Two Sides of the Same Coin

Unravelling the role of transcription factors and miRNAs in activated monocytes, macrophages and microglia

Karin Weigelt

Two Sides of the Same Coin

Unravelling the role of transcription factors and miRNAs in activated monocytes, macrophages and microglia

Karin Weigelt

The studies described in this thesis were performed at the Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands and at the Department of Human Genetics, University Hospital Regensburg, Regensburg, Germany.

The studies were financially supported by the European Commission EU-FP7-HEALTH-F2-2008-222963 'MOODINFLAME', ZonMW-TOP-91208018, The Pro Retina Foundation Germany and the German Research Foundation (DFG).

The printing of thesis was supported by J.E. Jurriaanse Stichting.

ISBN: 978-90-5335-586-2

Illustrations: Karin Weigelt, prof. dr. Thomas Langmann, Sandra de Bruin-Versteeg
Cover: Karin Weigelt, Ridderprint B.V., Ridderkerk, the Netherlands
Lay-out: Nikki Vermeulen, Ridderprint B.V., Ridderkerk, the Netherlands
Printing: Ridderprint B.V., Ridderkerk, the Netherlands

Two Sides of the Same Coin

Unravelling the role of transcription factors and miRNAs in activated monocytes, macrophages and microglia

Two Sides of the Same Coin

Het ontrafelen van de rol van transcriptiefactoren
en miRNA's in geactiveerde monocyten,
macrofagen en microglia

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
prof.dr. H.G. Schmidt
en volgens besluit van het College voor Promoties
De openbare verdediging zal plaatsvinden op
woensdag 7 november 2012 om 9.30 uur

door

Karin Weigelt

geboren te Neumarkt i. d. Opf., Duitsland

PROMOTIECOMMISSIE

Promotoren:	Prof.dr. H.A. Drexhage Prof.dr. T. Langmann
Overige leden:	Dr. P.J.M. Leenen Prof.dr. S. Bahn Prof.dr. S. Kushner

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

This thesis focusses on understanding the role of three transcriptions factors, namely ATF3, EGR3 and PU.1, and also microRNA-146a, involved in the activation of monocytes, macrophages and microglia in pathological conditions, which are thought to be driven – at least in part – by special inflammatory reactions. The diseases in question are severe psychiatric diseases, such as bipolar disorder, major depression, schizophrenia and postpartum psychosis and X-linked juvenile retinoschisis. In the introduction the immune cells, the diseases and the transcription factors + pathways will be introduced subsequently.

THE IMMUNE SYSTEM

With the immune system, mammals have developed the most complex and diverse mechanism to defend themselves against invading pathogens. Because of its high sensitivity and complexity this system is also susceptible to making mistakes. Therefore, besides providing protection agains different invading pathogens, self destruction of the host by immune responses has to be avoided at the same time.
The mammalian immune system is divided into two branches – the innate immue system that relies on germline encoded receptors and the adaptive immune system that has the ability to evolve with its tasks on the basis of receptors generated using genetic recombination. In this classical view, the innate immune system is the dominant system of host defense in most organisms, but it does not confer long-lasting immunity against a pathogen. The induction of a systemic adaptive immune response might not always be necessary, however it is favorable because the adaptive immune response will not only help to clear an acute infection, but it will also result in long-lasting antigen-specific immunological memory that provides protection for future contacts with the pathogen.
However, this classic dichotomy of innate versus adaptive immunity is currently challenged by studies demonstrating memory properties for natural killer (NK) cells and macrophages. This implicates that mammalian innate immunity also exhibits an immunological memory of past insults, for which the term “trained immunity” was proposed.[1]

INNATE IMMUNITY

The innate immune system is a first line of defence against pathogens. Therefore, a vast number of different receptors sensing diverse signals form a tight network that is not as specific as the adaptive immune response, meaning that these systems respond to pathogens in a generic way. The major components of the innate immune system are 1) barriers which are either chemical (tears or saliva) or mechanical (skin); 2) cells which comprise monocytes, monocyte-derived dendritic

cells and macrophages, neutrophils, natural killer cells and mast cells; and 3) soluble factors such as complement factors, cytokines and chemokines.

ADAPTIVE IMMUNITY

The key players of the adaptive immune response are B and T cells. In contrast to the receptors of the innate immune system, B cell receptors (BCR) and T cell receptors (TCR) are antigen-specific. The hallmark of the adaptive immune system is its ability to recognize and remember specific pathogens by facilitating the production of an unlimited spectrum of receptors. To provide immunological memory distinct B and T cell receptors are preserved in specialised memory B and T cells.

After activation of naive B cells by its specific antigen, the B cells develop into a long living memory B cells and antibody secreting plasma cells. The production of antibodies – the so-called humoral immunity – is important to neutralize extracellular bacteria, viruses and toxics, activate complement and facilitate pathogen phagocytosis. In contrast to B cells that promote their immune functions mainly through soluble antibodies, T cells are the key players for cell mediated adaptive immune responses. T cells form a heterogenous group of cells in which the expression of the TCR is the common feature of all subsets. Similar to B cells, each T cell is specific for a distinct antigen and undergoes many selection steps during its development to avoid the presence of autoreactive T cells. Effector $CD8^+$ T cells are cytotoxic and important in killing other cells of the body infected with virus, intracellular bacteria, or which are otherwise damaged or dysfunctional. The $CD4^+$ T cell subset is divided into several subtypes. Effector $CD4^+$ T helper cells (e.g. interferon γ (IFN-γ) producing TH1, interleukin 4 (IL-4) producing TH2 and interleukin 17 (IL-17) producing TH17) further activate immune responses such as the differentiation of B cells into plasma cells and memory B cells as well as the activation of macrophages.

Next to these effector and helper T cells, there are also T cells with regulatory functions. The main task of $CD4^+$ regulatory T cells (Treg) is the maintenance of tolerance (= antigen non-responsiveness) in the steady state, the supression of autoreactive T cells that escaped clonal selection in the thymus and the shutdown of adaptive immune responses after an infection has been cleared.

Both naive B and T cells are activated by antigen presenting cells (APC). In this way, the activation of the innate immune response will trigger the initiation of the adaptive immune response. Monocytes, macrophages and microglia are examples of APC but the most specialised ones are the dendritic cells (DC). DC pick up antigen, travel through lymphatics to the lymph node and present the collected antigen to cells of the adaptive immune system.

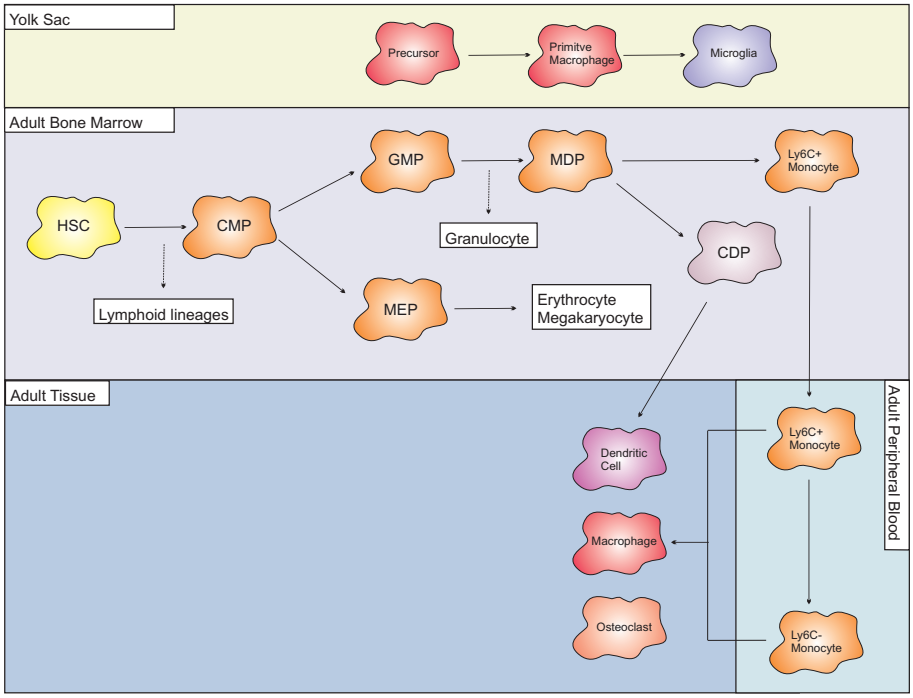


Fig. 1. *Adapted from Gordon S. and Taylor P.R. (2005) and Chow et al. (2011).*[5] ***Lineage of mononuclear phagocytes****. Commitment to differentation into a monocyte, macrophage or dendritic cell (DC) occurs at the stage of the macrophage and DC progenitor (MDP). MDPs can give rise to common DC progenitors (CDPs) or monocytes. CDPs give rise to DCs. Monocytes can give rise to DCs, macrophages or osteoclasts. In mice Ly6C is a marker of the 'inflammatory' population of monocytes. The concept of the MPS has come under scrutiny following the discovery of a separate embryonic phagocyte lineage in the yolk sac which gives rises to the resident microglia population in the brain.*[3,4] *CMP, common myeloid progentor; GMP, granulocyte and macrophage progenitor; HSC, hematopoietic stem cells*

THE MONONUCLEAR PHAGOCYTE SYSTEM (MPS)

The mononuclear phagocyte system is a family of cells that comprises committed precursors in the bone marrow, circulating blood monocytes, dendritic cells and tissue macrophages in every organ of the body.[2] Originally, the definition of the MPS was intended to separate cells of this family from lymphoid cells (T and B lymphocytes), granulocytes, and endothelial cells. In different tissues, they show significant heterogenity with respect to phenotype, homeostatic turnover and function. Microglia are the macrophages of the central nervous system (CNS) but they differ decisively from peripheral macrophages. Fate mapping recently identified microglia as an ontogenically distinct population of the mononuclear

phagocyte system.[3] A recent reinvestigation of macrophage development confirmed that microglia and other tissue macrophages define a lineage that derives from the yolk sac and is genetically distinct from hematopoietic stem cell (HSC) progeny.[4]
However, each of these separations described reflects the current status quo of research (depicted in **Figure 1**) and is under ongoing debate and investigation.[2]

PROGENITORS IN THE BONE MARROW

Myeloid differentiation is initiated by self-renewing HSC that give rise to multipotent precursors.[6, 7] These multipotent cells are lineage-associated marker negative (Lin^-), Sca-1^+, and $CD117^+$ (c-kit) and give rise to lineage-restricted common lymphoid progenitor cells (CLP)[8] and common myeloid progenitor cells (CMP).[9] CMP generate all subsets of myeloid cells, including macrophages, granulocytes, and DC by developing into granulocyte/macrophage progenitors (GMP), megakaryocyte/erythroid progenitors (MEP) and the recently identified macrophage-DC progenitors (MDP) which were isolated from bone marrow suspensions of $CX3CR1^{gfp/+}$ mice.[10] When introduced into bone marrow, MDPs give rise to $Ly6C^+$ and $CX3CR1^+$ bone marrow monocytes, which exit the bone marrow, partially guided by CCR2-dependent signals and differentiate into tissue DCs and macrophages.[10-13]

MONOCYTES

Circulating blood monocytes descend from GMP or MDP and their development depends on the macrophage colony-stimulating-factor (M-CSF) and its receptor, M-CSFR (CD115, c-fms, Csf1r). Deficiency in their cognate genes results in intensely reduced numbers of blood monocytes.[14-17] Monocytes circulate not only in blood, but also in bone marrow and spleen and do not proliferate in a steady state.[18, 19] They represent immune effector cells which produce inflammatory cytokines and engulf and take up antigen and cell debris. Therefore monocytes play important regulatory roles in both the innate and the adaptive immune response. Monocytes are equipped with chemokine receptors that mediate their migration from blood to tissues during infection.[20, 21] Additionally to their direct contribution to immune defense again microbial pathogens, circulating blood monocytes supply peripheral tissues with macrophage and DC precursors.[21] In response to a very wide range of different stimuli, monocytes are recruited into tissues and can differentiate into the various types of tissue macrophages, including bone-associated osteoclasts, as well as Langerhans cells and inflammatory DC.[13]
In humans, monocytes in peripheral blood have been in subdivided into three

subsets based upon certain surface markers, and especially based on discriminative CD14 (a component of the lipopolysaccheride (LPS) receptor complex) and CD16 expression (the low-affinity Fc receptor).[22] Similar to humans, a study based upon monocyte repopulation following toxic liposome administration suggested three monocyte populations in the mouse.[22] One of these three populations is represented by intermediate cells and can be found in humans and mice. However, the determination of the boundaries of these subpopulations remains difficult since unique markers for intermediate cells have not yet been identified.[22] During replenishment of blood monocyte pools following toxic liposome administration it was shown that the "inflammatory" ly6C^{hi} population appears first.[23] In a key experiment, Geissman et al demonstrated that - based upon monocyte adoptive transfer - ly6C^{hi}, CX3CR1^{lo} monocytes were selectively recruited to the peritoneal cavity as thioglycollate-elicited exudate cells. In contrast to that, most of the ly6C^{lo}, CX3CR1^{hi} cells were found in blood patrolling endothelium and noninflamed periphal organs, thereby replenishing resident tissue MPS populations.[24] The migration into the tissue and the differentiation of monocytes is likely to be determined by inflammatory stimuli in the surrounding (micro)environment[13] and in terms of their expression of key molecules, chemokine receptors and adhesion molecules that define the exact response to recruitment stimuli, blood monocytes should actually be regarded heterogenous. However, it is not yet clear to which extent monocytes subsets can be further subdivided with regard to their effector functions and fates, and whether distinct populations are recruited to distinct stimuli and distinct locations.

MACROPHAGES

Tissue macrophages are widely distributed throughout the body in various organs, including the dermis of the skin, the liver, the lungs, the brain and the spleen. The specific name of each type of macrophage is related to its location (**Figure 2**) and in most tissues macrophages represent a substantial number - up to 55%[25] - of the total cell population.

One must bear in mind that macrophages are not only cells of the immune system, but also have a central function in embryonic development[26, 27], homeostasis and wound repair. Characteristic features shared by macrophages are a high number of cytoplasmic granules, a defined cellular location, expression of surface markers and gene expression profiles.[28, 29] Macrophage heterogeneity and plasticity is very large – even within a single organ – and the set of marker combinations and subpopulations is essentially infinite.[30] Infectious agents and/or immunological stimuli attract macrophages that differ greatly depending on the precise nature of the challenge. So, through constitutive and induced endocytosis, phagocytosis, and

secretion of various products, including cytokines, growth factors, and metabolites, they perform both trophic and toxic functions. To define a simplified conceptual framework, classification of these phenotypes into functional categories of activation, called M1 and M2 macrophages has been proposed.[5, 31] The underlying differentiation processes are determined by the inflammatory stimuli from the microenvironment[32], resulting in *classically activated* (M1) or *alternatively activated* (M2) macrophages.

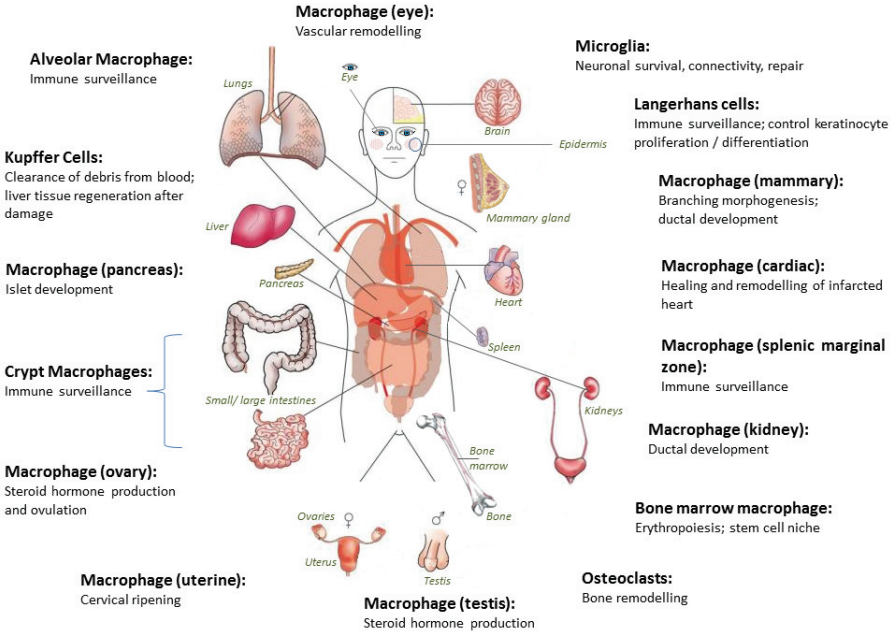


Fig. 2. *Adapted from D.A. Hume (www.macrophages.com). Macrophages can be found in every organ and in different tissues where they have specialised functions. Their role across the body varies and includes immune surveillance, development, repair, and remodelling.*

Upon inflammation, IFNγ induces differentiation of Ly-6C^{hi} classical monocytes into M1 macrophages. IFNγ is produced at early stages of infection by stimulated Th1 lymphocytes and NK cells in response to IL-12 production by DC and macrophages.[33] Mice lacking IFNγ are unable to mount macrophage-induced immune responses due to impaired NO and ROS production[34], thereby demonstrating the critical role of IFNγ. As a result, IFNγ$^{-/-}$ mice are unable to kill bacteria and die after challenge with a sublethal dose of *Mycobacterium bovis.*

Following activation, M1 macrophages produce high levels of oxidative metabolites, pro-inflammatory cytokines, such as *tumour necrosis factor* α (TNFα)

and IL-6, as well as phagocyte attracting chemokines, such as IL-8, CCL3 and CCL4, thereby promoting the recruitment of neutrophils. In the acute-phase response, TNFα, IL-1 and IL-6 contribute to the initiation of systemic responses including fever. Consequently these responses initiate sickness behaviour and make the body less conducive to pathogen replication. M1 macrophages not only target microbes, but are also tumoricidal and because these cells belong to the APCs, they also play an important role for the initiation of Th1 adaptive immune responses.[35] However, as a side effect, M1 macrophages can also damage healthy tissue, since M1-like macrophage-derived TNF, IL-18, IL-12 and IL-23 have been identified as important mediators in several chronic inflammatory and autoimmune diseases, including Crohn's disease, rheumatoid arthritis, multiple sclerosis and autoimmune hepatitis.[36-38] Furthermore, a subset of toll-like receptor (TLR) expressing CCR2-dependent macrophages was demonstrated to promote inflammation in the colon, during experimental colitis.[39]

Contrary to M1 macrophages, M2 or *alternatively activated* macrophages promote tissue remodeling and generally suppress destructive immune reactions. During steady state development, and in sterile wounds or tissue injury, the Th2 cytokines IL-4 and IL-13 amplify M2 induction from Ly6C^{lo} monocytes,[40] depending on, both *in vitro* and *in vivo*, IL4Rα1, the common IL-4 and IL-13 receptor alpha chain.[41] Next to the prototypical direct inducers of the M2 phenotype, other cytokines such as IL-33 and IL-25 can induce M2 indirectly, through Th2 cells[42] and recently, PPARγ activation was shown to skew human monocytes toward an anti-inflammatory M2 phenotype[43]. Richardson et al. demonstrated that an infection of macrophages with the respiratory syncytical virus (RSV) leads to an early induction of proinflammatory cytokines and COX-2.[44] After this early proinflammatory period, macrophages produce their own IL-4 and and IL-13 and differentiate into M2 macrophages through an IL-4Ra-, TLR4- and IFN-β-dependent mechanism to promote repair of damage caused by earlier inflammatory process and IL-10 acts to downregulate production of inflammatory cytokines.[45]

It is important to note, that phenotypes associated with classical (M1) and alternative (M2) activation of macrophages can be distinguished from the direct effects of microbial stimuli, such as lipopolysaccharide, which induce innate macrophage activation, often through TLRs.[46] Innate stimuli are able to synergize with classical activation of macrophages, to achieve full expression of macrophage effector pathways.[42] Next to microbial stimuli or T cell products, also pathogen-associated molecular patterns (PAMPs) induce macrophage activation. The initial sensing of infection is not only mediated by TLRs, but also C-type lectin receptors and cytoplasmic receptors of the RLR and the NLR family.[47] All these receptors

are known as innate pattern recognition receptors (PRRs) and recognize PAMPs.[47] Therefore, classical activation of macrophages might in fact be the consequence of synergistic interactions rather than single stimuli.

Consequently, the M1/M2 distinction - like the Th1/Th2 dichotomy - blurs on the boundaries when distinct types of stimuli and individual cells are compared. Recently Mosser and Edwards provided a more sustainable view which considers the range of macrophage phenotypes as the spectrum of colours on a colour wheel and infinite, because the "subsets" are defined as a function of the number of markers.[30] The concept that macrophages represent a spectrum of activated phenotypes, including DC phenotypes, rather than discrete stable subpopulations is supported by numerous studies which have documented flexibility in macrophage programming, with macrophages switching from one functional phenotype to another in response to the variable microenvironmental signals of the local milieu.[48-53] Similarly, though subpopluations have been characterized,[54] DCs should be considered to represent a spectrum of professional APCs rather than distinct cell types.

MICROGLIA

Microglia, the resident macrophages of the nervous system and the retina, were first described about 90 years ago by Rio-Hortega as a separate cell population of the brain.[55] Only later, however, their morphological[56-58] and cytochemical[59-62] properties have been studied in greater detail. Over the last decades, more and more studies have identified the important roles of microglia in immune regulation[63, 64] and neuronal homeostasis.[65, 66] Their special localization in the fragile neuronal environment and their morphological features clearly distinguish them from other peripheral macrophages.[67] Another important distinction is a consequence of their location, so due to residing behind the blood brain barrier (BBB) serum products represent danger signals indicating BBB breach.[68] Recently, an *in vivo* lineage tracing study in the mouse demonstrated that microglia derive from primitive myeloid progenitors that arise before embryonic day 8.[3] Nethertheless, microglia are part of the mononuclear phagocyte system and mice deficient in M-CSF (op/op) show reduced numbers of microglia with minor alterations in morphology.[69] Recently two *in vivo* studies demonstrated that ramified microglia – with their long protusions - perform a very active and continous surveillance function and it is estimated that the complete brain parenchyma could be monitored every few hours.[64, 70, 71] Thereby, microglia are thought to exhibit an actively repressed phenotype in which permanent tonic inhibitory inputs from neurons prevent microglial neurotoxicity.[72, 73]

Phenotype switching in microglia

Disturbances or loss of microglia-neuron cross-talk via cognate CD200-CD200 receptor (R) interactions[74, 75], or other ligangs constitutes a type of danger signal, indicating impairment of neuronal function and thereby contributing to microglial activation during pathological processes in both, the retina and the brain. The receptor CD200R is expressed on microglia and in mice a lack of CD200, normally expressed by neurons, leads to a functional transformation of ramified microglia with a variaty of effector functions, changes in the expression of MHC class II, and these changes also result in more severe disease in autoimmune encephalomyelitis[75] and retinal inflammation.[74] A number of other receptor-ligand pairs strenghten this point of view. Microglia express TREM-2 (*triggering receptor expressed on myeloid cells-2*) and this receptor signals via the adaptor molecule DAP12 (*DNAX-activating protein of 12 kDa*), thereby leading to inactivation of macrophages and microglia. Following stimulation with TLR agonists, the deletion of TREM-2 and DAP12 from macrophages induces identical phenotypes and enhanced cytokine production.[76] Homozygous deficiency of either TREM-2 or DAP12 in patients leads to adult-onset dementing leukoencephalopathy or Nasu-Hakola disease, thereby providing proof-of-priniciple that microglial functions are required for CNS homeostasis.[68] Also, local danger signals such as extracellular ATP[77], or neurotransmitter gradients[78, 79] have been shown to rapidly induce microglia activation. Similar to the continuum of activated phenotypes of peripheral macrophages, microglia are also thought to shift activity states, rather than proceeding from resting to activated (**Figure 3**). Excessive or prolonged microglial activation – also termed "microgliosis"[81] - preceeds neuronal degeneration under pathological conditions,[82-84] such as multiple sclerosis, Morbus Alzheimer and Morbus Parkinson[85] and results in an increased production of cytotoxic factors, such as nitric oxide (NO) or TNFα, thereby causing irreversible neuronal loss.[64, 86, 87] In eye research, a number of studies demonstrated that microglial activation is associated with retinal degeneration and photoreceptor apoptosis in the retina.[88-90] *In vitro* culture of 661W photoreceptor cells with microglia-conditioned medium induced apoptosis of these cells whereas Müller glia-conditioned medium did not.[91] During retinal degeneration in the retina of the rd1 mouse, microglia migrate into the outer nuclear layer of the retina. This migration is followed by an increased expression of chemokines and secretion of TNFα by the activated microglia before the onset of photoreceptor apoptosis.[89] The induction of apoptosis follows the release of *nerve growth factor* (NGF) by microglia and its binding to the *p75 neurotrophin receptor* (p75NTR) on the surface of photoreceptor cells, and increased mRNA levels of these two genes were found in the retina of the Royal College of Surgeons (RCS) dystrophic rat.[92]

Consequently, microglia should be able to sense subtle disturbances of retinal integrity or function at a very early stage of disease progression. [93]

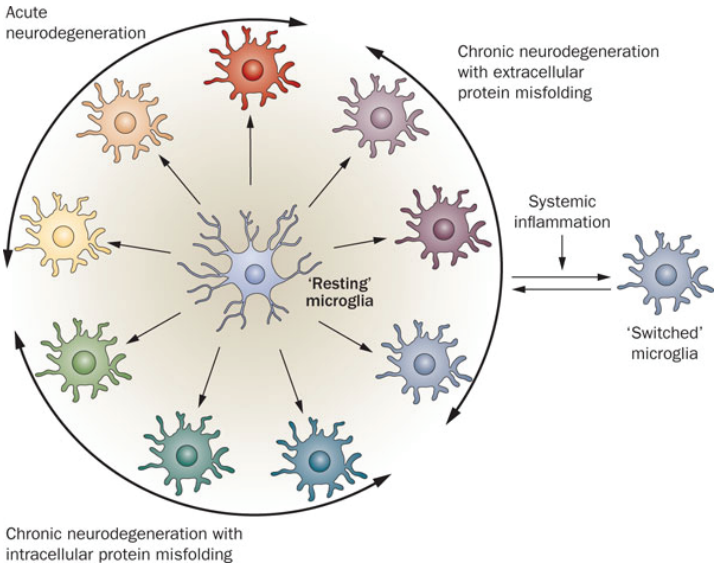


Fig. 3. *Adapted from Perry, V. H.* et al. *(2010).*[80] *Resident microglia can become activated to adopt one of many diverse phenotypes depending on the disease type, stage of disease, age of the patient and many other variables.*

DISORDERS

The gene regulatory aspects investigated in this thesis were analyzed in monocytes, macrophages and microglia. Due to their widespread functions, these cells are involved in various pathological conditions, such as histiocytosis, diabetis and cancer. The focus of this thesis is on major psychiatric disorders and X-linked juvenile retinoschisis which are also thought to be driven – at least partially - by deregulated MPS cells. Therefore background information about these disorders is provided in the following section.

