, and again the "negative" LADA cases had no signs of an abnormal pro-inflammatory monocyte set point. The childhood onset T1D cases are special in that they lack over expression of gene set B, but were positive in about two-third of cases for monocyte gene set C (and they also were positive in these cases for gene set D, the FABP5 associated cluster, i.e. an over expression of CD9 and FABP5 and a down regulation of HSPA1A).

In an attempt to enumerate more precisely the prevalence's of the various subsets of genes in the patient groups we arbitrarily defined positivity for the PDE4B correlating set cluster as positive for at least 6 of the 12 PDE4B genes, positivity for the FABP5 correlating set as positive for 2/3 genes and positivity for the chemotaxis/adhesion/cell assembly cluster as positive for at least 5 of the 9 genes. Table III gives these prevalence figures. As can be seen, 38% of AITD patients share an up regulation of the set of chemotaxis, adhesion and cell motility/assembly cluster genes (cluster C in figure 2) in monocytes with 60% of childhood onset T1D and 67% of LADA patients. They take an intermediate position in positivity for PDE4B correlating gene set (Cluster B in figure 2), i.e. 27% have such pro-inflammatory monocytes versus 50% of LADA patients and virtually none (3%) of childhood onset T1D patients. With regard to the FABP5 correlating gene sets (Cluster D in figure 2) AITD patients were hardly positive.

When we defined "fingerprint" positivity as a combination of 50% positivity for cluster B plus 50% positivity for cluster C (Table III) 24% of AITD, 50% of LADA and none of T1D childhood onset cases were positive. Interestingly, when we separated these 24% fingerprint positive AITD patients from the 76% fingerprint negative AITD patients (Table IV), we observed in the 24% of AITD cases virtually complete over expression of the various genes of the different clusters (supporting their mutual correlation, see also Figure 1), and also including 2 of the 4 in Affymetrix selected monocyte genes of AITD patients, i.e. FCAR and PPBP (i.e. CXCL7). In the 76% fingerprint negative patients significant over expression was still reached for some of the genes (IL-6, BCL2A1, NAB2, TNF, FABP5, PTPN7 and CDC42), suggesting that at least part of these "negative" cases, which do not have the full-blown fingerprint, have some weak expression of the fingerprint genes in their monocytes.

Table III. Prevalence of gene clusters in HC, AITD, T1D childhood onset and LADA patients legend Table 3.

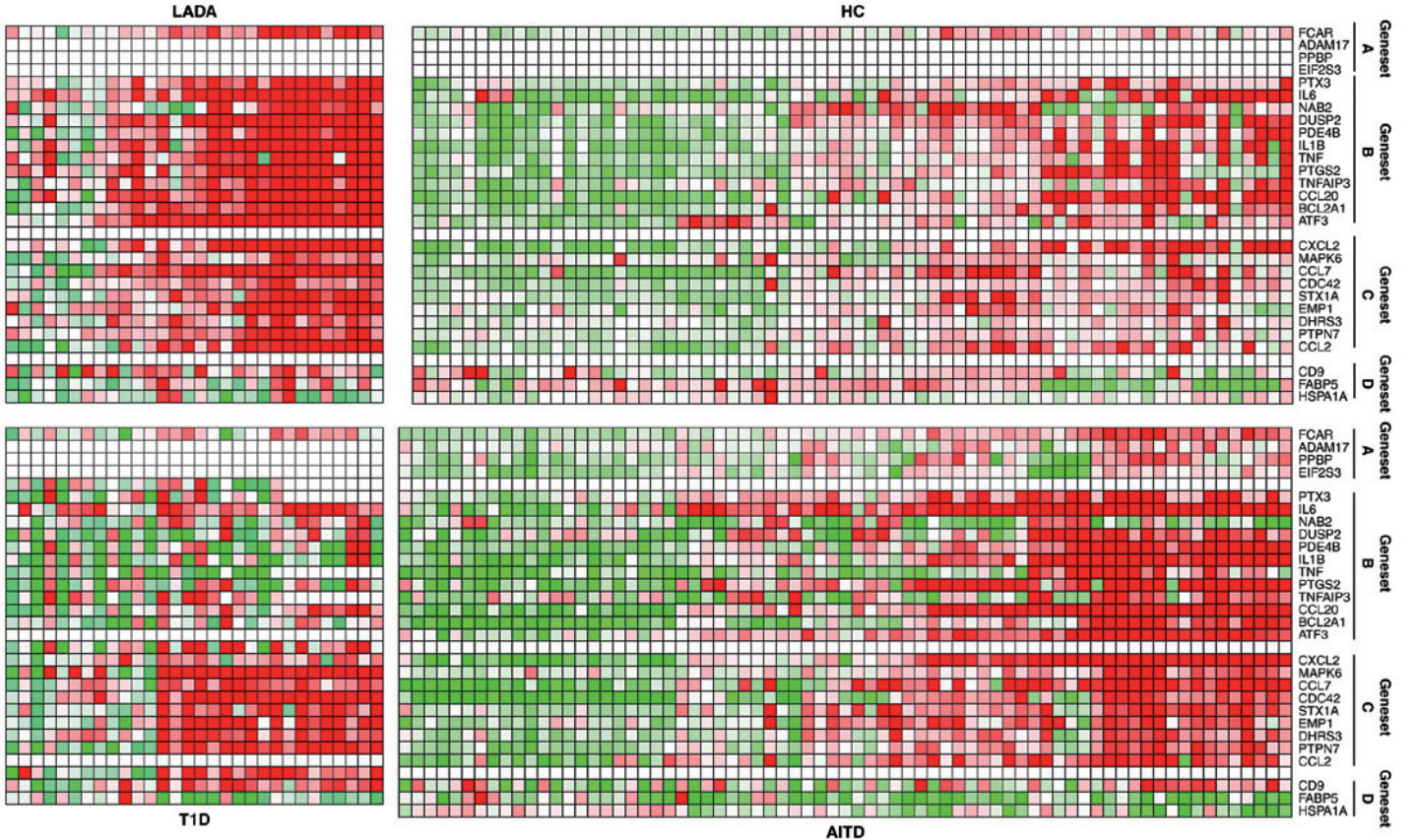
	HC	AITD	T1D	LADA
50% of PDE4B correlating genes PDE4B, CCL20, DUSP2, IL1B, PTGS2, IL6, BCL2A1, PTX3, ATF3, TNFAIP3, NAB2 and TNF	19% (13/70)	27% (18/67)	3% (1/30)	50% (15/30)
50% FABP5 correlating genes FABP5, CD9 and HSPA1A	1% (1/70)	7% (4/67)	50% (15/30)	23% (7/30)
50% chemotaxis, adhesion and cell assembly cluster STX1A, CCL2, CCL7, CDC42, MAPK6, EMP1, CXCL2, DHRS3 and PTPN7	14% (10/70)	38% (24/67)	60% (18/30)	67% (20/30)
50% PDE4B + 50% chemotaxis associated cluster	6% (4/70)	24% (16/67)	3% (1/30)	50% (15/30)

The presence of a PDE4B-associated, FABP5-associated and chemotaxis, adhesion and cell assembly molecular signature in healthy controls HC compared to AITD, T1D childhood onset and LADA patients.

Table IV. Fold change of individual molecules when comparing the 24% fingerprint positive AITD patients to the 76% negative AITD patients. Genes are considered differentially expressed when p< 0.05, Mann Whitney.

24% fingerprint positive AITD patients vs HC			76% negative AITD patients vs HC	
	Fold change	p level	Fold change	p level
Genes selected by whole genome screening in this study in autoimmune thyroid disease				
FCAR	4.81	<0.001	0.7	0.904
EIF2S3	1.47	0.076	1.15	0.381
ADAM17	1.81	0.068	1.52	0.753
PPBP	2.42	0.025	1.6	0.344
PDE4B correlating set: genes selected in a previous study on autoimmune diabetes (11)				
PDE4B	11.87	<0.001	2.24	0.395
CCL20	97.21	<0.001	10.72	0.663
DUSP2	7.67	<0.001	4.51	0.817
IL1B	31.41	<0.001	4.55	0.515
PTGS2	26.21	<0.001	4.73	0.109
IL6	119.61	<0.001	13.7	0.027
BCL2A1	8.99	<0.001	3.18	0.001
PTX3	16.96	<0.001	2.33	0.217
ATF3	10.61	<0.001	2.62	0.15
TNFAIP3	5.73	<0.001	2.91	0.971
NAB2	4.72	0.083	3.02	0.017
TNF	6.65	<0.001	3.09	0.002
FABP5 correlating set: genes selected in a previous study on autoimmune diabetes (11)				
CD9	2.77	0.017	5.07	0.512
HSPA1A	3.07	0.008	0.9	0.954
FABP5	2.72	0.003	4.25	0.001
Overlapping set of chemotaxis/adhesion/cell assembly genes selected in a previous study on autoimmune diabetes (11)				
PTPN7	4.52	<0.001	1.06	0.044
MAPK6	5.84	<0.001	0.4	0.479
DHRS3	5.65	<0.001	1.7	0.381
CCL7	57.92	<0.001	8.86	0.929
STX1A	12.62	<0.001	2.43	0.227
EMP1	6.81	<0.001	2.61	0.529
CDC42	5.63	<0.001	5.39	<0.001
CXCL2	31.65	<0.001	5.19	0.983
CCL2	19.05	<0.001	4.56	0.709

Figure 2. Heat map of monocyt gene expression in Healthy controls (HC), autoimmune thyroid (AITD) patients, LADA and T1D childhood onset patients.

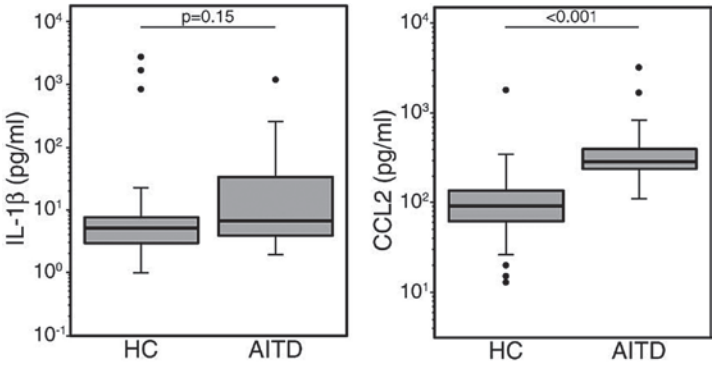


Representation of monocyte gene expression levels from the 28 genes identified in HC compared to autoimmune thyroid disease (AITD), latent autoimmune diabetes of the adults (LADA) and T1D childhood onset patients (T1D). Data represent ΔΔCT and is illustrated as heatmap by Treeview. Red indicates that the gene is higher expressed in the disease compared to HC, green represents that the gene lower expressed in patient than HC. Geneset A, genes selected by Affymetrix; Geneset B, PDE4B correlating set; Geneset C, overlapping set of chemotaxis/adhesion/cell assembly genes; Geneset D, FABP5 correlating set.

Expression at the serum protein level

We measured the serum protein expression levels of the gene set molecules IL-1β and IL-6 (which are part of the PDE4B associated pro-inflammatory gene set) and CCL2 and CCL7 (which are part of the set of chemotaxis, adhesion and cell motility/assembly genes). Only the serum CCL2 levels were significantly higher in AITD patients (Figure 3). Thus at the serum protein level there were only minor signs of monocyte/macrophage activation (since CCL2 is monocyte/macrophage-derived chemokine) and our serum cytokine level data again (see also 11) indicate that serum protein levels do correlate with the aberrant monocyte gene expression, but are only a weak reflection of it.

Figure 3. The protein expression of IL-1β and CCL2 in serum of thyroid autoimmune patients



Box plots of log transformed Il-1β and CCL2 serum levels of AITD patients. Data were log transformed to obtain a normal distribution. Only in case of CCL2 this resulted in a normal distribution, hence an ANOVA analysis was used, adjusted for age and gender. For Il-1β the non-parametric Mann-Whitney test was used. The boxes indicate the lower and upper quartiles. The lines in the boxes represent the median. The whiskers extend to the 2.5 and 97.5 percentiles; the outliers are characterized by the filed dots. Il-1β was tested on the same patients as described in Table 1. CCL2 was tested on more patients: HC n=157, 67% females, mean age 36 years, range 19-56 years, and AITD n=59, mean age 44 years, range 19-87 years.

Discussion

This study shows that monocytes of 24% of AITD patients show an inflammatory set point, a "finger print" of a set of 24 genes involved in inflammation, chemotaxis, adhesion and cell motility/assembly, similar to that previously described in 50 % of LADA patients: This study in addition revealed that two genes which had not been identified before, i.e. FCAR, the gene for the Fcα receptor I (FcαRI) and PPBP, the gene for CXCL7 were part of the pro-inflammatory monocyte fingerprint in these patients.

This study has limitations:
Firstly, the study does not include contrast hyper- and hypothyroid cases of non-autoimmune causes (such as patients with toxic nodular goiters, surgically removed thyroid glands or iodine deficient goiters) to formally rule out a clear effect of the thyroid hormonal state on the found pro-inflammatory state of monocytes. However, virtually the same pro-inflammatory finger print was found in hypothyroid HT and hyperthyroid GD cases (this study) and only one fingerprint gene showed a relation with the thyroid hormone level (i.e. EMP-1; EIF2S3 also showed a relation with the thyroid hormonal state but turned out not to be a significant part of the fingerprint). Also the same fingerprint was found in LADA patients in this study and previously in patients with bipolar disorder (15) with a normal thyroid function. From this we assume that the here described inflammatory state of monocytes is not determined by the thyroid hormonal state.
Secondly, the study does not include cases in follow-up, so we are not informed on the fluctuation of the fingerprint expression in monocytes over time during the disease course. Such information would tell us whether the 76% fingerprint negative cases represent a quiescent phase of the disease or reflect a complete different pathogenic route of thyroid autoimmune pathogenesis not involving abnormal monocytes/macrophages. With regard to the other patient groups, i.e. autoimmune diabetic patients and bipolar patients, showing the same or a similar monocyte pro-inflammatory gene fingerprint we have described that such monocyte fingerprints are already present in the pre-clinical stages of the disease (15) and are for a large part induced by environmental factors (16), yet detailed follow-up studies in AITD patients need to be performed.

It is tempting to speculate that the pro-inflammatory fingerprint of the above described sets of inflammatory, adhesion, chemotaxis and cell motility and assembly genes in monocytes of AITD and LADA patients leads to a set of functional abnormalities in monocyte inflammatory responses, adhesion, chemotaxis and motility. Indeed, we previously reported that monocytes and monocyte-derived cells of AITD and autoimmune diabetes patients display an array of adhesion and motility disturbances: The cells adhere and stretch abnormally on fibronectin (FN) and have a higher expression of the integrin CD11b (17). Interestingly, adherence to FN of monocytes of T1D patients induces a significantly increased production of MRP8/14 (a pro-inflammatory compound of the S100 family) and of the pro-inflammatory chemokine CCL2 (18), which was found at a higher level in this study in the serum of AITD patients too. Furthermore, monocytes of AITD patients have a reduced potency to form long cellular veiled protrusions (particularly after FN adherence) and such veiled cells are less capable to appropriately interact with T cells (8), putatively contributing to a faulty tolerance induction (19). Similar adhesion, cell motility/assembly and chemotactic abnormalities have been described of monocytes,

macrophages and dendritic cells in the NOD mouse. Clearly further functional studies with patient monocytes need to be performed particularly in the group of 24% with a positive inflammatory gene fingerprint. In particular knock-in and knock-out studies of the fingerprint genes will establish their role in the previously found functional adhesion and motility disturbances and to establish their role in misbalancing monocyte function towards a pro-inflammatory, non-tolerogenic phenotype.

This study also found that in conjunction and in correlation with the other over-expressed fingerprint genes, the monocytes of AITD and LADA patients showed a clear up regulation of FCAR, the gene for the FcαRI/CD89 and (to a lesser extent) for PPBP, the gene for CXCL7. The FcαRI is a bifunctional inhibitory/activating receptor mediating both anti- and pro-inflammatory functions of IgA (20). Sustained aggregation of the receptor (by e.g. IgA immune complexes) results in activation of monocytes, while absence of aggregation (monomeric IgA) leads to inhibition. There is a specific polymorphism of the FcαRI, the Gly^{248} allele, which in particular enhances the pro-inflammatory potential of serum IgA (21). Enrichment for this allele has been found in SLE, suggesting a pro-inflammatory route via this FcαRI allele in autoimmunity and preliminary studies of our team on another group of AITD patients also suggest an enrichment for this allele in AITD (to be published). It is also of interest to note that an over expression of the FcαRI has been found on white blood cells of MS patients (22). Clearly further studies are needed into the mutual involvement of mucosal infections, IgA immune complexes, FcαRI polymorphisms and activated monocytes/macrophages in AITD patients.
With regard to CXCL7, little is known about the role of this chemokine in monocytes. There are reports on an involvement of CXCL7 in the recruitment of monocytes from the circulation (23) and the resistance to mycobacteria (24). Again knock-in or knock-out studies in monocytes of AITD patients may unravel its role in the pathogenesis of AITD.

In conclusion monocytes of about a quarter of AITD patients (HT and GD alike) and about half of LADA patients share an inflammatory coherent gene-expression fingerprint consisting of at least 26 genes with a function in inflammation, chemotaxis, cell adhesion and cell motility/assembly, supporting the concept that autoimmune thyroiditis and autoimmune diabetes share a common pathogenic route as is indicated by the co-occurrence of both autoimmune disorders in patients, their families and in animal models of the disease.
But, perhaps the most important progress of this study is that it provides an important tool, i.e. the monocyte gene fingerprint, to determine a specific pro-inflammatory state of monocytes in patients and their family members. A similar pro-inflammatory state of the mononuclear phagocyte series exist in the NOD mouse and BB-DP rat prior to disease and

is one of the pillars underlying the pathogenesis of autoimmunity. Our fingerprint thus enables research into the presence of pro-inflammatory monocytes in humans in pre-stages of the disease (as in the animal models) and in addition enables identification of key fingerprint genes involved in the failure of mononuclear phagocytes to preserve tolerance and which could be targeted by drugs (e.g. PDE4 inhibitors, COX2 inhibitors, anti-TNF and N-acetyl cysteine) to correct their aberrant, pro-inflammatory, non-tolerogenic state.

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Chapter 7

The activation of monocyte and T cell networks in patients with bipolar disorder.

Short title: Immune activation and bipolar disorder

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Abstract

Objectives: We recently described a monocyte pro-inflammatory state in patients with bipolar disorder (BD). We hypothesized that the $CD4^+$T cell system is also activated and determined percentages of Th1, Th2, Th17 and $CD4^+CD25^{high}FoxP3^+$ regulatory T cells.

Methods: We carried out a detailed FACS analysis to determine the various T cell subsets and used frozen stored peripheral blood mononuclear cells (PBMC) of 38 BD patients (of whom we previously had tested monocytes for pro-inflammatory gene expression [1, 2]) and of 22 age/gender matched healthy controls (HC). In addition the cvtokines CCL2, IL-1β, IL-6, TNF-α, PTX3, IL-10, IFN-γ, IL-17A, IL-4, IL-5 and IL-22 were measured in serum.

Results:

a) Serum sCD25 levels and percentages of anti-inflammatory $CD4^+CD25^{high}FoxP3^+$ regulatory T cells were higher, the latter in BD patients < 40 years of age. Percentages of Th1, Th2 and Th17 cells were normal.

b) Of the pro-inflammatory monocyte cytokines CCL2 and PTX3 were raised in serum.

c) The monocyte pro-inflammatory state and the raised percentages of $CD4^+CD25^{high}FoxP3^+$ regulatory T cells occurred independently from each other.

d) In BD patients positive for thyroid autoimmune disease a significantly reduced percentage of $CD4^+CD25^{high}FoxP3^+$ regulatory T cells was found as compared to BD patients without AITD.

Conclusion:

Our data show an enhancement of pro-inflammatory monocyte and anti-inflammatory T cell forces in BD patients. A lack of anti-inflammatory T cell forces co-occurred with AITD in BD patients.

Introduction

There is accumulating evidence that activation of the immune system plays an important role in the pathogenesis of bipolar disorder (BD). In support of this view we recently described a higher expression of a coherent set of 34 inflammatory genes, an inflammatory gene expression "signature", in the circulating monocytes of 60-70% of BD patients [1, 2]. Apart from cells of the monocyte lineage, T cells are important contributors to the immune response, but literature on T cell numbers and cytokines in BD is scarce and the aim of the present study is to evaluate T cell related inflammatory networks in relation to monocyte activation state in patients with BD.

Previously we reported on higher numbers of $CD25^+$ and $CD71^+$ ("activated") T cells in the circulation of BD patients [3], but at present there are no data on the circulating numbers of the various $CD4^+$T helper cell subsets in BD, such as $CD4^+$T helper(h)1 and Th2 cells, though there are data on the serum levels of IFN-γ and IL-4, the hallmark cytokines of these two T cell subsets [4-6]. Both higher and unaltered levels of the cytokines have been described.

In recent years a new T helper subset has been discovered, the so-called $CD4^+$ "Th17 cells", which produce the pro-inflammatory cytokine IL-17 [7]. Th17 cells protect the host against bacteria and fungi by activating macrophages via the production of IL-17 (but also IL-21 and IL-22). In addition Th17 cells play a role in the pathogenesis of autoimmune diseases such as psoriasis and rheumatoid arthritis [7]. Functions of Th17 cells are thus very similar to those of Th1 cells.

The inflammation inducing effects of Th1 cells, Th17 cells and of monocytes and macrophages are controlled by a special class of T cells, the regulatory $CD4^+CD25^{high}FoxP3^+$ T cells [8, 9]. The main function of these natural, inborn, thymus-derived regulatory T cells is tempering the inflammatory response thereby maintaining homeostasis and tolerance to self-antigens. The cytokines involved in this anti-inflammatory action are thought to be TGF-β and IL-10 (though these cytokines are also produced by monocytes/macrophages). Patients lacking $CD4^+CD25^{high}FoxP3^+$ natural T regulatory (reg) cells due to a genetic mutation in the gene coding for FoxP3, suffer from a severe and rapidly lethal poly-endocrine auto-immune syndrome (IPEX, Immune dysregulation, Polyendocrinopathy, Enteropathy X-linked syndrome)[10].