PSYCHIATRIC DISORDERS AND THE MACROPHAGE THEORY OF DEPRESSION

In 1992, the concept of the "Macrophage or cytokine theory of depression and schizophrenia" was introduced[94,95] implicating that immune-induced aberrant brain development and function would allow various environmental factors (e.g. stress) to precipitate major psychiatric episodes (**Figure 4**). Our group identified immune activation in monocytes as a characteristic of schizophrenia,[96] bipolar disorder,[97] unipolar major depressive disorder and postpartum psychosis (unpublished data).

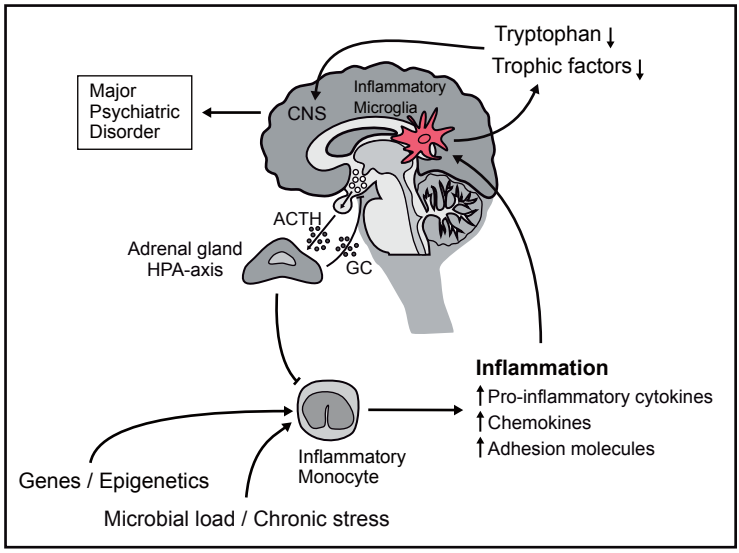


Fig. 4. ***The macrophage theory of depression***. *Chronic stress e.g. can activate the CNS system stress circuity and thereby lead to the activation of monocytes and peripheral macrophages, resulting in the release of inflammatory mediators that promote inflammation. Pro-inflammatory cytokines, in turn, can access the brain, induce inflammatory signalling pathways, decreased production of tryptophan and relevant trophic factors and activate microglia and the HPA-axis. Activated microglia release cytokines, chemokines and other inflammatory mediators and thereby amplify inflammatory signals within the CNS. Activated microglia also express high levels Indoleamine 2,3-dioxygenase (IDO). IDO breaks down tryptophan which is the primary precursor for serotonin.Activation of the HPA-axis leads to the release of cortisol to decrease the inflammmatory response. In the context of chronic stress and the influence of GC receptor function, activation of inflammatory pathways may become less sensitive to the inhibitory effects of cortisol, and the relative balance between the pro-inflammatory and the anti-inflammatory actions may be become seriously disturbed. CNS, central nervous system; HPA-axis, hypothalamic-pituitary-adrenal axis; GC, Glucocorticoid*

Major depressive disorder (MDD) or unipolar depression is one of the major psychiatric disorders. Standardized classification systems as DSM-IV[98] and ICD-10[99] are used to describe psychiatric disorders. Hallmarks of depression are depressed mood and or anhedonia. According to DSM-IV criteria at least 5 of 9 following symptoms must be present for at least 2 weeks: sleep disturbances, psychomotor retardation or agitation, fatigue, feelings of worthlessness or guilt, impaired thinking or concentration, change of appetite or weight and suicidal thoughts.[98] Depression has a lifetime prevalence of 2 to 15% and is associated with severe disability. In absence of treatment, depression tends to follow a chronic course and can severly impair the patient's functioning in society.[100]

In contrast to unipolar depression where patients only experience depressive

episodes as described above, patients with **bipolar disorder** (BD) also experience manic episodes. Mania describes the presence of elevated, expansive or irritable mood and therefore bipolar disorder was originally termed "manic-depressive illness" by Emil Kraepelin (1856-1926) in the late 19th century.[101] According to the DSM-IV classification bipolar disorder is a chronic disorder and characterized by the occurence of manic and depressive episodes with usually a full recovery between episodes. Bipolar Disorder is a major global health problem. The life time prevalence is estimated at 1 to 2% in the general population in the general population across ten European countries.[102] Together with MDD bipolar disorder belongs to the ten leading causes of disability worldwide[103] and is associated with high suicide rates.[104]

Postpartum psychosis (PP) is an acute psychiatric emergency, characterized by psychotic symptoms, mood symptoms and sometimes cognitive dysfunction. Postpartum psychosis is considered the most severe postpartum mood disorder.

The prevalence of postpartum psychosis in the general population is 1–2 per 1000 childbirths, and the rate is 100 times higher in women with bipolar disorder or a previous history of postpartum psychosis.[105]

One of the most consistent findings in neurobiological research in postpartum psychosis over the last decades is the vulnerability for postpartum psychosis in women with bipolar disorder. However, the majority of patients admitted with postpartum psychosis have no prior diagnosis of a psychiatric disorder.[106] Postpartum psychosis will often in retrospect be appreciated as the incipient presentation of bipolar disorder, while some patients experience symptoms only during the postpartum period. Some authors argue that postpartum psychosis might be a distinct diagnostic entity within the bipolar spectrum,[107] associated with the metabolic effects of abrupt hormonal withdrawal. Acute onset of mood symptoms and psychosis occurs within the first four weeks postpartum, thereby indicating that postpartum psychosis is a manifestation of a lifetime vulnerability to affective disorders with childbirth as the precipitating factor.[108]

Schizophrenia (SCZ) belongs to the major psychiatric disorders and was first described by Emil Kraepelin as Dementia Praecox.[101] A few years after the original description, Eugen Bleuler suggested rephrasing the disorder into schizophrenia ("fragmented mind").[109] Schizophrenia is - according to the DSM-IV - a mixture of positive and negative psychiatric signs and symptoms.[98] Positive symptoms reflect an excess or distortion of normal perceptive function such as delusions or hallucinations. Negative symptoms reflect a diminution or loss of normal function such as affective flattening and lack of initiative. In total five subtypes have been categorized.[98] Schizophrenia is present worldwide and the prevalence ranges

from 0.5% to 1.5%. The incidence is higher in urban areas[110] and migrant groups, since 1st and especially 2nd generation immigrants from Morocco and Surinam in the Netherlands have a 5.8 times and 2.8 higher chance to develop schizophrenia, respectively.[111] Usually, patients with schizophrenia are treated with antipsychotic medication for the positive symptoms such as psychosis and delusions.[110] So far, there is no medication for the negative symptoms and generally, the effect of medication is not totally satisfactory.[112]

As mentioned above, mounting evidence supports the involvement of the immune system in these major mental illnesses. A *postmortem* study on brains from schizophrenia patients, who had committed suicide during acute psychosis, revealed increased density of microglia.[113] The increased density could however be a consequence of death rather than pathophysiology in patients. Three other studies reported increased microglial activation in schizophrenia patients,[114-116] while another three did not confirm an activation state of microglia.[117-119] A qualitative study of HLA-DR expression showed increased expression of this surface marker on microglia of the hippocampus and prefrontal cortex of depressed patients.[116] The concept of MPS cell activation the brain of psychiatric patients is in line with the observation that there is an accumulation of monocytes and macrophages in the cerebrospinal fluid of patients with schizophrenia during acute psychotic episodes.[120]

More recently, increased expression of quinolinic acid (QUIN) has been identified in ramified microglia in subregions of the anterior cingulate cortex of severely depressed patients.[113] A similar trend was observed in bipolar disorder. QUIN is a terminal break down product of the tryptophan pathway primarily produced by activated microglial cells. A key enzyme for the production of QUIN in microglia is indoleamine 2,3-dioxygenase (IDO). IDO is activated by pro-inflammatory cytokines, including IFN-γ, IL-1β, IL-6 and TNF-α.[121] Furthermore, the PET-tracer ([11C]-PK11195) was employed successfully to investigate microglia activation in several patient and animal studies of neuropsychiatric disorders,[122] showing that immune activation ("inflammatory") lesions occur in brain regions related to the specific disease process. In schizophrenia microglia activation is found in the hippocampal area where functions (immediate memory, sensory/emotional integration) are impaired. Interestingly these focal changes are found only in acute psychotic patients with prominent cognitive impairment[123] and not in patients that recovered from psychosis,[124] these patients showed a global brain inflammatory effect. The mechanisms responsible for the activation of microglia in psychiatric disease however remain elusive.

With regard to the peripheral circulation, there are presently strong indications for activation of circulating monocytes in at least a proportion of patients with

psychiatric disease. Early reports showed that the number of circulating monocytes is aberrant in patients with schizophrenia. Rothermundt et al. reported a slight increase in the mean absolute and relative monocyte counts,[125] while we and others supported these observations, also finding a monocytosis and a higher number of $CD14^+$ cells in acute schizophrenia patients and children with psychosis.[126-128] In contrast to schizophrenia, higher numbers of $CD14^+$ monocytes could not be found in patients with bipolar disorder.[97, 126] Neither were differences found between the number of mature ($CD14^+CD16^+$) and immature ($CD14^+CD16^{neg}$) circulating monocytes in these bipolar disorder patients.[97]

Recently, we carried out two gene expression profiling studies[96, 97] on purified monocytes of psychiatric patients (56 bipolar and 27 schizophrenia patients) using Affymetrix analyses followed by confirmatory quantitative real time PCR. In sum, an upregulated expression of 34 genes was detected, forming a monocyte gene expression signature. The monocyte gene signature consisted of two main gene clusters:

Cluster 1, composed of mainly cytokines and inflammatory compounds, including notable factors such as IL1B, IL-6, TNF, PTGS2, PTX3 and inflammation regulators like PDE4B, DUSP2. We have indications that this sub-cluster is driven by the transcription factors/regulators ATF3 and EGR3. Some of the genes upregulated in this cluster are not always pro-inflammatory, but also anti-inflammatory (e.g. ATF3). The gene signature thus represents a situation of an activation of immune gene transcription, rather than a pro-inflammatory state.

Cluster 2, composed of mainly adhesion/motility factors and chemokines, such as CDC42, CCL2, CCL7, EMP-1 and STX1A, with PTPN7 and NAB2 being most likely the important transcription regulators of this cluster.

The majority, i.e. 50-60%, of patients with bipolar disorder showed an activated monocyte gene expression set point involving both cluster 1 and cluster 2 genes, 50-60% of schizophrenia patients showed an activated monocyte set point too, but of cluster 1 genes only.[96] Also, the over-expression of monocyte activation genes was particularly evident in active cases, i.e. in bipolar patients with an active mania or depression or schizophrenia patients with an active psychosis.[97]

There is evidence for an immune activation of microglia in acute bipolar and schizophrenia patients. It is thought that this immune activated microglia disturb the growth and function of important brain nuclei. The immune activation of microglia might be part of a systemic activation of the MPS, including circulating monocytes, in bipolar and schizophrenia patients with severe and active disease.

In sum, this systemic mononuclear phagocyte activation is considered to be driven by environmental factors upon a genetic background.

X-LINKED JUVENILE RETINOSCHISIS (RS)

In contrast to psychiatric disorders which represent complex multi-factorial diseases, X-linked retinoschisis is an inherited disease, but a common feature shared between all diseases is the activation of cells of mononuclear phagocyte system, particularly monocytes and microglia.

Retinoschisis is a recessively inherited retinal dystrophy which represents a common form of macular degeneration in males.[129-131] A characteristic feature of this disorder is a limited splitting of the retina at the level of the nerve fiber and ganglion cell layers, leading to cystic degeneration of the central retina but also may involve the periphery.[132-134] Blindness at birth was reported in severely affected individuals, although generally the clinical course is less progressive, with only moderate decrease in visual acuity.[129] RS is caused by loss-of-function mutations in RS1 gene on Xp22.13.[135] It encodes a retina-specific polypeptide of 24 kDa, termed retinoschisin, which is mainly secreted from photoreceptors as homo-oligomeric complex.[136, 137] The protein consists almost exclusively of a highly conserved discoidin domain frequently found in a wide range of membrane and extracellular proteins which have been implicated in cell adhesion and cellular signaling processes.[138] To study retinoschisin function and its role in the cellular pathology of RS, knockout mice deficient in *Rs1h*, the murine ortholog of *RS1,*[139] were generated.[140] The *Rs1h* knockout mouse displays retinal features typical for X-linked juvenile retinoschisis (RS), thereby representing an excellent model to study early molecular events in RS.[140] In 2007, genome-wide expression profiling was perfomed in retinal tissue of postnatal stages (P)7, 9, 11, and 14 to elucidate the molecular events that precede photoreceptor disease in the *Rs1h* knockout ($Rs1h^{Y/-}$) mouse.[90] Besides *Rs1h*, several differentially expressed transcripts with a functional annotation to adhesion, cytoskeleton, vesicular trafficking, and immune response were identified. Interestingly, many genes implicated in microglia/glia activity and inflammatory processes were upregulated before the onset of an appreciable expression of genes involved in apoptosis.[90]

Therefore, important molecules associated with MPS cell activation related to X-linked retinoschisis and/or psychiatric diseases will be introduced in the following section.

TREM-1/DAP12 AND TREM-2/DAP12 SIGNALLING

The membrane adaptor protein DAP12 (*DNAX activation protein of 12 kDa*) is expressed on several immune cells, contains an immunotyrosine-based activation motif (ITAM), and is essential for M-CSF-induced proliferation and survival of

macrophages.[141] Aoki et al demonstrated that the expression of DAP12 increased after LPS-induced differentiation of cells of the myeloid precursor cell line M1 into macrophages.[142] DAP12 associates with more than 20 different surface receptors to regulate immune responses[143] and although DAP12 has been shown to have a dual role by potentiating or attenuating immune cell functions, a mainly deactivating function has been postulated for myeloid cells.[144] Thus, together with *triggering receptor expressed on myeloid cells 2* (TREM-2), DAP12 blocks LPS/TLR-mediated cellular activation[76, 145] and enhances differentiation of macrophage precursors.[142] Furthermore, TREM-2 and DAP12 are expressed on brain microglia, but not in neurons, astrocytes, or oligodendrocytes and cooperatively control phagocytosis of apoptotic neurons.[146, 147] Loss or ablation of TREM-2 results in deficient removal of cellular debris from apoptotic cells but enhanced expression of inflammatory mediators (**Figure 5**).[148] As mentioned earlier in the introduction, deletions in the DAP12 or TREM-2 gene both lead to chronic neurodegenerative diseases (Nasu-Hakola[149] or polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy[150]). Nasu-Hakola disease is characterized by a combination of bone fractures and psychotic symptoms similar to schizophrenia, rapidly progressing to presenile dementia.[151, 152] DAP12-deficient ($DAP12^{-/-}$) mice develop osteopetrosis and a reduction of myelin accentuated in the thalamus. Additionally, $DAP12^{-/-}$ mice also display synaptic degeneration and impaired prepulse inhibition, which is commonly observed in several neuropsychiatric diseases in humans including schizophrenia.[153] Similar to the knockout method, also the knock-in method results in the disruption of the gene of interest. A study using DAP12 knock-in ($DAP12^{KI}$) mice[154] found impaired synaptic function in these mice.[146] A follow-up study by the same group showed that the mutation in the DAP12 gene induces delayed impairment of glutamatergic synaptic function via prenatal activation of microglia and that $DAP12^{KI}$ microglia overexpress genes for IL-1β, IL6 and NOS2, thereby demonstrating that a loss of DAP12 followed by prenatal inflammation is sufficient to induce synaptic alterations with delay.[155]

In the developing hippocampus, apoptotic neurons are contacted by microglia expressing both the integrin CD11b and the immunoreceptor DAP12. Developmental apoptosis decreases in mice deficient for CD11b or DAP12 and together both genes control the production of microglial superoxide ions, which kill the neurons, thereby implicating that the process of developmental neuronal death triggered by microglia is similar to the elimination of pathogenic cells by the innate immune cells.[156] Neurodegeneration is also prominent in mucopolysaccharidosis I und IIIB and the progression of these diseases are accompanied by microglia activation and increased expression of DAP12, thereby suggesting a role for DAP12

in the inflammatory component of these brain diseases.[157]

TREM-1 (*triggering receptor expressed on myeloid cells*) is an orphan immunoreceptor expressed on monocytes, macrophages and neutrophils.[142, 144, 158, 159] Several studies have characterized TREM-1 as an amplifier of the immune response that strongly potentiates the activation of leukocytes in response to microbial products.[158-160] Similar to LPS, TREM-1 activation via antibody-mediated receptor crosslinking upregulates chemokines, cytokines, matrix metalloproteases, and COX-2, consistent with a core inflammatory response.[159] Interestingly, TREM-1 acts synergistically with receptors for PAMPs, including both TLRs[158, 160, 161] and Nod-like receptors[162] and coligation of TREM-1 in concert with PAMP-mediated activation results in much more cytokine secretion than the sum of the responses induced by either TREM-1- or PAMP-mediated activation alone.[158] *In vivo* blockade of TREM-1[160, 163] improved the survival of mice during endotoxaemia or bacterial infection and confirmed a role for TREM-1 in activating cells, since improved survival was associated with decreased levels of circulating inflammatory cytokine concentrations. The transmembrane domain of TREM-1 associates with DAP12 via a transmembrane charge interaction, which is essential for TREM-1 surface expression and signaling (**Figure 5**).[144] By testing the response of DAP12$^{-/-}$ mice to LPS or bacterial infection, the role of TREM-1-associated DAP12 in activating myeloid cells was validated *in vivo*. Consistent with data from TREM1 blocking studies, abrogation of DAP12 signaling in DAP12$^{-/-}$ mice attenuated the inflammatory response to septic infection without compromising bacterial control.[164] Improved survival was accompanied by decreased levels of circulating TNF and IL-6, as well as an attenuated acute-phase response by the liver, further supporting an activating role for DAP12 *in vivo*. However, in contrast to studies demonstrating a role for DAP12 in amplifying inflammation during simple endotoxaemia and bacterial sepsis, DAP12 can also inhibit the response to TLR agonists, such as LPS.[165] In these studies, DAP12$^{-/-}$ macrophages were found to produce significant levels of cytokines in response to concentrations of LPS that induce little or no detectable cytokine production by wild-type macrophages. Following sensitization to low doses of LPS, DAP12$^{-/-}$ mice were more sensitive to d-galactosamine-potentiated endotoxaemia, a model of TNF-induced shock, *in vivo*, thereby implicating an inhibitory role for DAP12 and that the paradigm of the 'activating ITAM' is incomplete. At first glance, these data result in conflicting phenotypes observed in studies of DAP12$^{-/-}$ mice. However, the two models of endotoxaemia measure the function of the two different DAP12-associated receptors TREM-1 and TREM-2, each of which acts more prominently in one system versus the other: TREM-1–DAP12 exacerbates inflammation in the context of simple endotoxaemia whereas TREM-2–DAP12 attenuates inflammation

during d-galactosaminepotentiated endotoxaemia. In the case of TREM-1, expression of a multivalent ligand is induced by high doses of LPS, resulting in high-order clustering of the receptor, full DAP12 phosphorylation, activation and an increase in the magnitude of the inflammatory response. In case of TREM-2, low doses of LPS induce low-avidity ligands which are incapable of forming extensive receptor–ligand synapses, leading to a partial activation that is countered by inhibitory TREM-2 signalling, thereby attenuating the inflammatory response.[76, 166] TREM-2 functions are summarized in **Figure 5**.

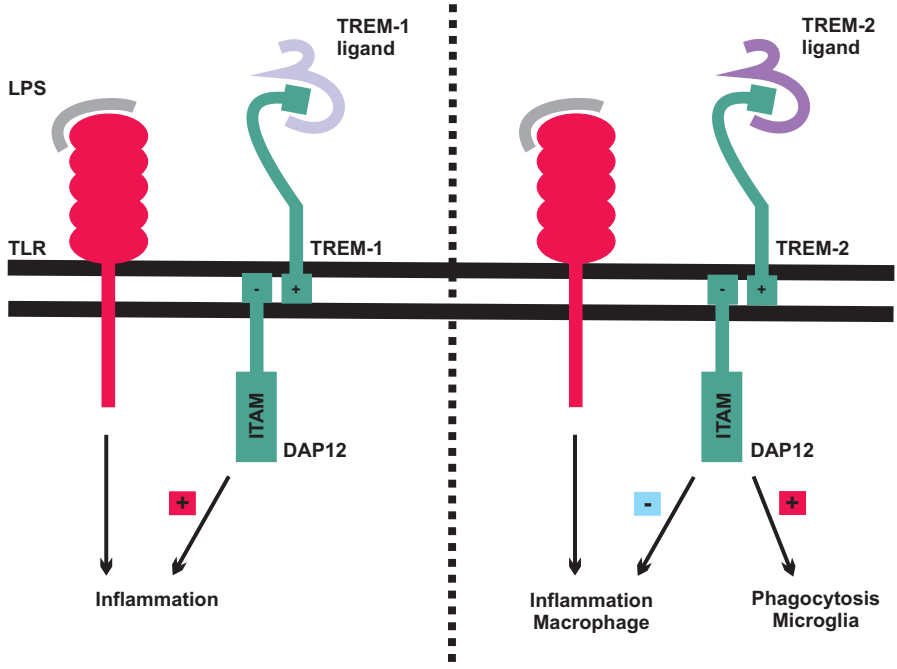


Fig. 5. *TREM-1 and TREM-2 function.* *TREM-1 amplifies TLR signaling (Nod-like receptor not shown) to mediate inflammatory responses, whereas TREM-2 inhibits TLR-induced cytokine production on macrophages. On microglia, TREM-2 potentiates phagocytosis. Adapted from Klesney-Tait J. et al. (2006).* [144]

Regarding transcriptional regulation of DAP12 and TREM-1 expression during inflammatory responses not much is known. It has been shown that retroviral re-expression of the transcription factor (TF) PU.1 in monocytic precursor cells induced a rapid induction of DAP12 expresison that was only found in the rescued cells, but not in PU.1 null cells.[167] Despite these findings, regulation of DAP12 expression and its association with resting or activated retinal microglia cells has not been

analyzed, and nothing is known about *cis*-acting elements controlling Dap12 transcription in any cell type. Similarly, increased expression of TREM-1 and the TF ATF3 was identified in human monocytes of healthy donors following antibody-mediated TREM-1 crosslinking[159] and LPS-stimulation.[168] In RAW264.7 macrophages overexpression of PU.1 led to inhibition of TREM-1 induction in response to LPS[169] but nothing is known about the TFs controlling the TREM-1 promoter in activated patient monocytes. Together with ATF3 the TF EGR3 was found upregulated in patient monocytes.[96, 97]

THE TRANSCRIPTION FACTOR ATF3

Activating transcription factor 3 (ATF3) – first described in 1991[170] - belongs to the ATF/cyclic AMP responsive element binding-protein (CREB) family of transcription factors.[171] This family is characterized by a basic region leucine zipper (bZip) motif. The basic region recognizes the ATF/CRE promoter segment (5' – TGACGTCA – '3) and ATF3 forms homo- or heterodimers with other CREB family members through its bZip domain, thereby either repressing or activating various genes depending on the cellular context.[172] Several subgroups within this relatively large family have been classified according to their level of homology both within and outside the bZip domain.[173] In contrast to the implication of its name, ATF3 as a homodimer functions as a transcriptional repressor, not an activator.[174] This property sets ATF3 apart from many of the other ATF/CREB proteins, which function as transcriptional activators. Up to date, also eight splice variants and five protein isoforms have been identified, but almost nothing is known the prevalence and the significance of *in vivo* of the products of the different splice variants of ATF3.[171]

ATF3 expression is maintained at low levels in quiescent cells. During stress response, ATF3 is induced by a broad range of stimuli including cytokines, genotoxic agents and physiological stresses.[175-178] Therefore, recently the idea was put forth that ATF3 is a hub of the cellular adaptive-response network to respond to signals perturbing homeostasis[176] and unlike other ATF family members, emerging evidence implicates ATF3 in host defence against invading pathogens.[178] In macrophages, ATF3 expression has been demonstrated to be induced by the TLR4 ligands, LPS and bacillus Calmette Guerin (BCG) and by IFNα in human PBMC.[179] Despite these observations, the role of ATF3 in innate immunity has only been recently described.[168, 180] Compared to wild type cells, ATF3-deficient bone-marrow-derived macrophages show elevated production of cytokines, such as IL-12p40, IL-6 and TNF, in response to LPS. After receiving LPS intraperitoneally, ATF3-deficient mice exhibited serum levels of IL-12p40, IL-6 and TNF significantly higher than did wild type mice. *In vivo* endotoxic shock experiments proved that

ATF3 could protect mice from LPS-induced endotoxic shock.[168] These experiments identified that ATF3 induction by TLR4 activation and its important role as a part of the negative feedback loop to modulate the TLR4-stimulated inflammatory response of macrophages. ATF3 can regulate the expression of many inflammation-related genes including IL-1ß, IL-6, IL12 and TNFa.[168]

One year later it was shown that ATF3 not only serves as a negative regulator in the TLR4-stimulated inflammatory response, but also in responses stimulated by TLR2/6 heterodimer, TLR3, TLR5, TLR7, and TLR9 pathways.[180] Kdo_2-Lipid A is sufficient to induce the endotoxin activity of LPS[181] and the induced activation of both NF-κB and JNK was affected in the absence of ATF3. TNF-induced activation of NF-κB and JNK however was unaffected and cell death was normal[182], thereby showing that both NF-κB and JNK activation are regulated by ATF3 via the TLR4 signaling pathway. Recently, the mechanism of ATF3 exertion of its negative regulation function was identified. ATF3 can interact with histone deacetylase HDAC1 in a LPS-induced pattern to attenuate transcription. ATF3 binds to the promoter, e.g. of IL-6, and recruits HDAC1, resulting in the closure of chromatin of chromatin to limit access to transcription factors, such as NF- KB.[183] Taken together, ATF3 plays an important role in the negative feedback for controlling cytokine toxicity in the innate response.

Also in the CNS, inflammation provides a stimulus for glial ATF3 upregulation and ATF3 was shown to be induced in macrophages and microglia by IL-6.[184] In mice, ATF3 deficiency leads to increased inflammation and brain injury after transient focal cerebral ischemia[185] and application of LPS to the motor cortex of rats induced ATF3 expression in the nuclei of many ganglial cells in the subcortical white matter.[186] However, inflammation around cell bodies of primary sensory neurons and retinal ganglion cells was shown to enhance the expression of neuronal growth-associated genes, thereby stimulating axonal regeneration,[187-189] thereby suggesting a neuroprotective role for ATF3.[190]

THE TRANSCRIPTION FACTOR EGR3

The early growth response (EGR) family consists of several immediate early gene transcription factors (EGR1, EGR2, EGR3 and EGR4) that are all important for neuronal responses[191]. EGR3 and the other members of the EGR family were first identified in the early 90's by several laboratories searching for genes whose expression was rapidly induced by growth factors.[192, 193] A defining feature of the EGR family is a highly conserved DNA-binding domain composed of three homologous zinc-finger motifs (Cys_2His_2).[192, 194] Together, these fingers recognize a GC-rich nine-base-pair segment of DNA (5'- GCGGGGGCG -3'), with each finger spanning

three nucleotides.[195-197] The transcription factor EGR3 was first described in 1991 by Patwardhan et al[198] and in 1994 it was shown that EGR3 mRNA is rapidly and transiently induced in neurons of the hippocampus and cortex by electroconvulsive seizure.[199] EGR3 mRNA levels peak 2 hr after seizure and remain elevated for as long as 8 hr. EGR3 mRNA is also rapidly induced in granule cells of the dentate gyrus by synaptic NMDA receptor activation elicited by patterned stimulation of the perforant pathway and by drugs that alter dopamine neurotransmission in the striatum. Basal levels of EGR3 mRNA in the cortex appear to be driven by natural synaptic activity. Besides aspects of the protein structure, sequence-specific DNA binding and transcriptional activity, the regulation of EGR3 is highly similar to another zinc-finger transcription factor, EGR1.[199] EGR1 and EGR3 are the most abundant EGR proteins in the brain, and their expression is upregulated by synaptic activity in the brain.[200] However it was shown that there is a sequential expression of EGR1 and EGR3 following electroconvulsive stimulation of hippocampal granule cells, indicating that EGR1 and EGR3 act in concert to mediate early and late phases, respectively, of the transcriptional response regulated by their cognate response element.[201] More recent work showed that EGR3 plays an essential role in neuronal development[202] as well as in learning and memory processing of both short- and long-term hippocampus-dependent memory; it also mediates adaptation to stress and novelty.[200 203]

Also, in the pathology of major psychiatric diseases the involvement of EGR3 was clearly shown in EGR3$^{-/-}$ mice. EGR3$^{-/-}$ mice display increased aggression in response to the stressful social stimulus of a foreign intruder. Chronic administration of clozapine significantly inhibits the observed aggressive behaviour in these animals, though they are more resistant to the side effects (sedating activity) of clozapine compared to their wild type littermates.[204] This decreased susceptibility to the side effects of antipsychotic medications is also found in SCZ patients. In fact, a broad range of characteristics of EGR3$^{-/-}$ mice is consistent with animal models of schizophrenia. These include e.g. hyperactivity, an accentuated response and abnormal adaptation to novelty and stress.[203] It is thus not surprising that EGR3 was recently identified as a susceptibility gene for schizophrenia and other neuropsychiatric disorders in humans.[191, 205-207] The expression of EGR3 has been reported to be significantly lower in postmortem hippocampus' of schizophrenic patients compared to the control subjects[208] and downregulated expression of EGR3 was also reported for the dorsolateral refrontal cortex of patients with schizophrenia.[208] In addition, EGR3 was highlighted as a key regulatory gene in a recent miRNA-transcription factor network study on SCZ genes.[209]

Interestingly, increased expression of EGR3 and pro-inflammatory molecules such as IL-1B and COX2 co-appeared in human leukocytes from individuals

that experienced social isolation for at least 3 years.[210] Increased mRNA levels of TREM-1 and EGR3 co-appeared in the severe immune deregulated Nrf2$^{-/-}$ mice[211], which are characterized by a proneness for inflammation[212], including that of the brain[213-216], thereby suggesting a regulatory role of EGR3 on the TREM-1 promoter. Furthermore, EGR3 was shown to be involved in the regulation of apoptosis by binding to the FasL promotor.[217]

THE TRANSCRIPTION FACTOR PU.1

PU.1 (*purine-rich box-1*) belongs to the family of the Ets factors, consists of 272 amino acids and is encoded by the *Sfpi1* (*SFFV proviral integration 1*) gene.

There are approx. 29 current members of the ETS family which are involved in the regulation of many cell biological processes such as growth, proliferation, transformation, differentiation, apoptosis and embryonal development[218], but also in the regulation of the immune system.[219] The members of this family share a DNA-binding domain at the carboxy terminus which consists of 85 amino acids and binds to sequences with the GGAA/T motif in its center. Different studies showed that PU.1 plays a central role in the development of myeloid cells and thereby showing specific expression patterns.[220-222] The highest expression can be found in myeloid cells, especially in neutrophils, eosinophils and monocytes, in B cells, but not in T cells. In human CD34 positive stem cells there is only little expression of PU.1 initially but during the hematopoetic development of these cells there is a dramatic increase of PU.1 expression levels. This upregulation of PU.1 expression correlates strongly with the myeloid differentation and maturation. Deregulation of the PU.1 function will therefore block the myeloid development. No granulocytes or monocytes can be found in PU.1 knock out mice[223], which indirectly negatively influences the development of the immune system. Meanwhile many studies show that genes associated with immune response are directly regulated by PU.1 and this was further investigated in our own studies.

The surface molecules CD11b and CD18 serve as markers for mature macrophages and cannot be detected on PU.1-deficient cells. Together these molecules form the the *complement receptor 3* (CR3). The CD11b subunit promotes the migration of macrophages and phagocytosis through binding extracellular proteins and different microbial antigens. The binding of PU.1 to the promoter of CD11b is essential for the expression of this gene and for the induction of CD18 expression PU.1 has to bind tot two binding sites in the CD18 promotor.

PU.1-deficient (PU.1$^{-/-}$) embryonic stem cells show a reduced or no epxression of G-CSF, M-CSF and GM-CSF.[224] G-CSF is expressed by monocytes and macrophages and after binding to its receptor on neutrophils it regulates chemotaxis, phagocytosis

and the oxidative burst.[225] Expression of the GM-CSF receptor can be found on all myeloid cells. Interaction with its ligand regulates the growth and differentiation of these cells.[226] Also the M-CSF promotor contains a binding site for PU.1. Changes in PU.1 protein levels directly influence the M-CSF dependent proliferation of macrophages and myeloid progenitor cells.[219] The Fc γ receptor I (FcγRI) CD64 can not be detected in PU.1-deficient cells which further strengthens the importance of this transcription factor for terminal myeloid differentiation.[224] Phagocytosis of IgG-bound antigens is regulated by CD64.[227] Furthermore, PU.1 might be essential for the epxression of macrophage inflammatory protein 1α (MIP1α) in mice. Macrophages and monocytes express this protein which has a chemotactic effect on monocytes, B cells, T cells, natural killer cells and eosinophils.[219] Within the promotor of the mannose receptor gene two PU.1 binding sites seem to be essential for the expression of this gene. Infiltrating microorganisms are recognized by this macrophage specific receptor, thereby initiating phagocytosis.[228]
In sum, the transcription factor PU.1 is essential for the development and the differentiation of myeloid cells, as well as this transcription factor PU.1 directly influences the immune response by directly regulating surface molecules. Therefore, PU.1 might be a key player for the activation of microglia and monocytes.

MICRORNA

Next to transcription factors, gene expression can also be regulated epigenetically or by microRNAs. Epigenetics are defined as heritable changes in gene expression without a change in the DNA sequence itself.[229] DNA methylation, histone methylation and aceylation status are important mechanisms in the area of epigenetics that control the accessibilty of chromatin and transcriptional activities inside a cell.[230]
MicroRNAs (miRNAs) are small RNA molecules, ~ 22 nucleotides long that can negatively control their target gene expression posttranscriptionally. It is only since 2001 that this level protein expression regulation became apparent.[231-233] MiRNAs mediate their regulatory effects by binding to the 3'–untranslated sequences of particular mRNAs through partially complementary sequences and prevent the mRNAs from being translated into proteins.[234, 235] However, also upregulation of translation by miRNAs has been reported.[236] A vast number of miRNAs is evolutionary conserved among species[237] and in mammals, miRNAs are predicted to control the activity of 50-60% of all protein-coding genes.[238, 239] Currently, there are 939 mature human miRNA sequences listed in the Sanger updated miRNA registry and estimates based upon computational predictions range around 1500 miRNAs in total in the human genome.[240] Various miRNAs have been shown

to play to key roles in pathways orchestrating immune responses and chronic inflammatory processes. [241]

AIM OF THIS THESIS

Transcription factors and microRNAs have been shown to tightly regulate gene networks involved in the differentiation, proliferation and activation of cells. The simultaneous regulation of a group of target genes is an important process to be clinically applied in the treatment of diseases. The potential to manipulate transcription factors and microRNAs for therapeutic purposes is currently still challenged by a lack of knowledge about the target genes and networks orchestrated by these molecules. In this way, defining the regulation of networks orchestrated by different transcription factors and microRNAs in different cell types has become a major goal in cell biology. This thesis describes studies aimed to understand the role of three transcriptions factors, namely ATF3, EGR3 and PU.1, and microRNA-146a, involved in the activation of monocytes, macrophages and microglia in relation to psychiatric diseases and X-linked retinoschisis. All these diseases are thought to be driven – at least partially - by special inflammatory reactions.

Chapter 2 and **3** describe the role of PU.1 in microglia and macrophage function. In **Chapter 2** genome-wide expression profiling from $Rs1h^{-/Y}$ and control microglia was performed and identified disease-related expression of DAP12 and the transcription factor PU.1 in activated retinal microglia cells. The regulation of DAP12 expression and its promoter by PU.1 was demonstrated *in vitro* by transfection of several DAP12 reporter constructs, *in vitro* and *in vivo* binding assays for PU.1 and *in vitro* knock-down or re-expression of PU.1. The studies on gene expression regulation were further extended in **Chapter 3** by combining bibliographic gene connections, binding site prediction and ChIP-Chip data with transciptomic analysis of $PU.1^{-/-}$ progenitors after restoration of PU.1 activity and a publicly available microarray dataset of PU.1 knockdown hematopoietic stem cells to define a multi-level PU.1 regulatory network with novel validated PU.1 targets in macrophages. **Chapter 4** and **5** describe the regulatory role of PU.1, ATF3, EGR3 and miR-146a in monocytes of patients with a psychiatric disease with a particular emphasis on the activation status of these cells in patients. In **Chapter 4** qPCR analysis from monocytes of schizophrenic and bipolar patients identified disease-related expression of TREM1 and *in vivo* binding of the transcription factors ATF3, EGR3 and PU.1 to its promoter in human monocytes. In **Chapter 5** a microRNA expression profiling approach was used to identify activation-related microRNA expression in patient monocytes. Finally, **Chapter 6** discusses the significance and implications of the studies described, and provides directions for future research.

LITERATURE

1. Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. *Cell host & microbe* 2011; **9**(5)**:** 355-361.
2. Hume DA. Macrophages as APC and the dendritic cell myth. *J Immunol* 2008; **181**(9)**:** 5829-5835.
3. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S *et al.* Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science (New York, NY* 2010; **330**(6005)**:** 841-845.
4. Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K *et al.* A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science (New York, NY* 2012; **336**(6077)**:** 86-90.
5. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 2005; **5**(12)**:** 953-964.
6. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science (New York, NY* 1988; **241**(4861)**:** 58-62.
7. Iwasaki H, Akashi K. Myeloid lineage commitment from the hematopoietic stem cell. *Immunity* 2007; **26**(6)**:** 726-740.
8. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* 1997; **91**(5)**:** 661-672.
9. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 2000; **404**(6774)**:** 193-197.
10. Fogg DK, Sibon C, Miled C, Jung S, Aucouturier P, Littman DR *et al.* A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science (New York, NY* 2006; **311**(5757)**:** 83-87.
11. Varol C, Landsman L, Fogg DK, Greenshtein L, Gildor B, Margalit R *et al.* Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *J Exp Med* 2007; **204**(1)**:** 171-180.
12. Chow A, Brown BD, Merad M. Studying the mononuclear phagocyte system in the molecular age. *Nat Rev Immunol* 2011; **11**(11)**:** 788-798.
13. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. *Science (New York, NY* 2010; **327**(5966)**:** 656-661.
14. Sasmono RT, Oceandy D, Pollard JW, Tong W, Pavli P, Wainwright BJ *et al.* A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. *Blood* 2003; **101**(3)**:** 1155-1163.
15. Dai XM, Ryan GR, Hapel AJ, Dominguez MG, Russell RG, Kapp S *et al.* Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood* 2002; **99**(1)**:** 111-120.
16. Cecchini MG, Dominguez MG, Mocci S, Wetterwald A, Felix R, Fleisch H *et al.* Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse. *Development* 1994; **120**(6)**:** 1357-1372.
17. Wiktor-Jedrzejczak W, Gordon S. Cytokine regulation of the macrophage (M phi) system studied using the colony stimulating factor-1-deficient op/op mouse. *Physiological reviews* 1996; **76**(4)**:** 927-947.
18. Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annual review of immunology* 2009; **27:** 669-692.
19. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P *et al.* Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science (New York, NY* 2009; **325**(5940)**:** 612-616.
20. Jia T, Serbina NV, Brandl K, Zhong MX, Leiner IM, Charo IF *et al.* Additive roles for MCP-1 and MCP-3 in CCR2-mediated recruitment of inflammatory monocytes during Listeria monocytogenes infection. *J Immunol* 2008; **180**(10)**:** 6846-6853.
21. Serbina NV, Jia T, Hohl TM, Pamer EG. Monocyte-mediated defense against microbial pathogens. *Annual review of immunology* 2008; **26:** 421-452.

22. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN *et al.* Nomenclature of monocytes and dendritic cells in blood. *Blood* 2010; **116**(16)**:** e74-80.
23. Sunderkotter CH, Tomimori-Yamashita J, Nix V, Maeda SM, Sindrilaru A, Mariano M *et al.* High expression of myeloid-related proteins 8 and 14 characterizes an inflammatorily active but ineffective response of macrophages during leprosy. *Immunology* 2004; **111**(4)**:** 472-480.
24. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 2003; **19**(1)**:** 71-82.
25. Dupasquier M, Stoitzner P, van Oudenaren A, Romani N, Leenen PJ. Macrophages and dendritic cells constitute a major subpopulation of cells in the mouse dermis. *The Journal of investigative dermatology* 2004; **123**(5)**:** 876-879.
26. Pollard JW. Trophic macrophages in development and disease. *Nat Rev Immunol* 2009; **9**(4)**:** 259-270.
27. Gow DJ, Sester DP, Hume DA. CSF-1, IGF-1, and the control of postnatal growth and development. *Journal of leukocyte biology* 2010; **88**(3)**:** 475-481.
28. Hume DA, Ross IL, Himes SR, Sasmono RT, Wells CA, Ravasi T. The mononuclear phagocyte system revisited. *J Leukoc Biol* 2002; **72**(4)**:** 621-627.
29. Hume DA. The mononuclear phagocyte system. *Current opinion in immunology* 2006; **18**(1)**:** 49-53.
30. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008; **8**(12)**:** 958-969.
31. Mosser DM. The many faces of macrophage activation. *J Leukoc Biol* 2003; **73**(2)**:** 209-212.
32. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci* 2008; **13:** 453-461.
33. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 2004; **75**(2)**:** 163-189.
34. Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science (New York, NY* 1993; **259**(5102)**:** 1739-1742.
35. Desmedt M, Rottiers P, Dooms H, Fiers W, Grooten J. Macrophages induce cellular immunity by activating Th1 cell responses and suppressing Th2 cell responses. *J Immunol* 1998; **160**(11)**:** 5300-5308.
36. Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA *et al.* Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med* 2003; **198**(12)**:** 1951-1957.
37. Smith AM, Rahman FZ, Hayee B, Graham SJ, Marks DJ, Sewell GW *et al.* Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. *J Exp Med* 2009; **206**(9)**:** 1883-1897.
38. Kawane K, Ohtani M, Miwa K, Kizawa T, Kanbara Y, Yoshioka Y *et al.* Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages. *Nature* 2006; **443**(7114)**:** 998-1002.
39. Platt AM, Bain CC, Bordon Y, Sester DP, Mowat AM. An independent subset of TLR expressing CCR2-dependent macrophages promotes colonic inflammation. *J Immunol* 2010; **184**(12)**:** 6843-6854.
40. Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* 1992; **176**(1)**:** 287-292.
41. Linehan SA, Coulson PS, Wilson RA, Mountford AP, Brombacher F, Martinez-Pomares L *et al.* IL-4 receptor signaling is required for mannose receptor expression by macrophages recruited to granulomata but not resident cells in mice infected with Schistosoma mansoni. *Lab Invest* 2003; **83**(8)**:** 1223-1231.
42. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. *Immunity* 2010; **32**(5)**:** 593-604.
43. Bouhlel MA, Derudas B, Rigamonti E, Dievart R, Brozek J, Haulon S *et al.* PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell metabolism* 2007; **6**(2)**:** 137-143.

44. Richardson JY, Ottolini MG, Pletneva L, Boukhvalova M, Zhang S, Vogel SN *et al.* Respiratory syncytial virus (RSV) infection induces cyclooxygenase 2: a potential target for RSV therapy. *J Immunol* 2005; **174**(7)**:** 4356-4364.
45. Shirey KA, Pletneva LM, Puche AC, Keegan AD, Prince GA, Blanco JC *et al.* Control of RSV-induced lung injury by alternatively activated macrophages is IL-4R alpha-, TLR4-, and IFN-beta-dependent. *Mucosal immunology* 2010; **3**(3)**:** 291-300.
46. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 2010; **11**(5)**:** 373-384.
47. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* 2010; **140**(6)**:** 805-820.
48. Hagemann T, Lawrence T, McNeish I, Charles KA, Kulbe H, Thompson RG *et al.* "Re-educating" tumor-associated macrophages by targeting NF-kappaB. *J Exp Med* 2008; **205**(6)**:** 1261-1268.
49. Rutschman R, Lang R, Hesse M, Ihle JN, Wynn TA, Murray PJ. Cutting edge: Stat6-dependent substrate depletion regulates nitric oxide production. *J Immunol* 2001; **166**(4)**:** 2173-2177.
50. Kawanishi N, Yano H, Yokogawa Y, Suzuki K. Exercise training inhibits inflammation in adipose tissue via both suppression of macrophage infiltration and acceleration of phenotypic switching from M1 to M2 macrophages in high-fat-diet-induced obese mice. *Exercise immunology review* 2010; **16:** 105-118.
51. Mylonas KJ, Nair MG, Prieto-Lafuente L, Paape D, Allen JE. Alternatively activated macrophages elicited by helminth infection can be reprogrammed to enable microbial killing. *J Immunol* 2009; **182**(5)**:** 3084-3094.
52. Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J Leukoc Biol* 2004; **76**(3)**:** 509-513.
53. Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK, Suttles J. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol* 2005; **175**(1)**:** 342-349.
54. Segura E, Villadangos JA. Antigen presentation by dendritic cells *in vivo*. *Current opinion in immunology* 2009; **21**(1)**:** 105-110.
55. del Rio-Hortega P. *Cytology and Cellular Pathology of the Nervous System* 1932**:** 481-534.
56. Mori S, Leblond CP. Identification of microglia in light and electron microscopy. *The Journal of comparative neurology* 1969; **135**(1)**:** 57-80.
57. Oehmichen M, Huber H. Supplementary cytodiagnostic analyses of mononuclear cells of the cerebrospinal fluid using cytological markers. *Journal of neurology* 1978; **218**(3)**:** 187-196.
58. Rieske E, Graeber MB, Tetzlaff W, Czlonkowska A, Streit WJ, Kreutzberg GW. Microglia and microglia-derived brain macrophages in culture: generation from axotomized rat facial nuclei, identification and characterization *in vitro*. *Brain research* 1989; **492**(1-2)**:** 1-14.
59. Perry VH, Hume DA, Gordon S. Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. *Neuroscience* 1985; **15**(2)**:** 313-326.
60. Giulian D, Baker TJ. Peptides released by ameboid microglia regulate astroglial proliferation. *The Journal of cell biology* 1985; **101**(6)**:** 2411-2415.
61. Hayes GM, Woodroofe MN, Cuzner ML. Characterisation of microglia isolated from adult human and rat brain. *Journal of neuroimmunology* 1988; **19**(3)**:** 177-189.
62. Schnitzer J. Enzyme-histochemical demonstration of microglial cells in the adult and postnatal rabbit retina. *The Journal of comparative neurology* 1989; **282**(2)**:** 249-263.
63. Hanisch UK. Microglia as a source and target of cytokines. *Glia* 2002; **40**(2)**:** 140-155.
64. Hanisch UK, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nature neuroscience* 2007; **10**(11)**:** 1387-1394.
65. Streit WJ. Microglia as neuroprotective, immunocompetent cells of the CNS. *Glia* 2002; **40**(2)**:** 133-139.
66. Streit WJ. Microglia and neuroprotection: implications for Alzheimer's disease. *Brain Res Brain Res Rev* 2005; **48**(2)**:** 234-239.
67. Giulian D, Li J, Bartel S, Broker J, Li X, Kirkpatrick JB. Cell surface morphology identifies microglia as a distinct class of mononuclear phagocyte. *J Neurosci* 1995; **15**(11)**:** 7712-7726.

68. Ransohoff RM, Perry VH. Microglial physiology: unique stimuli, specialized responses. *Annual review of immunology* 2009; **27:** 119-145.
69. Wegiel J, Wisniewski HM, Dziewiatkowski J, Tarnawski M, Kozielski R, Trenkner E *et al.* Reduced number and altered morphology of microglial cells in colony stimulating factor-1-deficient osteopetrotic op/op mice. *Brain research* 1998; **804**(1)**:** 135-139.
70. Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S *et al.* ATP mediates rapid microglial response to local brain injury *in vivo*. *Nature neuroscience* 2005; **8**(6)**:** 752-758.
71. Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma *in vivo*. *Science (New York, NY* 2005; **308**(5726)**:** 1314-1318.
72. Broderick C, Hoek RM, Forrester JV, Liversidge J, Sedgwick JD, Dick AD. Constitutive retinal CD200 expression regulates resident microglia and activation state of inflammatory cells during experimental autoimmune uveoretinitis. *The American journal of pathology* 2002; **161**(5)**:** 1669-1677.
73. Cardona AE, Pioro EP, Sasse ME, Kostenko V, Cardona SM, Dijkstra IM *et al.* Control of microglial neurotoxicity by the fractalkine receptor. *Nature neuroscience* 2006; **9**(7)**:** 917-924.
74. Dick AD, Carter D, Robertson M, Broderick C, Hughes E, Forrester JV *et al.* Control of myeloid activity during retinal inflammation. *J Leukoc Biol* 2003; **74**(2)**:** 161-166.
75. Hoek RM, Ruuls SR, Murphy CA, Wright GJ, Goddard R, Zurawski SM *et al.* Down-regulation of the macrophage lineage through interaction with OX2 (CD200). *Science (New York, NY* 2000; **290**(5497)**:** 1768-1771.
76. Turnbull IR, Gilfillan S, Cella M, Aoshi T, Miller M, Piccio L *et al.* Cutting edge: TREM-2 attenuates macrophage activation. *J Immunol* 2006; **177**(6)**:** 3520-3524.
77. Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan WB *et al.* The P2Y12 receptor regulates microglial activation by extracellular nucleotides. *Nature neuroscience* 2006; **9**(12)**:** 1512-1519.
78. Kaushal V, Schlichter LC. Mechanisms of microglia-mediated neurotoxicity in a new model of the stroke penumbra. *J Neurosci* 2008; **28**(9)**:** 2221-2230.
79. Taylor DL, Diemel LT, Pocock JM. Activation of microglial group III metabotropic glutamate receptors protects neurons against microglial neurotoxicity. *J Neurosci* 2003; **23**(6)**:** 2150-2160.
80. Perry VH, Nicoll JA, Holmes C. Microglia in neurodegenerative disease. *Nat Rev Neurol* 2010; **6**(4)**:** 193-201.
81. Block ML, Zecca L, Hong JS. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nat Rev Neurosci* 2007; **8**(1)**:** 57-69.
82. Gonzalez-Scarano F, Baltuch G. Microglia as mediators of inflammatory and degenerative diseases. *Annual review of neuroscience* 1999; **22:** 219-240.
83. Aschner M, Allen JW, Kimelberg HK, LoPachin RM, Streit WJ. Glial cells in neurotoxicity development. *Annual review of pharmacology and toxicology* 1999; **39:** 151-173.
84. Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. *Trends in neurosciences* 1996; **19**(8)**:** 312-318.
85. McGeer PL, Itagaki S, Boyes BE, McGeer EG. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology* 1988; **38**(8)**:** 1285-1291.
86. Schuetz E, Thanos S. Microglia-targeted pharmacotherapy in retinal neurodegenerative diseases. *Current drug targets* 2004; **5**(7)**:** 619-627.
87. Rivest S. Regulation of innate immune responses in the brain. *Nat Rev Immunol* 2009; **9**(6)**:** 429-439.
88. Zeiss CJ, Johnson EA. Proliferation of microglia, but not photoreceptors, in the outer nuclear layer of the rd-1 mouse. *Investigative ophthalmology & visual science* 2004; **45**(3)**:** 971-976.
89. Zeng HY, Zhu XA, Zhang C, Yang LP, Wu LM, Tso MO. Identification of sequential events and factors associated with microglial activation, migration, and cytotoxicity in retinal degeneration in rd mice. *Investigative ophthalmology & visual science* 2005; **46**(8)**:** 2992-2999.
90. Gehrig A, Langmann T, Horling F, Janssen A, Bonin M, Walter M *et al.* Genome-wide expression profiling of the retinoschisin-deficient retina in early postnatal mouse development. *Investigative ophthalmology & visual science* 2007; **48**(2)**:** 891-900.
91. Roque RS, Rosales AA, Jingjing L, Agarwal N, Al-Ubaidi MR. Retina-derived microglial cells induce photoreceptor cell death *in vitro*. *Brain research* 1999; **836**(1-2)**:** 110-119.

92. Srinivasan B, Roque CH, Hempstead BL, Al-Ubaidi MR, Roque RS. Microglia-derived pronerve growth factor promotes photoreceptor cell death via p75 neurotrophin receptor. *J Biol Chem* 2004; **279**(40)**:** 41839-41845.

93. Karlstetter M, Ebert S, Langmann T. Microglia in the healthy and degenerating retina: insights from novel mouse models. *Immunobiology* 2010; **215**(9-10)**:** 685-691.

94. Smith RS, Maes M. The macrophage-T-lymphocyte theory of schizophrenia: additional evidence. *Med Hypotheses* 1995; **45**(2)**:** 135-141.

95. Smith RS. A comprehensive macrophage-T-lymphocyte theory of schizophrenia. *Med Hypotheses* 1992; **39**(3)**:** 248-257.

96. Drexhage RC, van der Heul-Nieuwenhuijsen L, Padmos RC, van Beveren N, Cohen D, Versnel MA *et al.* Inflammatory gene expression in monocytes of patients with schizophrenia: overlap and difference with bipolar disorder. A study in naturalistically treated patients. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP)* 2010**:** 1-13.