Since BD patients have a higher risk to develop endocrine autoimmune diseases, including autoimmune thyroid disease (AITD) [11, 12], we had as second aim of this study to investigate whether BD patients with AITD have an imbalanced interplay between inflammation-prone monocytes and an abnormal tuned T cell system, notably abnormal numbers of natural Treg cells.

For this study we were able to use deep frozen leukocytes of 38 BD patients of the previously reported series of 56 BD patients tested for monocyte gene expression [1, 2, 13] and 22 age and gender matched healthy controls. This enabled us to relate the T cell state to the pro-inflammatory state of the monocytes of the BD patients. TPO-Abs were positive in 11 of the 38 cases (29%).

We determined the percentages of IFN-γ^{+}Th1, IL-4^{+}Th2, IL-17A^{+}Th17 and of CD4^{+}CD25^{high}FoxP3^{+} regulatory T cells using FACS analysis. Next to these cellular analyses we evaluated the serum levels of the monocyte cytokines PTX3, CCL2, TNF-α, IL-1β IL-6, IL-8, and IL-10, of the shed sCD25 (the IL-2 Receptor, highly expressed on CD4^{+}CD25^{high}FoxP3^{+} regulatory T cells) and of the T cell cytokines IFN-γ, IL-4, IL-5, IL-17A and IL-22.

Materials and Methods

Patients with bipolar disorder and controls:

The 38 BD subjects tested were the index cases of twin-pairs, which we had used in a previous study, aged 18 to 60 years, suffering from bipolar I or bipolar II disorder according to DSM-IV criteria [14] (we tested these index BD cases, since we had frozen samples left in store of only these individuals). Material of the co-twins was not used in this study. The recruitment procedure and inclusion criteria have previously been described in [15]. In short the BD diagnosis was confirmed via the Structured Clinical Interview for DSM-IV (SCID). The cases had no history of drug or alcohol dependency for the last half year and no severe medical illness, verified with a medical history inventory. Current mood state was assessed via the Young Mania Rating Scale (YMRS) and the Inventory for Depressive Symptomatology (IDS). All patients were euthymic at the time of blood collection, euthymia was defined as an YMRS score of ≤ 4 and IDS score ≤ 12. In addition, current and previous use of medication was assessed.

Healthy controls: The BD cases were compared to healthy control twins matched on zygosity, gender and age. The controls twins had no history of a major axis I psychiatric disorder such as schizophrenia, psychotic disorder, mood disorder, anxiety disorder or substance related disorder according to DSM-IV criteria confirmed with a SCID and also no history of a severe medical illness. Furthermore, they had no first degree relative with a history of a major axis I psychiatric disorder (DSM-IV).

the Medical Ethical Review Board of the UMC Utrecht had approved the study and all participants gave written informed consent after full explanation of the study aims and procedures. The demographics of the patients and controls are summarized in Table I.

Table I. Characteristics of patients with bipolar disorder and healthy controls.

		Bipolar Disorder		Healthy Controls	
Group size		38		22	
Age (years)1		41.1	+- 9.6	41.3	+- 9.5
Gender	Male	9	24%	3	14%
	Female	29	76%	19	86%
	Duration of illness (years)1	13.6	+-8.9		
TPO-antibodies	Positive	11	29%	7	32%
	Negative	27	71%	15	68%
Medication	None	1			
	Lithium	30			
	Valproate	6			
	Carbamazepine	3			
	Lamotrigine	1			
	Antidepressives	14			
	Antipsychotics	8			
	Benzodiazepines	11			

[1] Mean with standard deviation

Laboratory methods

Blood collection and preparation

Blood (drawn in the morning from 9.00-11.00 hrs) was collected in clotting tubes for serum preparation (stored at -80°C) and in sodium-heparin tubes for immune cell preparation. From the heparinised blood, peripheral blood mononuclear cell (PBMC) suspensions were prepared by low-density gradient centrifugation, as described in detail before [16], within 8 hours to avoid activation of the monocytes (erythrophagy). PBMCs were frozen in 10% dimethylsulfoxide and stored in liquid nitrogen. This enabled us to test patient and control immune cells in the same series of experiments later.

Flow cytometric analyses

FACS (fluorescence-activated cell sorting) analysis was used to measure intracellular cytokine content in PBMC of patients and age and gender matched HC. As hallmark intracellular cytokines we used: IFN-γ, IL-4 and IL-17A. To enable the enumeration of regulatory T cells we intracellular stained for FoxP3. Membrane-staining was done for CD3, CD4, CD25 and CD45RO. This enabled us to assign the cytokine staining to the enigmatic Th1, Th2 and Th17 cells in either the total population of $CD4^+$ cells or the memory population. It also enabled us to enumerate the enigmatic regulatory $CD4^+CD25^{high}FoxP3^+$ T cell population.

For the analysis PBMCs were suspended in complete culture medium. Cell suspensions were then stimulated with PMA (Sigma Aldrich, Missouri, USA), ionomycin (Sigma Aldrich, Missouri, USA) in the presence of Golgistop (Becton Dickinson, New Jersey, USA) for 4 hours in 37 C under a 5% CO2 environment for T effector cells, regulatory T cells were not stimulated. Cells were harvested; membrane staining was done with anti-CD3 (PerCP; BD Biosciences, California, USA), anti-CD4 (APC; BD Biosciences, California, USA, PerCP-Cy5.5; Becton Dickinson, New Jersey, USA) and CD25 (FITC; BD Biosciences, California, USA) via standard protocol. Following membrane staining, the cells were fixed and permeabilized according to the manufacturers instructions (eBioscience, California, USA) and then stained for FoxP3 (PE; Becton Dickinson, New Jersey, USA), IL-4 (PE; Becton Dickonson, New Jersey, USA), IFN-γ (APC; Becton Dickinson, New Jersey, USA), IL-17A (PE; eBioscience, California, USA).

Isotype antibody controls were used to confirm antibody specificity for CD25. Stained cells were analyzed by four colour flowcytometry (FACSCalibur, BD Biosciences, California, USA) as described previously and analyzed using FlowJo (Tree Star Inc. Ashland, Oregon, USA) research software. Supplementary figure 1 gives the gating strategy and the dot plots of the stainings and the definition of the cell populations.

mRNA gene expression in monocytes

The definition and determination of the mRNA gene expression fingerprints in monocytes has been previously described in detail [1].

Serum cytokine determinations

Serum cytokines (IFN-γ, IL-17A, IL-10, IL-6, IL-4, IL-5, IL-8, TNF-α, CCL2, and IL-1β) were measured using the Cytometric Bead Array kits (BenderMedSystems, California, USA) according to the manufacturer's protocol. Bead flow cytometry allows the simultaneous quantification of various proteins in the same test (http://www.ebioscience.com/media/pdf/literature/flowcytomix-multiple-analyte-detection.pdf). Twenty-five µls of serum per test were used. Samples were analyzed in a FACSCanto flow cytometer (BD Biosciences, California, USA) using the FlowCytomix Pro 2.3 Software (BenderMedSystems, California, USA). Results are expressed as picograms per milliliter.

For sIL-2R (R & D Systems, Minneapolis, USA) a commercially available ELISA was used according to the manufacturer's protocol. For PTX3 an in house ELISA was used.

Statistics

Statistical analysis was performed using the SPSS 15.0 package for Windows. Data were tested for normal distribution using the Kolmogorov-Smirnov test. Parametric tests were used when the data had a normal distribution. When normal distribution was not reached, data were log-transformed. If the data were still skewed after log-transformation non-

parametric tests were used. In the text, tables and figures the original data have been used. Levels of significance were set at p = 0.05 (2-tailed). The specific tests used are mentioned under tables and in legends.

Results

Th1 (CD4+IFNγ+), Th2 (CD4+IL4+) and Th17 (CD4+IL17A+) cells in BD patients and HC

We first analyzed by forward and side scatter in FACS (for gating strategy, see Fig 1) the percentages of total lymphocytes and monocytes in the PBMC preparations of the BD patients and healthy controls (HC). There were no differences in the percentages of monocytes and lymphocytes between BD and HC (data not shown).

Subsequently, we quantified after stimulation with PMA and ionomycin for 4 hours (a procedure to activate lymphocytes) the percentages of the Th1, Th2 and Th17 cells. There were no significant differences in the percentages of Th1 (BD: 10.88 +- 5.65; HC: 9.21 +-4.67, p = 0.373), Th2 (BD: 2.42 +- 1.14; HC: 2.25 +- 0.99, p = 0.679) and Th17 cells (BD: 0.55 +- 0.32; HC: 0.43 +- 0.24, p = 0.208) between BD patients and controls.

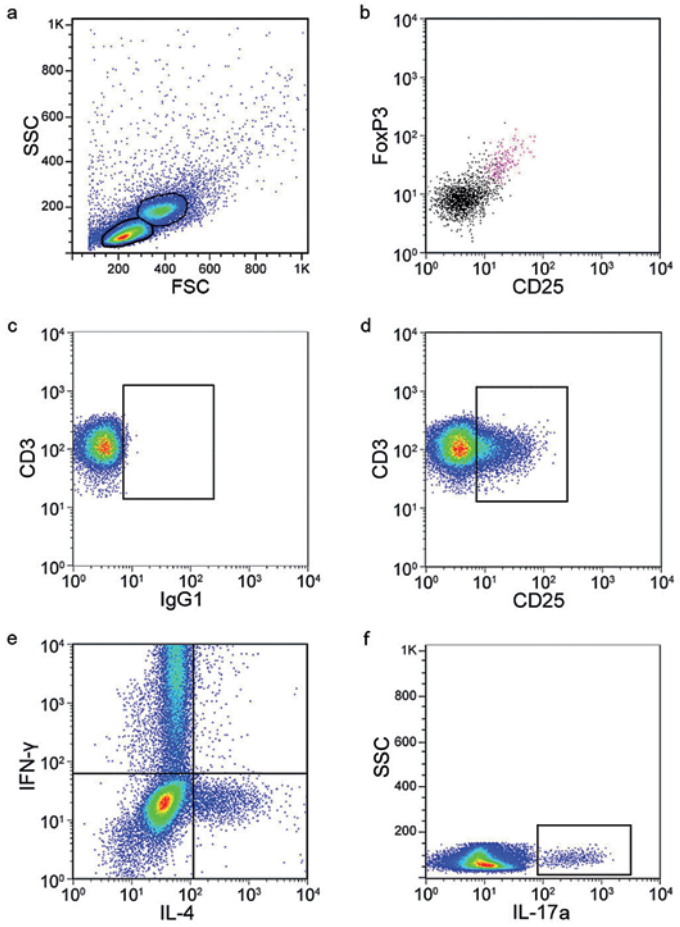
There was also no statistically significant influence of age and gender on the percentages of these T cell subsets.

CD4+CD25highFoxP3+ T cells and serum sCD25 in BD patients and HC

Thereafter we studied the percentages of $CD4^{+}CD25^{high}FoxP3^{+}$ T cells (natural T reg cells) and found these not statistically significantly different in BD patients as compared to the controls (BD: 2.36 +- 0.86; HC: 2.08 +- 0.65, p = 0.348). Age however had a positive significant influence on the $CD4^{+}CD25^{high}FoxP3^{+}$ T cells in healthy controls (r = 0.707, p = 0.001). Since there was this positive correlation with age, and since there are reports showing the same phenomenon [17], we post hoc divided the BD patients and controls arbitrarily into a younger age group (< 40 years, BD: n = 19, HC; n = 10) and an almost equally large older age group (> 40 years, BD: n = 19, HC: n = 12). After this procedure, we found clear differences in the younger BD patients compared to the younger controls and percentages of natural T reg cells were significantly higher in the younger BD patients compared to the younger HC (Fig. 2, BD < 40: median: 2.49%; min-max: 1.24-4.06%; BD > 40: median: 2.19%; min-max: 0.50-5.00%; HC < 40: median: 1.58%; min-max: 0.97-2.50%; HC > 40: median: 2.38%; min-max: 1.44-3.42%).

Finally, we measured the shed soluble form of CD25 in the serum and found higher levels of sCD25 in BD patients as compared to HC, irrespective of age (Fig. 2b, BD: median: 493IU/ml; min-max: 190-2223; HC: median: 386 iU/ml; min-max: 225-1040). sCD25 serum levels correlated positively to percentages of $CD25^{+}$ T cells (r = 0.250, p = 0.058), including the natural T reg cells.

Figure 1. FACS definition of different subsets and gating-strategy



The gating strategy in flow cytometry to detect the different subsets of CD4+ T cells in a given patient/control.

1a. Flow cytometric distinction between lymphocytes (lower left gate) and monocytes (upper right gate) by forward (FSC: proportional to cell size) and side scatter (SSC: proportional to cellular granularity).

1c+d. Flow cytometric analysis for surface expression of $CD25^{+}$ on $CD3^{+}$ lymphocytes in the lymphocyte gate of analysis 1a. The gate in 1d represents cells positive for CD25 in the population of $CD3^{+}$ lymphocytes. The isotype IgG1 antibody in 1c was used to specify antibody specificity for CD25. The cells outside the gates in 1c and 1d are the CD25 negative $CD3^{+}$ lymphocytes.

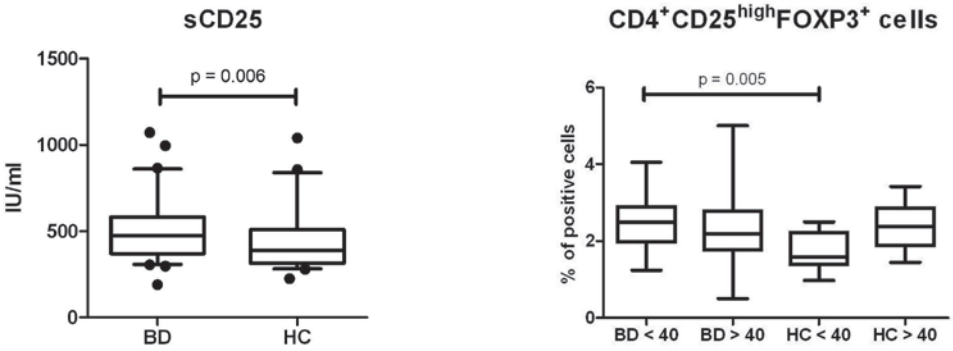
1b. Flow cytometric analysis for intracellular FOXP3 and surface CD25 within gated $CD3^{+}CD4^{+}$ lymphocytes. Pink cells represent $FOXP3^{high}$ and $CD25^{high}$ cells: The natural T regulatory cells. First $CD4^{+}$ were selected in $CD3^{+}$ lymphocytes, within this subset $CD25^{high}$ cells were selected and in this subset $FoxP3^{+}$ were gated as regulatory T cells.

1e. Flow cytometric analysis for intracellular IFN-γ^{+} and IL-4^{+} expression in $CD3^{+}CD4^{+}$ lymphocytes. The upper left quadrant (IFN-γ^{+} positive cells) represents the Th1 cells. The lower right quadrant (IL-4^{+} positive cells) represents the Th2 cells.

1f. Flow cytometric analysis for intracellular IL-17a expression in $CD3^{+}CD4^{+}$ lymphocytes. The gated cells represent the Th17 cells.

In 33 (87%) of the BD patients we had determined the gene expression signature in the monocytes previously [1, 2]. We correlated the expression levels of the various genes with the different T cell parameters studied: We did not find a correlation between the expression of the different genes of the pro-inflammatory monocyte gene expression signature and the different T cell parameters studied, in particular also not to the raised percentages of the natural T reg cells and the raised sCD25 level (data not shown).
Most patients were using psychotropic medication at the time of blood collection (Table I). The use or the class of psychotropic medication did not influence the percentages of the different studied T cell subsets and the sCD25 level in serum (data not shown).
A minority of the BD patients were BD2 patients (20%). Also a subdivision between BD1 and BD2 patients did not influence the percentages of the different studied T cell subsets or the sCD25 level in serum (data not shown).

Fig 2. Percentages of circulating $CD4^{+}CD25^{high}FoxP3^{+}$ T regulatory cells and serum sCD25 in BD patients

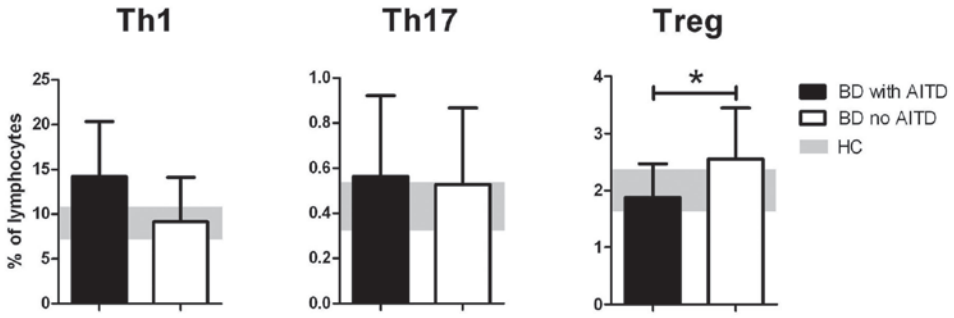


a) Box plots are given of the percentages of $CD4^{+}CD25^{high}FoxP3^{+}$ cells grouped according to age for patients with bipolar disorder and healthy controls. BD < 40 = patients aged under 40 years, BD > 40 = patients aged over 40 years, HC < 40 = healthy controls aged under 40 years, HC > 40 = healthy controls aged over 40 years
b) The serum sCD25 levels in IU/ml
Significant P levels are given between the indicated groups, because of the small groups p values were calculated by the non-parametric Mann-Whitney test.
Age had a positive significant influence on the $CD4^{+}CD25^{high}FoxP3^{+}$ T cells in healthy controls ($r = 0.707$, $p = 0.001$). Since there was this positive correlation with age, and since there are reports showing the same [17], we post hoc divided the BD patients and HC arbitrarily into a younger age group (< 40 years, BD: n = 19, HC; n = 10) and an almost equally large older age group (> 40 years, BD: n = 19, HC: n = 12). Age did not have a significant influence on the $CD4^{+}CD25^{high}FoxP3^{+}$ T cells in BD patients ($r = -0.125$, $p = 0.475$).

Relation of T cell subsets with the TPO-antibody status in BD patients and HC

TPO-Abs were positive in 11 of the 38 BD cases (29%). Interestingly, patients with BD and positive TPO-Abs had significant lower percentages of $CD4^{+}CD25^{high}FoxP3^{+}$ regulatory T cells and higher percentages of Th1 cells (though the latter not significant at p=0.10) as compared to BD patients without AITD (Fig. 3). Despite the significant differences in natural T reg cell state, the levels of sCD25 were not significantly lower in the TPO-Abs positive versus negative BD cases (532 ± 201 and 565 ± 382 IU/ml respectively, p=0.82).

Fig 3. Th1, Th17 and natural T reg cells in bipolar disorder patients with and without TPO-Abs



Percentages of Th1, Th17 and regulatory T cells are grouped according to the presence of TPO-Abs. Positivity for TPO-Abs was defined as values for TPO-Abs higher than 25 pg/ml measured by a commercially available ELISA.
Means ± standard deviations are given. BD with AITD = patients with bipolar disorder positive for TPO-abs, BD no AITD = patients with bipolar disorder negative for TPO-Abs, HC = healthy controls without TPO-Abs, grey areas are the 95% confidence interval of the healthy controls.
Because of the small groups p values were calculated by the non-parametric Mann-Whitney test. * = $p < 0.05$

Thirty-three BD patients were tested for both T cell and monocyte parameters, 9 of these were positive for TPO-Abs. With regard to monocyte gene profiling, the TPO-Abs positive BD patients had a weaker inflammatory gene expression in their monocytes as compared TPO-Abs negative BD patients (particularly in the cluster 1 genes and not so much in the cluster 2 genes) (Table II).

Of the TPO-Abs positive BD patients there were 3 patients with high TSH levels ("sub-clinical hypothyroidism"), there were no differences in T cell levels or inflammatory gene expression compared to patients with normal TSH levels. All patients had levels of fT4 in the normal range.

Table II. Monocyte gene expression levels in fold change versus the reference gene abl.

	BD with AITD [a]	BD no AITD [b]	HC [c]	a vs b p value	a vs c p value	b vs c p value
Cluster 1						
ATF3	2.42 (9.50)	3.58 (3.92)	2.79 (1.11)	0.380	0.825	0.066
PDE4B	2.62 (16.84)	**5.60 (17.37)**	3.23 (2.55)	0.357	1.000	**0.015**
DUSP2	1.86 (9.59)	4.37 (6.04)	2.23 (2.42)	0.428	0.875	0.103
IL1B	29.92 (557.28)	**82.16 (1028.66)**	11.31 (74.61)	0.273	0.149	**0.005**
IL6	0.01 (0.01)	0.01 (3.31)	0.01 (0.05)	0.220	0.681	0.212
TNF	5.71 (17.51)	7.57 (34.19)	3.56 (2.63)	0.564	0.325	**0.028**
PTX3	0.79 (1.92)	**0.95 (3.86)**	0.54 (0.61)	0.237	0.681	**0.016**
PTGS2	4.97 (16.28)	**8.87 (40.54)**	4.29 (5.62)	0.220	0.728	**0.026**
CCL20	0.03 (1.33)	**0.09 (8.22)**	0.03 (0.06)	0.220	0.325	**0.009**
TNFAIP3	2.72 (22.38)	**8.45 (30.81)**	1.84 (2.76)	0.654	0.076	**0.009**
Cluster 2						
MAPK6	**19.67 (564.35)**	**34.80 (93.67)**	2.40 (1.30)	0.848	**0.001**	**0.001**
NAB2	0.30 (0.70)	0.27 (0.44)	0.21 (0.65)	0.749	0.875	0.521
PTPN7	0.30 (0.31)	0.41 (0.25)	0.31 (0.38)	0.593	0.825	0.368
EMP1	0.76 (1.62)	0.80 (1.12)	0.54 (1.22)	0.983	0.636	0.484
CDC42	1.27 (0.95)	1.35 (1.30)	0.97 (0.80)	0.380	0.825	0.182
CCL2	0.45 (1.10)	**1.25 (2.28)**	0.38 (0.90)	0.094	0.636	**0.030**
CCL7	0.01 (0.15)	0.05 (0.96)	0.01 (0.05)	0.113	0.825	0.420

The determination of the mRNA gene expression fingerprints in monocytes has been previously described in detail [1] and the medians (+inter-quartile ranges between brackets) of some cluster 1 genes (inflammatory compound cluster) and cluster 2 genes (motility, adhesion, chemotaxis cluster) are given for the here tested 38 BD patients in this series of experiments. Medians are given for the relative expressions towards the reference gene abl, in bold are statistically significant values as indicated. As can be seen the pro-inflammatory state of monocytes is particularly evident in the BD cases without AITD and is in fact hardly present in the BD cases with AITD (except for MAPK6)

Cytokine measurements in BD patients and controls

Of the cytokines tested only the monocyte/macrophage cytokines CCL2 and PTX3 were statistically significantly higher in the serum of BD patients as compared to control serum (Table III); levels of the monocyte/macrophage cytokine IL-1β were higher in this series of BD patients, but not significant (p=0.10). In a previous study [2] involving 42 BD patients

(26 euthymic, 7 mania, 7 depressed and 2 unknown) IL-1β levels were significantly higher, while those of CCL2 were near significant. We were not able to find significant correlations between the levels of the in this study tested cytokines and the percentages of the various T cell subsets, the monocyte gene expression levels or use of psychotropic medication.