97. Padmos RC, Hillegers MH, Knijff EM, Vonk R, Bouvy A, Staal FJ *et al.* A discriminating messenger RNA signature for bipolar disorder formed by an aberrant expression of inflammatory genes in monocytes. *Arch Gen Psychiatry* 2008; **65**(4)**:** 395-407.

98. APA. Diagnostic and statistical manual of mental health disorders (4th edition). . 2000.

99. WHO. Internation statistical classification of diseases and related health problems 10th revision. 2007.

100. Moussavi S, Chatterji S, Verdes E, Tandon A, Patel V, Ustun B. Depression, chronic diseases, and decrements in health: results from the World Health Surveys. *Lancet* 2007; **370**(9590)**:** 851-858.

101. Kraepelin E. Manic-depressive insanity and paranoia. *Journal of Nervous & Mental Disease* 1921; **53**(4)**:** 350.

102. Pini S, de Queiroz V, Pagnin D, Pezawas L, Angst J, Cassano GB *et al.* Prevalence and burden of bipolar disorders in European countries. *Eur Neuropsychopharmacol* 2005; **15**(4)**:** 425-434.

103. Kupfer DJ. The increasing medical burden in bipolar disorder. *Jama* 2005; **293**(20)**:** 2528-2530.

104. Novick DM, Swartz HA, Frank E. Suicide attempts in bipolar I and bipolar II disorder: a review and meta-analysis of the evidence. *Bipolar disorders* 2010; **12**(1)**:** 1-9.

105. Stewart DE, Klompenhouwer JL, Kendell RE, van Hulst AM. Prophylactic lithium in puerperal psychosis. The experience of three centres. *Br J Psychiatry* 1991; **158:** 393-397.

106. Oates M. Perinatal psychiatric disorders: a leading cause of maternal morbidity and mortality. *British medical bulletin* 2003; **67:** 219-229.

107. Chaudron LH, Pies RW. The relationship between postpartum psychosis and bipolar disorder: a review. *J Clin Psychiatry* 2003; **64**(11)**:** 1284-1292.

108. Spinelli MG. Postpartum psychosis: detection of risk and management. *The American journal of psychiatry* 2009; **166**(4)**:** 405-408.

109. Bleuler E. Dementia praecox or the group of schizophrenias. *New York; International Universities Press* 1950.

110. van Os J, Kapur S. Schizophrenia. *Lancet* 2009; **374**(9690)**:** 635-645.

111. Veling W, Selten JP, Veen N, Laan W, Blom JD, Hoek HW. Incidence of schizophrenia among ethnic minorities in the Netherlands: a four-year first-contact study. *Schizophrenia research* 2006; **86**(1-3)**:** 189-193.

112. Muench J, Hamer AM. Adverse effects of antipsychotic medications. *American family physician* 2010; **81**(5)**:** 617-622.

113. Steiner J, Bielau H, Brisch R, Danos P, Ullrich O, Mawrin C *et al.* Immunological aspects in the neurobiology of suicide: elevated microglial density in schizophrenia and depression is associated with suicide. *Journal of psychiatric research* 2008; **42**(2)**:** 151-157.

114. Radewicz K, Garey LJ, Gentleman SM, Reynolds R. Increase in HLA-DR immunoreactive microglia in frontal and temporal cortex of chronic schizophrenics. *Journal of neuropathology and experimental neurology* 2000; **59**(2)**:** 137-150.

115. Wierzba-Bobrowicz T, Lewandowska E, Lechowicz W, Stepien T, Pasennik E. Quantitative analysis of activated microglia, ramified and damage of processes in the frontal and temporal lobes of chronic schizophrenics. *Folia neuropathologica / Association of Polish Neuropathologists and Medical Research Centre, Polish Academy of Sciences* 2005; **43**(2)**:** 81-89.

116. Bayer TA, Buslei R, Havas L, Falkai P. Evidence for activation of microglia in patients with psychiatric illnesses. *Neurosci Lett* 1999; **271**(2)**:** 126-128.

117. Arnold SE, Trojanowski JQ, Gur RE, Blackwell P, Han LY, Choi C. Absence of neurodegeneration and neural injury in the cerebral cortex in a sample of elderly patients with schizophrenia. *Arch Gen Psychiatry* 1998; **55**(3)**:** 225-232.

118. Falke E, Han LY, Arnold SE. Absence of neurodegeneration in the thalamus and caudate of elderly patients with schizophrenia. *Psychiatry research* 2000; **93**(2)**:** 103-110.

119. Togo T, Akiyama H, Kondo H, Ikeda K, Kato M, Iseki E *et al.* Expression of CD40 in the brain of Alzheimer's disease and other neurological diseases. *Brain research* 2000; **885**(1)**:** 117-121.

120. Nikkila HV, Muller K, Ahokas A, Miettinen K, Rimon R, Andersson LC. Accumulation of macrophages in the CSF of schizophrenic patients during acute psychotic episodes. *The American journal of psychiatry* 1999; **156**(11)**:** 1725-1729.

121. Myint AM. Kynurenines: from the perspective of major psychiatric disorders. *The FEBS journal* 2012; **279**(8)**:** 1375-1385.

122. Doorduin J, de Vries EF, Dierckx RA, Klein HC. PET imaging of the peripheral benzodiazepine receptor: monitoring disease progression and therapy response in neurodegenerative disorders. *Current pharmaceutical design* 2008; **14**(31)**:** 3297-3315.

123. Doorduin J, de Vries EF, Willemsen AT, de Groot JC, Dierckx RA, Klein HC. Neuroinflammation in schizophrenia-related psychosis: a PET study. *J Nucl Med* 2009; **50**(11)**:** 1801-1807.

124. van Berckel BN, Bossong MG, Boellaard R, Kloet R, Schuitemaker A, Caspers E *et al.* Microglia activation in recent-onset schizophrenia: a quantitative (R)-[11C]PK11195 positron emission tomography study. *Biol Psychiatry* 2008; **64**(9)**:** 820-822.

125. Rothermundt M, Arolt V, Weitzsch C, Eckhoff D, Kirchner H. Immunological dysfunction in schizophrenia: a systematic approach. *Neuropsychobiology* 1998; **37**(4)**:** 186-193.

126. Drexhage RC, Hoogenboezem TA, Cohen D, Versnel MA, Nolen WA, van Beveren NJ *et al.* An activated set point of T-cell and monocyte inflammatory networks in recent-onset schizophrenia patients involves both pro- and anti-inflammatory forces. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP)* 2011; **14**(6)**:** 746-755.

127. Zorrilla EP, Cannon TD, Gur RE, Kessler J. Leukocytes and organ-nonspecific autoantibodies in schizophrenics and their siblings: markers of vulnerability or disease? *Biol Psychiatry* 1996; **40**(9)**:** 825-833.

128. Falcone T, Fazio V, Lee C, Simon B, Franco K, Marchi N *et al.* Do systemic inflammation and blood-brain barrier failure play a role in pediatric psychosis? *Neurology, Psychiatry and Brain Research* 2009; **Volume 16**(Suppl 1).

129. Kellner U, Brummer S, Foerster MH, Wessing A. X-linked congenital retinoschisis. *Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie* 1990; **228**(5)**:** 432-437.

130. George ND, Yates JR, Moore AT. X linked retinoschisis. *The British journal of ophthalmology* 1995; **79**(7)**:** 697-702.

131. Puech B, Kostrubiec B, Hache JC, Francois P. [Epidemiology and prevalence of hereditary retinal dystrophies in the Northern France]. *Journal francais d'ophtalmologie* 1991; **14**(3)**:** 153-164.

132. Yanoff M, Kertesz Rahn E, Zimmerman LE. Histopathology of juvenile retinoschisis. *Archives of ophthalmology* 1968; **79**(1)**:** 49-53.

133. Manschot WA. Pathology of hereditary juvenile retinoschisis. *Archives of ophthalmology* 1972; **88**(2)**:** 131-138.

134. Condon GP, Brownstein S, Wang NS, Kearns JA, Ewing CC. Congenital hereditary (juvenile X-linked) retinoschisis. Histopathologic and ultrastructural findings in three eyes. *Archives of ophthalmology* 1986; **104**(4)**:** 576-583.

135. Sauer CG, Gehrig A, Warneke-Wittstock R, Marquardt A, Ewing CC, Gibson A *et al.* Positional cloning of the gene associated with X-linked juvenile retinoschisis. *Nature genetics* 1997; **17**(2)**:** 164-170.

136. Reid SN, Akhmedov NB, Piriev NI, Kozak CA, Danciger M, Farber DB. The mouse X-linked juvenile retinoschisis cDNA: expression in photoreceptors. *Gene* 1999; **227**(2)**:** 257-266.

137. Molday LL, Hicks D, Sauer CG, Weber BH, Molday RS. Expression of X-linked retinoschisis protein RS1 in photoreceptor and bipolar cells. *Investigative ophthalmology & visual science* 2001; **42**(3)**:** 816-825.

138. Baumgartner S, Hofmann K, Chiquet-Ehrismann R, Bucher P. The discoidin domain family revisited: new members from prokaryotes and a homology-based fold prediction. *Protein Sci* 1998; **7**(7)**:** 1626-1631.

139. Gehrig AE, Warneke-Wittstock R, Sauer CG, Weber BH. Isolation and characterization of the murine X-linked juvenile retinoschisis (Rs1h) gene. *Mamm Genome* 1999; **10**(3)**:** 303-307.

140. Weber BH, Schrewe H, Molday LL, Gehrig A, White KL, Seeliger MW *et al.* Inactivation of the murine X-linked juvenile retinoschisis gene, Rs1h, suggests a role of retinoschisin in retinal cell layer organization and synaptic structure. *Proc Natl Acad Sci U S A* 2002; **99**(9)**:** 6222-6227.

141. Otero K, Turnbull IR, Poliani PL, Vermi W, Cerutti E, Aoshi T *et al.* Macrophage colony-stimulating factor induces the proliferation and survival of macrophages via a pathway involving DAP12 and beta-catenin. *Nat Immunol* 2009; **10**(7)**:** 734-743.

142. Aoki N, Kimura S, Takiyama Y, Atsuta Y, Abe A, Sato K *et al.* The role of the DAP12 signal in mouse myeloid differentiation. *J Immunol* 2000; **165**(7)**:** 3790-3796.

143. Takaki R, Watson SR, Lanier LL. DAP12: an adapter protein with dual functionality. *Immunological reviews* 2006; **214:** 118-129.

144. Klesney-Tait J, Turnbull IR, Colonna M. The TREM receptor family and signal integration. *Nat Immunol* 2006; **7**(12)**:** 1266-1273.

145. Hamerman JA, Tchao NK, Lowell CA, Lanier LL. Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12. *Nat Immunol* 2005; **6**(6)**:** 579-586.

146. Roumier A, Bechade C, Poncer JC, Smalla KH, Tomasello E, Vivier E *et al.* Impaired synaptic function in the microglial KARAP/DAP12-deficient mouse. *J Neurosci* 2004; **24**(50)**:** 11421-11428.

147. Takahashi K, Rochford CD, Neumann H. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. *J Exp Med* 2005; **201**(4)**:** 647-657.

148. Takahashi K, Prinz M, Stagi M, Chechneva O, Neumann H. TREM2-transduced myeloid precursors mediate nervous tissue debris clearance and facilitate recovery in an animal model of multiple sclerosis. *PLoS medicine* 2007; **4**(4)**:** e124.

149. Kuroda R, Satoh J, Yamamura T, Anezaki T, Terada T, Yamazaki K *et al.* A novel compound heterozygous mutation in the DAP12 gene in a patient with Nasu-Hakola disease. *Journal of the neurological sciences* 2007; **252**(1)**:** 88-91.

150. Colonna M. TREMs in the immune system and beyond. *Nat Rev Immunol* 2003; **3**(6)**:** 445-453.

151. Paloneva J, Kestila M, Wu J, Salminen A, Bohling T, Ruotsalainen V *et al.* Loss-of-function mutations in TYROBP (DAP12) result in a presenile dementia with bone cysts. *Nature genetics* 2000; **25**(3)**:** 357-361.

152. Lanier LL, Bakker AB. The ITAM-bearing transmembrane adaptor DAP12 in lymphoid and myeloid cell function. *Immunology today* 2000; **21**(12)**:** 611-614.

153. Kaifu T, Nakahara J, Inui M, Mishima K, Momiyama T, Kaji M *et al.* Osteopetrosis and thalamic hypomyelinosis with synaptic degeneration in DAP12-deficient mice. *J Clin Invest* 2003; **111**(3)**:** 323-332.

154. Tomasello E, Desmoulins PO, Chemin K, Guia S, Cremer H, Ortaldo J *et al.* Combined natural killer cell and dendritic cell functional deficiency in KARAP/DAP12 loss-of-function mutant mice. *Immunity* 2000; **13**(3)**:** 355-364.

155. Roumier A, Pascual O, Bechade C, Wakselman S, Poncer JC, Real E *et al.* Prenatal activation of microglia induces delayed impairment of glutamatergic synaptic function. *PLoS One* 2008; **3**(7)**:** e2595.

156. Wakselman S, Bechade C, Roumier A, Bernard D, Triller A, Bessis A. Developmental neuronal death in hippocampus requires the microglial CD11b integrin and DAP12 immunoreceptor. *J Neurosci* 2008; **28**(32)**:** 8138-8143.

157. Ohmi K, Greenberg DS, Rajavel KS, Ryazantsev S, Li HH, Neufeld EF. Activated microglia in cortex of mouse models of mucopolysaccharidoses I and IIIB. *Proc Natl Acad Sci U S A* 2003; **100**(4)**:** 1902-1907.

158. Bouchon A, Dietrich J, Colonna M. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J Immunol* 2000; **164**(10)**:** 4991-4995.

159. Dower K, Ellis DK, Saraf K, Jelinsky SA, Lin LL. Innate immune responses to TREM-1 activation: overlap, divergence, and positive and negative cross-talk with bacterial lipopolysaccharide. *J Immunol* 2008; **180**(5)**:** 3520-3534.

160. Bouchon A, Facchetti F, Weigand MA, Colonna M. TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature* 2001; **410**(6832)**:** 1103-1107.

161. Bleharski JR, Kiessler V, Buonsanti C, Sieling PA, Stenger S, Colonna M *et al.* A role for triggering receptor expressed on myeloid cells-1 in host defense during the early-induced and adaptive phases of the immune response. *J Immunol* 2003; **170**(7)**:** 3812-3818.

162. Netea MG, Azam T, Ferwerda G, Girardin SE, Kim SH, Dinarello CA. Triggering receptor expressed on myeloid cells-1 (TREM-1) amplifies the signals induced by the NACHT-LRR (NLR) pattern recognition receptors. *J Leukoc Biol* 2006; **80**(6)**:** 1454-1461.

163. Gibot S, Kolopp-Sarda MN, Bene MC, Bollaert PE, Lozniewski A, Mory F *et al.* A soluble form of the triggering receptor expressed on myeloid cells-1 modulates the inflammatory response in murine sepsis. *J Exp Med* 2004; **200**(11)**:** 1419-1426.

164. Turnbull IR, McDunn JE, Takai T, Townsend RR, Cobb JP, Colonna M. DAP12 (KARAP) amplifies inflammation and increases mortality from endotoxemia and septic peritonitis. *J Exp Med* 2005; **202**(3)**:** 363-369.

165. Hamerman JA, Jarjoura JR, Humphrey MB, Nakamura MC, Seaman WE, Lanier LL. Cutting edge: inhibition of TLR and FcR responses in macrophages by triggering receptor expressed on myeloid cells (TREM)-2 and DAP12. *J Immunol* 2006; **177**(4)**:** 2051-2055.

166. Turnbull IR, Colonna M. Activating and inhibitory functions of DAP12. *Nat Rev Immunol* 2007; **7**(2)**:** 155-161.

167. Henkel GW, McKercher SR, Maki RA. Identification of three genes up-regulated in PU.1 rescued monocytic precursor cells. *International immunology* 2002; **14**(7)**:** 723-732.

168. Gilchrist M, Thorsson V, Li B, Rust AG, Korb M, Roach JC *et al.* Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature* 2006; **441**(7090)**:** 173-178.

169. Zeng H, Ornatowska M, Joo MS, Sadikot RT. TREM-1 expression in macrophages is regulated at transcriptional level by NF-kappaB and PU.1. *Eur J Immunol* 2007; **37**(8)**:** 2300-2308.

170. Hsu JC, Laz T, Mohn KL, Taub R. Identification of LRF-1, a leucine-zipper protein that is rapidly and highly induced in regenerating liver. *Proc Natl Acad Sci U S A* 1991; **88**(9)**:** 3511-3515.

171. Hunt D, Raivich G, Anderson PN. Activating transcription factor 3 and the nervous system. *Frontiers in molecular neuroscience* 2012; **5:** 7.

172. Nakagomi S, Suzuki Y, Namikawa K, Kiryu-Seo S, Kiyama H. Expression of the activating transcription factor 3 prevents c-Jun N-terminal kinase-induced neuronal death by promoting heat shock protein 27 expression and Akt activation. *J Neurosci* 2003; **23**(12)**:** 5187-5196.

173. Hai T, Hartman MG. The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. *Gene* 2001; **273**(1)**:** 1-11.

174. Chen BP, Liang G, Whelan J, Hai T. ATF3 and ATF3 delta Zip. Transcriptional repression versus activation by alternatively spliced isoforms. *J Biol Chem* 1994; **269**(22)**:** 15819-15826.

175. Tamura K, Hua B, Adachi S, Guney I, Kawauchi J, Morioka M *et al.* Stress response gene ATF3 is a target of c-myc in serum-induced cell proliferation. *The EMBO journal* 2005; **24**(14)**:** 2590-2601.

176. Hai T, Wolford CC, Chang YS. ATF3, a hub of the cellular adaptive-response network, in the pathogenesis of diseases: is modulation of inflammation a unifying component? *Gene expression* 2010; **15**(1)**:** 1-11.

177. Lu D, Wolfgang CD, Hai T. Activating transcription factor 3, a stress-inducible gene, suppresses Ras-stimulated tumorigenesis. *J Biol Chem* 2006; **281**(15)**:** 10473-10481.

178. Thompson MR, Xu D, Williams BR. ATF3 transcription factor and its emerging roles in immunity and cancer. *Journal of molecular medicine (Berlin, Germany)* 2009; **87**(11)**:** 1053-1060.

179. Hai T, Wolfgang CD, Marsee DK, Allen AE, Sivaprasad U. ATF3 and stress responses. *Gene expression* 1999; **7**(4-6)**:** 321-335.

180. Whitmore MM, Iparraguirre A, Kubelka L, Weninger W, Hai T, Williams BR. Negative regulation of TLR-signaling pathways by activating transcription factor-3. *J Immunol* 2007; **179**(6)**:** 3622-3630.

181. Raetz CR, Garrett TA, Reynolds CM, Shaw WA, Moore JD, Smith DC, Jr. *et al.* Kdo2-Lipid A of Escherichia coli, a defined endotoxin that activates macrophages via TLR-4. *Journal of lipid research* 2006; **47**(5)**:** 1097-1111.

182. Kim EY, Shin HY, Kim JY, Kim DG, Choi YM, Kwon HK *et al.* ATF3 plays a key role in Kdo2-lipid A-induced TLR4-dependent gene expression via NF-kappaB activation. *PLoS One* 2010; **5**(12)**:** e14181.

183. Litvak V, Ramsey SA, Rust AG, Zak DE, Kennedy KA, Lampano AE *et al.* Function of C/EBPdelta in a regulatory circuit that discriminates between transient and persistent TLR4-induced signals. *Nat Immunol* 2009; **10**(4)**:** 437-443.

184. Dominguez E, Mauborgne A, Mallet J, Desclaux M, Pohl M. SOCS3-mediated blockade of JAK/STAT3 signaling pathway reveals its major contribution to spinal cord neuroinflammation and mechanical allodynia after peripheral nerve injury. *J Neurosci* 2010; **30**(16)**:** 5754-5766.

185. Wang L, Deng S, Lu Y, Zhang Y, Yang L, Guan Y *et al.* Increased Inflammation and Brain Injury after Transient Focal Cerebral Ischemia in Activating Transcription Factor 3 Knockout Mice. *Neuroscience* 2012.

186. Hossain-Ibrahim MK, Rezajooi K, MacNally JK, Mason MR, Lieberman AR, Anderson PN. Effects of lipopolysaccharide-induced inflammation on expression of growth-associated genes by corticospinal neurons. *BMC neuroscience* 2006; **7:** 8.

187. Shokouhi BN, Wong BZ, Siddiqui S, Lieberman AR, Campbell G, Tohyama K *et al.* Microglial responses around intrinsic CNS neurons are correlated with axonal regeneration. *BMC neuroscience* 2010; **11:** 13.

188. Lu X, Richardson PM. Inflammation near the nerve cell body enhances axonal regeneration. *J Neurosci* 1991; **11**(4)**:** 972-978.

189. Yin Y, Cui Q, Li Y, Irwin N, Fischer D, Harvey AR *et al.* Macrophage-derived factors stimulate optic nerve regeneration. *J Neurosci* 2003; **23**(6)**:** 2284-2293.

190. Beveridge NJ, Cairns MJ. MicroRNA dysregulation in schizophrenia. *Neurobiology of disease* 2012.

191. Kim SH, Song JY, Joo EJ, Lee KY, Ahn YM, Kim YS. EGR3 as a potential susceptibility gene for schizophrenia in Korea. *Am J Med Genet B Neuropsychiatr Genet* 2010; **153B**(7)**:** 1355-1360.

192. Gashler A, Sukhatme VP. Early growth response protein 1 (Egr-1): prototype of a zinc-finger family of transcription factors. *Progress in nucleic acid research and molecular biology* 1995; **50:** 191-224.

193. Mages HW, Stamminger T, Rilke O, Bravo R, Kroczek RA. Expression of PILOT, a putative transcription factor, requires two signals and is cyclosporin A sensitive in T cells. *International immunology* 1993; **5**(1)**:** 63-70.

194. Chavrier P, Lemaire P, Revelant O, Bravo R, Charnay P. Characterization of a mouse multigene family that encodes zinc finger structures. *Mol Cell Biol* 1988; **8**(3)**:** 1319-1326.

195. Christy B, Nathans D. DNA binding site of the growth factor-inducible protein Zif268. *Proc Natl Acad Sci U S A* 1989; **86**(22)**:** 8737-8741.

196. Pavletich NP, Pabo CO. Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A. *Science (New York, NY* 1991; **252**(5007)**:** 809-817.

197. Nardelli J, Gibson TJ, Vesque C, Charnay P. Base sequence discrimination by zinc-finger DNA-binding domains. *Nature* 1991; **349**(6305)**:** 175-178.

198. Patwardhan S, Gashler A, Siegel MG, Chang LC, Joseph LJ, Shows TB *et al.* EGR3, a novel member of the Egr family of genes encoding immediate-early transcription factors. *Oncogene* 1991; **6**(6)**:** 917-928.

199. Yamagata K, Kaufmann WE, Lanahan A, Papapavlou M, Barnes CA, Andreasson KI *et al.* Egr3/Pilot, a zinc finger transcription factor, is rapidly regulated by activity in brain neurons and colocalizes with Egr1/zif268. *Learning & memory (Cold Spring Harbor, NY* 1994; **1**(2)**:** 140-152.

200. Li L, Yun SH, Keblesh J, Trommer BL, Xiong H, Radulovic J *et al.* Egr3, a synaptic activity regulated transcription factor that is essential for learning and memory. *Mol Cell Neurosci* 2007; **35**(1)**:** 76-88.

201. O'Donovan KJ, Wilkens EP, Baraban JM. Sequential expression of Egr-1 and Egr-3 in hippocampal granule cells following electroconvulsive stimulation. *J Neurochem* 1998; **70**(3)**:** 1241-1248.

202. Eldredge LC, Gao XM, Quach DH, Li L, Han X, Lomasney J *et al.* Abnormal sympathetic nervous system development and physiological dysautonomia in Egr3-deficient mice. *Development* 2008; **135**(17)**:** 2949-2957.

203. Gallitano-Mendel A, Izumi Y, Tokuda K, Zorumski CF, Howell MP, Muglia LJ *et al.* The immediate early gene early growth response gene 3 mediates adaptation to stress and novelty. *Neuroscience* 2007; **148**(3)**:** 633-643.

204. Gallitano-Mendel A, Wozniak DF, Pehek EA, Milbrandt J. Mice lacking the immediate early gene Egr3 respond to the anti-aggressive effects of clozapine yet are relatively resistant to its sedating effects. *Neuropsychopharmacology* 2008; **33**(6)**:** 1266-1275.

205. Yamada K, Gerber DJ, Iwayama Y, Ohnishi T, Ohba H, Toyota T *et al.* Genetic analysis of the calcineurin pathway identifies members of the EGR gene family, specifically EGR3, as potential susceptibility candidates in schizophrenia. *Proc Natl Acad Sci U S A* 2007; **104**(8)**:** 2815-2820.

206. Kim SH, Song JY, Joo EJ, Lee KY, Ahn YM, Kim YS. EGR3 as a potential susceptibility gene for schizophrenia in Korea. *Am J Med Genet B Neuropsychiatr Genet*; **153B**(7)**:** 1355-1360.

207. Tabares-Seisdedos R, Rubenstein JL. Chromosome 8p as a potential hub for developmental neuropsychiatric disorders: implications for schizophrenia, autism and cancer. *Molecular psychiatry* 2009; **14**(6)**:** 563-589.

208. Mexal S, Frank M, Berger R, Adams CE, Ross RG, Freedman R *et al.* Differential modulation of gene expression in the NMDA postsynaptic density of schizophrenic and control smokers. *Brain Res Mol Brain Res* 2005; **139**(2)**:** 317-332.

209. Guo AY, Sun J, Jia P, Zhao Z. A novel microRNA and transcription factor mediated regulatory network in schizophrenia. *BMC systems biology* 2010; **4:** 10.

210. Cole SW, Hawkley LC, Arevalo JM, Sung CY, Rose RM, Cacioppo JT. Social regulation of gene expression in human leukocytes. *Genome biology* 2007; **8**(9)**:** R189.

211. Thimmulappa RK, Lee H, Rangasamy T, Reddy SP, Yamamoto M, Kensler TW *et al.* Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. *J Clin Invest* 2006; **116**(4)**:** 984-995.

212. Kong X, Thimmulappa R, Kombairaju P, Biswal S. NADPH oxidase-dependent reactive oxygen species mediate amplified TLR4 signaling and sepsis-induced mortality in Nrf2-deficient mice. *J Immunol* 2010; **185**(1)**:** 569-577.

213. Johnson DA, Amirahmadi S, Ward C, Fabry Z, Johnson JA. The absence of the pro-antioxidant transcription factor Nrf2 exacerbates experimental autoimmune encephalomyelitis. *Toxicol Sci* 2010; **114**(2)**:** 237-246.

214. Innamorato NG, Lastres-Becker I, Cuadrado A. Role of microglial redox balance in modulation of neuroinflammation. *Current opinion in neurology* 2009; **22**(3)**:** 308-314.

215. Innamorato NG, Rojo AI, Garcia-Yague AJ, Yamamoto M, de Ceballos ML, Cuadrado A. The transcription factor Nrf2 is a therapeutic target against brain inflammation. *J Immunol* 2008; **181**(1)**:** 680-689.

216. Rojo AI, Innamorato NG, Martin-Moreno AM, De Ceballos ML, Yamamoto M, Cuadrado A. Nrf2 regulates microglial dynamics and neuroinflammation in experimental Parkinson's disease. *Glia* 2010; **58**(5)**:** 588-598.

217. Mittelstadt PR, Ashwell JD. Cyclosporin A-sensitive transcription factor Egr-3 regulates Fas ligand expression. *Mol Cell Biol* 1998; **18**(7)**:** 3744-3751.

218. Wasylyk B, Hahn SL, Giovane A. The Ets family of transcription factors. *European journal of biochemistry / FEBS* 1993; **211**(1-2)**:** 7-18.

219. Gallant S, Gilkeson G. ETS transcription factors and regulation of immunity. *Arch Immunol Ther Exp (Warsz)* 2006; **54**(3)**:** 149-163.

220. Scott EW, Fisher RC, Olson MC, Kehrli EW, Simon MC, Singh H. PU.1 functions in a cell-autonomous manner to control the differentiation of multipotential lymphoid-myeloid progenitors. *Immunity* 1997; **6**(4)**:** 437-447.

221. Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science (New York, NY* 1994; **265**(5178)**:** 1573-1577.

222. DeKoter RP, Walsh JC, Singh H. PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors. *The EMBO journal* 1998; **17**(15)**:** 4456-4468.

223. McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H *et al.* Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *The EMBO journal* 1996; **15**(20)**:** 5647-5658.

224. Olson MC, Scott EW, Hack AA, Su GH, Tenen DG, Singh H *et al.* PU. 1 is not essential for early myeloid gene expression but is required for terminal myeloid differentiation. *Immunity* 1995; **3**(6)**:** 703-714.

225. Boneberg EM, Hartung T. Molecular aspects of anti-inflammatory action of G-CSF. *Inflamm Res* 2002; **51**(3)**:** 119-128.

226. Martinez-Moczygemba M, Huston DP. Biology of common beta receptor-signaling cytokines: IL-3, IL-5, and GM-CSF. *The Journal of allergy and clinical immunology* 2003; **112**(4)**:** 653-665; quiz 666.

227. Indik ZK, Park JG, Hunter S, Schreiber AD. The molecular dissection of Fc gamma receptor mediated phagocytosis. *Blood* 1995; **86**(12)**:** 4389-4399.

228. Eichbaum Q, Heney D, Raveh D, Chung M, Davidson M, Epstein J *et al.* Murine macrophage mannose receptor promoter is regulated by the transcription factors PU.1 and SP1. *Blood* 1997; **90**(10)**:** 4135-4143.

229. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. *Nature* 2004; **429**(6990)**:** 457-463.

230. Feng S, Jacobsen SE, Reik W. Epigenetic reprogramming in plant and animal development. *Science (New York, NY* 2010; **330**(6004)**:** 622-627.

231. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. *Science (New York, NY* 2001; **294**(5543)**:** 853-858.

232. Lau NC, Lim LP, Weinstein EG, Bartel DP. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science (New York, NY* 2001; **294**(5543)**:** 858-862.

233. Lee RC, Ambros V. An extensive class of small RNAs in Caenorhabditis elegans. *Science (New York, NY* 2001; **294**(5543)**:** 862-864.

234. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nature reviews* 2008; **9**(2)**:** 102-114.

235. Novina CD, Sharp PA. The RNAi revolution. *Nature* 2004; **430**(6996)**:** 161-164.

236. Vasudevan S, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. *Science (New York, NY* 2007; **318**(5858)**:** 1931-1934.

237. Grimson A, Srivastava M, Fahey B, Woodcroft BJ, Chiang HR, King N *et al.* Early origins and evolution of microRNAs and Piwi-interacting RNAs in animals. *Nature* 2008; **455**(7217)**:** 1193-1197.

238. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. *Nature reviews* 2010; **11**(9)**:** 597-610.

239. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome research* 2009; **19**(1)**:** 92-105.

240. Duroux-Richard I, Presumey J, Courties G, Gay S, Gordeladze J, Jorgensen C *et al.* MicroRNAs as new player in rheumatoid arthritis. *Joint Bone Spine* 2011; **78**(1)**:** 17-22.

241. Tsitsiou E, Lindsay MA. microRNAs and the immune response. *Current opinion in pharmacology* 2009; **9**(4)**:** 514-520.

CHAPTER 2

DAP12 EXPRESSION IN ACTIVATED MICROGLIA FROM RETINOSCHISIN-DEFICIENT RETINA AND ITS PU.1-DEPENDENT PROMOTER REGULATION

Karin Weigelt,* Wolfgang Ernst,* Yana Walczak, Stefanie Ebert, Thomas Loenhardt, Maja Klug, Michael Rehli, Bernhard H. F. Weber, and Thomas Langmann
* These authors contributed equally to this work

Journal of Leukocyte Biology, 2007 Dec;82(6): 1564 –1574

ABSTRACT

Several alterations in the expression of immune-related transcripts were identified recently in the degenerating retina of the retinoschisin knockout (Rs1h$^{-/Y}$) mouse, including the strong expression of the adaptor protein DAP12. As DAP12 is found in leukocytes, we hypothesized that its disease-related expression may be confined to activated retinal microglia cells. To test this hypothesis, we established a procedure for isolation and culture of retinal microglia cells and performed genome-wide expression profiling from Rs1h$^{-/Y}$ and control microglia. While retaining their activated state in culture, *ex vivo* microglia expressed high levels of DAP12 and the transcription factor PU.1. The activation-dependent induction of DAP12 was also confirmed in the microglia cell line BV-2 following *in vitro* stimulation. To examine the transcriptional regulation of DAP12 further, macrophage cell lines were transfected with several DAP12 reporter constructs. Promoter deletion assays and site-directed mutagenesis experiments demonstrated an essential role of evolutionarily conserved PU.1 consensus sites in the proximal –104/+118 DAP12 promoter. *In vitro* and *in vivo* binding of PU.1 to this promoter region was demonstrated using EMSA and chromatin immunoprecipitation. Knockdown of PU.1 by RNA interference caused a significant reduction of endogenous DAP12 expression and re-expression, and activation of PU.1 in PU.1$^{-/-}$ progenitor cells induced DAP12 transcription. Taken together, our results indicate that activated microglia from degenerating retinae express high levels of DAP12 and PU.1, and PU.1 controls the myeloid-specific regulation of DAP12 directly and may also play a general role in microglia gene expression during retinal degeneration.

INTRODUCTION

Microglia cells are resident mononuclear phagocytes required for neuronal homeostasis in the CNS. The immune surveillance function of microglia includes the clearance of tissue debris by inflammation-independent phagocytosis and the secretion of various neurotrophic factors.[1] However, chronic activation can lead to local, uncontrolled, inflammatory reactions often associated with neurodegeneration.[2] Although the initial triggers may be quite diverse, retinal dystrophies share the common hallmarks of over-activated microglia and photoreceptor apoptosis.[3] To characterize the molecular activation pathways, large-scale gene-expression profiling has been carried out in several studies.[4-7] Related to this, highly activated microglia cells and a strong overexpression of DAP12 (also termed Tyrobp or Karap) have been described recently in the retina of retinoschisin-deficient (Rs1h$^{-/Y}$) mice,[8] a murine model of human X-linked juvenile retinoschisis.[9]

The membrane adaptor protein DAP12 is expressed on several immune cells, contains an immunotyrosine-based activation motif, and associates with more than 20 different surface receptors to regulate immune responses.[10] Although DAP12 has been shown to have a dual role by potentiating or attenuating immune cell functions, a mainly deactivating function has been postulated for myeloid cells.[11] Thus, together with triggering receptor expressed on myeloid cells 2 (TREM-2), DAP12 blocks LPS/TLR-mediated cellular activation[12, 13] and enhances differentiation of macrophage precursors.[14] Furthermore, TREM-2 and DAP12 are expressed on brain microglia and cooperatively control phagocytosis of apoptotic neurons.[15, 16] Despite these findings, regulation of DAP12 expression has not been analyzed in resting or activated retinal microglia cells, and nothing is known about *cis*-acting elements controlling DAP12 transcription in any cell type.

Therefore, our objective in this study was to investigate whether the increased DAP12 expression in the dystrophic retina of Rs1h$^{-/Y}$ mice can be attributed to activated microglia cells. To achieve this goal, *ex vivo* isolation and culture of retinal microglia from Rs1h$^{-/Y}$ mice and an *in vitro* model of microglia activation were established. Detailed mRNA expression studies were performed in both cell systems using DNA microarrays and real-time quantitative RT-PCR (qRT-PCR).

Moreover, as a first step towards an understanding of the transcriptional regulation of DAP12 in mononuclear phagocytes, luciferase reporter gene assays, EMSA, chromatin immunoprecipitation (ChIP), RNA interference, and re-expression experiments were carried out. These experiments establish PU.1 as a major regulator of DAP12 expression in macrophages.

MATERIAL AND METHODS

Animals

Animals were maintained in an air-conditioned environment on a 12-h light–dark schedule at 20–22°C and had free access to food and water. The health of the animals was monitored regularly, and all procedures adhered strictly to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The Rs1h$^{-/Y}$ mouse has been described previously.[9] These mice were on a C57BL/6 background and backcrossed for 12 generations.

Isolation and culture of retinal microglia

Retinal tissue from wild-type and Rs1h$^{-/Y}$ mice at Postnatal Day 14 (P14) was isolated from eye bulbs and purified from contaminating vitreous body and retinal pigment epithelium (RPE)/choriocapillaris. Pools of four retinae each were cut into small pieces and incubated for 40 min at 37°C in 1 ml PBS with 1 mg/ml collagenase type I (Sigma Chemical Co., St. Louis, MO, USA), 0.3 mg/ml DNase I (Roche, Indianapolis, IN, USA), and 0.2 mg/ml hyaluronidase (Sigma Chemical Co.). The cell suspension was filtered through a 70-µm cell strainer (Becton Dickinson, San Jose, CA, USA). Cells were washed twice with 10 ml DMEM/10% FCS and finally subjected to Ficoll density gradient centrifugation for 20 min at 2000 rpm (690 *g*, without brake) in a Heraeus centrifuge for the isolation of mononuclear cells. The interphase was removed carefully and washed with 10 ml DMEM/FCS. The cells were cultured for 11 days in 75 cm^2 flasks containing DMEM/10% FCS, supplemented with 50 ng/ml recombinant human M-CSF (R&D Systems, Minneapolis, MN, USA), and phase contrast micrographs were taken with a Nikon Eclipse TE2000-S microscope.

Culture of cell lines

HeLa and RAW264.7 cells were obtained from American Type Culture Collection (Manassas, VA, USA), and BV-2 cells were a gift from Professor Ralph Lucius (Clinic of Neurology, Christian Albrechts University, Kiel, Germany). HeLa and RAW264.7 cells were cultured in DMEM, supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin and incubated in 10% CO_2 in air at 37°C. The culture of BV-2 cells has been described elsewhere.[17] PUER cells[18] were cultivated in IMDM (Invitrogen, Carlsbad, CA, USA), supplemented with penicillin/streptomycin (10,000 U/ml), glutamin (200 mM), β-ME (50 µM), mouse IL-3 (5 ng/ml, Biosource, Camarillo, CA, USA), puromycin (1 µg/ml), and 10% FCS. To activate the PU.1-ER fusion protein, PUER cells were centrifuged and resuspended in medium containing 0.1 µM tamoxifen [4-hydroxytamoxifen (OHT)].

Activation and phagocytosis assay of BV-2 cells

To keep BV-2 cells in a ramified and resting state, cells were incubated in serum-free medium for 24 h. For the gradual transformation into amoeboid cells, 5% and 10% FCS was added to the culture medium, followed by further incubation for 48 h. The phagocytic activity of amoeboid cells cultured in the presence of 10% FCS was assessed as reported earlier.[19] Briefly, 1 µm blue Latex beads from polystyrene (Sigma Chemical Co.) were added to the wells at a concentration of 1 µl beads/ml, and cells were washed with PBS after overnight incubation. The phagocytosis potential was monitored by counting Latex blue bead-positive cells using microscopy.

RNA isolation and RT

Total RNA was extracted from retinae or cultured microglia cells according to the manufacturer's instructions using the RNeasy Protect Midi kit or Micro kit, respectively (Qiagen, Valencia, CA, USA). Purity and integrity of the RNA were assessed on the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip® reagent set (Agilent Technologies, Santa Clara, CA, USA). The RNA was quantified spectrophotometrically and then stored at -80°C. First-strand cDNA synthesis was performed with the RT system from Promega (Madison, WI, USA), according to the manufacturer's instructions.

DNA microarray analysis

Generation of ds-cDNA, preparation and labeling of cRNA, hybridization to Affymetrix 430 2.0 mouse genome arrays, washing, and scanning were performed according to the Affymetrix standard protocol. Duplicate microarrays were carried out with pooled RNA from *ex vivo* microglia isolated at P14 from wild-type and Rs1h$^{-/Y}$ retinae, which were cultured for 11 days in M-CSF medium. Data analysis was carried out as described previously[8, 20] and minimal information about a microarray experiment criteria was met.[21] Functional annotation of coregulated transcripts was performed using BiblioSphere pathway edition (Genomatix Software GmbH, Germany) and the GenMap annotator and pathway profiler (Gladstone Institutes, San Francisco, CA, USA). The microarray dataset of this study is publicly available at the National Center for Biotechnology Information Gene Expression Omnibus (http://www. ncbi.nlm.nih.gov/geo/).

Real-time qRT-PCR

Amplifications of 50 ng cDNA were performed with the iCycler iQTM real-time PCR detection system (Bio-Rad, Munich, Germany) in triplicates in 25 µl reaction

mixtures containing 1X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 200 nM primers (**Table 1**), and 0.25 µl dual-labeled probe (Roche Probe Library). The reaction parameters were as follows: 2 min, 50°C hold; 30 min, 60°C hold; and 5 min, 95°C hold, followed by 45 cycles of 20 s, 94°C melt, and 1 min, 60°C anneal/extend. Relative quantification with normalization to various reference genes was performed as described earlier.[22]

Table 1. *Primer Sequences and Probe IDs for Real-Time qRT-PCR Using the Roche Probe Library.*

Gene	Acc. No.	F-Primer (5'–3')	R-Primer (5'–3')	Probe ID
Atp5b[a]	NM_016774	GGCACAATGCAGGAAAGG	TCAGCAGGCACATAGATAGCC	63
Casp11	NM_007609	GATCGGGCAACCTTGACA	TGAGATTCAGTTGCTTGTTGC	72
Cd68	NM_009853	CTCTCTAAGGCTACAGGCTGCT	TCACGGTTGCAAGAGAAACA	22
Clec7a	NM_020008	GGAAATTCCTGGTATGGAAGTAAG	GACGATGTTTGGCTTTCAATG	25
Cralbp	NM_020599	CCCCTCGGATCTCAAGAAG	TTTGAACCTGGCTGGGAAT	1
Dap12	NM_011662	CGTACAGGCCCAGAGTGAC	CACCAAGTCACCCAGAACAA	17
Gfap	NM_010277	ACAGACTTTCTCCAACCTCCAG	CCTTCTGACACGGATTTGGT	49
Gusb[a]	NM_010368	GTGGGCATTGTGCTACCTC	ATTTTTGTCCCGGCGAAC	16
Hprt1[a]	NM_013556	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC	71
PU.1	NM_011355	GGAGAAGCTGATGGCTTGG	CAGGCGAATCTTTTTCTTGC	94
Rp14[a]	NM_024212	GATGAGCTGTATGGCACTTGG	CTTGTGCATGGGCAGGTTA	28

[a] Reference genes. F, Forward; R, reverse; Cralbp, cellular retinaldehyde-binding protein; Gfap, glial fibrillary acidic protein

Reporter gene assays

Forward primers containing restriction sites for *Xho*I for the amplification of three deletion constructs of the murine DAP12 promoter (-860/+118, -308/+118, -104/+118) were designed on the genomic sequence retrieved by Gene2Promoter (Accession Number GXP_44406, Genomatix Software GmbH). The sequences of the forward primers were 5'-CTCGAGACTAAAATAGTC-CCAACCTCCTCTC-3', 5'-CCGAGCTCTCAAGGCCCAGAGAAGTAGCG-3', 5'-GAGCTCCTTTCTGCGGCCATG-CTATAGTTCC-3', respectively, and the unique reverse primer was 5'-CCCAAGCTTACTACCCCCACAGTCAGG-3'. Murine genomic DNA served as a template for the amplification of the promoter sequences with PfuUltra II Fusion HS DNA polymerase (Stratagene, La Jolla, CA, USA). Digested PCR promoter fragments were cloned by ligation into the *Xho*I and *Hin*dIII restriction sites of the pGL4.10 vector (Promega). The identity of the subcloned DNA fragments was confirmed by DNA sequencing. A promoterless vector served as a negative control, and the CMV promoter was used as a positive control.

Clone –860/+118 was used as template for site-directed mutagenesis at the –80 and 26 PU.1 sites. The QuikChange site-directed mutagenesis kit (Stratagene) was applied, according to the manufacturer's recommendations, with the following mutagenic primers: PU.1(1), 5′-GCGGCCATGTCTATACG-T**AAA**TCCTCCCTGCTGC-3′, and PU.1(2), 5′-CCACCACCCACCTCACA-C**AAA**CTCCTTCACTTGGTTGG-3′. Double PU.1 site mutations were generated by applying both primers in a single reaction. Altered nucleotides are depicted in bold and underlined.

RAW264.7, BV-2, and HeLa cells were transfected using 6 µl Fugene6 reagent (Roche) with 3 µg each reporter plasmid in six-well plates. The transfected cell lines were harvested after incubation for 48 h. Cell lysates were assayed for protein concentration using the Bradford assay, and firefly luciferase activity was measured with the Luciferase assay system (Promega) on a FLUOstar OPTIMA (BMG Labtech, Germany). Each experiment was carried out three times, and each measurement was done in triplicate.

EMSA

Nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL, USA). Double-stranded oligonucleotides were end-labeled with T4 polynucleotide kinase (Fermentas, Ontario, Canada) and [γ-^{32}P]ATP. An equivalent of 40,000 cpm double-stranded oligonucleotide probe containing the DAP12 promoter sequence was incubated with 5 µg nuclear extract from BV-2 or RAW264.7 cells as described previously[23] in a buffer containing 50 mM HEPES/HCl, pH 7.9, 6 mM $MgCl_2$, 50 mM DTT, 100 µg/ml BSA, 0.01% Nonidet P-40 (NP-40), and 1 µg poly(dI-dC) at room temperature for 20 min.

In vitro-translated PU.1 (IVT-PU.1) or a control reaction was generated from a pGEM plasmid containing the open-reading frame of PU.1 in sense or antisense orientation,[24] respectively, and the TNT Quick Coupled Transcription/Translation system (Promega). [^{35}S]Methionine PU.1 was analyzed by SDS-PAGE and autoradiographed to confirm its correct molecular mass of 38 kDa. Supershift analysis was carried out with 2 µl antisera (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against PU.1 (SC352X) and Ets1/2 (SC351X). In competition experiments, nuclear extracts were preincubated with a 100-fold molar excess of wild-type or mutant competitor for 10 min prior to the addition of the radiolabeled probe. The sequences of the oligonucleo-tides used in EMSA analysis are shown (see Fig. 7). DNA–protein complexes were electrophoretically resolved on a native 8% polyacrylamide gel, dried, and audioradiographed with Kodak BioMax MR films at –80°C.

ChIP assay

RAW264.7 cells ($10X10^6$) were cross-linked for 10 min with formaldehyde to achieve a final concentration of 1% and then quenched with glycine (0.125 M final concentration). After washing with PBS, the cells were incubated on ice for 10 min in 1 ml lysis buffer [10 mM HEPES, pH 7.9, 85 mM KCl, 1 mM EDTA, 1 mM PMSF, 1X protease inhibitor (Roche), 1% NP-40]. Nuclei were pelleted and resuspended in nuclear lysis buffer [50 mM Tris/HCl, pH 7.4, 1% SDS, 0.5% Empigen BB, 10 mM EDTA, 1 mM PMSF, 1X protease inhibitor (Roche)]. Subsequently, sonication was carried out with four pulses of 10 s on a Fisher sonicator. The lysate was cleared by centrifugation for 5 min at 16,000 *g*. After 1:2 dilution with buffer (20 mM Tris/HCl, pH 7.4, 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 1X protease inhibitor), a fraction was kept as input for normalization. The lysates were precleared with 5 µg salmon sperm DNA/Sepharose CL-4B beads and precipitated with 2.5 µg antibody (rabbit polyclonal PU.1 antibody and IgG rabbit isotype control, Santa Cruz Biotechnology) overnight at 4°C. Complexes were recovered with protein A/protein G sepharose beads, preincubated with salmon sperm DNA, and washed as described previously.[25] After reversal of cross-linking overnight at 65°C, the DNA was purified with Qiaquick PCR purification columns. The DNA was analyzed by qPCR using the Applied Biosystems Power SYBR® Green PCR Master Mix with primers, which amplify both PU.1 sites in the DAP12 promoter (forward, 5′-TCAAGGCCCAGAGAAGCTAA-3′; reverse, 5′-CATGAGCT-GAGGACACAG-3′). A region in the early growth response 1 (Egr1) promoter, which does not bind PU.1, was amplified to control for variability between individual precipitations using the following primers: forward, 5′-GGCCGGTCCTTCCATATTAG-3′; reverse, 5′-GTGGGTGAGTGAGGAAAGGA-3′. In an independent set of experiments, ChIP-Chip assays were performed with Affymetrix-GeneChip® mouse promoter 1.0R arrays. Three arrays were hybridized each with RAW264.7 DNA from PU.1 and IgG precipitations, respectively. Retrieval of a significantly enriched promoter was performed using Genomatix ChipInspector software.

RNA interference

Knockdown of PU.1 in RAW264.7 cells was achieved with two independently annealed, ds-small interfering (si)RNAs (Qiagen). The siRNA sequences of murine PU.1 were: PU.1(1), 5′-(GGAUGUUACAGGCGUGCAA)dTdT-3′ (sense), and PU1.(2), 5′-(GCA AGAAGAUGACCUACCA)dTdT-3′ (sense). BLAST searches confirmed that these sequences were not homologous to any nuclear genes other than PU.1. GAPDH siRNA represented a positive control and a scrambled siRNA molecule a negative control. RAW264.7 cells at 80% confluence in 12-well plates were transfected with

75 ng each of the above siRNAs using the HiPerFect transfection reagent (Qiagen), according to the manufacturer's protocol. The transfected cells were incubated at 37°C for 48 h before isolation of total RNA and qRT-PCR analysis to confirm the knockdown effect.

Bioinformatic transcription factor binding site and promoter analysis

Promoter sequences from transcripts induced significantly in activated microglia were retrieved with Gene2Promoter (Genomatix Software GmbH) and DataBase of Transcriptional Start Sites (http://dbtss.hgc.jp). Approximately 1 kb DNA of the upstream regulatory regions was analyzed for putative transcription factor binding sites using Matinspector (Genomatix Software GmbH) and Transfac (BIOBASE GmbH, Germany). Only matrices predicted with algorithms and a core similarity of 1.0/a matrix similarity >0.75 were included in the regulatory network analysis. Transcription start sites were determined by the cap analysis of gene expression (CAGE) resource for comprehensive promoter analysis (http://fantom3.gsc.riken.jp).[26]

Statistical analysis

Data are presented as means, and error bars indicate the SD of the mean. Statistical significance was calculated using the Student's *t*-test, and $P < 0.05$ was significant.

RESULTS

High levels of DAP12 and PU.1 expression in *ex vivo* Rs1h$^{-/Y}$ microglia

Reactive microglia and up-regulated DAP12 mRNA levels have been identified recently in the dystrophic Rs1h$^{-/Y}$ retina.[8] Based on these findings, we first aimed to analyze whether retinal DAP12 expression in Rs1h$^{-/Y}$ mice can be ascribed to activated microglia. We established a procedure for *ex vivo* isolation and M-CSF-dependent *in vitro* culture of retinal microglia cells from wild-type and Rs1h$^{-/Y}$ mice at P14. At this time-point, prominent microglia proliferation and activation have been observed in the Rs1h$^{-/Y}$ retina.[8]

We adapted a protocol for the isolation of colonic macrophages[27] and obtained pure cultured microglia cells without visible contaminations of Müller glial cells, RPE cells, or astrocytes (**Fig. 1**). The cells proliferated in distinct, large colonies when cultured for 3–5 days in medium supplemented with M-CSF. A clear difference in the morphological phenotype was observed between wild-type (**Fig. 1, A and C**) and Rs1h$^{-/Y}$ (**Fig. 1, B and D**) mice after 11 days in culture. Although wild-type microglia showed long protrusions, typical for resting cells (**Fig. 1C**), most microglia from Rs1h$^{-/Y}$ retinae displayed an amoeboid morphology characteristic

of activated phagocytes (**Fig. 1D**). This morphological difference was lost when cells were cultured more than 2 weeks (data not shown), indicating an adaptation to the culture conditions after prolonged culture.

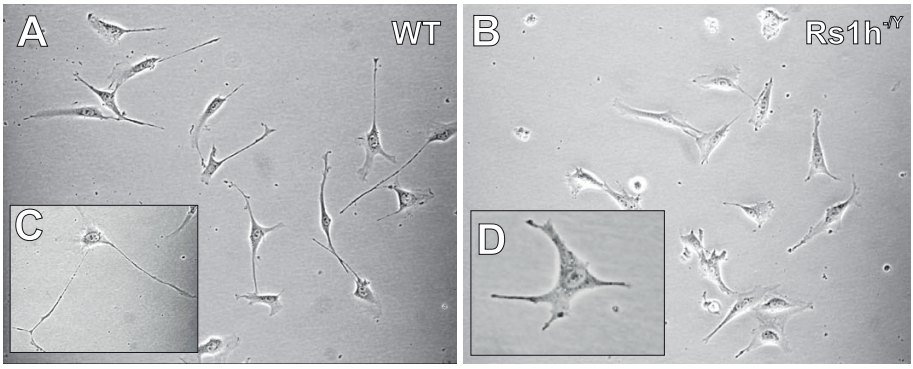


Fig.1. *Morphology of ex vivo microglia cells from wild-type (WT) and Rs1h$^{-/Y}$ retinae. Phase contrast micrographs were taken from retinal microglia isolated at P14 and cultured in the presence of 50 ng/ml M-CSF for 11 days. (A and C) Ramified morphology of resting, nonactivated cells (wild-type C57BL/6 littermates). (B and D) Ameboid cell shape typical for activated microglia cells (Rs1h$^{-/Y}$ mice). (A and B) Originial magnification, X100: (C and D) original magnification, X200.*

To estimate the purity of activated *ex vivo* microglia from Rs1h$^{-/Y}$ retinae, marker transcripts for different retinal cell types were analyzed in isolated cultured cells and total retina by real-time qRT-PCR (**Fig. 2A**). The specific myeloid marker CD68 and the RPE/Müller cell marker Cralbp were highly enriched in microglia or in retina (**Fig. 2A**, first and last bars). Transcript levels of DAP12, Clec7a, and Casp11 were markedly higher in microglia cells compared with retina when using the same amount of template from both RNA sources. In contrast, Gfap, which has been detected previously in the dystrophic Rs1h$^{-/Y}$ retina, shows lower abundance in isolated microglia, suggesting expression in microglia and also in other retinal cell types. In summary, these data indicate that we have isolated and cultured a pure cell population well suited to detect microglia-specific molecular events in retinal degeneration.