Table III. Cytokine levels in serum of patients with bipolar disorder

		Bipolar disorder		Healthy Controls		
		Mean	Range	Mean	Range	P-value
Monocyte activation	CCL2	1084.31	494.51	867.21	522.85	.009
	IL-8	38.95	89.18	33.94	74.50	.678
	IL1b	3.43	11.270	0	0	.106
	IL-6	0	0	2.65	12.15	.203
	TNF-α	1.67	4.120	1.00	3.160	.520
	PTX3	166.66	144.380	112.62	187.050	.021
Th1	IFN-γ	0.70	4.07	0	0	.432
Th2	IL-4	1.11	6.50	0	0	.432
	IL-5	4.40	20.93	16.93	77.60	.895
Th17	IL17A	6.19	27.87	0	0	.262
	IL-22	125.47	217.24	69.46	101.44	.354
Regulatory T cells	IL-10	2.37	13.80	15.60	71.49	.708

Levels of cytokines are expressed as mean with range. All values are in pg/ml. Normal distribution was not obtained after log-transformation. Therefore P values were calculated by Mann-Whitney tests.

Of the cytokines tested only the monocyte cytokines CCL2 and PTX3 were statistically significantly higher in the serum of BD patients as compared to control serum.

For the other cytokines many of the readings were below the detection level of this CBA assay, this was most evident for IL-6, IFN-γ, IL17A and IL-10 (where more than % of the readings were below the detection level). Thus although for IL17A and IL-22 (Th17 cytokines) mean readings were increased and for IL-10 (a monocyte/T reg cell cytokine) mean readings were decreased in BD patients, this was not at all statistically significant.

Discussion

This study shows significantly higher levels of sCD25 and higher percentages of circulating $CD4^{+}CD25^{high}FoxP3^{+}$ regulatory T cells in the circulation of BD patients, the latter only in BD patients < than 40 years of age and in BD patients without signs of AITD.
The BD patients with AITD lacked the increase in $CD4^{+}CD25^{high}FoxP3^{+}$ regulatory T cells and were additionally characterized by high circulating percentages of Th1 cells (though the latter not significant). Although in this relatively small series of BD patients the presence of AITD was not statistically significantly correlated to a reduced level of sCD25, we previously found in a larger series of BD patients that a reduced serum level of sCD25 was significantly related to AITD: Padmos et al described in a series of 239 BD patients that those with AITD had significantly reduced serum levels of CD25 (be it mildly) in accord with the here described reduced level of natural T reg cells [12].
Our present observation on a relative lack of T regulator cells and our previous observation on a reduced sCD25 in BD patients with AITD point to an imbalance within the T cell system as an important determinant for AITD to develop in BD patients.
Similar T cell imbalances have been found by us 1st) in siblings of AITD patients, who have a heightened risk to develop AITD: These at risk individuals exhibited reduced percentages of natural T regulator cells as compared to healthy control individuals [18], and 2nd) in a small group of patients (n=17, a preliminary not-published study) with recent onset AITD (and no BD); in this group we found higher levels of Th1 and Th17 cells as compared to age-and gender matched healthy controls with regulatory T cell levels in the range of the healthy controls. These findings are in accord that imbalances In the T cell system, i.e. (relative) increases of effector Th1 and Th17 cells or (relative) decreases of $CD4^{+}CD25^{high}FoxP3^{+}$ T regulatory cells are of importance in AITD development (be it with or without BD).
With regard to such imbalances in the T cell system, there is ample evidence that regulatory T cells are capable of dampening the effects of pro-inflammatory Th1/Th17 cells [19] and of pro-inflammatory monocytes/macrophages [20]. It is thus of note that we found the pro-inflammatory state of the monocytes earlier reduced than enhanced in BD patients with AITD (see this report) and it is thus likely that in particular the activation state of Teffector cells is an important effector driving force in AITD development in BD (and not so much the activation state of monocytes/macrophages).

The here presented study has limitations. First, we had no frozen PBMC left in store of BD patients in an active phase of the disease and all tested BD patients were patients in a euthymic phase at the time of blood collection. In the previous report of our group we found that the monocyte pro-inflammatory activation state was more outspoken when blood was collected during a manic or depressive episode [1, 2] and the literature also

indicates that pro-inflammatory cytokines are higher when the illness is active such as in mania or depression [5]. It might thus be possible that also inflammatory T cell numbers are higher during an episode. This being said, the advantage of the present study is that the found T cell abnormalities can be regarded as trait rather than state phenomena.
Second, we have tested for a couple of associations in a relative low number of patients. Therefore, the found association between reduced natural T reg cell percentages and TPO-Abs may be false positive and confirmation is in principle needed in an independent and larger series. However in a previous larger series of 239 BD patients significantly reduced levels of sCD25 did correlate to the presence of AITD, thus strengthening the present observation of weakened T reg cell forces in co-occurrent AITD.
Third, our study was naturalistic and all BD patients were receiving psychotropic drugs, the majority was on lithium. Thus, we cannot rule out that some of the findings are influenced by the medications, although we could not establish correlations between the major types of medication (lithium, anti-depressants, anti-psychotics and valproate) and the various immune parameters in this study. Regarding lithium, this medication is considered in general to be anti-inflammatory [21-23].
A final limitation is that our FACS data represent percentages, not the number of cells within the lymphocyte population. We were unable to give absolute cell numbers of circulating cells, since we had not performed a leukocyte count in the same blood sample as used for FACS, although leukocyte counts routinely performed in the patient at the same time of blood sampling did not show abnormalities.

Despite these limitations our study is the first report on a detailed FACS analysis of various $CD4^+$ helper cell subsets and natural T reg cells in BD patients. Previous literature concentrated on measuring T cell cytokines in serum and on in vitro production of T cell cytokines by isolated blood cells.
The majority of these previous papers showed, in accord with our data, a tendency for a proliferative activation of the IL-2/CD25 system in BD patients: sCD25 higher in 2 reports [3, 24], IL2 higher in 1 report [5], IL-2 normal in 2 reports [25, 26]. Our data however extend these IL-2/sCD25 data showing that it is mainly the anti-inflammatory regulatory T cell force, which is more numerous. This higher activation of the T regulatory cell compartment was however not reflected in our study in significantly higher serum cytokine levels of the (partly) T reg cell derived anti-inflammatory cytokine IL-10, (though the relative low number of patients tested and the relative insensitivity of the used CBA assay may have played a role here, Table III).
With regard to literature data on Th1 (IFN-γ) and Th2 (IL-4, IL-5) related cytokines, a higher IL-4 level was found in lithium treated patients [26]. We did not find higher or reduced levels of typical Th1 or Th2 cytokines in our BD patients, nor did we find abnormal levels

of Th1 or Th2 cells, although the majority of our patients were treated with lithium (here again the relatively low number of patients and insensitivities of the CBA assay might have played a role).

The higher serum levels of CCL2 and PTX3 (this report) and of IL-1β (previous report [2]) shown in this cohort of BD patients strongly support the view that the monocyte-macrophage system is pro-inflammatory activated in BD (a view also expressed in our previous reports, [1, 2]). However the altered T cell set point reported here, i.e. an increase in circulating $CD4^{+}CD25^{high}FoxP3^{+}$ regulatory T cells and sCD25, was found independent of the pro-inflammatory monocyte gene expression state and did also not correlate to the higher levels of CCL2 and PTX3, strengthening a view that T cell and monocyte-macrophage activation are independent phenomena in BD patients. And thus the question arises whether the T cell activation products as described here are capable of inducing mood symptoms independent from monocyte-derived pro-inflammatory compounds? Infusion of IL-2 in cancer patients has been reported to result in reduced energy, impaired confidence, depressed mood and confusion [27], while infusion of the pro-inflammatory monocyte-related cytokine IL-1β leads to the so-called sickness behavior [28]. In an animal model infusion of IL-2 or IL-1β during the direct post-natal period lead to distinct behaviors in adult rats, i.e. active locomotor behavior or increased startle response/decreased acoustic prepulse inhibition/increased social interaction respectively [29]. This indicates that T cell and monocyte related cytokines might induce distinct behavioral abnormalities. Taken together, our observations combined with those in the literature thus suggest that both the monocyte-macrophage system and the T cell system are activated in BD patients and that both types of activation might play independent roles in the pathophysiology of the illness. If this were the case, data from this report would also suggest that in BD patients with AITD such monocyte and T cell activation is reduced in comparison to BD patients without AITD and it becomes interesting to speculate about differences in psychiatric phenotype between BD with and without AITD. In a first report [30] on the association between TPO-Ab positivity and psychiatric illness we found that the most significant association was between TPO-Ab positivity (in particular of high titer and/or with TSH > 4.0 mU/l) and rapid cycling of bipolar disorder. In a later study [11] we could not confirm this association with rapid cycling, yet the association of TPO-Ab positivity and BD in general was confirmed. Suffice to say that further studies are needed to investigate whether differences exist in psychiatric phenotype between BD patients with varying involvements of the monocyte/ macrophage and T cell system.

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All other authors declare that they have no conflicts of interest.

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Chapter 8

Monocyte gene-expression profiles associated with childhood-onset type 1 diabetes and disease risk: A study of identical twins.

Running title: **monocyte gene-expression in childhood-onset diabetes**

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H. Beyan and R.C. Drexhage each contributed equally to this paper.

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Abstract

Objective: Monocytes in childhood-onset type 1 diabetes show distinct gene expression. We hypothesize that monocyte activation in monozygotic (MZ) twin pairs discordant for childhood-onset type 1 diabetes could reflect distinct stages of the disease process including diabetes susceptibility (differences between twins, both diabetic and non-diabetic, and controls) and/or disease progression (differences between diabetic and non-diabetic twins).

Research Design and Methods: We studied patterns of inflammatory gene expression in peripheral blood monocytes of MZ twin pairs (n=10 pairs) discordant for childhood-onset type 1 diabetes, normal control twin pairs (n=10 pairs) and healthy control subjects (n=51) using quantitative-PCR (Q-PCR). We tested the 24 genes previously observed by whole genome analyses and verified by Q-PCR in autoimmune diabetes and performed a hierarchical cluster analysis.

Results: Of 24 genes abnormally expressed in childhood-onset type 1 diabetes, we re-validated abnormal expression in 16 of them in diabetic twins, including distinct sets of down-regulated ($p<0.03$) and up-regulated genes ($p<0.02$). Of these 16 genes: 13 were abnormally expressed in non-diabetic twins, implicating these genes in diabetes susceptibility ($p<0.044$ for all). Cluster analysis of monocyte gene-expression in non-diabetic twins identified two distinct, mutually exclusive clusters, while diabetic twins had a network of positively correlated genes.

Conclusions: Patients with childhood-onset type 1 diabetes show abnormal monocyte gene-expression levels with an altered gene-expression network due to gene-environment interaction. Importantly, perturbed gene-expression clusters were also detected in non-diabetic twins, implicating monocyte abnormalities in susceptibility to diabetes.

Introduction

The destructive autoimmune process associated with type 1 diabetes involves both the innate and adaptive immune response represented by monocytes, dendritic cells, macrophages and T lymphocytes which infiltrate the islets at disease onset (1). Patients with type 1 diabetes show functional abnormalities of monocytes and monocyte-derived cells (2-8), which are assumed to promote the immunogenic potential of the cells.

Recently, we reported that type 1 diabetes patients show abnormal monocyte gene-expression profiles involving 24 inflammatory-related genes (5). Two distinct sets of correlating genes were found. One cluster consisted of down-regulation of 12 core "inflammatory cytokine/compound" genes strongly correlated to the expression of PDE4B (the "PDE4B-associated" cluster). A second cluster was most prevalent in childhood-onset type 1 diabetes and consisted of 10 up-regulated genes strongly correlated to the expression of FABP5 (the "FABP5-associated" cluster).

These aberrant expression profiles in monocytes could be either familial, through shared genetic and/or environmental factors, or disease-associated. To resolve this issue we studied monocytes of monozygotic (MZ) twin pairs discordant for childhood-onset type 1 diabetes, that is one twin had type 1 diabetes and the other did not. Gene-expression abnormalities associated with type 1 diabetes susceptibility, would be found in both type 1 diabetes twins and their non-diabetic twins, (such similarities between MZ twins being due to shared genes, shared environment, or both), but not in normal healthy individuals (twin pair case-control design). Changes associated with type 1 diabetes progression, would only be found in the type 1 diabetes twin (co-twin case control design).

Research design and methods

Twins and Controls

Monozygotic twin pairs were selected from the British Diabetic Twin Study (For details see reference 10). Of 451 twin pairs we selected MZ pairs discordant for type 1 diabetes eligible according to the following criteria: 1) European origin, 2) affected twins had type 1 diabetes (diagnosed according to American Diabetes Association guidelines), 3) both twins of each pair were available for study, 4) neither twin was receiving drugs other than human insulin in the index case, 5) the non-diabetic twin had a low disease risk, that is a risk less than 2% based on lack of diabetes-associated antibodies (9, 10). Of the selected 10 MZ pairs discordant for type 1 diabetes (n=10, mean age 32 years, range 18-50 years, 3 males), the diabetic twins were treated from the time of diagnosis with insulin and were taking highly purified human insulin at least twice daily; mean (Standard Deviation) (SD) duration of diabetes 22 (11) years in the diabetic twin. Monozygosity was established in twin pairs, using both clinical data and at least 22 blood groups, as described (9, 10). Control MZ twin pairs (n=12, mean age 37 (11) years, range 17-53 years, 4 male) were recruited from the local population through advertising. Childhood-onset type 1 diabetes singletons (n=30, mean age 24 years, range 5-50 years, 11 males) were identified from our previous study (5). Healthy control (HC) singletons (n=51, mean age 39 years, range 21-67 years, 23 male) were recruited from enrolling laboratory staff, medical staff and medical students. The inclusion criteria for the healthy controls were: no family history of diabetes or other autoimmune disease, no illness at the time of testing or for at least two weeks prior to the blood withdrawal, including acute infections and allergic reactions, taking no drugs, and on a normal diet. All the subjects gave informed consent. The ethics committees of Bart's and The London NHS Trust and Royal Hospital Trusts, Heinrich-Heine University, Dusseldorf, and Erasmus HC Medical Centre, Rotterdam have approved the study.

Blood collection and monocyte isolation

Peripheral blood mononuclear cell (PBMC) was isolated from Heparinized blood on standard Ficoll procedure (11). Purified PBMCs were then frozen in 10% dimethylsulfoxide and stored in liquid nitrogen. This enabled us to store the samples in order to batch samples for assays. CD14 positive monocytes were isolated by positive selection as described from frozen PBMCs using magnetic cell sorting system (Miltenyi Biotec, UK); monocyte purity was > 95% (determined by morphological screening after trypan blue staining and FACS).

Quantitative RT–PCR

In this present study, RNA was isolated from monocytes using RNeasy columns (Qiagen, Hilden, Germany) and both this method and quantitative RT-PCR has been described

in detail elsewhere (11). Genes under study are the sets of genes previously described as aberrantly expressed in type 1 diabetes and type 2 diabetes. All Taqman probes and consensus primers were pre-formulated and custom designed by Applied Biosystems (for details see 12). PCR amplification of the housekeeping gene *ABL* was performed for each sample to allow normalization between the samples. For details of primers and probes used see Supplementary Table 1. The quantitative value obtained from Q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from the normalized CT values (CT gene - CT housekeeping gene).

Statistical Analysis

Data were assembled using Microsoft Excel software and are expressed as mean (standard deviation)(SD). Data were analyzed using SPSS/the Prism statistical package (SPSS 15.0 package for Windows/GraphPad Prism, Inc. Version 3). Data were tested for normal distribution using the Kolmogorov-Smirnov test. Hierarchical gene analysis and heat maps were determined using a Pearson correlation matrix. Depending on the distribution pattern and the total number of subjects, parametric (normal distribution and ≥ 50 subjects) or non-parametric tests (skewed distribution or <50 subjects) were used. Levels of significance were set at $p < 0.05$ (2-tailed).

Results

Q-PCR analysis A preliminary observation and validation study identified 24 genes abnormally expressed in monocytes from type 1 diabetes patients (5). These 24 genes were examined in this re-validation study of MZ twins and the gene expression levels of 16 of these 24 monocyte genes were abnormally expressed in twins with childhood-onset type 1 diabetes when compared with 12 normal MZ twin pairs and 51 normal singletons (Figure 1, Table I). These 16 abnormally expressed genes were: PDE4B, IL-1B, PTGS2, BCL2A1, TNFAIP3, TNF, CXCL2, HSP1A (all down-regulated)(<p0.04 for all) and CCL2, CCL7, STX1A, EMP1, PTPN7, CD9, FABP5, DHRS3 (all up-regulated)(<p0.02 for all)(Figure 1, Table 1).

To determine whether the changes were familial, i.e. due to gene-environment interaction independent of diabetes and hyperglycaemia, we examined the non-diabetic MZ twins of these patients, selected to be at low disease-risk. Of 16 genes that were altered in the validation study in the index twins, 13 were also altered in the non-diabetic twins compared with controls: PDE4B, IL-1B, PTGS2, BCL2A1, TNFAIP3, TNF, CXCL2, HSP1A (all down-regulated) ($p<0.04$ for all) and CCL7, EMP1, PTPN7, CD9, FABP5 (all up-regulated) ($p<0.044$ for all)(Figure 1)(Figure 1,Table 1); 2 additional genes were abnormally expressed in non-diabetic, but not diabetic, twins i.e. DUSP2 and PTX3 (both down-regulated, $p<0.05$

for each)(Table 1).Only 4 genes (STX1A, CDC42, PTPN7, CD9) were abnormally expressed in diabetic compared with both non-diabetic twins and controls ($p<0.041$ for all), consistent with a diabetic effect, these 4 genes are also abnormally expressed in monocytes of type 2 diabetes patients, consistent with a metabolic effect (5).

Table I

INDEX DIABETIC TWIN			NON-DIABETIC CO-TWIN			
	fold change	p value vs HC		fold change	p value vs HC	p value vs co-twin
PDE4B	**0,29**	0,001	**PDE4B**	**0,32**	0,001	0,741
CCL20	0,45	0,482	**CCL20**	0,27	0,129	0,425
DUSP2	0,74	0,067	**DUSP2**	**0,44**	0,018	0,142
IL-1B	**0,16**	0,001	**IL-1B**	**0,12**	0,001	0,650
PTGS2	**0,23**	0,014	**PTGS2**	**0,25**	0,014	0,899
IL-6	1,99	0,448	**IL-6**	0,86	0,999	0,216
BCL2A1	**0,42**	0,021	**BCL2A1**	**0,29**	0,001	0,076
PTX3	0,66	0,129	**PTX3**	**0,46**	0,027	0,360
ATF3	0,86	0,224	**ATF3**	1,00	0,697	0,654
TNFAIP3	**0,54**	0,007	**TNFAIP3**	**0,35**	0,001	0,266
NAB2	1,26	0,499	**NAB2**	1,75	0,601	0,569
TNF	**0,35**	0,026	**TNF**	**0,48**	0,039	0,561
CXCL2	**0,41**	0,035	**CXCL2**	**0,29**	0,016	0,364
CCL7	**75,58**	0,009	**CCL7**	**43,71**	0,033	0,425
STX1A	**13,83**	0,002	**STX1A**	9,00	0,081	**0,019**
CCL2	**14,83**	0,017	**CCL2**	8,94	0,074	0,380
EMP1	**5,43**	0,001	**EMP1**	**4,56**	0,007	0,544
CDC42	2,19	0,329	**CDC42**	1,60	0,754	**0,015**
PTPN7	**3,36**	0,004	**PTPN7**	**2,22**	0,043	**0,023**
MAPK6	1,75	0,106	**MAPK6**	0,77	0,218	0,082
DHRS3	**6,11**	0,017	**DHRS3**	7,06	0,203	0,741
CD9	**3,94**	0,005	**CD9**	**2,81**	0,014	**0,040**
HSP1A	**0,28**	0,001	**HSP1A**	**0,18**	0,001	0,108
FABP5	**7,84**	0,001	**FABP5**	**2,89**	0,002	0,064

Q-PCR analysis of monocytes of MZ twins with and without type 1 diabetes and healthy control (HC) MZ twin pairs. The quantitative values are expressed as fold changes: in essence the quantitative value obtained from Q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from normalized CT values, as previously described (5). Data were standardized to 51 healthy control singletons (used as the calibrator). Significance was tested by univariate analysis of covariance (ANCOVA), so that values >1 (in red) reflect higher expression and <1 (in green) lower expression in twins than in these control singletons.

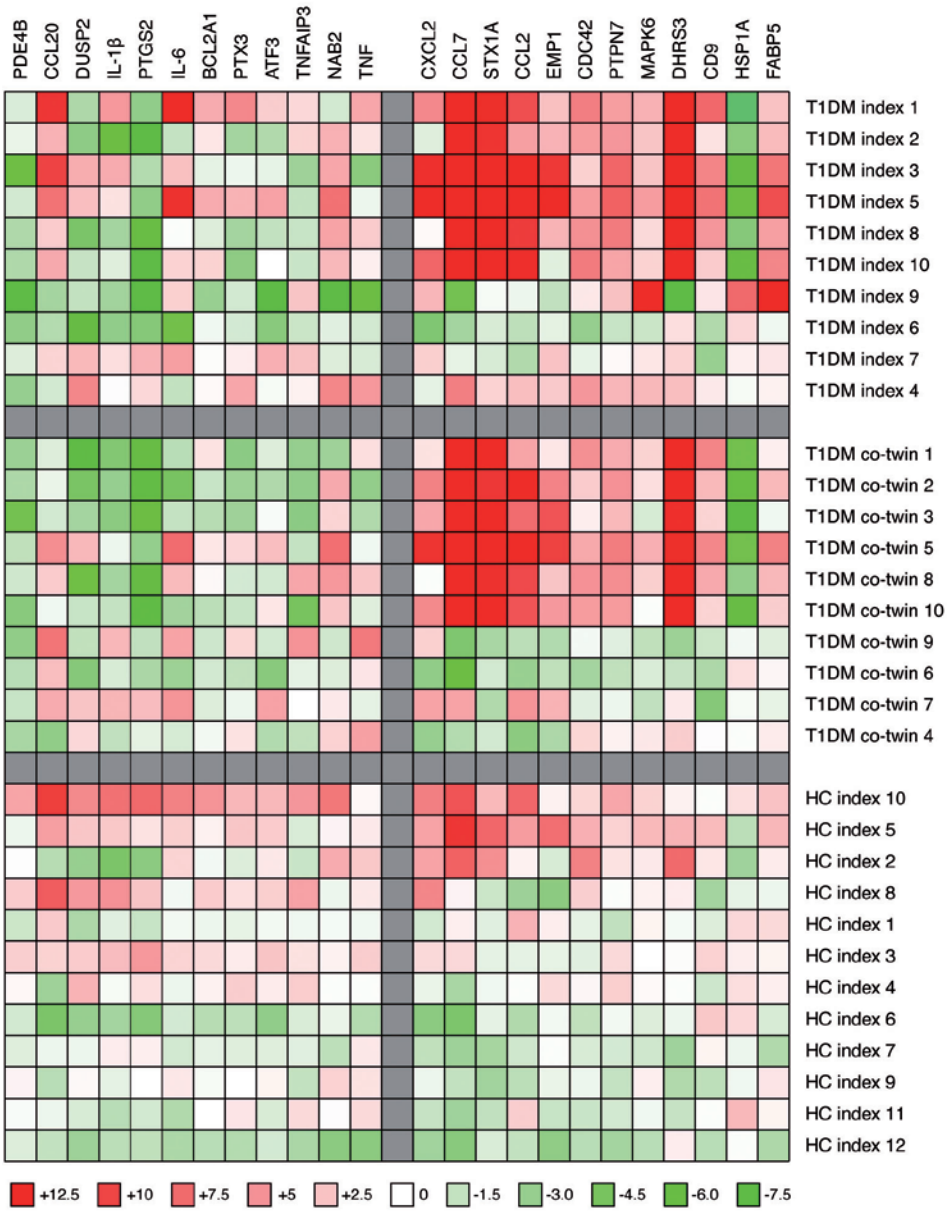
Cluster analysis Diabetic twins had a similar gene-expression profile and gene-order in cluster analysis to that found previously in childhood-onset type 1 diabetes singletons (Figure 2a,c); strongly positively correlated genes clustered in both diabetic groups, the dominant cluster extending from CCL2 to NAB2 (Figure 2a,c). Non-diabetic twins showed similar abnormalities of gene-expression (13 of 16) (data not shown) and gene-order to their diabetic twins, and a similar positively correlating dominant gene cluster (Figure 2b); by contrast, the gene-order differed from control twins (data not shown), and the latter had no comparable dominant cluster (Figure 2d). Both diabetic twins and singletons had two genes (PTGS2, HSPA1A) strongly negatively correlated with the dominant cluster (Figure 2a,c); in the non-diabetic twins these negatively correlated genes extended into a cluster from PDE4B to CCL20 (Figure 2b). The two gene-expression clusters evident in the non-diabetic twins broadly corresponded to the PDE4B-associated and FABP5-associated gene clusters, this latter previously reported by us as the major gene cluster in childhood-onset type 1 diabetes (5). No dominant cluster in diabetic and non-diabetic twins or diabetic singletons was found in control twins. Importantly, the disease discordant twin pairs showed significant correlations for 12 of 16 abnormally expressed genes (r (range) 0.68-0.95; for all 12 $p<0.05$)(data not shown) consistent with a strong shared gene-environment interaction.

Discussion

We previously reported that monocyte gene-expression profiles are abnormal in type 1 diabetes, and vary according to age at diabetes onset, suggesting heterogeneity in disease pathogenesis (5). We re-validated abnormal monocyte gene expression levels for 16 genes in twins with childhood-onset type 1 diabetes. Cluster analysis found that the gene order was similar in both twins and singletons with childhood-onset type 1 diabetes, but distinct from that found in control twins and singletons. Diabetic twins and singletons showed a dominant cluster of 12 abnormally expressed monocytes genes, including FABP5. Our failure to re-validate all the abnormally expressed monocyte genes initially reported, could be due to the relatively small numbers of highly-selected twin pairs, including selection of only childhood-onset type 1 diabetes patients, which will have limited the study power.

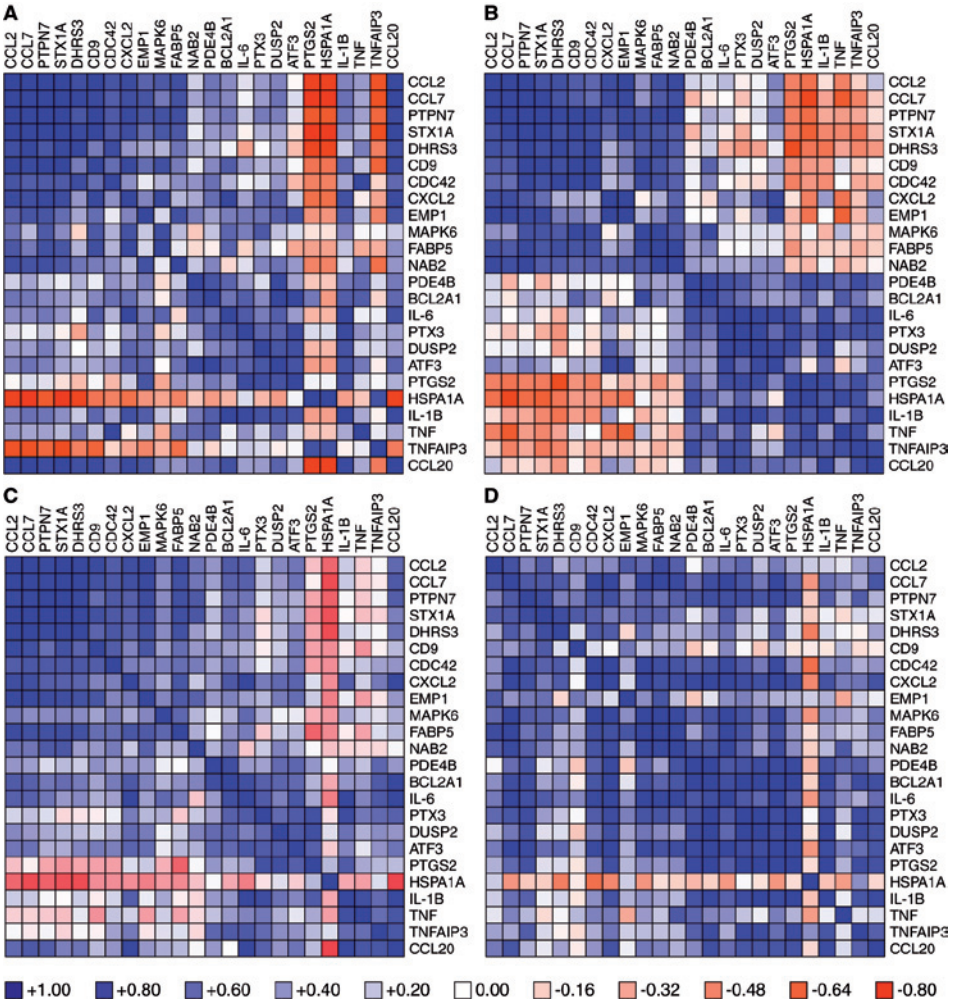
The present observations in type 1 diabetes indicate that much of the altered monocyte gene-expression profile is due to shared gene-environment interaction, since similar changes were detected in their genetically identical twins who were not diabetic and selected to be at low disease risk. Such similarities between MZ twins are not necessarily due to shared genes, as shared environmental effects, or shared gene/environment interaction, would give the same result. Changes in these non-diabetic twins likely reflect monocyte changes associated with disease susceptibility, without necessarily predicting type 1 diabetes. Of 16 monocyte genes abnormally expressed in type 1 diabetes twins,

Figure 1



This Figure shows monocyte gene-expression levels from diabetic index twins, non-diabetic co-twins and the index twin of a healthy control (HC) twin pair. Fold changes (analysed as in table I) are presented similarly with increased (red) or decreased (green) expression levels (the colour intensities code for the strength of the expression levels). A grey line separates two distinct gene-expression profiles (PDE4B- and FABP5-associated clusters). For the majority, diabetic and non-diabetic twins show similar increased or decreased gene-expression levels compared with HC twins

Figure 2



This Figure shows a heat map of correlations between gene-expression levels for 24 genes initially observed to be abnormally expressed in type 1 diabetes in: index diabetic twins (A), their non-diabetic co-twins (B), diabetic singletons (C) and healthy control twins (D). Correlations in gene-expression level are presented as positive (blue) or negative (red) expression levels (the colour intensities code for the strength of the correlations). The hierarchy of gene expression (found in cluster analysis) in all figures is ordered according to that in the index diabetic twins; two distinct clusters are noted in non-diabetic co-twins.

13 were abnormal in their non-diabetic twins and gene-expression levels for the majority were strongly correlated between diabetic and non-diabetic twins. Moreover, in contrast to controls, the gene order and dominant gene cluster on cluster analysis were broadly similar in co-twins. It follows from our observations that most abnormalities in monocyte gene-expression in childhood-onset type 1 diabetes are associated with familial predisposition to the disease, consistent with a previous study of monocyte responses in disease-discordant twins (7). Resolution of the degree of genetic contribution to altered monocyte gene-expression must await a classic twin study; but this would require substantially more MZ twins discordant for type 1 diabetes, as well as matched dizygotic twins, than we studied.

Cluster analysis showed a striking disparity between non-diabetic twins and other groups, in that they had two clear gene-expression clusters, one cluster negatively correlated with the other. The dominant monocyte gene-expression cluster, with an up-regulated FABP5, included genes involved in adhesion, chemotactic and metabolic factors, while the less prominent negatively correlated and down-regulated PDE4B-associated cluster involved pro-inflammatory cytokines and chemokines (5). These two gene clusters were perturbed in the diabetic twins into a network of predominantly positively correlated genes, with a weaker down-regulation of the PDE4B-associated gene cluster, potentially representing a progression marker to clinically overt type 1 diabetes. Longitudinal studies of at-risk individuals could clarify if such changes reflect disease progression.

Our aberrant gene expression data implicates altered monocyte function in the pathogenesis of childhood-onset type 1 diabetes through gene-environment interaction. The character of the genes and previous studies (6, 7, 12) suggest that these functional changes include altered monocyte integrin expression, adhesion to endothelial cells and fibronectin, lipid raft and immune synapse formation, cell motility and assembly and induction of pro-inflammatory cells. In-depth functional studies using transfection or si-RNA regulating the expression of selected key genes in human monocytes will need to be performed to define their exact function in diabetes pathogenesis. A substantially expanded classic twin study, also using dizygotic twins, would be required to determine to what extent genetic factors alone determine the altered gene expression. Nevertheless, identification of key monocyte genes, involved in the perturbed gene-expression networks predisposing to type 1 diabetes, suggests possible therapeutic targets to prevent progression to clinical disease.

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Supplementary Table 1. Techniques, primers and probes used in Q-PCR analysis

Public ID Code (NCBI)	Gene Symbol	Applied Biosystems gene expression assays (Hs)/ Primer/Probe 5'-FAM-3'
NM_001037339	*PDE4B*	Hs00387320_m1
NM_000600	*IL6*	Hs00174131_m1
NM_000576	*IL1B*	Hs00174097_m1
NM_002852	*PTX3*	Hs00173615_m1
NM_000963	*PTGS2*	Hs00153133_m1
NM_000594	*TNF*	Hs00174128_m1
NM_006290	*TNFAIP3*	Hs00234712_m1
NM_013448	*BAZ1A*	Hs00203772_m1
NM_006273	*CCL7*	Hs00171147_m1
NM_004591	*CCL20*	Hs00355476_m1
NM_002089	*CXCL2*	Hs00236966_m1
NM_002982	*CCL2*	Hs00234140_m1
NM_044472	*CDC42*	Hs00741586_mH
NM_001769	*CD9*	Hs00233521_m1
NM_004603	*STX1A*	Hs00270282_m1
NM_004049	*BCL2A1*	Hs00187845_m1
NM_001423	*EMP1*	Hs00608055_m1
NM_002832	*PTPN7*	Hs00160732_m1
NM_004418	*DUSP2*	Hs00358879_m1
NM_001030287	*ATF3*	Hs00231069_m1
NM_005967	*NAB2*	Hs00195573_m1
NM_002748	*MAPK6*	Hs00833126_g1
NM_004753	*DHRS3*	Hs00191073_m1
NM_001444	*FABP5*	Hs02339439_m1
NM_005345	*HSPA1A*	Probe 1.TTCGAGAGTGACTCCCGTTGTCCCA FPrimer 2.GCTGCGACAGTCCACTACCTT RPrimer 3.GGTTCGCTCTGGGAAGCC
NM_005157	*ABL*	Probe 1.TGGAGATAACACTCTAAGCATAACTAAAGGT FPrimer 2.CCATTTTTGGTTTGGGCTTCACACCATT RPrimer 3.GATGTAGTTGCTTGGGACCCA

Chapter 9

Discussion

Discussion

Our data strongly support the presence of an activated immune response involving both pro- and anti-inflammatory forces of the monocyte and T cell system in patients with bipolar disorder, schizophrenia, autoimmune thyroid disease, juvenile onset type 1 diabetes (T1D) and individuals genetically at risk to develop juvenile T1D, i.e. monozygotic co-twins of a diabetic twin, who however did not develop diabetes.

Although the set points of the immune system were activated in all patients and at risk individuals, set points differed between the various disorders and conditions. Set points were in addition modulated by disease activity, disease duration, medication and/or life style factors.

The majority of studies in this thesis focus on the gene expression of circulating monocytes to identify a pro-inflammatory state of these cells. In all individuals tested we were able to find that monocyte gene activation occurred in real life in two main patterns or gene clusters:

1. Cluster 1 with mainly pro-inflammatory cytokines and compounds, with IL-1β, IL-6, TNF, PTGS2, PTX3, various pro-inflammatory chemokines and inflammation regulators as PDE4B, DUSP2 as eye-catching factors. This sub-cluster is likely driven by the transcription factors/regulators ATF3, EGR3, MXD1 and MAFF.
2. Cluster 2 with mainly adhesion/motility factors and chemokines, such as CDC42, CCL2, CCL7, EMP1 and STX1A. PTPN7 and NAB2 are most likely important transcription regulators for this sub-cluster.

For T cell activation we tested for the proportions of CD3+ CD25+T cells, Th1 cells, Th2 cells, Th17 cells and natural CD4+CD25^{high}FOXP3+ Treg cells.

Cytokine studies in the serum of the patients completed the studies; e.g. serum levels of the innate immune cytokines TNF-α, IL-1β, IL-6, IL-10 and PTX3 were measured as well as the T cell proliferation indicator sCD25 and the T cell cytokines IFN-γ, IL-4 and IL-17.

Limitations of our studies

Small study samples

A strong point of our studies is that we carried out our studies in well-defined psychiatric patients. Nevertheless studies involved relative low numbers of patients and healthy controls (but never less than 25) and the studies turned out to have enough power to detect differences between disease and the healthy state. Although we were able to match patients and healthy controls for age and gender, it was not always possible to fully correct for confounding factors such as activity of the disease, medication, BMI, smoking, and the metabolic syndrome, of which factors we showed that they do influence serum cytokine levels and the inflammatory state of the immune cells. Splitting up the groups

along these lines, when possible, often resulted in too low numbers of patients with the specific characteristic making correlation studies less reliable.
Therefore our studies should be confirmed in larger study samples of well-defined patients and healthy controls, also taking into account the BMI, other variables of the metabolic syndrome, disease duration, medication and disease activity making correction for the different confounders possible.

Peripheral blood samples

All our studies were carried out on peripheral blood samples and we are not informed on the immune cells in the actual affected organs. We thus do not know whether the same aberrant activated set points are present in the immune cells in the brain, the thyroid or the pancreas. However in recent onset schizophrenia PET scan studies indicated that the microglia were activated in total gray matter [1], and particularly in the hippocampal area during acute psychosis [2]. This observation was taken as evidence for a "low grade inflammation" of the brain. In addition a recent paper investigated T cell subsets in the CSF and the peripheral blood of patients with a major psychiatric disorder and found an overlapping "low grade" inflammation in CSF as well as in the peripheral blood [3]. This indicates that up to a certain extent studies on immune cells in the peripheral blood reflect aberrancies in the target organs, but it is needless to say that our studies should be completed with investigations on the cerebrospinal fluid, the brain, the thyroid gland and/or the pancreas of patients. Such investigations might be feasible via scans or small needle biopsies of the target glands (when possible).

Only percentages have been given for CD4+ T cell subsets

In our studies only percentages for CD4+ T cells subsets were given, since we were not informed on the actual blood cell counts in the same sample (all of the samples had been frozen as PBMC). However there was in routine screening at intake of the patients (tested for a limited number of samples) no leukocytosis or abnormal differentiation. Another weak point of our way of enumeration is that functional studies on the CD4+T cell subsets are lacking; this is particular relevant for the natural T regulator cells, which might be normal or even increased in number, yet functional defective.

Influence of medication.

Most patients in our studies were using medication and – as discussed above - medication has an effect on the immune system.
With regard to the effects of psychiatric medication it must be noted that effects of lithium and anti-psychotics are generally anti-inflammatory in character [4-6] and the data presented in this thesis, as well as previous data from our group, support such an immune suppressive action of lithium and anti-psychotics and show that, if any, these medications do not induce but correct the described high inflammatory set point of patient monocytes and T cells.

For patients with T1D it is in general more difficult to rule out an effect of medication, because all patients need insulin to survive. However, we were able to test individuals at risk to develop T1D (without having the disease or being on insulin). In both groups of tested individuals (patients with juvenile onset T1D and their non-affected monozygotic co-twin) a good correlation could be established between patients and healthy co-twin regarding the core-signature of autoimmune diabetes (cluster 2), which rules out an effect of medication on the induction of this cluster in monocytes by insulin. Insulin might however have an effect on cluster 1 expression and future studies should address this.
With regard to the disease state of patients with autoimmune thyroid disease we showed that there were only minimal differences in monocyte and T cell aberrancies between patients with hyper- or hypothyroidism and we interpreted the findings as indicating that there exists an immune aberrancy in monocytes and T cells that is shared between GD and HT and that too low or too high thyroid hormone levels did not influence the detected immune activation. Patients with AITD were very recent onset patients with hardly any drug treatment.

Summary of data in the endocrine autoimmune diseases

Taken the limitations into account, we established for autoimmune thyroid disease:

- That only a minority (24%) of AITD patients (mean age 41 yrs, ranging from 18-88 yrs, females 76%) had an activated monocyte set point, i.e. a monocyte pro-inflammatory gene expression signature (this thesis), which was however very similar to that found in about 50-60% of patients with LADA (this thesis) [7]. When splitting the signature in "presence of cluster 2 genes" 38% of AITD patients were positive, when splitting the signature in "presence of cluster 1 genes" 27% of AITD patients were positive (this thesis).
- That the serum levels of IL-1β, IL-6 and TNF-α were not raised, which is in accord with the in general low inflammatory gene cluster 1 state of the monocytes. However the serum levels of CCL2 were higher in patients suggesting an adhesion/infiltration activity of monocytes and in accord with the higher prevalence of gene cluster 2 genes in monocytes.

We established for juvenile onset type 1 diabetes mellitus (T1D):

- That activation of cluster 2 genes was present in 5/10 juvenile onset T1D index twin cases and also in a paired fashion in the monocytes of their monozygotic, discordant co-twins (this thesis). This shows that activation of cluster 2 genes is a familial trait and probably linked to a higher risk to develop T1D. These findings are in accord with our previous findings that around 40% (13/30) of T1D patients had a monocyte activation set point formed by cluster 2 genes only [7].

  Activation of cluster 1 genes was not present in the monocytes of the index juvenile

T1D cases; there was even a downregulation of some of the cluster 1 genes. This downregulation of cluster 1 genes was in particular prominent in the monocytes of monozygotic, discordant co-twins of T1D index twins (this thesis). This suggests that an anti-inflammatory set point of monocytes for cluster 1 genes might be a mechanism to prevent islet autoimmunity in the discordant co-twins.

We conclude for endocrine autoimmune diseases:

A. That it is unlikely that a pro-inflammatory state of circulating monocytes via cluster 1 genes is an important causal factor for the development of AITD and juvenile onset T1D disease. If any, downregulation of these genes in monocytes might be a factor in the prevention of the disease. Preliminary data of our group also show a strong downregulation of cluster 1 genes in monocytes of recent onset cases of juvenile T1D, again showing that this type of monocyte activation cannot play a positive causal role in the outbreak of endocrine autoimmune disease.