Our next goal was to capture and compare the transcriptional profile of microglia cells from Rs1h$^{-/Y}$ versus wild-type retinae using Affymetrix Mouse Genome 430 2.0 GeneChips. Duplicate microarray analyses from two independent animal pools were performed. Applying high-stringency criteria with at least 2.0-fold difference and a minimum signal intensity of 50, we have identified 143 differentially regulated genes (**Supplementary Table 1**). It is interesting that the majority of these genes (n=94) was induced significantly in Rs1h$^{-/Y}$ microglia compared with control cells.

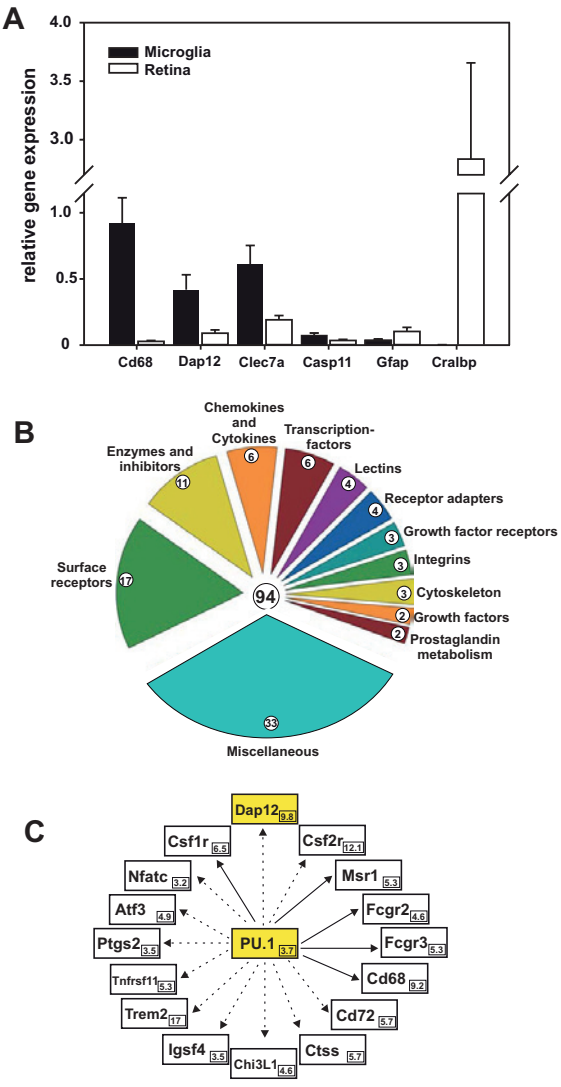


Fig.2. *Gene expression profiles of isolated microglia and PU.1 regulatory network. (A) qRT-PCR analysis of selected marker genes in Rs1h$^{-/Y}$-derived microglia and total retina. The relative expression of each marker gene was analyzed in 50 ng RT cDNA (total RNA equivalent) and calculated for microglia (solid bars) and retina (open bars), respectively. The data shown are the means of two independent cell/tissue preparations from four retinae each. Cd68, DAP12, Clec7a, Casp11, Gfap, and Cralbp transcript abundance was normalized to a set of reference genes (Atp5b, Gusb, Hprt1, and Rp14), which were not significantly different in both RNA sources. Error bars indicate the SD of the mean. (B) Pie chart displaying functional categories represented by the 94 significantly up-regulated genes in Rs1h$^{-/Y}$ versus wild-type microglia. Functional annotation of the up-regulated genes (presented in Supplementary Table 1) was carried out with BiblioSphere Pathway edition (Genomatix Software GmbH) and GenMapp (Gladstone Institutes). Circled numbers indicate the quantity of genes in each segment. (C) PU.1-dependent transcriptional regulatory network. Sixteen genes significantly induced in Rs1h$^{-/Y}$ versus wild-type microglia contain conserved binding motifs for PU.1/Ets. Among these, Csf1r, Msr1, Fcgr2, Fcgr3, and CD68 are known PU.1 targets[28-31] (solid-line arrows). All other genes, including DAP12, have not been shown to be regulated by PU.1 (dashed-line arrows). Gene symbols and the fold change of regulation identified by DNA microarrays are indicated in small boxes.*

Molecular annotation of functions and pathways of the 94 up-regulated genes revealed a strong representation of the categories “Surface receptors,” “Enzymes and inhibitors,” “Chemokines and cytokines,” and “Transcription factors,” strongly

reminiscent of activated macrophages (**Fig. 2B**). In contrast, a significant number of genes could not be grouped into larger, immune-related pathways and have not been connected previously to microglia activation. Also, many genes with no detectable expression difference in the total retina of Rs1h$^{-/Y}$ versus wild-type mice[8] were now significantly different in isolated and enriched microglia. Likewise, we have detected a significant 3.7-fold mRNA increase of the transcription factor PU.1 in activated microglia (**Fig. 2C**, yellow centered box). As nuclear factors are of particular importance for transcriptional responses, regulatory network analysis was performed with all up-regulated gene clusters and the identified transcription factors PU.1, Runx3, ATF3, Msx2, Lmo7, and peroxisome proliferator-activated receptor γ. Bioinformatic binding-site analysis and literature data mining exclusively revealed a PU.1-dependent network consisting of 16 genes, including five known PU.1 targets (**Fig. 2C**, solid-line arrows). There were also 11 novel, putative, PU.1-regulated genes (**Fig. 2C**, dashed-line arrows), including DAP12.

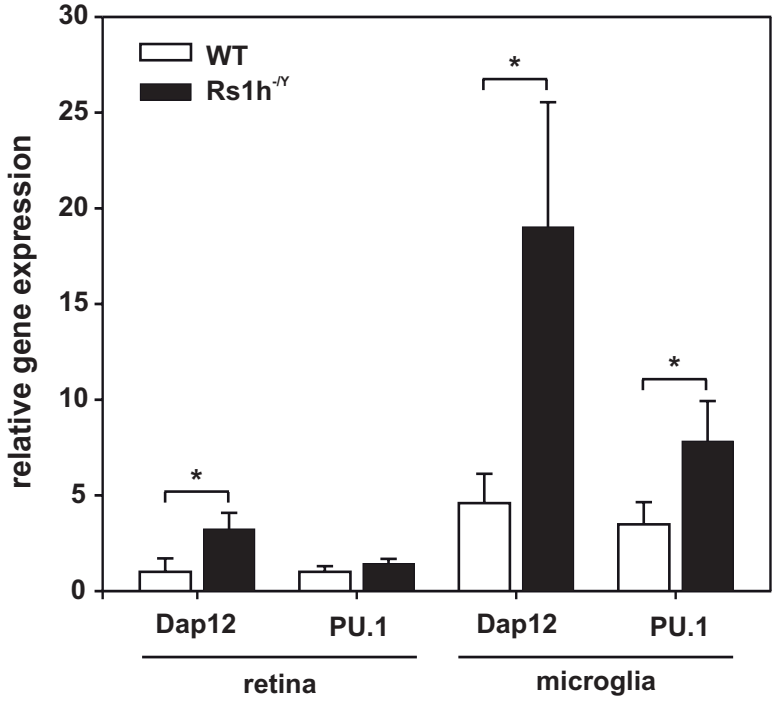


Fig.3. *Increased DAP12 and PU.1 expression in total retinae and isolated microglia from Rs1h$^{-/Y}$ mice. qRT-PCR analysis of DAP12 and PU.1 in Rs1h$^{-/Y}$ retinae and ex vivo microglia compared with wild-type mice. The data shown are mean values of two independent experiments representing transcript levels from a pool of four retinae or ex vivo microglia preparations each. Error bars indicate the SD of the mean. *, P 0.05 Rs1h$^{-/Y}$, versus wild-type using Student's t-test.*

To confirm the DNA microarray data, DAP12 and PU.1 transcript levels were determined in total retinae and isolated microglia from Rs1h$^{-/Y}$ and wild-type mice by real-time qRT-PCR. As shown in **Figure 3**, Rs1h$^{-/Y}$ retinae showed a threefold higher expression of DAP12 than wild-type tissue, and a more than fivefold difference of DAP12 mRNA levels was observed in Rs1h$^{-/Y}$ compared with wild-type microglia. Although the amount of PU.1 transcripts was not different in total retinae (most likely as a result of an overall low expression level), enriched, activated microglia expressed significantly higher amounts of PU.1 mRNA than cells from wild-type mice (**Fig. 3**). These data indicate that DAP12 and PU.1 expression is correlated with the activation state of microglia.

To investigate whether the overexpression of DAP12 is a direct consequence of microglia activation, an independent *in vitro* model (BV-2 microglia cell line) was established. BV-2 cells are converted into a quiescent state when cultured in the absence of serum for 24 h (**Fig. 4A**). Addition of 5% and 10% FCS caused a gradual, morphological change in the majority of cells, such as loss of cytoplasmic protrusions and rounding of the cells (**Fig. 4, B and C**). Experiments measuring the overnight uptake of Latex beads demonstrated a strong phagocytic activity of cells kept in medium with 5% FCS and 10% FCS (**Fig. 4, D and E**) compared with resting cells in serum-free conditions. Therefore, these morphological changes and functional characteristics suggest an activated state of BV-2 cells kept in full culture medium. In line with our findings from the *ex vivo* microglia, an elevated DAP12 expression was detected in BV-2 cells when transformed from the ramified to the amoeboid state by increasing the FCS concentration in the culture medium (**Fig. 4F**). It is interesting that the mouse macrophage cell line RAW264.7 showed a BV-2-comparable amount of DAP12 transcripts (**Fig. 4F**) and high levels of PU.1 mRNA (data not shown). These cells were therefore used for the majority of the following promoter assays.

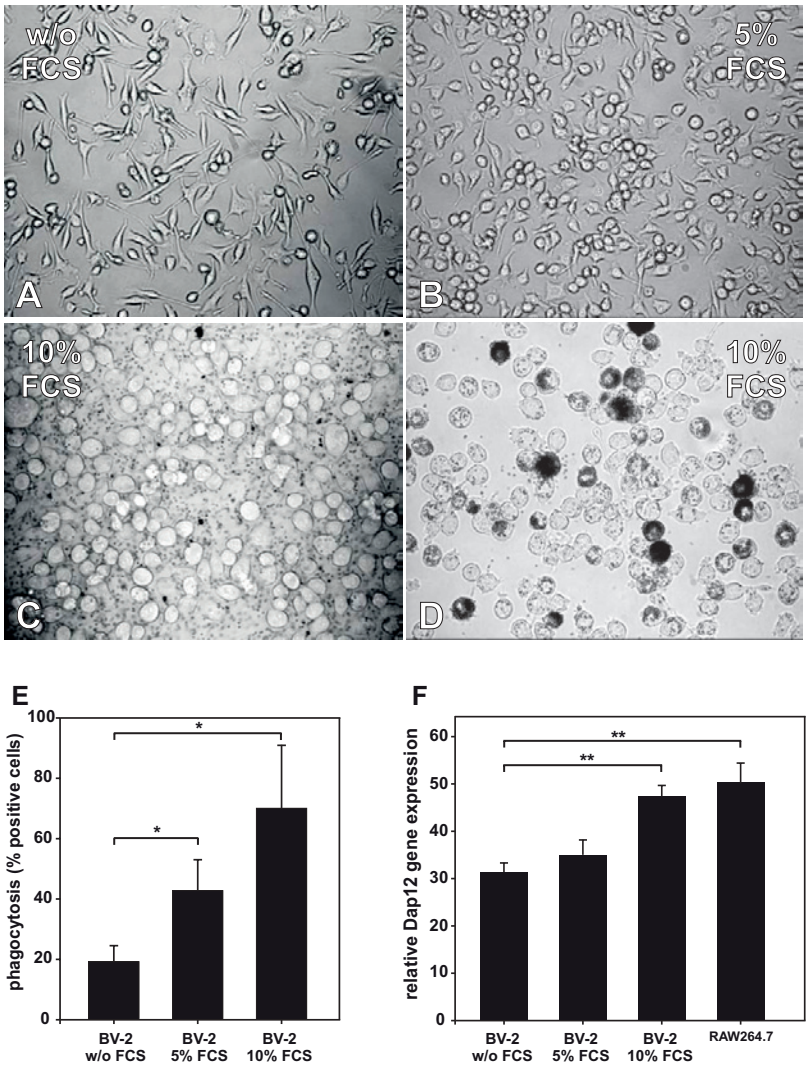


Fig.4. *Induction of DAP12 expression during in vitro activation of BV-2 cells. (A–E) Phase contrast micrographs of BV-2 cells cultured in the absence of FCS (A, ramified morphology), 5% FCS (B, partially activated), or 10% FCS (C and D, fully activated). Activated BV-2 cells incubated with 1 µl Latex blue beads for 1 h (C) and overnight (D). Original magnification, X100. (E) Quantification of phagocytosis by microscopic counting of Latex blue bead-positive cells after overnight incubation. A strong, phagocytic activity was detected in cells cultured in the presence of 10% FCS compared with resting cells. *, P < 0.05, BV-2 10% FCS and BV-2 5% FCS versus BV-2 without (w/o) FCS using Student's t-test. (F) qRT-PCR analysis of DAP12 in in vitro-activated BV-2 cells and RAW264.7 cells. The data shown are mean values of three independent experiments, and error bars indicate the SD of the mean. **, P < 0.01, BV-2 10% FCS and RAW264.7 versus BV-2 without FCS using Student's t-test.*

Myeloid-specific promoter activity of DAP12 depends on proximal PU.1 sites

To further study myeloid DAP12 gene expression and to characterize the potential regulation by PU.1 at the promoter level, we cloned the murine DAP12 upstream regulatory region and determined the transcription start sites with the help of macrophage data from the CAGE library.[26] Two close transcription initiation sites were found in independent mouse macrophage mRNA pools (**Fig. 5**, *). Five 5′-upstream regions from mouse, rat, cow, human, chimpanzee, and rhesus monkey DAP12 revealed a 90-bp promoter region with two highly conserved blocks for PU.1-binding sites (**Fig. 5**). There was no strong sequence homology further upstream of this region, suggesting that the perfectly conserved motif for the myeloid transcription factor PU.1 at position –26 and the less conserved, second PU.1 site at –80 might be functionally important for several species. A VDR/RXR motif with lower similarity score was also identified (**Fig. 5**).

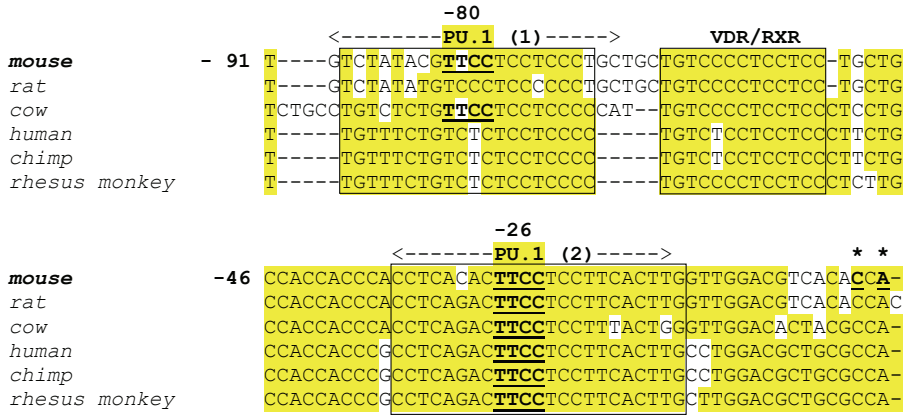


Fig.5. *Evolutionary conservation of the proximal Dap12 promoter. A ClustalW alignment is shown using DAP12 promoter sequences from Mus musculus (mouse), Rattus norvegicus (rat), Bos taurus (cow), Homo sapiens (human), Pan troglodytes (chimp), and Macaca mulatta (rhesus monkey). The boxed sequences contain predicted binding sites for PU.1 (–80 and –26) and vitamin D receptor (VDR)/retinoid X receptor (RXR). The core binding sequences for PU.1 are indicated in bold and underlined. The transcription start sites identified using the mouse CAGE library [26] are shown as asterisks, and the location of the sequences used for EMSA analysis is marked by arrows over the conservation blocks.*

Transient transfections of a pGl4.10 luciferase reporter construct containing 860 bp of the DAP12 promoter and extending until the end of the first exon (–860/+118) revealed a strong promoter activity in BV-2 and RAW264.7 cells, whereas the nonmyeloid cell line HeLa (cervical carcinoma) displayed no significant activity (**Fig. 6A**). Analysis of the two deletion constructs, –308/+118 and –104/+118, in

RAW264.7 (**Fig. 6B**) and BV-2 cells (data not shown) indicated the presence of a negative regulatory element between –308 and –104. These findings locate the myeloid-specific core promoter region 104 bp proximal to the most upstream transcription start site.

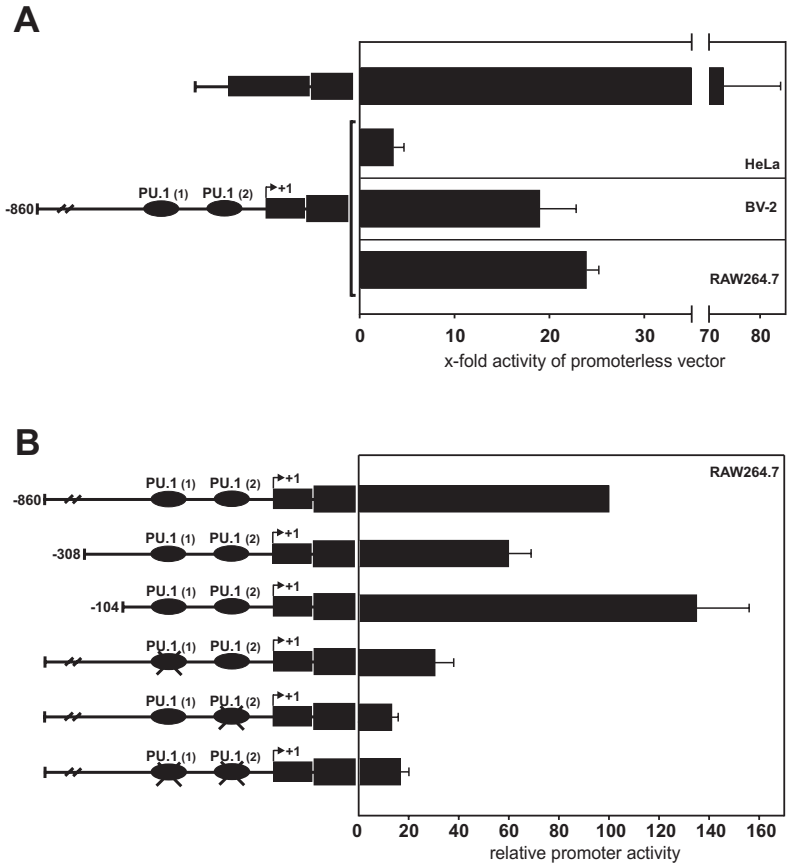


Fig.6. *The DAP12 promoter is only active in myeloid cells and requires intact PU.1/Ets binding sites. (A and B, left sides) Overview of luciferase (Luc) reporter plasmids. (A) CMV-Luc-positive control, a promoterless luciferase vector, and the –860/+118 DAP12 promoter luciferase construct were transiently transfected into the nonmyeloid HeLa cell line and into the microglia/macrophage cell lines BV-2 and RAW264.7. Luciferase activity is calculated relative to the empty reporter vector. (B) Deletion and mutagenesis analysis of the DAP12 promoter in RAW264.7 cells. Luciferase activity is calculated relative to the longest wild-type construct –860/+118, which was set to 100%. Values are means from three independent experiments, and error bars indicate the SD of the mean.*

The proximal DAP12 promoter region lacks a classical TATA box, initiator sequences, or GC-rich motif (**Fig. 5**) but contains purine-rich sequences characteristic for myeloid-specific promoters.[32] We therefore tested whether the two 5′-GGAA-3′ binding motifs for PU.1 at –80 and –26 are required for DAP12 promoter activity. Mutant reporter gene constructs changing GGAA to TTTA at both sites were created by site-directed mutagenesis and analyzed by transient transfection. Mutation of the upstream PU.1 site [PU.1(1)] reduced the activity of the full-length promoter by 70% while mutating the PU.1(2) site diminished promoter activity by over 85% (**Fig. 6B**). Modifying both PU.1 sites in the same construct had a similar effect, resulting in an over 80% loss of reporter activity. These results demonstrate that both PU.1 sites are essential for the activity of the DAP12 promoter in myeloid cells.

PU.1 interacts with the DAP12 proximal promoter *in vitro* and *in vivo*

To study *in vitro* binding of PU.1 to the functionally important ETS/PU.1 motifs, EMSA analysis with nuclear extracts from RAW264.7 and BV-2 cells as well as IVT-PU.1 were performed. As shown in **Figure 7**, prominent PU.1 binding activity was detected for the –90/–68 PU.1(1) site and the –37/–15 PU.1(2) site (Lanes 2). Complex formation at both sites was abolished completely by competition with an excess of unlabeled oligonucleotides containing the wild-type motif but not a mutated PU.1 site (**Fig. 7, A**, Lanes 3 and 4, and **B**, Lanes 4 and 5). Each DNA–protein complex was confirmed to contain PU.1 by supershift analysis with a polyclonal anti-PU.1 antibody, resulting in a slowly migrating, high molecular complex (**Fig. 7, A**, Lane 5, and **B**, Lanes 3 and 7). Furthermore, IVT-PU.1 strongly bound to the PU.1(1) and PU.1(2) sequences in a dose-dependent manner, whereas the IVT-negative control did not show specific binding (**Fig. 7, A**, Lanes 7–10, and **B**, Lanes 8–11). Complex formation was similar when using RAW264.7 nuclear extract or BV-2 extract (**Fig. 7B**, Lanes 6 and 7), which express high levels of PU.1 mRNA and protein. It is notable that the band at the PU.1(1) site was not supershifted completely by PU.1. Addition of an anti- Ets1/2 antibody diminished complex formation significantly, suggesting the presence of Ets1/2 protein in this complex. These results suggest that PU.1 is the only binding protein at site –37/–15 and that the region –90/–68 might contain an additional component, most likely Ets1 or Ets2 We also analyzed the evolutionarily conserved VDR/RXR binding element located between both PU.1 sites using EMSA (data not shown). However, no specific binding to this DAP12 promoter region could be detected in RAW264.7 or BV-2 cells, indicating that myeloid expression of DAP12 is not mediated via this sequence motif.

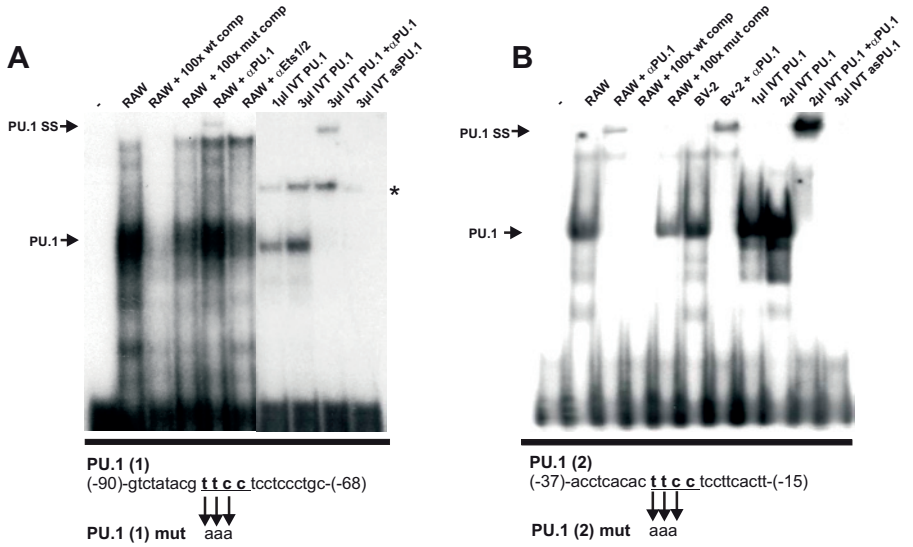


Fig.7. *PU.1 interacts with both PU.1/Ets sites in the proximal DAP12 promoter. Labeled,dsPU.1 oligonucleotides PU.1(1) and PU.1(2), containing the sequences shown (lower), were used in EMSA with nuclear extracts from RAW264.7 cells, BV-2 cells, or IVT-PU.1 protein. (A) EMSA with the PU.1(1) oligonucleotide and RAW264.7 nuclear extracts (Lanes 2–6). Competition analysis was carried out using 100-fold molar excess of cold wild-type (Lane 3) or cold mutant (Lane 4) probe. Antibody supershift analyses with specific PU.1 (Lane 5) or Ets1/2 antibodies (Lane 6) are indicated. IVT-PU.1 and a corresponding negative control (asPU.1) were used in Lanes 7–10. (B) EMSA with the PU.1(2) oligonucleotide and RAW264.7 extract (Lanes 2–5), BV-2 extracts (Lanes 6 and 7), and IVTPU.1 (Lanes 8–11). Competition experiments were performed using 100-fold molar excess of wild-type or mutant oligonucleotides. Supershift analyses were done in the presence of 2µl of the indicated antisera. Specific PU.1-containing complexes are indicated by arrows, and nonspecific bands are marked (*).*

By ChIP analysis, we next investigated whether PU.1 interacts with the proximal DAP12 promoter region *in vivo*. Chromatin was prepared from RAW264.7 cells, which expressed highest levels of DAP12 and PU.1, and immunoprecipitation was achieved with specific antibodies against PU.1 and IgG isotype as a control. After reversal of cross-linking, real-time qPCR analysis was carried out on ChIP–DNA using primers flanking the PU.1 sites in the DAP12 proximal promoter. The EGR1 gene was amplified as a genomic control, as it has no obvious PU.1 binding motif in its regulatory region. As shown in **Figure 8A**, the DAP12 core promoter region was strongly and specifically immunoprecipitated by anti-PU.1 antibody (**Fig. 8**, left bar), whereas the IgG isotype control shows only background binding (right bar). We further studied *in vivo* binding of PU.1 to the DAP12 regulatory region using ChIP-Chip analysis. Mouse promoter tiling arrays were hybridized with DNA

from PU.1 and IgG immunoprecipitations, respectively, and 5 kb upstream and 1 kb downstream of the DAP12 gene were analyzed for significant hybridization signals. **Figure 8B** displays that four adjacent probes located in the proximal DAP12 promoter region exhibit strong PU.1-specific signals compared with the IgG control (red bars). No additional significant signals were detected further upstream or in the first intron of the DAP12 locus. Taken together, both ChIP assays provide clear evidence for *in vivo* binding of PU.1 to the proximal DAP12 promoter in myeloid cells.