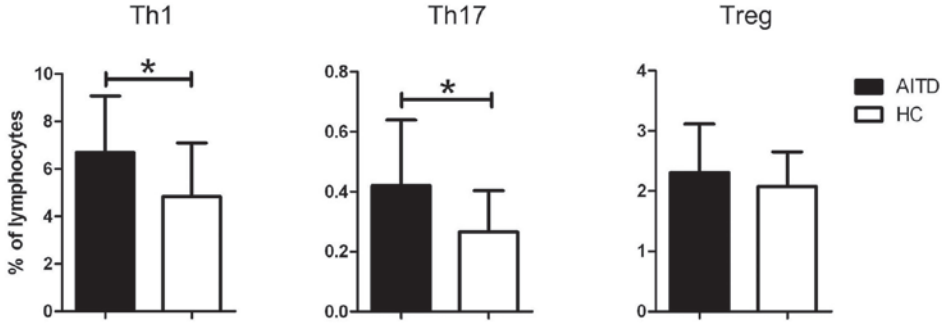
B. That, however, monocytes activated via cluster 2 genes (the adhesion, motility and chemotaxis genes) might be causally important in the development of at least a proportion of juvenile onset T1D and AITD cases. This gene expression pattern probably points to an abnormal adhesion, motility and chemotaxis of monocytes and this view is congruent with the observations on abnormal monocyte adhesive and motility function in AITD patients, T1D patients and in the NOD mouse model of these diseases (see Introduction):In patients with AITD and T1D monocytes show a higher adhesion to endothelial cells and fibronectin (FN). After such adhesion the monocytes become less motile and more pro-inflammatory activated producing more of CCL2 and the inflammatory compound S100A [8, 9]. The monocytes also show a reduced migration towards the chemokines CCL2 and CCL3.

In the NOD mouse model monocytes also show an increased adhesion to fibronectin, in particular the expanded mature (Ly6C low) population of circulating monocytes, while recruitment of monocytes, macrophages and dendritic cells into inflammatory sites is hampered pointing to an abnormal chemotactic response, which can indeed be shown by a reduced migration towards CCL2. Interestingly more of FN is present in the vascular pole of the dysmorhogenic pre-diabetic islets of the NOD mouse [10]. Monocytes and macrophages adhere in particular to these spots [10] and would thus be strongly pro-inflammatory activated. Indeed Wildenberg et al from our group [106] were able to show that such tissue DC lacked the expression of CCR5 and that this reduced CCR5 expression leads to an over production of IL-12, a cytokine capable of skewing the T cell response in a damaging Th1 direction.

It remains to be seen whether also in AITD and T1D patients disease-pre-existing abnormalities exist in the ECM of islets and the thyroid, similar to those of the NOD mouse. Such abnormalities would aggravate the induced higher local pro-inflammatory state of abnormally adhesive monocytes and their descendent cells (the macrophages and DC) in

individuals at risk to develop endocrine autoimmunity.
C. However, it must be noted that not only adhesive monocyte-macrophage abnormalities dictate the autoimmune process in humans and that – similar to the animal models of endocrine autoimmunity – concurrent T cell abnormalities probably play a prominent role in the pathogenesis. It is therefore relevant to note that we detected clear abnormal set points of the T cell system both in recent onset AITD and in recent onset childhood T1D.

Figure 1. Preliminary data on CD4+ T cell subsets in patients with autoimmune thyroid disease.



Percentages (mean and standard deviation) of circulating IFN-γ+Th1, IL17+Th17 and CD4+CD25^{high}FoxP3+ T regulatory cells in patients with recent onset autoimmune thyroid disease (n=15, including 8 autoimmune hypothyroidism and 9 Graves' disease patients) are shown. For FACS techniques see Chapters 5 and 7. Patients samples were from the group of AITD patient studied in Chapter 6.
Significant P levels are given between the indicated groups, because of the small groups p values were calculated by the non-parametric Mann-Whitney test. * = $p<0.05$.

For AITD we found that the percentages of Th1 and Th17 cells were higher in the circulation of the cohort of recent onset AITD patients studied in Chapter 6, while the percentages of activated T cells (CD3+CD25+ cells) and in particular of CD4+CD25^{high}FOXP3+ regulatory T cells were normal (Figure 1). We have to recall here that we also reported a reduced number of T cells with a T regulator phenotype in a study on individuals at risk to develop AITD (family members of AITD patients) and we took these data as suggestive for a reduction in T cell tolerance mechanism in these at risk individuals [11].
We also tested very recently a small series of recent onset Dutch T1D children the first week after diagnosis (n=5) and 6 months after diagnosis (n=10) and compared data to those of healthy control children (n=6). Patients were recruited from the diabetes clinic DIABETER in Rotterdam (courtesy Dr H.J. Aanstoot). In this series of recent onset Dutch T1D children we also found the percentages of Th1 and Th17 cells raised in the first half year after onset of the disease, however here percentages of CD4+CD25^{high}FOXP3+ regulatory T cells were raised too in the circulation (though there are data in the literature that such cells are functionally defective, see introduction).

These T cell data suggest that at the time of clinical outbreak of thyroid and islet autoimmunity the destructive process is probably driven, besides by an abnormal inflammatory state of the monocytes/macrophages and DC in the target-gland-to-be - by Th1 and Th17 effector cells, which are apparently out of control of T cell regulator forces, though we found these only numerically decreased in at risk individuals and not in recent onset cases.

Table I. Summary of monocyte and T cell data for the endocrine autoimmune diseases under study

	AITD	At risk AITD	T1D	At risk T1D	LADA
Monocyte gene cluster 1	↑	nd	↓	↓	↑
Monocyte gene cluster 2	↑	nd	↑	↑	↑
T effector cells	↑	nd	↑	nd	nd
T regulator cells	=	↓	=	nd	nd

(AITD=Autoimmune thyroid diseases, T1D=type 1 diabetes, LADA= Latent autoimmune diabetes of the adults). Higher (↑), lower (↓), or the same (=) expression or percentage as in healthy controls. nd=not determined

Table I summarizes the monocyte and T cell data and figure 2 illustrates a hypothetical scheme for the development of human endocrine autoimmune disease (present state of the art) integrating the data presented in this thesis with our previous animal and human data and the previous data from the literature. In the Introduction we summarized the data found in endocrine autoimmune diseases in figure 5, and showed that they were very similar to those found in the NOD mouse and BB-DP rat. These data are depicted here again under "old" in figure 2. In summary these data at the start of my studies show that there were signs of

1. An abnormal adhesive state of circulating monocytes (gene cluster 2 upregulated, more adhesiveness to FN and endothelial cells) leading to an over production of pro-inflammatory factors (CCL2 and S100A)
2. A defective generation of veiled cells and dendritic antigen presenting cells (APC) from monocytes resulting in APC with a poor capability of stimulating tolerogenic T cells or to induce AITCD
3. A resistance of T cells to undergo apoptosis in AITCD
4. A defective function of natural regulatory T cells, and
5. A reduced number of regulatory T cells in individuals at risk for endocrine autoimmunity.

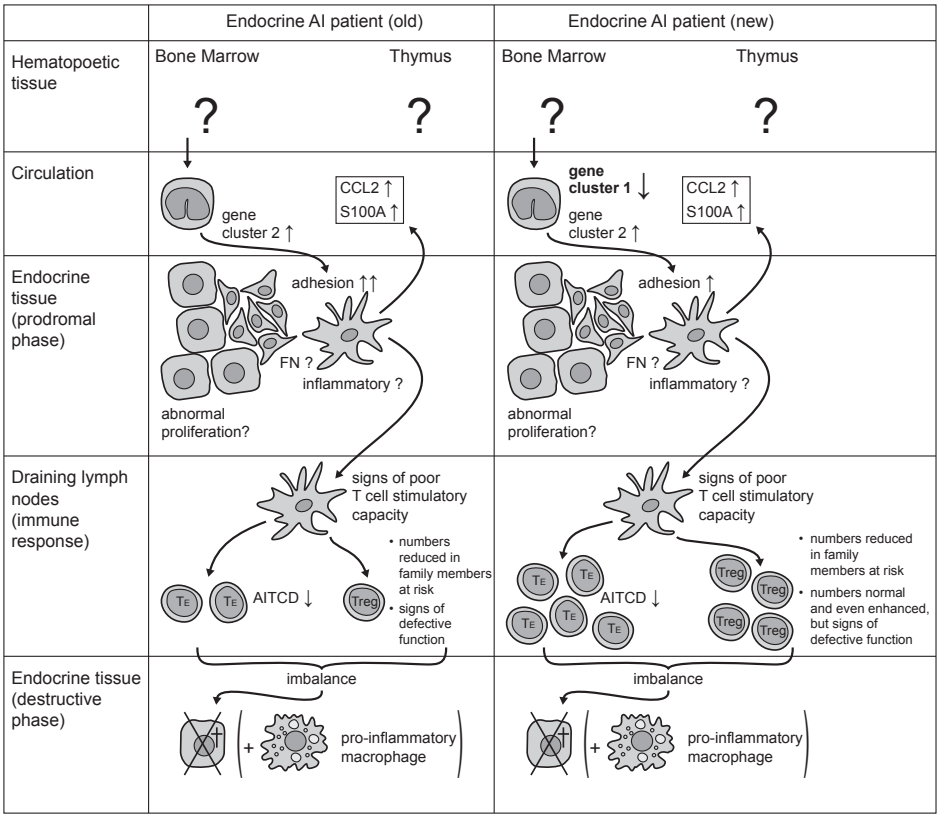
The new findings presented in this thesis include (figure 2 "new"):

6. That the abnormal state of the circulating monocytes hardly includes an upregulation of cluster 1 inflammatory genes and often includes even a downregulation of the

cluster 1 genes. This was described in chapter 6 for AITD, where there was only a limited expression of this gene cluster in patients as compared to the expression of cluster 2 genes in monocytes. For childhood onset T1D (chapter 8) we also found a slightly downregulation of cluster 1 genes in index cases of T1D twins and the expression level of these cluster 1 genes was positively correlated to the expression level of cluster 2 genes. Interestingly in the non-diabetic co-twins there was even a stronger downregulation of cluster 1 genes that was tightly negatively correlated to the expression level of cluster 2 genes in the monocytes, suggesting an anti-inflammatory counter regulatory mechanism in monocytes of at risk individuals who do not develop autoimmunity.

7. That T effector cells are often increased in number in recent onset cases of T1D and AITD (preliminary data described above in the discussion section and see figure 1)
8. That even regulatory T cells may be increased in recent onset cases (preliminary data in T1D).

Figure 2. An update on the suggested immune mechanism playing a role in endocrine autoimmunity.



Summary of data in the psychiatric disorders

We established for schizophrenia:

- That the majority of patients with a recent onset acute schizophrenia (mean age 27, range 17-59 yrs, 81% male, Chapter 3) had an activated monocyte set point, i.e. a pro-inflammatory gene expression signature
    - o With a high expression of cluster 1 genes, but
    - o Lacking cluster 2 genes.
- That the serum levels of pro-inflammatory monocyte cytokines and chemokines were not higher in this cohort of relatively young acute patients.
- That the percentages of monocytes, activated T cells (CD3+CD25+ cells), CD4+CD25^{high}FOXP3+ regulatory T cells and (memory) Th17 were higher in the circulation of this cohort of relatively young acute patients, as well as the serum level of sCD25 (Chapter 5);
- That there was no correlation between the monocyte and T cell activation parameters;
- That the levels of IL-1β, IL-6, TNF-α and CCL2 were higher in a cohort of older patients with chronic schizophrenia (mean age 40 yrs, range 18 to 65 yrs, Chapter 4) of whom 51/145 (35%) suffered from the metabolic syndrome (visceral obesity, hypercholesterolemia, and diabetes).

Interestingly preliminary data on 11 patients of this cohort of older chronic schizophrenic patients show a rather limited monocyte pro-inflammatory gene expression, in which only ATF3, MAFF, MXD1, PTGS2, EREG and THBD were over expressed (data not shown).

- That in this older cohort of patients with chronic schizophrenia and the higher prevalence of the metabolic syndrome the serum level of raised CCL2 was negatively correlated with reduced HDL levels, one of the traits of the metabolic syndrome.

We established for bipolar disorder:

- That the majority of patients (mean age 42, range 26-61 yrs, 61% female, of whom one third had active disease) had an activated monocyte set point involving both cluster 1 and cluster 2 genes.
- That the serum levels of IL-1β, PTX3 and CCL2 were raised in these patients
- That the overexpression of the monocyte gene expression signature was particularly evident in active cases, i.e. in patients with mania or with an active depression.
- That the percentages of activated T cells (CD3+CD25+ cells) and CD4+CD25^{high}FOXP3+ regulatory T cells as well as the sCD25 in serum were higher in the circulation of patients under 40 yrs of age. The numbers of (memory) Th1 and Th17 cells tended to be higher (but differences did not reach statistical significance).
- That the serum levels of the innate immune cytokines TNF-α, IL-10 and TGFβ and the T cell cytokines IFN-γ, IL-4 and IL-17 were not different between patients and controls.
- That there was no correlation between the monocyte and T cell activation parameters, indicating separate activation mechanisms.

Table II. Summary of monocyte and T cell data for schizophrenia (SCZ) and bipolar disorder (BD)

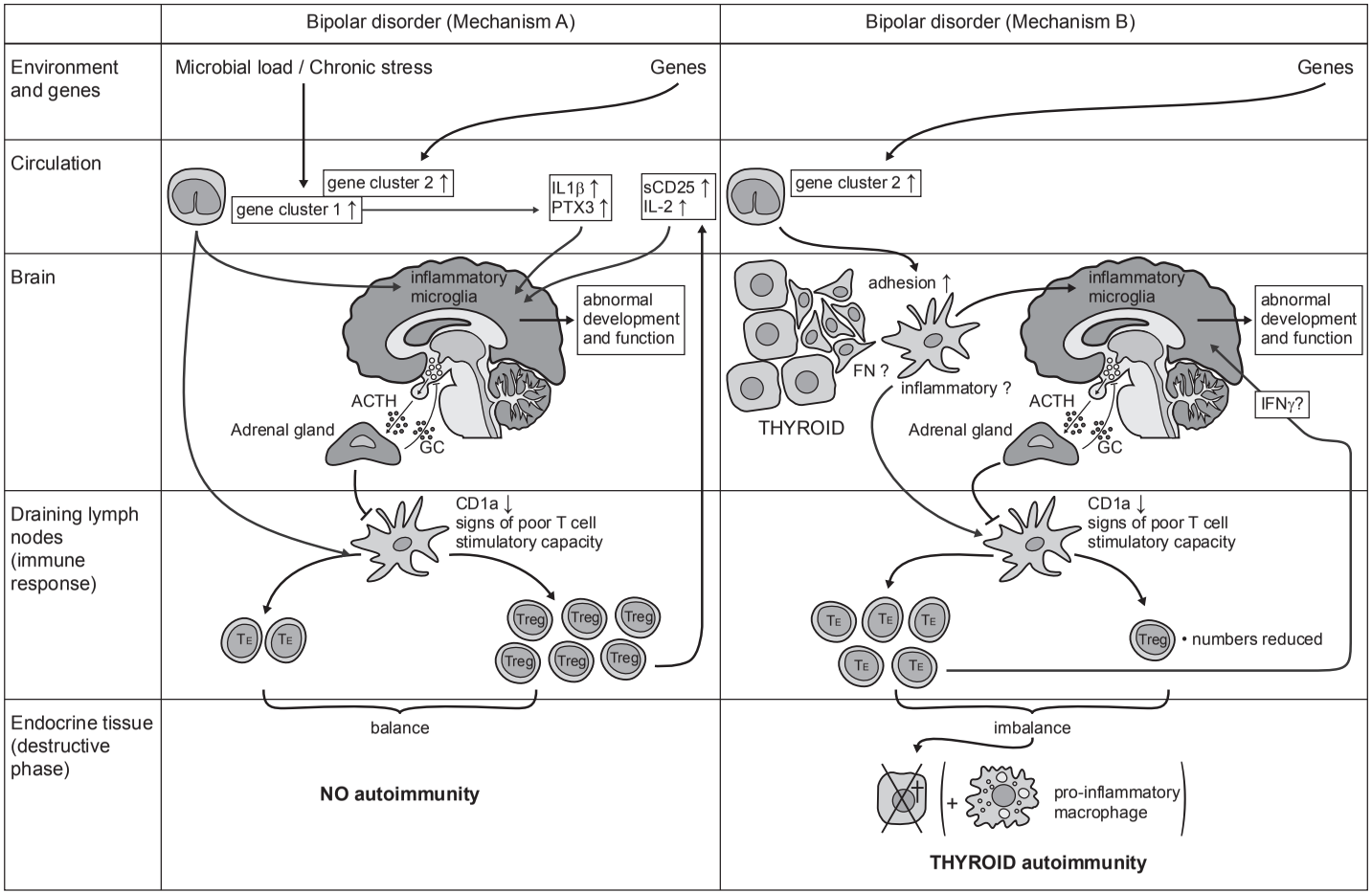
	SCZ	BD
Monocyte gene cluster 1	↑ (B)	↑ (A)
Monocyte gene cluster 2	↓	↑
T effector cells	↑	=
T regulator cells	↑	↑

Higher (↑), lower (↓), or the same (=) expression or percentage as in healthy controls. nd=not determined

We conclude for schizophrenia and bipolar disorder (Table II) that our monocyte gene expression studies, our T cell FACS analysis and cytokine studies suggest that
1. An upregulation of subset 1A and 1B monocyte genes and products of both T effector and T regulator cells are involved in the activation of the MPS and microglia perturbing brain development and function in SCZ.
2. An upregulation of subset 1A monocyte genes, but in particular also of subset 2 monocyte genes, is involved in the activation of the MPS and microglia perturbing brain development and function in BD. In addition products of T regulator cells may play a role in the elicitation of psychiatric symptoms in BD.

Figure 3 represents a hypothetical scheme of the different immune set points relevant for the development of both disorders.
Monocytes in circulation are 'angry', that is to say, they have a pro-inflammatory transcriptome, which differs between bipolar disorder (both gene cluster 1 and 2) and schizophrenia (only cluster 1). There is indication from twin studies that cluster 1 overexpression is determined by environmental factors, whereas genes determine for a part gene cluster 2 overexpression [12].
It can be envisaged that inflammatory monocytes, upon arrival in the limbic system (the "emotional brain") as inflammatory microglia, will display an abnormal interaction with neurons and deregulate synaptic function and neuronal sprouting, and are perhaps even cytotoxic to these cells. This will lead to vulnerability for psychiatric behavior.
A higher level of pro-inflammatory cytokines in the circulation reflects the inflammatory state of the MPS, at least in bipolar disorder and *chronic* schizophrenia. These cytokines may penetrate the brain and aggravate the neuronal deregulations of the brain.
It can also be envisaged that inflammatory monocytes, when differentiated to dendritic cells in the tissues and after having traveled to the draining lymph nodes will abnormally stimulate the T cells in the lymph nodes. Indeed there are signs of an abnormal differentiation of monocytes of bipolar patients to dendritic cells, these dendritic cells have a reduced T cell stimulatory capacity [35].

Figure 3. A hypothesis on the immune mechanisms of recent onset schizophrenia and bipolar disorder.



With regard to the expansion of the T cells: In the circulation of schizophrenia patients both Th17 cells and T regulator cells are over represented, in the circulation of bipolar patients only T regulator cells are more numerous. As a sign of higher T cell proliferation sCD25 is higher in the serum of both recent onset schizophrenia and bipolar disorder patients. Since there is both a stimulation of the anti- and pro-inflammatory T cell forces autoimmunity does not develop yet.

Differences between the immune status in young patients with acute and older patients with chronic schizophrenia.

Despite the clear overexpression of genes for the inflammatory cytokines in the circulating monocytes of young schizophrenic patients we found the level of the inflammatory cytokines not raised in the serum of these patients. Previously we also noted in our BD patients that serum pro-inflammatory cytokine levels were only mild reflections of the expression levels of their respective genes in the circulating monocytes of the patients [12], and over the last decade it has become clear that there is a whole level of complex regulation from gene expression to protein expression, a.o. by microRNA's.

In contrast to the young patients we did find many pro-inflammatory cytokines elevated in the serum of our cohort of older SCZ patients (mean age 40 yrs, range 18 to 65 yrs, Chapter 4), while there was only a limited expression of the pro-inflammatory gene expression signature in their circulating monocytes (see before, preliminary data). Recent data (Beumer at all, to be published) show that although the higher serum levels of these cytokines in older SCZ patients are clearly linked to the disease state that they are in addition strongly determined by the BMI of the patients and other components of the metabolic syndrome, such as the lipid profile.

It is tempting to speculate that the higher serum levels of the pro-inflammatory cytokines in older SCZ individuals is mainly due to a higher production of these cytokines by tissue macrophages (such as in the adipose tissue, the microglia and/or –atherosclerotic-endothelial cells) and not by circulating monocytes. It is known that older SCZ patients are in particular at risk for morbid obesity and atherosclerosis and perhaps the medication (inducing obesity) might play a role as well in switching the pro-inflammatory cytokine production from circulating immune cells to the sessile macrophages in fat (though we found anti-psychotics to be mainly anti-inflammatory).

Immune system to brain communication in psychiatric illnesses: A key role for microglia.

In this thesis we clearly showed an activation of the immune system in patients with psychiatric disorders. But how does this impact the brain?

There is clear evidence from animal models that an activation of the immune system

influences the brain causing an altered behavior. The nicest model in this respect is in our vision the "maternally induced inflammation model", which highlights several aspects of the immune to brain communication. Several studies have shown that intra-peritoneal injection of a pregnant rodent at late gestational age with LPS gives the pups a long lasting activation of the immune system together with behavioral changes [13]. These behavioral changes include learning disabilities and reduced social behavioral performances and are often referred to as schizophrenia-like behavior. The LPS treatment of the pregnant rodents is known to elevate levels of various pro-inflammatory cytokines in the serum, fetal liver and amniotic fluid of the pregnant rodent, but also TNF-α and IL-10 have been found higher in the fetal brain, which are thought to be involved in the abnormal brain development and behavior of the pup. Blocking antibodies against IL-6 given to the pregnant rodents prevent the later altered behavior in the pups [13].