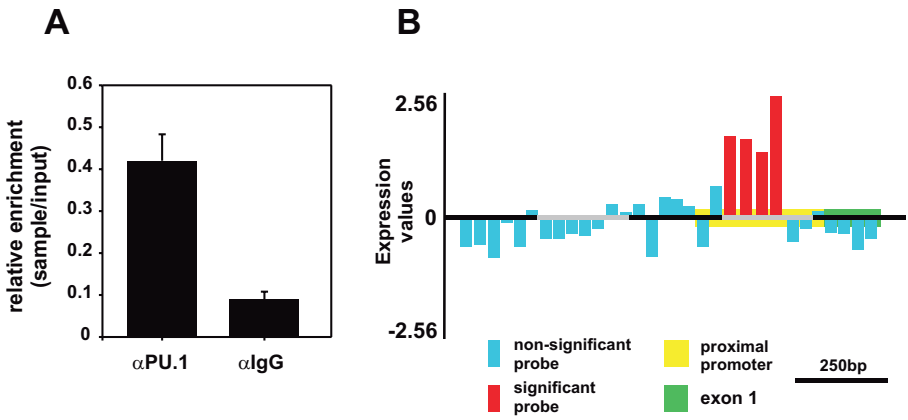


Fig.8. *In vivo binding of PU.1 to the proximal DAP12 promoter. Chromatin prepared from RAW264.7 cells was immunoprecipitated with antibodies against PU.1 (αPU.1) or IgG isotype control. (A) qPCR was performed with specific primers against the proximal DAP12 promoter region, and an internal control region from the EGR1 promoter was used as reference to calculate relative enrichment. The data shown are mean values of two independent experiments measured in triplicates, and error bars indicate the SD of the mean. (B) ChIP-Chip analysis of the DAP12 upstream regulatory region. Log2 expression values of tiled probes spanning 1 kb upstream of the first Dap12 exon are depicted. Significant probes are shown in red; nonsignificant probes are shown in blue. Specific in vivo binding of PU.1 to the proximal DAP12 promoter region spanning both PU.1 sites is indicated by four adjacent, significant probes.*

PU.1 is required for DAP12 expression in RAW264.7 cells and PU$^{-/-}$-progenitors

Following an alternative strategy to demonstrate that endogenous PU.1 regulates DAP12, we have applied specific RNA interference with two different, PU.1-specific siRNAs in RAW264.7 cells. The GAPDH-positive control led to a 65% decrease of endogenous GAPDH transcripts, and a specific PU.1 knockdown was achieved by reducing its gene expression to 40% and 38% of basal PU.1 levels, respectively (**Fig. 9**). Simultaneously, expression levels of DAP12 mRNA decreased to 63% and 55%, respectively, in the cells transfected with PU.1 siRNAs. In another set

of experiments we used a PU.1$^{-/-}$ progenitor cell line with retroviral re-expression of the OHT-inducible PU.1-ER fusion protein (PUER cells).[18] In these cells, which inducibly activate PU.1 nuclear translocation and promoter binding in the presence of OHT, we could show that DAP12 expression is nearly absent in cells lacking transcriptionally active PU.1 (**Fig. 10**, 0 h). After addition of 100 nM OHT, which leads to PU.1-ER activation, DAP12 transcription was up-regulated following a short lag phase of 6 h and further, increased strongly with culture time in the presence of OHT (**Fig. 10**). It is notable that DAP12 transcripts in 24 h OHT-induced PUER cells reached comparable levels present in RAW264.7 cells, which express PU.1 protein abundantly (data not shown). Taken together, these findings provide further *in vivo* evidence for a direct regulatory function of the transcription factor PU.1 in DAP12 gene expression.

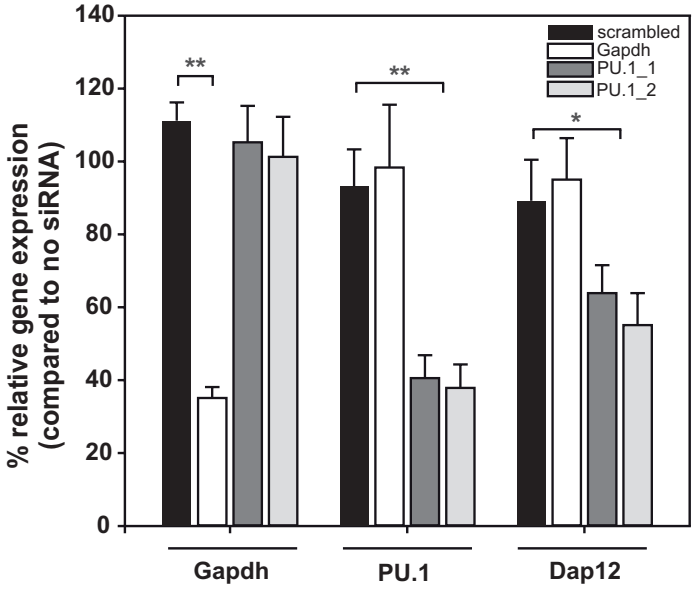


Fig.9. *PU.1 knockdown down-regulates endogenous DAP12 expression. RNA interference of PU.1 in RAW264.7 cells was carried out with two independent siRNAs against PU.1 (PU.1_1 and PU.1_2), GAPDH, and a nonsilencing, scrambled siRNA. Transfected cells were harvested after 48 h, and qRT-PCR analysis for GAPDH (positive control), PU.1, and DAP12 was carried out to quantify the knockdown effect. Results are displayed as relative gene expression compared with untransfected cells. The data shown are mean values of three independent experiments, and error bars indicate the SD of the mean. **, P < 0.01; *, P < 0.05, specific siRNA versus scrambled siRNA using Student's t-test.*

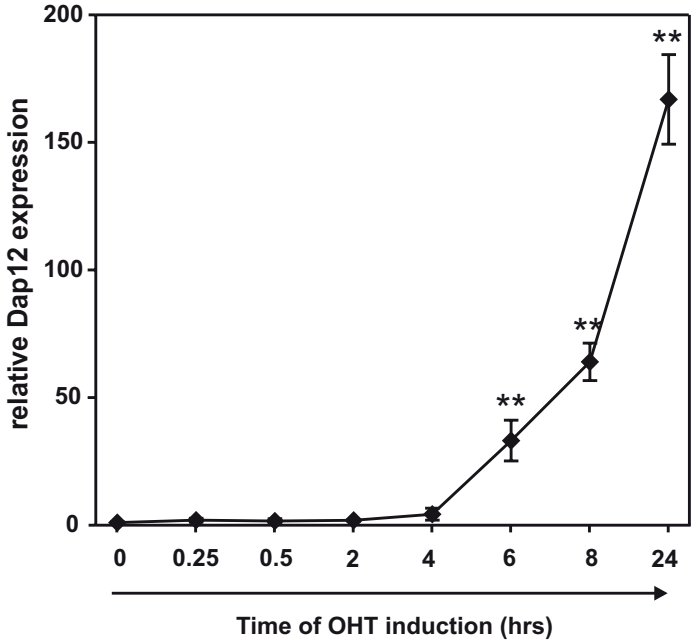


Fig.10. *PU.1 re-expression and activation in PU.1$^{-/-}$ cells up-regulate endogenous DAP12 expression. PUER cells were cultured in the presence or absence of 100 nM OHT for the indicated time-points, and DAP12 mRNA levels were determined using qRT-PCR. Results are displayed as relative gene expression levels compared with noninduced PUER cells. The data represent mean values of two independent experiments, and error bars indicate the SD of the mean. **, P < 0.01, OHT-induced versus noninduced using Student's t-test.*

DISCUSSION

In this study, we have isolated and cultured activated retinal microglia cells from Rs1h$^{-/Y}$ mice to characterize their phenotypic properties and to monitor their gene expression profiles. Overexpression of the adaptor protein DAP12 and the myeloid transcription factor PU.1 was identified specifically in microglia from Rs1h$^{-/Y}$ mice as well as in the BV-2 microglia *in vitro* cell system. Based on DNA microarray results and promoter prediction algorithms, we identified a PU.1-dependent regulatory network including DAP12. Furthermore, we performed a detailed characterization of the DAP12 upstream regulatory region and have shown that the murine DAP12 promoter is highly active in myeloid but not in nonmyeloid cells. The DAP12 core promoter activity is dependent on conserved PU.1/ETS DNA segments in the proximal region, and PU.1 binds to these sites *in vitro* and *in vivo*. Finally, a siRNA approach to downregulate PU.1 expression and PU.1 re-expression and activation in deficient progenitor cells revealed that endogenous DAP12 transcription is

regulated directly by PU.1-dependent transactivation mechanisms.

Our results provide the first report of whole genome transcript profiling in *ex vivo* microglia from a murine retinal degeneration model. In a pioneering work, Roque and Caldwell[33] established isolation and culture of retinal microglia from 8-week Royal College of Surgeons rats and performed a morphological, phenotypic characterization of these cells. In our initial experiments to transfer this method to 14-day-old Rs1h$^{-/Y}$ and wild-type mice, we were not successful in obtaining larger amounts of highly pure cells. We therefore modified and adapted a protocol initially used for the isolation of colonic macrophages.[27] As a crucial step, Percoll separation and prolonged culture in M-CSF-supplemented medium were required to achieve a highly enriched microglia population without significant contaminations of other retinal cell types. It is remarkable that ramified and amoeboid cell shapes were maintained over the culture period, and the gene expression profiles of resting and activated cells could be clearly distinguished. Also, a significant overlap to transcript patterns found in total retinae from Rs1h$^{-/Y}$ and wild-type mice[8] was established, demonstrating an excellent comparability of the cultured microglia to the in situ situation in the retina. In addition to our data, the power of DNA microarray analysis to study microglia/ macrophage activation in the nervous system has been shown recently by Albright and Gonzalez-Scarano.[34] In their study, specific expression of chemokines and surface receptors, which partially correspond to our microarray data, could be identified in mixed glial cultures of HIV-induced, degenerating brains. However, as microglia constitute only 60% of the mixed glial cultures,[34] these data may still be biased by the presence of mRNA molecules of nonmyeloid cell types. An elegant solution to obtain highly pure microglia from neuronal tissues without further selective culture would be the use of transgenic mice with fluorescent markers in the microglia population for cell sorting and isolation. Pursuant to this idea, breeding of Rs1h$^{-/Y}$ mice with MacGreen mice expressing enhanced GFP under a macrophage-selective promoter[35] is currently underway in our laboratory. This will greatly facilitate the isolation and further characterization of activated microglia cells in retinal degeneration.

Our DNA microarray and qRT-PCR profiling data have shown significant mRNA induction and a potential functional role of DAP12 in activated microglia cells from degenerating Rs1h$^{-/Y}$ retinae. It is interesting that as an adaptor protein, DAP12 is known to cooperate with several cell-surface receptors to control leukocyte functions. DAP12-interacting receptors include members of the TREM family.[11, 36] Stimulation of one of these receptors, TREM-2, causes DAP12 phosphorylation and increased migratory and phagocytic activity of brain microglia. Conversely, knockdown of TREM-2 leads to impaired clearance of apoptotic neurons and

increased secretion of proinflammatory cytokines.[16] Showing altered synaptic functions in the brain of DAP12-deficient mice, Roumier et al.[15] also identified predominant expression of DAP12 in amoeboid microglia during hippocampal development. Thus, DAP12 and its partner TREM-2 may fulfill a regulatory role in microglia to control phagocytosis of dying neurons without strong concomitant immune activation. In support of this hypothesis, Hamerman et al.[12] and Turnbull et al.[13] found that DAP12 can attenuate macrophage functions and cytokine production in response to TLR agonists such as LPS. We have also found a dramatic increase of TREM-2 mRNA levels in activated microglia by DNA microarray analysis. However, whether DAP12 and TREM-2 have an anti-inflammatory and microglia-controlling function in the retina remains to be determined. To address this question will be especially important, as DAP12 might have activating and inhibitory functions, depending on selective associations with other receptors and/or receptor/ ligand avidities caused by different triggers.

In activated microglia, we have identified a PU.1-dependent regulatory network consisting of 16 genes including DAP12. In addition to the bioinformatic evidence of PU.1 binding sites in the conserved promoter regions of a number of species, several lines of evidence from our *in vitro* and *in vivo* promoter assays suggest that the DAP12 promoter is a direct target for PU.1. Supporting our data, Henkel et al.[37] found that retroviral re-expression of PU.1 into PU.1 null monocytic precursor cells leads to a strong and fast induction of DAP12 mRNA levels as identified by substractive hybridization. There was no detectable expression of DAP12 in PU.1-deficient cells, and the specific rescue of PU.1 was sufficient to restore DAP12 expression, indicating direct interaction of PU.1 with the DAP12 promoter. Furthermore, similar cellular phenotypes and antigen expression of mature macrophages in ectopic DAP12- and PU.1-expressing myeloid progenitors indicate that DAP12 may be a target of PU.1 in the control of macrophage maturation and activity. [14]

In addition to our mRNA data showing increased expression of PU.1 in activated microglia from Rs1h$^{-/Y}$ retinae, little information is available about PU.1 expression in microglia. In line with our observations, high expression levels of PU.1 immunoreactivity were identified in the hemispheres of hypoxia-injured hippocampus of rats.[38] It is notable that the authors speculate that the increased PU.1 protein levels in the infarcted cortex may be more likely a result of microglia proliferation than activation. However, gene expression patterns of PU.1 target genes and activation markers would be useful to fully understand the role of PU.1 in hypoxia-induced brain injury. Based on our data and the data from the literature, we propose that PU.1 is critical for the myeloid-specific transcriptional regulation

of the murine DAP12 gene. Moreover, as indicated by our gene expression profiles, PU.1 may also play a general role in the regulation of microglia genes during retinal degeneration. Therefore, further characterization of the PU.1 regulatory network in microglia cells is required to better understand the molecular mechanisms of microglia activation in degenerative disorders.

ACKNOWLEDGMENTS

Work in the laboratory of M. R. is supported by the Deutsche Forschungsgemeinschaft (Re1310/7). The project was funded by the grant "Microglia activation in retinal degeneration" from the Deutsche Forschungsgemeinschaft (La1203/4-1) to T. Langmann. We are grateful to Harinder Singh for providing PUER cells and to Prof. Ralph Lucius for providing the BV-2 cell line. We thank M. Seifert, A. Hahn, and C. Zinser (Genomatix Software GmbH) and C. Moehle and T. Stempfl (Competence Center for Fluorescent Bioanalysis Regensburg) for support in ChIP-Chip analysis.

LITERATURE

1. Streit WJ. Microglia as neuroprotective, immunocompetent cells of the CNS. *Glia* 2002; **40**(2)**:** 133-139.
2. Schuetz E, Thanos S. Microglia-targeted pharmacotherapy in retinal neurodegenerative diseases. *Current drug targets* 2004; **5**(7)**:** 619-627.
3. Langmann T. Microglia activation in retinal degeneration. *J Leukoc Biol* 2007; **81**(6)**:** 1345-1351.
4. Ahmed F, Brown KM, Stephan DA, Morrison JC, Johnson EC, Tomarev SI. Microarray analysis of changes in mRNA levels in the rat retina after experimental elevation of intraocular pressure. *Investigative ophthalmology & visual science* 2004; **45**(4)**:** 1247-1258.
5. Chen L, Wu W, Dentchev T, Zeng Y, Wang J, Tsui I *et al.* Light damage induced changes in mouse retinal gene expression. *Experimental eye research* 2004; **79**(2)**:** 239-247.
6. Steele MR, Inman DM, Calkins DJ, Horner PJ, Vetter ML. Microarray analysis of retinal gene expression in the DBA/2J model of glaucoma. *Investigative ophthalmology & visual science* 2006; **47**(3)**:** 977-985.
7. Vazquez-Chona F, Song BK, Geisert EE, Jr. Temporal changes in gene expression after injury in the rat retina. *Investigative ophthalmology & visual science* 2004; **45**(8)**:** 2737-2746.
8. Gehrig A, Langmann T, Horling F, Janssen A, Bonin M, Walter M *et al.* Genome-wide expression profiling of the retinoschisin-deficient retina in early postnatal mouse development. *Investigative ophthalmology & visual science* 2007; **48**(2)**:** 891-900.
9. Weber BH, Schrewe H, Molday LL, Gehrig A, White KL, Seeliger MW *et al.* Inactivation of the murine X-linked juvenile retinoschisis gene, Rs1h, suggests a role of retinoschisin in retinal cell layer organization and synaptic structure. *Proc Natl Acad Sci U S A* 2002; **99**(9)**:** 6222-6227.
10. Takaki R, Watson SR, Lanier LL. DAP12: an adapter protein with dual functionality. *Immunological reviews* 2006; **214:** 118-129.
11. Klesney-Tait J, Turnbull IR, Colonna M. The TREM receptor family and signal integration. *Nat Immunol* 2006; **7**(12)**:** 1266-1273.
12. Hamerman JA, Tchao NK, Lowell CA, Lanier LL. Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12. *Nat Immunol* 2005; **6**(6)**:** 579-586.
13. Turnbull IR, Gilfillan S, Cella M, Aoshi T, Miller M, Piccio L *et al.* Cutting edge: TREM-2 attenuates macrophage activation. *J Immunol* 2006; **177**(6)**:** 3520-3524.
14. Aoki N, Kimura S, Takiyama Y, Atsuta Y, Abe A, Sato K *et al.* The role of the DAP12 signal in mouse myeloid differentiation. *J Immunol* 2000; **165**(7)**:** 3790-3796.
15. Roumier A, Bechade C, Poncer JC, Smalla KH, Tomasello E, Vivier E *et al.* Impaired synaptic function in the microglial KARAP/DAP12-deficient mouse. *J Neurosci* 2004; **24**(50)**:** 11421-11428.
16. Takahashi K, Rochford CD, Neumann H. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. *J Exp Med* 2005; **201**(4)**:** 647-657.
17. Blasi E, Barluzzi R, Bocchini V, Mazzolla R, Bistoni F. Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. *Journal of neuroimmunology* 1990; **27**(2-3)**:** 229-237.
18. Walsh JC, DeKoter RP, Lee HJ, Smith ED, Lancki DW, Gurish MF *et al.* Cooperative and antagonistic interplay between PU.1 and GATA-2 in the specification of myeloid cell fates. *Immunity* 2002; **17**(5)**:** 665-676.
19. Kim DC, Kim SH, Jeong MW, Baek NI, Kim KT. Effect of rottlerin, a PKC-delta inhibitor, on TLR-4-dependent activation of murine microglia. *Biochem Biophys Res Commun* 2005; **337**(1)**:** 110-115.
20. Langmann T, Moehle C, Mauerer R, Scharl M, Liebisch G, Zahn A *et al.* Loss of detoxification in inflammatory bowel disease: dysregulation of pregnane X receptor target genes. *Gastroenterology* 2004; **127**(1)**:** 26-40.
21. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C *et al.* Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nature genetics* 2001; **29**(4)**:** 365-371.
22. Langmann T, Mauerer R, Zahn A, Moehle C, Probst M, Stremmel W *et al.* Real-time reverse transcription-PCR expression profiling of the complete human ATP-binding cassette transporter superfamily in various tissues. *Clinical chemistry* 2003; **49**(2)**:** 230-238.

23. Langmann T, Porsch-Ozcurumez M, Heimerl S, Probst M, Moehle C, Taher M *et al.* Identification of sterol-independent regulatory elements in the human ATP-binding cassette transporter A1 promoter: role of Sp1/3, E-box binding factors, and an oncostatin M-responsive element. *J Biol Chem* 2002; **277**(17)**:** 14443-14450.
24. Rehli M, Poltorak A, Schwarzfischer L, Krause SW, Andreesen R, Beutler B. PU.1 and interferon consensus sequence-binding protein regulate the myeloid expression of the human Toll-like receptor 4 gene. *J Biol Chem* 2000; **275**(13)**:** 9773-9781.
25. Pham TH, Langmann S, Schwarzfischer L, El Chartouni C, Lichtinger M, Klug M *et al.* CCAAT enhancer-binding protein beta regulates constitutive gene expression during late stages of monocyte to macrophage differentiation. *J Biol Chem* 2007; **282**(30)**:** 21924-21933.
26. Kawaji H, Kasukawa T, Fukuda S, Katayama S, Kai C, Kawai J *et al.* CAGE Basic/Analysis Databases: the CAGE resource for comprehensive promoter analysis. *Nucleic Acids Res* 2006; **34**(Database issue)**:** D632-636.
27. Rogler G, Hausmann M, Vogl D, Aschenbrenner E, Andus T, Falk W *et al.* Isolation and phenotypic characterization of colonic macrophages. *Clin Exp Immunol* 1998; **112**(2)**:** 205-215.
28. Feinman R, Qiu WQ, Pearse RN, Nikolajczyk BS, Sen R, Sheffery M *et al.* PU.1 and an HLH family member contribute to the myeloid-specific transcription of the Fc gamma RIIIA promoter. *The EMBO journal* 1994; **13**(16)**:** 3852-3860.
29. Moulton KS, Semple K, Wu H, Glass CK. Cell-specific expression of the macrophage scavenger receptor gene is dependent on PU.1 and a composite AP-1/ets motif. *Mol Cell Biol* 1994; **14**(7)**:** 4408-4418.
30. O'Reilly D, Quinn CM, El-Shanawany T, Gordon S, Greaves DR. Multiple Ets factors and interferon regulatory factor-4 modulate CD68 expression in a cell type-specific manner. *J Biol Chem* 2003; **278**(24)**:** 21909-21919.
31. Zhang DE, Hetherington CJ, Chen HM, Tenen DG. The macrophage transcription factor PU.1 directs tissue-specific expression of the macrophage colony-stimulating factor receptor. *Mol Cell Biol* 1994; **14**(1)**:** 373-381.
32. Tenen DG, Hromas R, Licht JD, Zhang DE. Transcription factors, normal myeloid development, and leukemia. *Blood* 1997; **90**(2)**:** 489-519.
33. Roque RS, Caldwell RB. Isolation and culture of retinal microglia. *Current eye research* 1993; **12**(3)**:** 285-290.
34. Albright AV, Gonzalez-Scarano F. Microarray analysis of activated mixed glial (microglia) and monocyte-derived macrophage gene expression. *Journal of neuroimmunology* 2004; **157**(1-2)**:** 27-38.
35. Sasmono RT, Oceandy D, Pollard JW, Tong W, Pavli P, Wainwright BJ *et al.* A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. *Blood* 2003; **101**(3)**:** 1155-1163.
36. Turnbull IR, Colonna M. Activating and inhibitory functions of DAP12. *Nat Rev Immunol* 2007; **7**(2)**:** 155-161.
37. Henkel GW, McKercher SR, Maki RA. Identification of three genes up-regulated in PU.1 rescued monocytic precursor cells. *International immunology* 2002; **14**(7)**:** 723-732.
38. Walton MR, Gibbons H, MacGibbon GA, Sirimanne E, Saura J, Gluckman PD *et al.* PU.1 expression in microglia. *Journal of neuroimmunology* 2000; **104**(2)**:** 109-115.

Supplemental Table 1. List of significantly regulated genes analyzed by DNA-microrrays in Rs1h$^{-/Y}$ versus wild-type *ex vivo* microglia.