Interestingly there is a human correlate: The relation between prenatal infections and the development of schizophrenia has been described for a long time. Mothers suffering from influenza in the first trimester of pregnancy have a 7 times higher chance for the development of schizophrenia in their offspring, this was 3 times higher for an infection in the second trimester. Another study shows that mothers sero-positive for herpes simplex virus (HSV)-2 in pregnancy have a 2 times higher chance of schizophrenia development in their offspring. Moreover in a cohort study IgG antibodies to Toxoplasma were 2 times higher in mothers who gave birth to a child with schizophrenia [14]. These data are in general interpreted that the microbial pathogens are crossing the placenta and cause congenital brain anomalies and this has been proven for Toxoplasma, HSV, rubella and cytomegalovirus. However a role for inflammation and immune activation in general should not be neglected, it has also been shown that levels of pro-inflammatory cytokines were higher in the serum of mothers during the pregnancy from which a child was born who later developed a psychiatric disorder [14].

Via which routes does a peripherally activated immune system influence brain development and function? Two main pathways have been described:

1. An altered inflammatory set point of the brain milieu and in particular of the microglia.

Pro-inflammatory cytokines can enter the brain via the circumventricular organs and the choroid plexus via an active transport mechanism. Although infiltrated cytokines may influence neuronal networks directly, there are no histological reports on the expression of pro-inflammatory cytokine networks in the brain of BD and SCZ patients. There is a report on an increased ICAM-1 expression in gray and white matter of the anterior cingulated cortex in post mortem brains of BD patients (this overexpression was absent in that of SCZ patients), suggesting a low grade inflammation of the anterior cingulated cortex of patients with bipolar disorder [15].

We however like to suggest that the microglia, the "macrophage/dendritic cell of the brain"

is the important intermediate in the process of immune system to brain communication. A direct role of microglia was recently shown by Chen et al [16] where compulsive-like behavior in a mouse model had a hematopoietic origin: The Hoxb8 mutant mouse showed excessive grooming and hair removal and this is originated from mutant microglia of bone marrow origin [16].

Also the "maternally induced mouse inflammation model" is very instructive for a key role of microglia in peripheral immune system-brain communication: In recent experiments carried out in our group via the EU-consortium MOODINFLAME Beumer et al and Bessis et al (to be published) showed that LPS injection of pregnant mice induces a monocyte gene expression profile very similar to the monocyte gene expression profile reported here in psychiatric patients. In the mouse model this resulted in an identical pro-inflammatory gene expression profile in the fetal microglia, which clearly impacted the growth and development of neurons in the hippocampal area. At an earlier occasion Bessis et al [17] had already shown that pro-inflammatory activated microglia alter glutamatergic synaptic function in the brain.

Is microglia activated in the brain of psychiatric patients?

With regard to the number of microglial cells and their activation, histological post mortem reports are limited and controversial. A post-mortem study on the brains of SCZ patients, who had committed suicide, revealed increased densities of microglia [2]; two other studies also reported on increased microglial activation in SCZ patients [18, 19]. However, 3 studies refuted an activation state of microglia [20-22]. A drawback of these post mortem studies is their character, since they are normally performed on old to very old individuals (after a process of dying) and studies on brain material collected in these individual might not reflect the acute disease stages happening years before the post mortem examination. Currently one does not need to rely on post mortem studies, developments in the field of positron emission tomography (PET) allow researchers to study microglia activation in real-time in live patients. A PET-tracer ([11C]-PK11195) binds to the peripheral benzodiazepine receptor (PBR), which expression is increased in activated microglia and interestingly also on pro-inflammatory activated monocytes (to be published), thereby visualizing microglia and microglia activation. This technique has already successfully been applied in several patient and animal studies on Parkinson's disease and recent-onset schizophrenia [1, 23].

2. Depletion of tryptophan and its metabolite serotonin and the production of neurotoxic metabolites from an altered tryptophan break down pathway.

Tryptophan can be broken down via 2 metabolic pathways

1. The Methoxyindole pathway leading to the formation of the important neurotransmitter 5-HT or serotonin
2. The Kynurenine pathway leading to kynurenic acid and quinolonic acid via the

production of kynurenine. Quinolonic acid has neurotoxic effects, kynurenic acid has a neuroprotective effect. Plasma kynurenic acid is lower in patients with MDD, as well as the ratio kynurenic acid/kynurenine.

The rate-limiting enzyme for the second tryptophan-kynurenine pathway is IDO (99%). IDO activity is particularly induced at sites of inflammation in immune cells. The IDO pathway is a key regulatory element responsible for induction and maintenance of peripheral immune tolerance in normal physiological situations [24]. Pro-inflammatory cytokines, e.g. produced by macrophages, induce IDO in a variety of immune cells [25]. Inflammation (and probably pro-inflammatory monocytes and microglia) promotes the second pathway, thereby depriving the first pathway of fuel leading to a decrease in serotonin synthesis [26]. Serotonin deprivation is an important determinant for the development of depression. Serotonin reuptake inhibitors are commonly used in patients with depression. Also a meta-analysis shows that acute tryptophan depletion decreases mood in healthy controls with a family history of MDD and patients with MDD in remission but not in population healthy controls [27].

Not only there is a deprivation of fuel for the serotonin pathway, but there is also a shift towards formation of kynurenines, which have an apoptotic, neurotoxic, and pro-oxidative effect. Interestingly a higher expression of quinolate has been seen in activated microglia in the brains of suicide victims [2]. Moreover the second pathway may lead to an upregulation of inducible nitric oxide synthase (iNOS), phospholipase A2, arachidonic acid, prostaglandin, 5-lipoxygenase, and the leukotriene cascade which may lead to the development of metabolic syndrome [25].

The origin of the activated immune system: Genes or environment?

The effect of the environment on the immune activation

A couple of years ago Padmos et al [28] from our group carried out a case-control study using the monocytes of bipolar twins to determine the contribution of genetic and environmental influences on the expression of the monocyte pro-inflammatory gene signature. It was found that the association of the pro-inflammatory monocytes gene signature with bipolar disorder was primarily the result of common shared environmental factors, apart however for some of the cluster 2 genes, which were also genetically determined. In her thesis Roos Padmos extensively described the various environmental factors, which could play a role in this influence and highlighted the role of the microbiome, acute and chronic stress and dietary factors. Also for endocrine autoimmunity these environmental factors have been described as important determinants.

Our data on the relation between high CCL2 levels and a reduced HDL in older patients with chronic SCZ illustrate the role of a western style diet with too much unsaturated fat in the higher set point of the immune system in psychiatric disease.

The effect of genes on the immune activation.

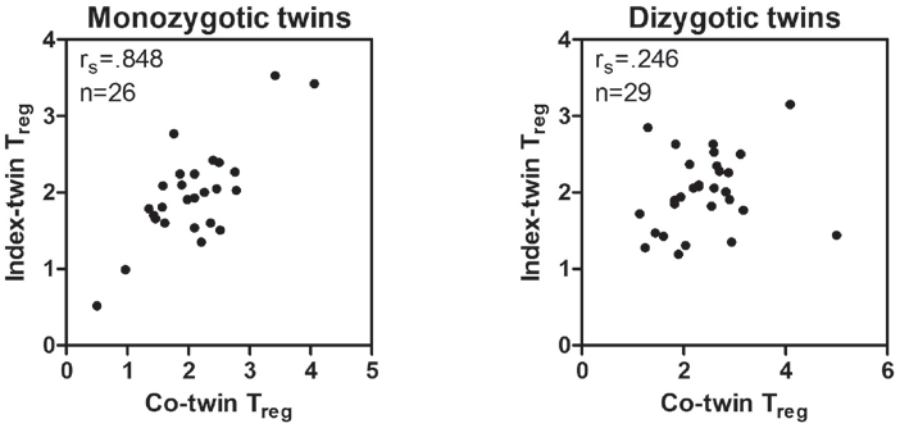
Genome wide association studies (GWAS) have been performed in the last decade in large cohorts of patients with BD and SCZ with overall disappointing results. It turned out to be virtually impossible to consistently find specific genes linked to the disorders and to replicate findings for individual studies. The problem is that psychiatric diseases are probably from a pathogenesis point of view very heterogeneous and certainly in general not the result of a mutation in a single or a few genes. Large meta-analyses were needed to complete the GWAS studies and presently a few genetic markers with a limited risk have been identified. Amongst these markers is the MHC complex in SCZ [29] and the TNF gene in major depression [30]. A new approach is to find in these large studies molecular pathways, which are affected in psychiatric disease. This approach is somewhat more successful and found for SCZ for instance an involvement of the glutamate metabolism pathway and the TNFR1 pathway [31]. These data point indeed towards a role for an activated mononuclear phagocyte system (MPS, including circulating monocytes and brain microglia) in interaction with neurons in psychiatric disease, as discussed above, yet also show that the contribution of genetic polymorphisms to the activation of the MPS is limited.

This might be different for the activation of the T cell system in psychiatric disease. An extension of our twin studies involving Treg cell enumeration (to be published) shows that Treg cell numbers are under the influence of genes (see Figure 4). These studies need now to be extended for the other T cell populations, but at least show for an important T cell population in autoimmunity that genes are involved. The genes consistently found to be involved in endocrine autoimmunity are the MHC-complex, CTLA4 and PTPN22 and point to an important contribution of disturbances in T cell activation and downregulation in endocrine autoimmunity [32]. It is of interest to note that the gene molecular pathway studies mentioned above also delivered the TGF-β signaling pathway in SCZ; TGF-β is an important Treg cell cytokine and T regs are elevated in SCZ (see this thesis).

The role of epigenetic imprinting in the immune activation

Epigenetic imprinting can certainly be a mechanism via which the above-described environmental factors create long lasting pro-inflammatory set points in the MPS of psychiatric patients and via which even fetal/childhood influences impact cellular functions later in life. Of chronic severe stress (child abuse) it has been reported that it induces epigenetic changes in the glucocorticoid receptor in the brain [33]. Glucocorticoid resistance is an important phenomenon in at least the T cell activation of psychiatric patients [34]. Epigenetic modulations of important cluster 1 and 2 signature genes therefore deserve further study.

Figure 4. Preliminary data on the percentages of natural T regulator cells in monozygotic and dizygotic twins, either with an index twin with bipolar disorder or a healthy index twin of matched age and gender.



Twins were from a study previously published by Vonk et al [41]. As can be seen from the figure monozygosity strongly determines the correlation between the percentages of CD4+CD25highFoxP3+ regulatory T cells in index twin and co-twin, while the presence or absence of bipolar disorder is of limited influence. This suggests that in particular genes play a role in the numbers of circulating natural regulatory T cells. Clearly further studies are needed.

A relation of a partial immune deficiency, the commensal microbial environment and a pro-inflammatory set point of the immune system in severe psychiatric disease?

A previous study of our group showed that the dendritic cells of patients with bipolar disorder are poor T cell stimulatory antigen presenting cells [35]. Theodoropoulou et al made a similar observation in medicated and not medicated patients with schizophrenia and showed that patients had a diminished autologous mixed lymphocyte reaction [36]. These poor presentation and T cell stimulatory skills of patient antigen presenting cells may lead to difficulties in immunization towards commensal microbes such as streptococci, staphylococci and yeasts like candida. Indeed patients with schizophrenia have diminished delayed type hypersensitivity skin reactions to such commensals, showing a partial T cell immune deficiency towards these commensal microbes [37]. Perhaps this partial T cell deficiency is also linked to the MHC gene linkage of SCZ and BD (see before), since the MHC complex determines the host's capability to optimally respond to the commensal bacterial flora. It is thus tempting to speculate that patients with BD and SCZ have a reduced T cell immunity to commensal (and non-commensal) bacteria, making patients more vulnerable to infections with commensal micro-organisms (perhaps even from birth onwards).

There is indeed circumstantial evidence for this view in studies measuring antibodies against various common bacteria and viruses. Many of these studies do find higher titers to commensal microbes in patients with SCZ or BD compared to healthy controls, suggesting a higher exposition level to these microbes [38]. This would allow factors produced by these microbes, such as LPS, peptidoglycan and viral DNA/RNA, to continuously stimulate the immune system through pattern recognition receptors, such as the Toll-Like Receptors (TLRs), leading to a pro-inflammatory set point as described in this thesis.

The Poly-endocrine Glandular Syndrome (PGS) extended to the Poly-Neuro-Endocrine Syndrome (PNES): The association of bipolar disorder with thyroid autoimmunity/T1D.

AITD and T1D occur more frequently together than normal within patients and families. The co-occurrence of these two organ specific autoimmune diseases is described as subtype 3A of a syndrome called Poly-endocrine Glandular Syndrome [39]. This syndrome was initially defined as a multiple endocrine gland insufficiency associated to an autoimmune disease in a patient, involving AITD, T1D, atrophic gastritis and Addison 's disease (adrenal cortex insufficiency). These associations were noted not to be at random but to occur in particular combinations.

However not only autoimmune endocrine gland insufficiencies cluster mutually together in patients and their families, but also together with mood disorders.

First, hypothyroidism is commonly accompanied by depressive symptoms. Second, subjects positive for TPO-antibodies were shown to have a higher risk to develop mood disorders. And, finally, bipolar patients are more prone to develop thyroid autoimmunity (see for references the Introduction).

Also children of bipolar patients are at higher risk to develop endocrine autoimmunity as was demonstrated by a study conducted in our group [40]. In this research, there was a higher prevalence of TPO-antibody positivity than in healthy controls especially in female bipolar offspring (16% vs. 4%). Furthermore, three children had developed T1D (i.e. 2% versus 0.4% in the general population). In addition, a twin study of our group showed that healthy co-twins of bipolar index twins show a similar high prevalence in TPO-antibody positivity, irrespective of mood symptoms [41]. Thus family members of BD patients are more vulnerable to develop thyroid and islet autoimmunity, but these endocrine autoimmune diseases do develop independently from the vulnerability to develop aberrancies of the mood state. These findings refute the concept that mood disorders and thyroid/islet autoimmunity are cause or consequence of each other. This is further strengthened by our observation in pregnant and postpartum women where postpartum depression was related to TPO-antibody positivity rather than to thyroid dysfunction [42] and by the observation that the depression seen in TPO-antibody positive post-partum

women was not corrected by thyroid hormone treatment [43].
Collectively these data imply the presence of a shared common vulnerability factor for mood disorders, thyroid autoimmunity and possibly also T1D.
We hypothesized that in particular the immune activation state is important as shared vulnerability factor and asked ourselves the question what the key immune abnormality is which runs in the patients and their family members and which raises the chance for acquiring a psychiatric disorder and/or an endocrine autoimmune disease. Table III summarizes the monocyte and T cell findings in BD with and without AITD.
We like to discuss two possibilities:
1. Both psychiatric and endocrine autoimmune disorders are driven by pro-inflammatory set point of circulating monocytes.
In BD and SCZ cluster-1-activated pro-inflammatory monocytes are present in 50-60% of patients, particularly in those with active disease (Chapter 3). However in AITD only 24% of circulating monocytes show this pro-inflammatory profile (Chapter 6). Moreover in BD patients with AITD monocytes are, if any, less cluster-1-pro-inflammatory activated as in BD patients without AITD (Chapter 7). This makes it less likely that the cluster 1 gene activation state of circulating monocytes is the factor for the higher co-occurrence of both psychiatric and endocrine autoimmune disorders (see also Table III).
2. Both psychiatric and endocrine autoimmune disorders are driven by a pro-inflammatory set point of monocyte-descendent cells in the tissues, respectively in the brain and the thyroid.
The serum level of CCL2 is raised in BD and AITD, indicating a migratory activity of monocytes and their descendant cells in the tissues (Chapters 4 and 6). PET indicates a pro-inflammatory state of microglia in SCZ (see before), but we are not informed on the pro-inflammatory activation state of microglia in BD and AITD, let alone on the pro-inflammatory activation state of monocytes and DC infiltrating the thyroid or the islets in BD patients or individuals at risk for or with an endocrine autoimmune disease.
However, if analogies exist with the animal models of autoimmune thyroiditis and insulitis (see Introduction) a role of an enhanced adherence of abnormally sticky monocytes/dendritic cells to an abnormal ECM with a high FN content might play a role in an enhanced pro-inflammatory set point of both the microglia in the brain (in such view activated by an abnormal ECM compartment in the brain) and DC in the thyroid/islets of patients. This stickiness and resulting local inflammatory state of monocytes/macrophages and DC would then be the shared vulnerability mechanism between BD and AITD and indeed Table III shows that cluster-2 activation in monocytes is the shared vulnerability factor between BD and AITD.

Table III. Summary of monocyte and T cell data for the bipolar disorder (BD) with and without autoimmune thyroid disease (AITD)

	BD without AITD	BD with AITD
Monocyte gene cluster 1	↑ (A)	=
Monocyte gene cluster 2	↑	↑
T effector cells	=	↑
T regulator cells	↑	↓

Higher (↑), lower (↓), or the same (=) expression or percentage as in healthy controls. nd=not determined

It would also mean that there are in fact two immune mechanisms that may lead to BD (see Figure 5):

a) One primarily based on a pro-inflammatory activation of peripheral mononuclear phagocytes, including monocytes via subset 1 genes. These genes are predominantly induced by environmental factors [28], and
b) The other based on a stickiness of mononuclear phagocytes, including monocytes, to extracellular matrix components (as indicated by the upregulation of subset 2 genes), which would render the cells in the tissues pro-inflammatory. The latter would be also genetically determined [28], and the one linked to thyroid autoimmunity.

Five-year view

Various issues will be addressed in the coming 5 years regarding the activated immune system in schizophrenia, bipolar disorder and endocrine autoimmunity.

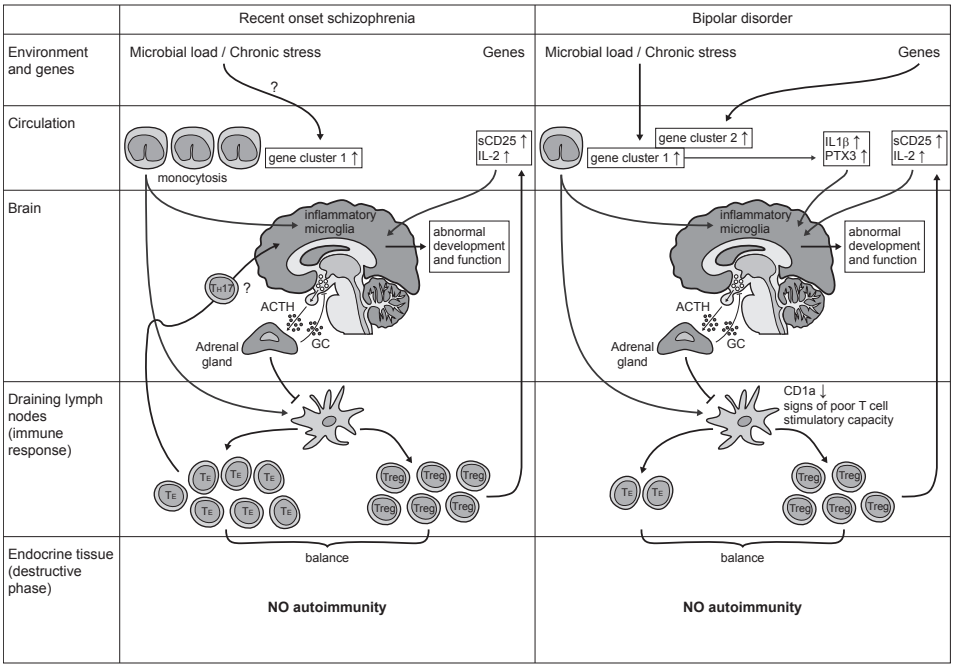
1. The development of array systems other than those described in this thesis both on the transcriptomic and proteomic level to more reliably detect the activated state of monocytes and T cells in schizophrenia, bipolar disorder and endocrine autoimmunity.

At present also other research groups perform transcriptomic arrays on T cells and various proteomic arrays on the cytosol of circulating blood cells and on serum/plasma (Sabine Bahn, personal communication). The first indications are that with arrays of limited numbers of 10-20 analytes (many comprising growth and immuno-neuro-endorine factors) on serum assays may be developed that are able to make distinctions between the disease states and healthy controls.

2. The potential of the above described transcriptomic and proteomic array systems to objectively identify and subdivide major psychiatric illnesses, such as schizophrenia, bipolar disorder and major depressive disorder.

Here it must also be noted that only part of the patients (about 50-60%) in the different psychiatric categories are positive for pro-inflammatory ("angry") monocytes in the RQ-PCR arrays. The question must be addressed whether this indicates heterogeneity in disease pathogenesis, as we suggest in Figure 5.

Figure 5. The hypothetical mechanisms for two types of immune pathogenesis of bipolar disorder.



Mechanism A is as described for bipolar disorder in Figure 3.
Mechanism B is more of an "autoimmune type" and is associated with thyroid autoimmunity. We like to suggest that in fact the pathogenic mechanism is followed for endocrine autoimmunity as illustrated in endocrine autoimmune patients and the animal models of endocrine autoimmunity. That is to say that the myeloid precursors of the bone marrow show a deviant maturation. This deviant maturation results in

1) An abnormal adhesive state of circulating monocytes (gene cluster 2 upregulated, more adhesiveness to FN and endothelial cells) leading to an over production of pro-inflammatory factors (CCL2 and S100A). This abnormal adhesive state would be more determined by genes than environment.
2) An abnormal maturation of microglia from these sticky monocytes resulting in microglia with an intrinsic high inflammatory set point. This microglia will display an abnormal interaction with neurons and deregulate synaptic function and neuronal sprouting, and is perhaps even cytotoxic to these cells. This will lead to vulnerability for psychiatric behavior.
3) With regard to the thyroid DC developing from sticky monocytes see Figure 2.
4) In bipolar patients with autoimmune thyroid disease there is a clear imbalance between Th1 and regulatory T cells (in favor of the Th1 cells) as compared to those without autoimmune thyroid disease. Interferon-γ produced by these Th1 cells might also play a role in the deregulation of brain function by inducing IDO and interfering in the tryptophan metabolism.

If autoantibodies play a role in the induction of psychiatric symptoms Mechanism B would be the pathogenic mechanism in which they are the most likely produced.

3. The power of the transcriptomic and proteomic arrays to identify psychiatric patients, who will benefit from adjunctive anti-inflammatory therapy targeting important fingerprint genes.

Adjunctive therapy with anti-inflammatory agents, such as COX-2 inhibitors, can alleviate symptoms in mood disorders and schizophrenia (44). Also patients treated with TNF antagonists for immune diseases such as psoriasis, Crohn's disease or rheumatoid arthritis not only describe improvements in somatic symptoms but also in depression scores and quality of life (45). Drugs inhibiting the activity of PDE4 have anti-depressive and anti-inflammatory potential (46-47), as do have strong anti-oxidants, such as N-acetylcysteine (NAC) (48). It is to be expected that in particular the psychiatric patients with " angry" monocytes showing strong elevations of pro-inflammatory genes (about 50% of the patients) will benefit from these treatments.

4. The prognostic potential of the transcriptomic and proteomic array systems to identify at risk individuals for schizophrenia, bipolar disorder and endocrine autoimmunity. In particular family and twin studies will be instrumental here. At the same time environmental influences (stress, infections) inducing the abnormal inflammatory state of the immune cells and preceding the psychiatric illnesses can be studied via epidemiological approaches in these individuals at risk.

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Summary

Although there were already reports of an involvement of the immune system in major mental illnesses in the first decade of the 20th century, it took until the last decade of the 20th century before detailed studies on an immune involvement became more numerous. These studies reported aberrant levels of pro-inflammatory cytokines in the serum, plasma and cerebrospinal fluid of patients with schizophrenia and major mood disorders. On the basis of these reports, it was hypothesized that a pro-inflammatory state of the cytokine network induces psychopathologic symptoms, and is involved in the pathogenesis and pathophysiology of these major mental illnesses.
Activated cells of the immune system, such as activated endothelial cells, cells of the mononuclear phagocyte system (MPS; e.g., monocytes, monocyte-derived dendritic cells and macrophages) and T cells primarily produce these pro-inflammatory cytokines. The realization that such cells must be involved led to the 'macrophage–T-cell theory of depression and schizophrenia', which was proposed in 1992 and adapted in 1995. In this theory, chronically activated macrophages (and their related cells in the brain, i.e., the microglia) and T cells produce cytokines and inflammatory compounds, which destabilize the brain in such a way that other genetic and environmental influences are able to precipitate the signs and symptoms of schizophrenia and mania/depression. Indeed, receptors for inflammatory cytokines are present in various brain nuclei and, via their triggering, deregulations of important neurotransmitter and neurodevelopmental systems are introduced, facilitating the development of psychiatric signs and symptoms. Microglia is particularly important for synaptogenesis, synaptic functioning and neuronal sprouting. Under steady state conditions, microglia promotes neurogenesis by the production of neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) that support the survival of neurons. However when microglia switches from a steady state to a pro-inflammatory set point, it impacts the growth of neurons in the hippocampus and synaptic function.

The major mental illnesses studied in this thesis are bipolar disorder (BD) and schizophrenia (SCZ). According to the DSM-IV classification BD is a chronic, episodic illness of the mood with usually a full recovery between episodes. Patients have to suffer from at least one manic episode to be diagnosed with bipolar disorder, with or without having had a depressed episode. In this aspect bipolar disorder differs from unipolar depression since in that disorder patients do not experience episodes of elevated mood but only episodes of a depressed mood. According to the DSM IV SCZ is a mixture of positive and negative psychiatric signs and symptoms. Positive symptoms reflect an excess or distortion of normal perceptive function such as delusions and hallucinations. Negative symptoms reflect a diminution or loss of normal function such as affective flattening and lack of initiative.

In a previous study on a large sample of outpatients with BD we found the prevalence of autoimmune thyroiditis (as evidenced by a higher prevalence of antithyroid antibodies) and of thyroid failure (as evidenced by a raised serum TSH) higher than in the general population and disease controls. Autoimmune thyroiditis is a major cause of thyroid failure, and is marked by the presence of antibodies to the thyroid, in particular TPO-antibodies. Mild forms of autoimmune thyroiditis with a clinically euthyroid state are not uncommon: Antithyroid antibodies occur in 5–15% of the normal population, predominantly in women and older people.
Since autoimmune thyroiditis was not associated with lithium exposure in the BD cases and since there were also no differences in the prevalence rates of autoimmune thyroiditis between euthymic bipolar patients and bipolar patients in a manic or depressive episode, we considered thyroid autoimmunity more likely to be a "trait marker" of BD than a "state marker" of an episode and these findings raised the question whether thyroid autoimmunity is related to the disease itself or to the vulnerability for BD. For that reason we studied co-twins of a bipolar index case and offspring of a bipolar parent for the presence of autoimmune thyroiditis. Both studies revealed that co-twins and offspring were not only vulnerable to develop mood disorders but also more vulnerable to develop autoimmune thyroiditis independently from the mood disorders. These findings refute the concept that mood disorders and thyroid autoimmunity are cause or consequence of each other, but that they are more likely part of a larger neuro-immuno-endocrine syndrome and are rooted in the same, shared immune abnormality.
In later studies BD patients not only appeared to have a higher prevalence of TPO-antibodies, but also a higher prevalence of antibodies to glutamic acid decarboxylase-65 (GAD-65). Antibodies to GAD-65 are frequently present in Type 1 diabetes (T1D), another typical organ specific endocrine autoimmune disease. In T1D an autoimmune process destroys the insulin producing β cells of the islets of Langerhans in the pancreas. Because of this β cell destruction, the production of insulin diminishes and finally comes to an end. This will lead to well-known hyperglycemia of T1D. Apparently also T1D is part of the extended neuro-immuno-endocrine syndrome.

This thesis deals with the immune aspects of bipolar disorder, schizophrenia, autoimmune thyroid disease (AITD) and T1D and also tries to find the shared immune abnormality underlying these disorders in the extended neuro-immuno-endocrine syndrome.

Chapter 1 firstly gives an introduction to the immune system and some of its important cells. In particular the cells of the mononuclear phagocyte system are addressed, how these cells traffic and behave under steady state conditions, how they are involved in the inflammatory immune response under 'danger' conditions, and which are the most

important adhesion molecules, cytokines and chemokines the cells produce in these processes.
Thereafter the various aberrancies in monocyte, macrophage, DC, T cell and endocrine cell functions in the three animal models of spontaneous endocrine autoimmunity are summarized. Collectively these aberrancies are highly suggestive for a basic underlying inborn defect in growth and development of myeloid, lymphoid, epithelial and mesenchymal cell precursors. This defect is characterized by a reduced proliferation and a reduced apoptosis resistance, but an accelerated and deviant maturation.
For myeloid cells these growth and developmental defects result in a mature (partly inflammatory) set point of circulating monocytes with an enhanced adhesiveness to ECM and to monocyte-derived macrophages and DC with an intrinsic high inflammatory set point skewing T cell responses away from tolerogenic to effector immunogenic responses. For lymphoid cells the growth and developmental defects result in imbalances between the various T cell tolerogenic and autoimmunization differentiation routes, again leading to a skewing towards effector immunogenic T cell responses.

The experimental questions (**Chapter 2**) concentrate
a) On the pro-inflammatory aspects of circulating monocytes in BD, SCZ, autoimmune thyroid disease (AITD) and T1D, and
b) On the presence of effector and suppressor T cell populations in BD, SCZ, AITD and T1D

to establish a putative pro-inflammatory state of the monocyte and T cell system in these disorders as well as trying to find the shared vulnerability factor between these disorders.

The questions addressed are:

Q1. Do patients with schizophrenia and AITD have a pro-inflammatory monocyte gene expression signature and is there a difference or overlap with the previously found monocyte pro-inflammatory gene expression signature in patients with bipolar disorder?
In a previous study Padmos et al found a pro-inflammatory gene expression signature in the monocytes of patients with bipolar disorder and in the monocytes of LADA patients. The gene expression fingerprint consisted of 2 partly overlapping gene clusters, a PDE4B associated cluster (consisting of 12 core pro-inflammatory cytokine/compound genes), a "FABP5 associated" cluster (3 core genes) and a set of 9 overlapping chemotaxis, adhesion and cell assembly genes (the "overlapping cluster") correlating to both PDE4B and FABP5.

In **Chapter 3** we extended these studies to patients with recent-onset schizophrenia and aimed at detecting a specific inflammatory monocyte gene expression signature in SCZ

and compare such signature with the inflammatory monocyte gene signature in BD and relate it to the use of medication. We first searched for "schizophrenia-related monocyte genes" via Affymetrix whole genome analysis on pools of monocytes of patients with schizophrenia and thereafter carried out a quantitative-PCR case-control gene expression study on monocytes of 27 SCZ patients and compared to outcomes collected in 56 BD patients (most patients were drug-treated). For Q-PCR we used 9 "SCZ specific genes" (found in Affymetrix whole genome analysis) and the 24 genes described above.
Monocytes of recent onset SCZ patients had (similar to those of BD patients) a high inflammatory set point. This set point was composed of three sub-sets of strongly correlating genes characterized by different sets of transcription and MAPK regulating factors:

1) Sub-set 1A, characterized by ATF3 and DUSP2. This sub-set was previously identified as the PDE4B associated cluster. It was upregulated in the monocytes of 67 % of SCZ patients and 34 % of BD patients.
2) Sub-set 1B, characterized by EGR3 and MXD1. This sub-set was de novo identified in Affymetrix analysis of the pooled monocytes of SCZ. This sub-set was upregulated in 41% of SCZ patients and only 34% of BD patients.
3) Sub-set 2, characterized by PTPN7 and NAB2. This sub-set was previously identified as the "overlapping cluster" Sub-set 2 was *upregulated* in the monocytes of 62% of BD, but *downregulated* in the monocytes of 48% of SCZ patients.

With regard to mood state: Only during depression (and not in the manic phase) there was a significant upregulation of the gene expression signature and only of sub-set 2 genes.
With regard to medication: Both lithium and anti-psychotics downregulated many of the signature genes, but only one of the genes significantly, i.e. PDE4B, a cluster 1A gene.
Our approach shows that monocytes of SCZ and BD patients overlap in inflammatory gene expression (regarding sub-sets 1), but also differ in inflammatory gene expression (regarding sub-set 2). The Q-PCR assay system to detect the fingerprints was shown to be robust and the different fingerprint patterns detected with this system will open new avenues for the possibility for a nosological distinction between schizophrenia and bipolar disorder based on a biochemical blood test.

In **Chapter 6** we studied whether the monocyte inflammatory fingerprint as found by Padmos et al in BD and LADA is also present in autoimmune thyroid disease (AITD). We again first searched for "AITD-related monocyte genes" in Affymetrix analysis on pools of monocytes of patients with AITD and thereafter carried out a Q-PCR analysis for 28 genes in monocytes of 35 AITD patients and 38 healthy controls (HC). The tested 28 genes were the 24 genes previously found abnormally expressed in monocytes of BD and LADA plus 4 "extra" genes found in the Affymetrix whole genome analysis of monocytes of AITD patients.

Monocytes of a considerable proportion of AITD patients showed an abnormal inflammatory fingerprint:

a) In 57% of the patients characterized by the expression of subset 2 genes (the set of genes involved in chemotaxis, adhesion and cell motility/assembly (i.e. by CCL7, EMP-1, CDC42, STX1A, PTPN7, MAPK6, DHRS3), but
b) In only 34% of the patients characterized by some of the cluster 1A genes (the PDE4B associated inflammatory compound genes, i.e. by PDE4B, IL-6, ATF3 and NAB2).
c) Of the 4 in Affymetrix identified genes only FCAR was found to be significantly over expressed in Q-PCR in the 35 AITD patients. FCAR expression correlated as strongly to the expression of sub-set 2 as sub-set 1A genes.

We concluded in the discussion section that the monocytes of 50-60% of AITD patients show an inflammatory gene expression fingerprint with an over representation of cluster 2 genes similar to that previously described by us in the monocytes of 60% of childhood onset T1D cases and 67% of LADA patients, the latter in addition characterized by a strong overexpression of sub-set 1A genes.

Q2. Do monozygotic discordant co-twins of an index twin with juvenile-onset T1D have the same pro-inflammatory monocyte gene expression signature?

Padmos et al previously described that monocytes of patients with juvenile onset T1D have monocytes with a comparable gene expression fingerprint as LADA patients, but importantly lacking cluster 1 (which contains most of the pro-inflammatory cytokines and compounds) while cluster 2 (containing predominantly adhesion and motility factors and chemokines, like CCL2 and CCL7) was present in 60%.

In **Chapter 8** we investigated the presence of the pro-inflammatory mRNA gene expression signature in the monocytes in monozygotic co-twins of index twins with a juvenile onset T1D, who were discordant for T1D for already for many years. We hypothesized that monocyte activation in monozygotic (MZ) twin pairs discordant for childhood-onset type 1 diabetes could reflect distinct stages of the disease process including diabetes susceptibility (differences between twins, both diabetic and non-diabetic, and controls) and/or disease progression (differences between diabetic and non-diabetic twins).

We therefore studied patterns of inflammatory gene expression in peripheral blood monocytes of MZ twin pairs (n=10 pairs) discordant for childhood-onset type 1 diabetes, normal control twin pairs (n=10 pairs) and healthy control subjects (n=51) using quantitative-PCR (Q-PCR). We tested the 24 genes previously observed by whole genome analyses and verified by Q-PCR in LADA and childhood onset T1D and performed a hierarchical cluster analysis.

Of the 24 genes abnormally expressed in childhood-onset type 1 diabetes, we re-

validated abnormal expression in 16 of them in diabetic twins, including distinct sets of downregulated sub-set 1A genes ($p<0.03$) and up regulated sub-set 2 genes ($p<0.02$). Of these 16 genes: 13 were abnormally expressed in non-diabetic twins, implicating these genes in diabetes susceptibility ($p<0.044$ for all). Cluster analysis of monocyte gene-expression in non-diabetic twins identified that the two distinct sub-sets 1A and 2 were mutually exclusive clusters (i.e. there was a strong correlation between the upregulation of sub-set 2 genes and the downregulation of sub-set 1A genes), while diabetic twins had a network of positively correlated genes (i.e. sub-set 2 genes were up regulated and although sub-set 1A genes were not significantly up regulated the mutual exclusiveness was lost).

We concluded that patients with childhood-onset T1D show abnormal monocyte gene-expression levels with an altered gene-expression network due to gene-environment interaction. Importantly, perturbed gene-expression clusters were also detected in non-diabetic twins, implicating monocyte abnormalities in susceptibility to diabetes, particularly sub-set 2 genes.

Q3. Do patients with bipolar disorder and schizophrenia have aberrancies in their Th1, Th2, Th17 and natural T regulator cell compartment?

Since there is evidence for the involvement of the T cell system in the pathogenesis of schizophrenia and bipolar disorder, we investigated the frequency of CD3+CD25+T cells, IFN-γ+Th1, IL-4+Th2, IL-17A+Th17 CD4+ lymphocytes and CD4+CD25highFoxP3+ natural regulatory T cells in the circulation of patients with schizophrenia and bipolar disorder using detailed FACS analysis.

We related T cell outcomes to the pro-inflammatory state of the monocytes in the same patients, i.e. to the presence of the pro-inflammatory mRNA gene expression signature described above.

None of our SCZ patients was positive for thyroid autoimmune disease, i.e. TPO-antibodies. The prevalence of TPO-antibodies in our BD cohort was however 29%, thus about 3X higher than the general population. Thus only in the BD patients we were able to correlate the presence of TPO-antibodies to the T cell (and monocyte pro-inflammatory) state.

We detected for **schizophrenia (Chapter 5)**:

(a) Higher percentages of pro-inflammatory-prone monocytes, activated CD3+CD25+ T cells and pro-inflammatory Th17 cells in patients, but also higher percentages of anti-inflammatory CD4+CD25highFoxP3+ regulatory T cells and IL-4+ lymphocytes;
(b) That this activated T-cell set point was reflected in significantly raised serum levels of sCD25;
(c) That the upregulation of IL-4+-containing lymphocytes was predominantly found in patients characterized by a monocyte pro-inflammatory set point; and

(d) That regulatory T-cell and Th17-cell numbers were higher in patients irrespective of the pro-inflammatory state of the monocytes.

We detected for **bipolar disorder (Chapter 7)**:

a) That percentages of anti-inflammatory CD4+CD25highFoxP3+ regulatory T cells were higher, the latter only in BD patients < 40 years of age. Percentages of Th1, Th2 and Th17 cells were normal.
b) The monocyte pro-inflammatory state and the raised percentages of CD4+CD25highFoxP3+ regulatory T cells occurred independently from each other.
c) In BD patients positive for thyroid autoimmune disease a significantly reduced percentage of CD4+CD25highFoxP3+ regulatory T cells was found as compared to BD patients without AITD, as well as a near significant rise in the percentages of Th1 cells.
d) Curiously in the BD patients positive for TPO-antibodies the pro-inflammatory monocyte signature was different from those without TPO-antibodies: There was a clear and significant decrease of sub-set 1 expression.

We overall conclude in the discussion section that our data do not support the concept that the T-cell system is in a simple pro-inflammatory state in recent-onset SCZ and euthymic BD patients, but do show that the monocyte and T-cell networks are activated independently from each other and that the T cell networks involve both pro- and anti-inflammatory forces. Our data suggest T regulatory cell control over an activated inflammatory system in SCZ and BD patients.

In BD patients with AITD there was a lack of anti-inflammatory regulatory T cell forces, suggesting that these cells are important to prevent AITD from developing in BD patients.

Q4. Is an altered inflammatory set point of the monocytes and T cells in patients with bipolar disorder, schizophrenia and AITD reflected in higher serum levels of pro-inflammatory and T cell cytokines/chemokines and how are these levels related in patients with chronic forms of schizophrenia to the presence of the metabolic syndrome, a common co-morbidity of chronic schizophrenia?

Levels of monocyte and T cell cytokines were investigated in the serum of the patients with AITD, the group of patients with euthymic bipolar disorder (used for T cell analysis) and the patients with recent-onset acute schizophrenia using a cytometric bead array (CBA). Serum levels of the cytokines were correlated with the monocyte gene expression signatures and the T cell state in the same patients. Various relevant T-cell cytokines/ shedding products, e.g. sCD25, IFN-γ, IL-17A and IL-4 were measured in serum by the multiplex CBA. In addition the monocyte/macrophage cvtokines CCL2, IL-1β, IL-6, TNF-α, PTX3 and IL-10 were measured in the CBA.

We found that:

a) In **AITD** cases only the pro-inflammatory monocyte cytokine CCL2 was raised. In previous studies we found the T cell shedding product sCD25 downregulated in cases at risk to develop AITD.
b) In **euthymic bipolar disorder** cases the pro-inflammatory monocyte cytokines CCL2 and PTX3 and the T cell shedding product sCD25 were raised.
c) In **recent onset schizophrenia** none of the pro-inflammatory monocyte cytokines were raised, but the T cell shedding product sCD25 was raised.

For **chronic schizophrenia** pro-inflammatory monocyte cytokines have been described in literature as raised in serum. The serum level of the chemokine CCL2 had however not been studied in such patients, despite a reported polymorphism of its gene being over represented in psychiatric patients. CCL2 has also been implicated in diabetes (DM), obesity (OS) and the metabolic syndrome (MS), co-morbidities of chronic schizophrenia. We therefore investigated **(Chapter 4)** the serum CCL2 level in chronic schizophrenic patients (145 patients and 105 healthy controls, HC) and related the level with the use of anti-psychotics, with the presence of DM, OB and MS and with the level of typical monocyte pro-inflammatory cytokines and sCD25.

We found:

a) That the serum level of CCL2 was increased in patients as compared to HC
b) That CCL2 levels were not related to the use of anti-psychotics, the presence of diabetes or obesity, but were dependent on the presence of chronic schizophrenia and to the presence of the metabolic syndrome, and within the syndrome primarily to HDL-cholesterol.
c) That the levels of IL-1β, IL-6, TNF-α and sCD25 were also raised in patient serum. The levels of CCL2 correlated with these pro-inflammatory cytokine levels, but not with the sCD25 level.

We overall conclude for the cytokines:

1) That serum sCD25 is invariably raised in both recent onset and chronic psychiatric disease in fact reflecting the raised numbers of circulating CD3+CD25+ T cells, but in particular of anti-inflammatory CD4+CD25highFoxP3+ regulatory T cells.
2) That pro-inflammatory monocyte cytokines are not raised in recent onset cases of psychiatric disease (despite clear signs of pro-inflammatory monocyte activation at the gene level), but are raised in chronic cases of psychiatric disease and that levels of these cytokines are in these chronic cases co-determined by the presence of the metabolic syndrome. We hypothesize that intrinsically inflammation prone macrophages (due to the psychiatric disease) when accumulated in the vasculature or in the adipose tissue in these chronic cases are the source of these raised serum cytokines in these chronic SCZ cases.

3) That serum CCL2 is raised in endocrine autoimmune disease, but that sCD25 tends to be reduced, the latter reflecting a T cell tolerance defect.

With regard to the actual protein levels in serum of the inflammatory compounds and cytokines of the transcriptomic monocyte fingerprint and of the T cell activation state, we found the data from serum protein assays less robust and inconsistent, apart from the sCD25, which was a robust reflection of the CD25 expression on T cells, most notably in regard to the CD25 expression on T regulator cells. For the other pro-inflammatory monocyte and T cell cytokines, protein levels did not reach the same high levels as the genes in cells or the products in cells; e.g. in our studies on BD patients the approximately six fold raised expression of IL1B at the monocyte mRNA level was only reflected in a twofold raised IL-1β protein level in the serum of bipolar patients, while CCL2, CCL7 and IL-6 serum levels were not significantly higher. Clearly a regulation at the protein transcription level is operating in the pro-inflammatory 'angry' monocytes of psychiatric patients to ensure a close-to-normal (but still somewhat raised) protein production, and the question rises as to what is the role of epigenetic imprinting for these 'angry' monocytes and which environmental or endogenous conditions will create a failure of these angry monocytes to keep control over their aberrant gene transcript expressions, avoiding a high actual production of the pro-inflammatory compounds.

Our collective data suggest that an overexpression of sub-set 2 monocyte genes and a high T effector cell/T regulator cell ratio are mechanisms involved in (the proneness) for endocrine autoimmunity. In contrast: A raised number of T regulator cells and a downregulation of sub-set 1 monocyte inflammatory genes might protect from endocrine autoimmunity to develop.

For BD the data suggest that an upregulation of sub-set 1A monocyte genes, but in particular also of subset 2 genes, is involved in the activation of the MPS and microglia perturbing brain development and function in BD, while also products of T regulator cells may play a role in the elicitation of psychiatric symptoms.

For SCZ the data suggest that in particular an upregulation of sub-set 1B monocyte genes and products of both T effector and T regulator cells are involved in the activation of the MPS and microglia perturbing brain development and function in SCZ.

Figures 2 and 3 in the discussion section represent cartoons of these suggested immune mechanisms crucial for the development of endocrine autoimmunity and psychiatric disease.

Table III of the discussion section finally addresses the question what the shared vulnerability factor might be of BD and AITD. The most likely shared vulnerability factor is formed according to this table by the monocyte cluster 2 gene overexpression in monocytes of

both BD and AITD patients. This would mean that the chemotactic, motility and adhesion behaviour of circulating monocytes might be a crucial factor for both diseases developing in the same patient and that the local higher adhesiveness to endothelial cells and extracellular matrix proteins after infiltration of the monocytes to the thyroid or the brain and the consequent higher local inflammatory set point of local thyroid DC and microglia created by this adhesive interaction would constitute a general mechanism by which thyroid autoimmunity and "low grade inflammation" of the brain leading to BD would be linked. It would also mean that there are in fact two immune mechanisms that may lead to BD (see Figure 4 of the discussion section):

a) One primarily based on a pro-inflammatory activation of peripheral mononuclear phagocytes, including monocytes. This activation would predominantly be induced by environmental factors and
b) The other based on a (in part genetically-determined) stickiness of mononuclear phagocytes, including monocytes, to extracellular matrix components, which would render the cells in the tissues pro-inflammatory. The latter would be the one linked to thyroid autoimmunity.

Abbreviations

Abs	Antibodies
AITCD	Activated immune T cell death
AITD	Autoimmune thyroid disease
ANOVA	Analysis of variance
APC	Antigen presenting cells
APS	Autoimmune Polyendocrine Syndrome
ATF3	Activating transcription factor 3
BB-DP	Biobreeding diabetes prone
BB-DR	Biobreeding diabetes resistant
BCL2A1	BCL2- related protein A1
BD	Bipolar disorder
BDNF	Brain derived neurotrophic factor
BMI	Body mass index
CBA	Cytometric bead array
CCL2	Chemokine (C-C motif) ligand 2
CCR2	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CDC42	Cell division control protein 42
CI	Confidence interval
CMV	Cytomegalovirus
COX2	Cyclo-oxygenase 2
CSF	Cerebrospinal fluid
CTLA4	Cytotoxic T-lymphocyte antigen 4
CXCL	Chemokine (C-X-C) ligand
DC	Dendritic cells
DSM	Diagnostic and statistical manual of mental disorders
DUSP2	Dual specificity phosphatase 2
DZ	Dizygotic
EAE	Experimental allergic encephalitis
EAT	Experimental allergic thyroiditis
ECM	Extra cellular matrix
EGR3	Early growth response 3
EIF2S3	Eukaryotic translation initiation factor 2, subunit 3
ELISA	Enzyme-linked immunosorbent assay
EMP1	Epithelial membrane protein 1
EREG	High epiregulin gene

FABP	Fatty acid binding protein
FACS	Fluorescence activated cell sorting
FcαR	Fc fragment of IgA receptor
FN	Fibronectin
FOXP3	Forkhead box P3
FSH	Follicle-stimulating hormone
GAD65	65 kDA glutamic acid decarboxylase
GD	Graves' disease
GH	Growth hormone
GITR	Glucocorticoid-induced tumor necrosis factor receptor
GM-CSF	Granulocyte monocyte colony stimulating factor
GWAS	Genome wide association studies
HDL	High density lipoprotein
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
HT	Hashimoto's thyroiditis
IA2	Insulinoma-associated protein 2
ICA 69	69 kDa islet cell antigen
ICAM	Intercellular adhesion molecule
ICD	International Statistical Classification of Diseases
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IGF	Insulin like growth fractor
IL	Interleukin
IRS	Inflammatory response system
LADA	Latent autoimmune diabetes of the adults
LDL	Low density lipoprotein
LFA-1	Lymphocyte function associated-antigen 1
LH	Luteinizing hormone
LPS	Lipopolysaccharide
LTA	Lipotichoic acid
MACS	Magnetic cell sorting system
MAFF	V-maf musculoaponeurotic fibrosarcoma oncogene family
MAPK	Mitogen-activated protein kinase
MDD	Major depressive disorder
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reactions
MPS	Mononuclear phagocyte system

MXD	MAX dimerization protein
MZ	Monozygotic
NAB2	NGFI-A-binding 2
NAC	N-acetyl cysteine
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NK cell	Natural killer cell
NOD	Non obese diabetic
NOS	Not otherwise specified
OS	Obese strain
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PDE4B	Phosphodiesterase 4B
PET	Positron emission topography
PTGS2	Prostaglandin-endoperoxide synthase 2
PTPN7	Protein tyrosine phosphatase, non-receptor type 7
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
PTX	Pentraxin
ROS	Reactive oxygen species
RTE	Recent thymic emigrants
SCID	Structured clinical interview for DSM-IV axis I
SCZ	Schizophrenia
SDR	Short chain dehydrogenase/reductase 1
SFBN	Stanley foundation bipolar network
sIL-2R	soluble IL-2 receptor
STX	Syntaxin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
Tg	Thyroglobulin
TGF	Tumor growth factor
THBD	Thrombomodulin
THBS	Thrombospondin
Th	T helper cells
TLR	Toll like receptors
TNF	Tumor necrosis factor
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
TPO	Thyroid peroxidase
Treg	regulatory T cells

TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptor
VNTR	Variable number tandem repeats
YMRS	Young mania rating scale
ZFAT	Zinc finger gene in autoimmune thyroid disease

Populair wetenschappelijke samenvatting

Depressie of psychose door een ontstoken brein?

Ontsteking is nuttig. Het afweersysteem komt in actie tegen een probleem in het lijf en ruimt het op. Helaas is het in het echt niet zo eenvoudig. We weten al lang dat ons immuunsysteem soms ook zelf problemen veroorzaakt. Denk aan jeugd-diabetes, schildklieraandoeningen en andere auto-immuunziekten. In deze gevallen is de ontstekingsreactie volkomen onnuttig en schadelijk voor het lichaam. Toch wordt bij psychische problemen nog niet zo snel aan ontsteking gedacht.

Misschien komt daar nu verandering in. In de nog altijd voortdurende zoektocht naar oorzaken van psychiatrische ziektebeelden zijn wij nu iets interessants en belangwekkends op het spoor: problemen in het immuunsysteem als oorzaak van ernstige psychiatrie zoals schizofrenie, manisch-depressiviteit (bipolaire stoornis) en ernstige depressie.

Het idee van een mogelijke relatie tussen ontsteking en hersenaandoeningen is niet helemaal nieuw. Er wordt al een tijdje naar gezocht:
Een opmerkelijke link werd gevonden in het feit dat psychiatrische aandoeningen vaak samengaan met afweerstoornissen. Schizofrenen hebben in verhoogde mate last van hart- en vaatproblemen die door een ontsteking worden veroorzaakt. Ook auto-immuunziektes zoals diabetes en de ziekte van Crohn komen vaker voor bij mensen met depressie. En bipolairen hebben drie keer zo vaak een chronische auto-immuunschildklierontsteking als gezonde mensen.
Gevalletje kip of ei werpen sceptici tegen. Van de gevolgen van een chronische ziekte kun je behoorlijk depressief raken. Bovendien zijn medicijnen tegen schizofrenie slecht voor het hart. En lithium, dat bij bipolaire stoornis geslikt wordt, werkt ook op de schildklier. Dit klopt maar voor een deel. Toch hebben wij in dit proefschrift bewijs gevonden dat psychiatrische aandoeningen onderdeel zijn van chronische ontstekingsziekten. De chronische ontstekingsziekten kwamen namelijk ook meer voor bij niet-psychiatrische familieleden of wanneer er geen of andere medicijnen geslikt werden.

Hoe kan het immuunsysteem dan het functioneren van de hersenen verstoren?
Dit proefschrift beschrijft dat. De witte bloedcellen regelen onze afweer. We hebben er verschillende van, waarvan voor dit verhaal de monocyten het belangrijkst zijn. Monocyten komen via de bloedbaan in alle weefsels terecht. Daar specialiseren ze zich tot macrofagen. En macrofagen zijn de stofzuigers van het afweersysteem. Zij zuigen storende elementen op en vernietigen ze. Ze hebben echter nog een belangrijke rol: zorgen dat het immuunsysteem niet op hol slaat en de eigen lichaamscellen gaat aanvallen. Ook in het brein zitten deze macrofagen en daar heten ze microglia. Naast het opruimen van indringers

zorgen deze microglia ook voor het goed functioneren van de neuronen (zenuwcellen). Ze helpen onder andere bij het vormen van nieuwe synapsen (verbindingen) en nieuwe neuronen. Zo spelen ze ook een rol bij de vorming van de belangrijke neurotransmitter serotonine. Het is dus niet zo gek dat een afwijking in deze microglia een verstoring van de hersenen kan veroorzaken (*RC Drexhage et al, Expert Reviews Neurotherapy* 10(1) 2010).

Naast de microglia zijn de voorlopers, de cellen van het immuunsysteem in het bloed, de monocyten, dus heel belangrijk en veel gemakkelijker te bestuderen, omdat een beetje bloed snel en veilig kan worden afgenomen. In de witte bloedcellen begint het probleem en mogelijk kunnen zij gebruikt worden bij het diagnosticeren en hopelijk ook voorkomen van ziekte. Naar deze cellen hebben wij voor dit proefschrift vooral onderzoek gedaan en wij hebben gevonden dat bij patiënten met schizofrenie en met bipolaire stoornis (manisch-depressiviteit) veranderingen te zien zijn in het bloed, onder andere in de monocyten.

Bij deze patiënten zijn de monocyten anders dan bij gezonde mensen. Dit is heel interessant, omdat deze cellen daardoor gebruikt kunnen worden in de diagnostiek. De monocyten hebben een zogenaamde genetische handtekening. En deze blijkt bij mensen met schizofrenie net een beetje anders dan bij mensen met bipolaire stoornis. Zo kan je dus een onderscheid maken dat gebaseerd is op een biologische test in plaats van alleen af te gaan op de symptomen. De DSM-4, die nu nog wordt gebruikt om patiënten in te delen in de psychiatrie, is nog gebaseerd op symptomen. Hopelijk kan in de toekomst de biologische etiologie meer een rol gaan spelen (artikel hierover in *International Journal of neuropsychopharmacology* juni 2010).

Daarnaast zijn in het bloed zowel de pro-ontstekingslymfocyten als de anti-ontstekingslymfocyten (andere soorten van witte bloedcellen) verhoogd. 'Het eerste betekent dat er al sprake is van ontsteking. Het tweede betekent dat er ook sprake is van regulatie van het immuunsysteem. En dit is voor ons belangrijk. Bij deze mensen is het immuunsysteem als het ware anders afgesteld dan bij een gezond persoon. Er is sprake van een ander setpoint.' (artikel hierover in *Expert Reviews Neurotherapy* 10(1), 2010).

Zo'n ander setpoint van het afweersysteem is al eerder gezien. Namelijk bij kinderen van ouders met een bipolaire stoornis. In 2007 onderzochten wij samen met Manon Hillegers en Willem Nolen 140 van deze kinderen en wij doen nu nog verder onderzoek aan deze kinderen. Zij hebben een verhoogde kans op bipolaire stoornis. En ook zij bleken afwijkende cellen in hun bloed te hebben, al voordat ze überhaupt een ziektebeeld ontwikkelden. In een aantal gevallen veroorzaakten deze cellen uiteindelijk auto-immuun

schildklierproblemen, in een aantal gevallen stemmingsstoornissen, soms beide en soms helemaal niks (*European Neuropsychopharmacology* juni 2007).
Dit onderzoek is van groot belang. Hierdoor weten we dat het afwijkende immuunsysteem zeer waarschijnlijk oorzaak is van de psychische problemen en niet andersom. Daarnaast kan bij deze kinderen onderzocht worden of preventief ingrijpen werkt. We kunnen het systeem bij hen als het ware weer terug zetten. Dat kan hopelijk met sterke anti-oxidanten zoals n-acetyl cysteïne. De verwachting/hoop is dat er dan geen klachten zullen ontstaan.

Belangrijk ondersteunend voor dit werk in het proefschrift is dat andere onderzoekers onlangs ook een direct verband hebben gevonden tussen een ontspoord immuunsysteem en een afwijking in de hersenen dat leidde tot een veranderd ziekelijk gedrag.
Een onderzoeksteam uit Utah ontdekte dat muizen met een bepaald gendefect zo erg bezig waren met het verwijderen van lichaamshaar dat ze kale plekken en diepe wonden bij zichzelf veroorzaakten. Dit gedrag is vergelijkbaar met een vorm van dwangneurose (obsessief-compulsieve stoornis) bij de mens, namelijk trichotillomanie, het uittrekken van eigen haar. Bij nader onderzoek bleek dit gendefect een verandering te geven in de vorming van monocyten, de voorlopers van de microglia in de hersenen. Deze muizen bleken dan ook afwijkingen te hebben in hun microglia.
Het opvallendste deel van dit onderzoek is dat de muizen vervolgens een beenmergtransplantatie kregen. Daardoor maakten ze weer gezonde bloedcellen aan, de microglia werden weer normaal en wat bleek: binnen vier maanden stopte het dwangmatige gedrag en huid en haar herstelden (*Cell*, mei 2010).
Waarom is deze ontdekking nou zo belangrijk? Wel, dit is de eerste keer dat er een direct verband is gelegd tussen microglia en gedrag. En daarbij door in te grijpen in de microglia was het gedrag bij een muis te veranderen. Nu willen we natuurlijk weten of dat ook bij mensen lukt.
Overigens is het niet zo dat iedere psychiatrische aandoening dus altijd door een soort chronische ontsteking wordt veroorzaakt. Onderzoek in dit proefschrift suggereert de kans op vijftig tot zestig procent. Dit betekent wel dat veel psychiatrische patiënten nu een niet-optimale behandeling krijgen. Als ons afweersysteem de veroorzaker is, hebben psychiatrische middelen en psychoanalyses maar een beperkte werking. Ontstekingsremmende behandelingen zouden dan een goede aanvulling zijn.

Om terug te komen op de chronische ontstekingziekten die vaak voorkomen samen met stemmingsstoornissen, in dit proefschrift is ook onderzoek gedaan naar het setpoint van de monocyten en de lymfocyten bij jeugd-diabetes en schildklieraandoeningen. Dit gaf ons de mogelijkheid om overeenkomsten en verschillen te bestuderen met de setpoints bij psychiatrische ziekten en om er achter te komen waarom de aandoeningen zo frequent bij

elkaar voorkomen. Dit proefschrift beschrijft experimenten die aantonen dat de setpoints bij jeugd-diabetes en autoimmuun schildklierziekten vergelijkbaar en overlappend zijn met de setpoints gevonden bij stemmingsstoornissen, maar niet identiek zijn. Juist het overlappende gedeelte en de afwijkingen die daarbij horen geven ons meer inzicht in het ontstaan van deze autoimmuunziekten.

Om nu voor een grote groep psychiatrische patiënten precies op een rijtje te krijgen wat er gebeurt in het immuunsysteem, hoe de overlap is met auto-immuun ziekten en andere chronische ontstekingsziekten en of er immuuntherapie mogelijk is, loopt er een onderzoek bij 400-600 patiënten in Europa gefinancierd door de EU: *MoodInflame*.
Wij willen samen met de andere Europese onderzoekers verder onderbouwen dat patiënten met een ernstige stemmingsstoornis (dat zijn bijvoorbeeld de bipolaire stoornis, ernstige depressie en postpartum psychose) een afwijking hebben in hun afweersysteem en daardoor een lichte chronische ontsteking in de hersenen en ook chronische ontstekingsprocessen elders in het lichaam. De bedoeling is om van deze patiënten een uitgebreid profiel te maken met de genetische handtekening van de monocyten, de vorming van andere witte bloedcellen en van de cytokinen. Hiervoor moeten verdere bloedtests en hersenscans ontwikkeld worden. Daarnaast worden er dierproeven gedaan en ook zullen er clinical trials gedaan worden, waarbij patiënten naast hun gewone medicatie behandeld worden met ontstekingsremmers (Cox-2-remmers). De verwachting is dat zij sneller van hun klachten af zijn dan zonder (http://moodinflame.eu). De planning is om vóór 2013 definitieve resultaten te hebben. Interessant detail: Antidepressiva en antipsychotica werken sowieso al ontstekingsremmend.

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About the author

Roosmarijn Catherine Drexhage was born in Amsterdam in 1981. She grew up in Ouderkerk aan de Amstel and in Rotterdam. She attended secondary school at the Montessori Lyceum in Rotterdam. She then started Medical School at the Erasmus University of Rotterdam in 2001 and graduated in September 2007. Consecutively she was appointed as a PhD student at the Department of Immunology under the supervision of Dr. M.A. Versnel and Prof. Dr. H. Hooijkaas. During her PhD-training she also worked as a resident in Internal Medicine in the Maasstad Hospital under the supervision of Dr. Berghout and Dr. Van de Dorpel. As of January 2011 she works as a resident in Internal Medicine in the Ikazia Hospital under the supervision of Prof. Dr. Van Saase, Dr. Zandbergen and Dr. Dees. Roos lives happily together with Thijs Lingsma in Rotterdam.

Publications

The activation of monocyte and T cell networks in patients with bipolar disorder.
Drexhage RC, Hoogenboezem TH, Versnel MA, Berghout A, Nolen WA, Drexhage HA.
Brain Behav Immun. 2011 Mar 25.

TREM-1 and DAP12 expression in monocytes of patients with severe psychiatric disorders. EGR3, ATF3 and PU.1 as important transcription factors.
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Prevalence of autoimmune thyroid dysfunction in postpartum psychosis.
Bergink V, Kushner SA, Pop V, Kuijpens H, Lambregtse-van den Berg MP, Drexhage RC, Wiersinga W, Nolen WA, Drexhage HA.
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Int J Neuropsychopharmacol. 2011 Jan 24:1-10.

Inflammatory gene expression in monocytes of patients with schizophrenia: overlap and difference with bipolar disorder. A study in naturalistically treated patients.
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Monocyte gene-expression profiles associated with childhood-onset type 1 diabetes and disease risk: a study of identical twins.
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An inflammatory gene-expression fingerprint in monocytes of autoimmune thyroid disease patients.
van der Heul-Nieuwenhuijsen L, Padmos RC, Drexhage RC, de Wit H, Berghout A, Drexhage HA.
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Expert Rev Neurother. 2010 Jan;10(1):59-76. Review.

Distinct monocyte gene-expression profiles in autoimmune diabetes.
Padmos RC, Schloot NC, Beyan H, Ruwhof C, Staal FJ, de Ridder D, Aanstoot HJ, Lam-Tse WK, de Wit H, de Herder C, Drexhage RC, Menart B, Leslie RD, Drexhage HA; LADA Consortium.
Diabetes. 2008 Oct;57(10):2768-73. Epub 2008 Jul 3.

Patients with schizophrenia show raised serum levels of the pro-inflammatory chemokine CCL2: association with the metabolic syndrome in patients?
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Schizophr Res. 2008 Jul;102(1-3):352-5. Epub 2008 May 16. No abstract available.

PhD Portfolio Summary

Roosmarijn Drexhage

	Year	ECTS
Research skills		
Biostatistics for Clinicians (NIHES)	2008	0.5
Introduction to Clinical Research(NIHES)	2008	0.5
Regression Analysis for Clinicians (NIHES)	2009	0.5
General Academic skills		
Management voor promovendi en postdocs	2008	1.0
English Biomedical Writing and Communication	2009	4.0
The Photoshop CS3 Workshop for PhD-students and other researchers	2010	0.25
In-dept courses		
Molecular Immunology, Molmed	2009	2.0
Symposia and conferences		
Annual Symposium Dutch Thyroid Club, Amsterdam	2007+2008+2010	1.0
European Association for the study of Diabetes, Amsterdam	2007	1.0
Molecular Medicine Day, Rotterdam	2008+2009+2010	1.0
Kick-off Moodinflame, Rotterdam	2008	0.5
Schizophrenia and Bipolar Disorder, Keystone, USA	2009	1.0
NVDO jonge onderzoekersbijeenkomst, Amersfoort	2009	0.5
Diabetesdag ErasmusMC, Rotterdam	2009+2010	0.5
Boerhaave Cursus, Leiden	2010	1.0
8e Symposium Neuro-Endocrinologie, Utrecht	2010	0.25
AACAP, New York, USA	2010	1.0
Presentations		
Kick-off meeting Moodinflame, Rotterdam	2008	1.0
NVDO jonge onderzoekersbijeenkomst, Amersfoort	2009	1.0
Molecular Medicine Day, Rotterdam	2009	1.0
Psychiatrie in 't Dolhuys, Haarlem	2009	1.0

Boerhaave Cursus, Leiden	2010	1.0
Grand Rounds, Cleveland Clinic, Cleveland, USA	2010	1.0

Teaching

Casuistiek Immunologie, Geneeskunde 2e jaars studenten	2008+2009+2010	7.5
Dagje Proefstuderen, scholieren VWO	2008+2009+2010	3.0