Nr.	Unigene	Name	Full gene name	Fold change
1	Mm.3020	Pparg	peroxisome proliferator activated receptor gamma	147,0
2	Mm.46561	Lect1	leukocyte cell derived chemotaxin 1	97,0
3	Mm.1282	Ccl3	chemokine (C-C motif) ligand 3	39,4
4	Mm.218981	Lmo7	LIM domain only 7	34,3
5	Mm.329663	Tmem151	transmembrane protein 151	32,0
6	Mm.235998	S100b	S100 protein, beta polypeptide, neural	29,9
7	Mm.20079	Calml3	calmodulin-like 3	26,0
8	Mm.45071	Comp	cartilage oligomeric matrix protein	17,1
9	Mm.261623	Trem2	triggering receptor expressed on myeloid cells 2	17,1
10	Mm.118034	Bmp5	bone morphogenetic protein 5	16,0
11	Mm.153911	Lcp1	lymphocyte cytosolic protein 1	13,0
12	Mm.235324	Csf2rb1	colony stimulating factor 2 receptor, beta 1	12,1
13	Mm.12966	S3-12	plasma membrane associated protein, S3-12	11,3
14	Mm.371583	Slpi	secretory leukocyte peptidase inhibitor	11,3
15	---	Cspg4	chondroitin sulfate proteoglycan 4	11,3
16	Mm.1763	Msx2	homeo box, msh-like 2	10,6
17	Mm.248327	Clec4e	C-type lectin domain family 4, member e	10,6
18	Mm.46301	Tyrobp	TYRO protein tyrosine kinase binding protein	9,8
19	Mm.18628	Cd36	CD36 antigen	9,8
20	Mm.416152	Cd200r1	CD200 receptor 1	9,2
21	Mm.103765	Clec5a	C-type lectin domain family 5, member a	9,2
22	Mm.15819	Cd68	CD68 antigen	9,2
23	Mm.329997	Itga8	integrin alpha 8	8,6
24	Mm.214554	Grb14	growth factor receptor bound protein 14	8,6
25	Mm.244263	Ccl4	chemokine (C-C motif) ligand 4	8,0
26	Mm.179747	Itga7	integrin alpha 7	7,5
27	Mm.3460	Cd14	CD14 antigen	7,5
28	Mm.250256	Enpp2	ectonucleotide pyrophosphatase/phosphodiesterase 2	7,0
29	Mm.28209	Perp	PERP, TP53 apoptosis effector	7,0
30	Mm.378894	Runx3	runt related transcription factor 3	6,5
31	Mm.22574	Csf1r	colony stimulating factor 1 receptor	6,5
32	Mm.2068	Ncf4	neutrophil cytosolic factor 4	6,1
33	Mm.185355	Cd300lb	CD300 antigen like family member B	6,1
34	Mm.19287	Cart1	cartilage homeo protein 1	5,7

Supplemental Table 1. List of significantly regulated genes analyzed by DNA-microrrays in Rs1h$^{-/Y}$ versus wild-type *ex vivo* microglia. *(Continued)*

Nr.	Unigene	Name	Full gene name	Fold change
35	Mm.188157	Cd72	CD72 antigen	5,7
36	Mm.269747	Ltbp1	latent transforming growth factor beta binding protein 1	5,7
37	Mm.24130	Cd52	CD52 antigen	5,7
38	Mm.3619	Ctss	cathepsin S	5,7
39	Mm.235193	P2ry6	pyrimidinergic receptor P2Y, G-protein coupled, 6	5,3
40	Mm.239291	Msr1	macrophage scavenger receptor 1	5,3
41	Mm.15383	Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b	5,3
42	Mm.22119	Fcgr3	Fc receptor, IgG, low affinity III	5,3
43	Mm.23208	Shroom4	shroom family member 4	4,9
44	Mm.245151	Ltc4s	leukotriene C4 synthase	4,9
45	Mm.57035	Itga3	integrin alpha 3	4,9
46	Mm.2706	Atf3	activating transcription factor 3	4,9
47	Mm.20916	Wfs1	Wolfram syndrome 1 homolog	4,6
48	Mm.27154	Vnn1	vanin 1	4,6
49	Mm.38274	Chi3l1	chitinase 3-like 1	4,6
50	Mm.3900	Ltbp2	latent transforming growth factor beta binding protein 2	4,6
51	Mm.34159	Cobll1	Cobl-like 1	4,6
52	Mm.425062	Fcgr2b	Fc receptor, IgG, low affinity IIb	4,6
53	Mm.3064	Cfp	complement factor properdin	4,3
54	Mm.289824	Il1rl1	interleukin 1 receptor-like 1	4,3
55	Mm.276440	Ifitm6	interferon induced transmembrane protein 6	4,0
56	Mm.2899	Penk1	preproenkephalin 1	4,0
57	Mm.2923	Il2rg	interleukin 2 receptor, gamma chain	4,0
58	Mm.4938	Fmn1	formin 1	4,0
59	Mm.4871	Timp3	tissue inhibitor of metalloproteinase 3	3,7
60	Mm.1302	Sfpi1	SFFV proviral integration 1	3,7
61	Mm.234832	Igsf4a	immunoglobulin superfamily, member 4A	3,5
62	Mm.31748	Nox4	NADPH oxidase 4	3,5
63	Mm.289681	Hbegf	heparin-binding EGF-like growth factor	3,5
64	Mm.292547	Ptgs2	prostaglandin-endoperoxide synthase 2	3,5
65	Mm.35806	Crct1	cysteine-rich C-terminal 1	3,5
66	Mm.45436	Lyzs	lysozyme	3,5

Supplemental Table 1. List of significantly regulated genes analyzed by DNA-microrrays in Rs1h$^{-/Y}$ versus wild-type *ex vivo* microglia. *(Continued)*

Nr.	Unigene	Name	Full gene name	Fold change
67	Mm.46455	Osgin2	oxidative stress induced growth inhibitor family member 2	3,2
68	Mm.329560	Nfatc1	nuclear factor of activated T-cells	3,2
69	Mm.232930	Nrn1	neuritin 1	3,2
70	Mm.211096	Sorbs2	sorbin and SH3 domain containing 2	3,2
71	Mm.134093	Sema3e	semaphorin 3E	2,8
72	Mm.245851	Cd200	Cd200 antigen	2,8
73	Mm.335020	Bmper	BMP-binding endothelial regulator	2,8
74	Mm.34885	Hspb6	heat shock protein, alpha-crystallin-related, B6	2,8
75	Mm.269254	Ccrl1	Chemokine (C-C motif) receptor-like 1	2,6
76	Mm.2675	Pdgfa	platelet derived growth factor, alpha	2,6
77	Mm.1442	Bdnf	brain derived neurotrophic factor	2,6
78	Mm.30466	Trps1	trichorhinophalangeal syndrome I	2,6
79	Mm.316210	Fat4	FAT tumor suppressor homolog 4	2,6
80	Mm.38002	Rarres1	retinoic acid receptor responder (tazarotene induced) 1	2,3
81	Mm.265264	Ank	progressive ankylosis	2,3
82	Mm.181074	Ccdc80	coiled-coil domain containing 80	2,3
83	Mm.41423	Endod1	endonuclease domain containing 1	2,3
84	Mm.244215	Gls	glutaminase	2,3
85	Mm.20973	Lgals7	lectin, galactose binding, soluble 7	2,3
86	Mm.381	Adfp	adipose differentiation related protein	2,3
87	Mm.338790	Prg1	proteoglycan 1	2,3
88	Mm.290991	Cpne8	copine VIII	2,1
89	Mm.266341	Nrp2	Neuropilin 2	2,1
90	Mm.410189	Txnip	thioredoxin interacting protein	2,1
91	Mm.23837	Lrrc8d	leucine rich repeat containing 8D	2,0
92	Mm.247265	Cd59a	CD59a antigen	2,0
93	Mm.340818	Atp6v0a1	ATPase, H+ transporting, lysosomal V0 subunit A1	2,0
94	Mm.248615	Lgals3	lectin, galactose binding, soluble 3	2,0
1	Mm.390681	P2ry5	purinergic receptor P2Y, G-protein coupled, 5	-2,1
2	Mm.196382	Rsnl2	restin-like 2	-2,3
3	Mm.392123	Pml	promyelocytic leukemia	-2,5
4	Mm.275893	Mpa2l	macrophage activation 2 like	-2,5
5	Mm.423137	Arrdc3	arrestin domain containing 3	-2,5

Supplemental Table 1. List of significantly regulated genes analyzed by DNA-microrrays in Rs1h$^{-/Y}$ versus wild-type *ex vivo* microglia. *(Continued)*

Nr.	Unigene	Name	Full gene name	Fold change
6	Mm.27757	Bmp1	bone morphogenetic protein 1	-2,5
7	Mm.426080	Add3	adducin 3 (gamma)	-2,5
8	Mm.170521	Glis3	GLIS family zinc finger 3	-2,5
9	Mm.9474	Twist2	twist homolog 2 (Drosophila)	-2,5
10	Mm.278689	Slfn2	schlafen 2	-2,8
11	Mm.212395	Rai14	retinoic acid induced 14	-3,0
12	Mm.333375	C1r	complement component 1, r subcomponent	-3,0
13	Mm.132196	Zfp202	zinc finger protein 202	-3,0
14	Mm.68819	Uaca	uveal autoantigen with coiled-coil domains and ankyrin repeats	-3,0
15	Mm.426079	Ifit3	interferon-induced protein with tetratricopeptide repeats 3	-3,5
16	Mm.28498	Nod1	nucleotide-binding oligomerization domain containing 1	-3,5
17	Mm.261122	Plekha2	pleckstrin homology domain-containing, family A member 2	-3,5
18	Mm.172346	Tgfbr2	transforming growth factor, beta receptor II	-3,7
19	Mm.274926	Emb	embigin	-3,7
20	Mm.29597	Cd248	CD248 antigen, endosialin	-3,7
21	Mm.24208	Il13ra1	interleukin 13 receptor, alpha 1	-4,0
22	Mm.368330	Il13ra2	interleukin 13 receptor, alpha 2	-4,3
23	Mm.6718	Ifit1	interferon-induced protein with tetratricopeptide repeats 1	-4,3
24	Mm.4660	Cxcl5	chemokine (C-X-C motif) ligand 5	-4,3
25	Mm.146194	Irak3	interleukin-1 receptor-associated kinase 3	-4,6
26	Mm.27385	Gsdmdc1	gasdermin domain containing 1	-4,6
27	Mm.271275	Ifi27	interferon, alpha-inducible protein 27	-4,6
28	Mm.46662	Dact1	dapper homolog 1, antagonist of beta-catenin	-4,9
29	Mm.29798	Cd34	CD34 antigen	-4,9
30	---	Islr	immunoglobulin superfamily containing leucine-rich repeat	-5,3
31	---	Iigp2	interferon inducible GTPase 2	-5,3
32	Mm.271854	Lrp1	low density lipoprotein receptor-related protein 1	-5,7
33	Mm.89940	Adam19	a disintegrin and metallopeptidase domain 19	-7,0
34	Mm.303231	Cxcl12	chemokine (C-X-C motif) ligand 12	-7,5
35	Mm.112933	Adamts5	aggrecanase-2	-7,5
36	Mm.271897	Sned1	sushi, nidogen and EGF-like domains 1	-8,6

Supplemental Table 1. List of significantly regulated genes analyzed by DNA-microrrays in Rs1h$^{-/Y}$ versus wild-type *ex vivo* microglia. *(Continued)*

Nr.	Unigene	Name	Full gene name	Fold change
37	Mm.260733	Lphn1	latrophilin 1	-8,6
38	Mm.5264	Fez1	fasciculation and elongation protein zeta 1	-8,6
39	Mm.19155	Sfrp2	secreted frizzled-related protein 2	-9,2
40	Mm.434465	Ror1	receptor tyrosine kinase-like orphan receptor 1	-9,2
41	Mm.247695	Dtx4	deltex 4 homolog	-9,2
42	Mm.154660	Plat	plasminogen activator, tissue	-9,2
43	Mm.28888	Irx2	Iroquois related homeobox 2	-9,2
44	Mm.218846	Lbp	lipopolysaccharide binding protein	-12,1
45	Mm.292983	Vit	vitrin	-13,0
46	Mm.42029	Ccl8	chemokine (C-C motif) ligand 8	-14,9
47	Mm.882	Il1rn	interleukin 1 receptor antagonist	-14,9
48	Mm.182359	Il33	interleukin 33	-16,0
49	Mm.653	Cfb	complement factor B	-26,0

CHAPTER 3

TRANSCRIPTOMIC PROFILING IDENTIFIES A PU.1 REGULATORY NETWORK IN MACROPHAGES

Karin Weigelt, Monika Lichtinger, Michael Rehli, and Thomas Langmann

Biochemical and Biophysical Research Communications, 2009 Mar 6;380(2):308-12

ABSTRACT

PU.1 is a key transcription factor for hematopoiesis and macrophage differentiation. Using chromatin immunoprecipitation we have previously identified several PU.1 target genes in macrophages and microglia. With the aim to complement these studies, we performed a transcriptomic analysis of PU.1$^{-/-}$ progenitors after restoration of PU.1 activity. PUER cells committed to macrophage differentation were analyzed with novel Affymetrix exon 1.0 ST arrays and Affymetrix 430 2.0 genome arrays for crosswise validation. We combined these genome-wide expression data with a publicly-available microarray dataset of PU.1-knockdown hematopoietic stem cells for an integrated analysis. Bibliographic gene connections, binding site prediction and ChIP-Chip data were used to define a multi-level PU.1 regulatory network in macrophages. Moreover, an alternative transcript of the novel PU.1 target gene Ptpro was identified by exon arrays and PU.1 binding to an intronic promoter was demonstrated. In conclusion, we present a PU.1 transcriptional network with novel validated PU.1 target genes.

INTRODUCTION

Hematopoietic lineage development is regulated by a sophisticated interplay of a limited set of specific transcription factors.[1] PU.1, a member of the ETS family of transcription factors encoded by the gene *Sfpi1*, is required for the generation of early myeloid and lymphoid progenitors.[2, 3] PU.1 also directs the differentiation of committed myeloid progenitor cells into macrophages and neutrophils.[4, 5] Reduced PU.1 expression and lack of function are associated with a block in hematopoietic differentiation and increased proliferation leading to leukemia in humans[6] and mouse models.[7]

PU.1 is also critically involved in the regulation of macrophage-specific basal transcription,[8] including expression of the macrophage colony-stimulating factor (M-CSF) receptor encoded by the c-fms protooncogene.[9] Within the macrophage lineage, distinct cellular subsets can be defined based on the localization in specific tissues, activation status, effector functions, or marker proteins.[10] According to a new concept, macrophage cells have an extremely large plasticity, which is controlled by a regulatory network of transcription factor, including PU.1 and various other nuclear proteins.[11]

Microglia form an important population of macrophages in the CNS. However, little is known about the role of PU.1 in transcriptional programming of microglia activation. A previous study from our laboratory identified high PU.1 levels and several bioinformatically predicted PU.1 target genes in activated retinal microglia from retinoschisin-deficient ($Rs1h^{-/Y}$) mice.[12] Moreover, Walton et al. detected strong microglial PU.1 expression after hypoxic-ischemia brain injury.[13] The analysis of retinal or brain microglia from PU.1-deficient animals is hampered by the fact that null mutation of the *Sfpi1* gene in mice results in perinatal lethality.[2, 14] Walsh et al. recently developed a tunable system in which transcriptional regulation by PU.1 can be investigated using PU.1-deficient myeloid progenitors and a conditionally activatable PU.1-estrogen receptor binding domain fusion protein PUER.[15] These myeloid progenitors undergo rapid cell cycle arrest and differentiate into macrophages upon addition of 4-hydroxytamoxifen (OHT).[16]

Using chromatin immunoprecipitation coupled to microarrays (ChIP-Chip) we have previously identified 1202 putative PU.1 target genes in RAW264.7 mouse macrophages,[17] which partially overlap with differentially expressed genes of activated microglia.[12] To complement these previous studies we performed an integrated genome-wide analysis of the PU.1-dependent transcriptome in PUER

cells committed to macrophage differentation using Affymetrix mouse exon 1.0 ST arrays and Affymetrix 430 2.0 mouse genome arrays. We combined our data with a publicly-available microarray dataset of PU.1-knockdown hematopoietic stem cells and defined a novel PU.1 regulatory network in macrophages. Moreover, the *in vivo* binding of PU.1 to an intronic *protein tyrosine phosphatase receptor type O* (Ptpro) promoter was validated by ChIP and the corresponding alternative transcript was verified by quantitative real-time RT-PCR.

MATERIALS AND METHODS

Cell culture

PUER cells were cultured in IMDM (Invitrogen, Carlsbad, CA, USA), supplemented with penicillin/streptomycin (10,000 U/ml), glutamin (200 mM), β-ME (50 µM), mouse IL-3 (5 ng/ml, Biosource, Camarillo, CA, USA), puromycin (1 µg/ml), and 10% FCS as described earlier.[15] To activate the PU.1-ER fusion protein, PUER cells were cultured in medium containing 0.1 µM 4-hydroxy-tamoxifen (OHT) for the indicated time points. RAW264.7 cells were cultured in DMEM, supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin and incubated in 10% CO2 in air at 37°C.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as described previously[12, 17] using 2.5 µg polyclonal PU.1 antibody or IgG rabbit isotype control (Santa Cruz Biotechnology, Sata Cruz, CA, USA). Enrichment was analyzed by PCR with primers amplifiying the alternative Ptpro promoter region (forward, 5´-CCC AAA TGT ATG TGG TGC AA-3´; reverse, 5´-ATG TGG GCT GGG GAG AAT A-3´).

RNA isolation and reverse transcription

Total RNA was extracted according to the manufacturer's instructions using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Purity and integrity of the RNA were assessed on the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip® reagent set (Agilent Technologies, Santa Clara, CA, USA). The RNA was quantified spectrophotometrically and then stored at -80°C. First-strand cDNA synthesis was performed with the Reverse Transcription System from Stratagene (La Jolla, CA, USA).

Quantitative real-time RT-PCR

Amplifications of 50 ng cDNA were performed with the Taqman 7900HT real-time PCR detection system (Applied Biosystems, Foster City, CA, USA) in 20 µl reaction

mixtures containing 1x TaqMan Gene Expression Master Mix (Applied Biosystems), 200 nM primers, and 0.25 µl dual-labeled probe (Roche Universal Probe Library). Measurements were performed in triplicates and the results were analyzed with the $\Delta\Delta C_t$ method for relative quantitation. Normalization to three stable reference genes was performed as described earlier.[18] The primers for amplification of full-length Ptpro were F, 5´-GCC AGA AAC AGA AGG AGA GC-3´, and R, 5´-ACA GGT TTG CTT GAG TTC ACC-3´. The Ptpro short transcript form was quantified with primers F, 5´-TCC CAG TGT CCC TAC ATT CAT-3´, and R, 5´-CAC CAC GTT AGG GTT CAC CT-3´.

DNA-microarray analysis

Generation of probes, hybridization to Affymetrix GeneChip Mouse Genome 430 2.0 Arrays and Affymetrix GeneChip Mouse Exon ST 1.0 Arrays, washing, and scanning were performed according to the Affymetrix standard protocol. Triplicate microarrays of both types were carried out with RNA from PUER cells isolated before (0 h) and after (24 h) induction of the PU.1-ER fusion protein with 100nM OHT. Minimum information about a microarray experiment (MIAME) criteria were met.[19] The microarray datasets of this study are publicly available at the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) as series records GSE13125 and GSE9011.

Bioinformatic data analysis

The Affymetrix Expression Console Software Version 1.0 was used to create summarized expression values (CHP-files) from 3´ expression array and exon array feature intensities (CEL-files). The Robust Multichip Analysis (RMA) algorithm was applied to both microarray types. Exon arrays were analyzed at the extended gene level limiting the analysis to transcripts with exon-level probe sets that map to cDNA alignments. Integrative analysis of genome-wide expression activities from PUER cells and PU.1 knockdown preleukemic hematopoietic stem cells (HSCs) (GEO accession number GSE 5654) was performed with the Gene Expression Dynamics Inspector (GEDI), a Matlab (Mathworks, Natick, MA) freeware program which uses self-organizing maps (SOMs) to translate high-dimensional data into a 2D mosaic.[20] Each tile of the mosaic represents an individual SOM cluster and is color-coded to represent overexpression or underexpression of the cluster's genes, thus identifying the underlying pattern.

Differentially regulated transcripts in 24h OHT stimulated versus non-treated PUER cells were retrieved with the Genomatix ChipInspector program (Genomatix Software GmbH, Munich, Germany), applying the Significance Analysis of Microarray (SAM) algorithm. Overlapping gene groups between exon, expression

and promoter arrays were identified using Microsoft Access. Functional annotation of transcripts was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID)[21] and the Bibliosphere pathway edition (Genomatix). Identification and analysis of alternative transcripts and differential promoter usage was performed with Genomatix RegionMiner. Bibliosphere Pathway Edition which combines bibliographic co-citation with transcription factor binding site prediction and microarray expression data was used to create PU.1-, EGR2- and Gfi-1-directed regulatory networks.

RESULTS AND DISCUSSION

Genome-wide identification of PU.1 target genes using complementary microarray datasets

With the aim to identify PU.1-regulated genes in differentiating macrophages at the genome level, we performed microarray analysis of PUER cells before and after 24h induction with 100 nM OHT. These two conditions reflect PU.1-deficiency and restored PU.1 activity in the same cellular background and thereby allow dynamic monitoring of the direct transcriptomic effects of PU.1. The newest generation of Affymetrix mouse exon 1.0 ST arrays covering all exons and standard Affymetrix 430 2.0 mouse genome arrays, which detect transcritps via probes mainly in the 3´ region of mRNAs were hybridized with three replicates from each condition. The summarized expression values of PUER cells ± OHT were calculated from both array types using the RMA algorithm for extended gene level analysis (**Fig. 1**). The complete RMA datasets and all raw data (Affymetrix CEL-files) were stored in the NCBI GEO repository as records GSE13125 and GSE9011. A complimentary microarray dataset for a PU.1-knockdown (KD) transcriptome in FACS-sorted preleukemic hematopoitic stem cells (HSC) published by Steidl et al.[22] was retrieved from NCBI GEO for a direct comparative analysis. We used the Gene Expression Dynamic Inspector (GEDI) to determine the global patterns of gene expression in our PUER data and the PU.1 KD dataset. GEDI is based on self-organizing maps to identify genome-wide transcriptome activity via `gestalt´ recognition.[20] GEDI is sample-oriented rather than gene-oriented, which facilitates the recognition of genome-wide patterns. Each mosaic tile in the GEDI map represents a gene cluster that is expressed at similar levels, with red color indicating a high level and blue corresponding to low expression. In **Fig. 1A**, the GEDI maps from both Exon 1.0 ST and 430 2.0 arrays clearly show a dynamic induction of gene expression after 24 h activation of PU.1 in myeloid progenitors (**Fig. 1A**, white circles). The same positions in the HSC dataset display an inverse gene regulation, i.e. diminished expression in these gene clusters following PU.1 knockdown (**Fig. 1A**, white circles). These results

demonstrate that induction of PU.1 activity in PUER cells or PU.1 silencing have a major impact on the global pattern of gene expression in macrophage precursors and hematopoietic progenitor cells.

As obvious from **Fig. 1A**, the mosaic pictures from both microarray types hybridized with the same PUER RNA samples were not completely identical, with differences in the upper right corners of the mosaics. A larger area of highly expressed clusters was noticed in the Exon array data (**Fig. 1A**). We therefore reasoned that the performance of the two microarray platforms might be different and the number of detected transcripts could be higher with exon arrays. We used an MvA plot to compare the magnitude of change (M, log2 ratio) on the y-axis versus the average log2 signal (A) on the x-axis for both platforms after OHT induction of PUER cells (**Fig. 1B**). The grey scale of the plots indicates a higher density of differentially expressed probes on Exon 1.0 ST arrays (**Fig. 1B**). The relationship between M and A can also be described by their correlation. The Pearson´s correlation value was 0.91 for Affymetrix Exon 1.0 ST arrays and 0.96 for Affymetrix 430 2.0 data, showing more dynamic expression changes in the experiments performed with Exon 1.0 ST arrays.

We have previously shown that a single probe analysis of Affymetrix microarrays using the Genomatix ChipInspector tool with the Significance Analysis of Microarray (SAM) algorithm significantly improves detection of regulated transcripts.[17] ChipInspector analysis of data from both array types applying a false discovery rate (FDR) of 0.1%, a minimal probe coverage of 3, and a minimum log2 ratio of 2 resulted in 546 differentially expressed genes in PUER cells induced with OHT compared to control cells (**Fig. 1C**). An unexpectedly large number of 270 PU.1-regulated genes was exclusively found with Exon 1.0 ST arrays, 229 genes were identified with both arrays and only 47 genes were solely covered by 430 2.0 arrays (**Fig. 1C**). When discriminating between up-regulated and down-regulated transcripts, significantly more induced genes were found with Exon 1.0 ST arrays, which is in good accordance with our GEDI data. The higher overall number of induced versus repressed genes after OHT treatment indicates that PU.1 re-activation mainly causes stimulation of transcription. We next compared the log2 ratio distribution of the differentially expressed genes identified with Exon 1.0 ST arrays (499 genes) and 430 2.0 arrays (276 genes). In **Fig. 1D**, Exon 1.0 ST array measured transcripts were specifically enriched in the log2 ratio bins –(3-2), +(2-3), and +(3-4), whereas 430 2.0 arrays covered more strongly regulated genes. These data show that exon arrays detect more significant expression changes most likely by their broader probe coverage.

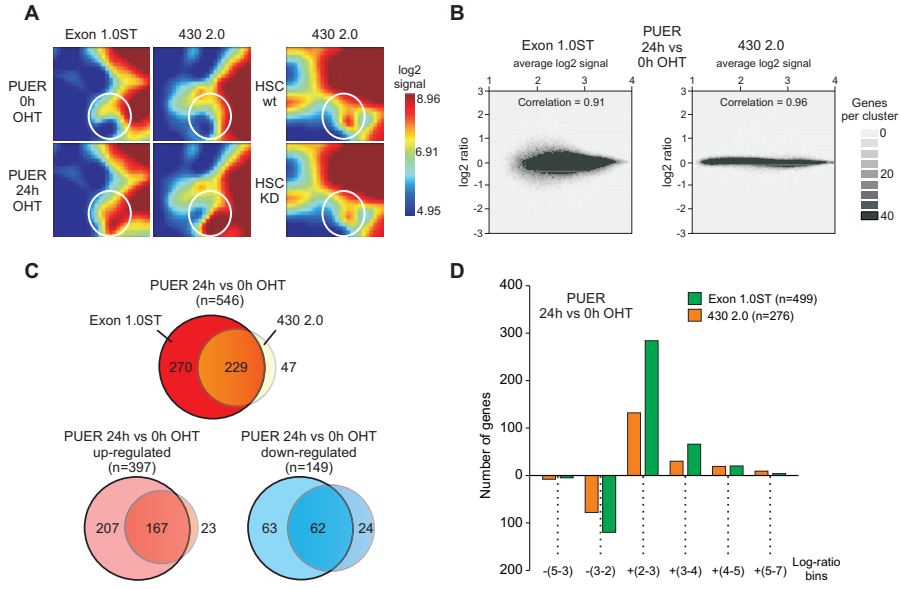


Fig.1. *Microarray expression analysis after modulation of PU.1 activity. (A) Comparative GEDI analysis of Affymetrix microarray expression signals from PUER cells before and after 24 h OHT induction (left panel) and PU.1 knockdown (KD) in hematopoietic stem cells (right panel). The HSC dataset was retrieved from GEO accession number GSE5654. (B) MvA plot of mean expression data from triplicate Exon 1.0 ST or 430 2.0 arrays from PUER cells before and after OHT treatment. (C) Genomatix ChipInspector single probe analysis of differentially expressed genes in PUER cells using data from Exon 1.0 ST or 430 2.0 arrays with a FDR <0.1%, 3 significant probes and log2 ratios > 2. (D) Log2 ratio distribution of differentially expressed genes of Exon 1.0 ST versus 430 2.0 arrays.*

Biological pathways and transcriptional network regulated by PU.1

Our next aim was to put the newly identified PU.1 regulated expression patterns into a specific biological context. We used the 229 overlapping differentially expressed genes from both Exon 1.0 ST and 430 2.0 arrays to perform a classification into functional categories with the Database for Annotation, Visualization, and Integrated Discovery (DAVID). In the up-regulated gene clusters, the major enriched functional categories were all associated with immune response, whereas gene ontology terms identified with the few down-regulated genes were related to cell cycle (**Table 1**). This implicates that the activation of a PU.1-related transcriptome promotes macrophage differentiation and cell cycle arrest, which is in agreement with the main functions of PU.1 described in the literature.[1] Various common macrophage markers including *Csf1r*, *CD14*, *F4/80*, *Btk*, *Chi3l3,* which were also identified by Laslo et al. in PUER cells treated with OHT for 4 days,[16] were strongly

induced by short-term restoration of PU.1 activity, indicating that PU.1 also activates gene expression to promote the early differentiation phase of myeloid progenitors into macrophages.

Table 1. *DAVID-classification of PU.1-regulated genes.*

Up-Regulated			
Enriched GO Term	**Genes**	**Enrichment**	***P*-value**
Immune response	23	5,31	<0.001
Immune system process	31	4,62	<0.001
Defense response	16	1,99	<0.001
Cell communication	50	1,50	<0.001
Down-Regulated			
Enriched GO Term	**Genes**	**Enrichment**	***P*-value**
Mitotic cell cycle	3	15,75	0.015
Inflammatory response	4	5,74	0.031
Response to external stimulus	6	3,94	0.016
Cell adhesion	6	2,88	0.052

In a further step, we sought to determine whether the newly identified genes regulated by re-activation of PUER cells are direct transcriptional targets of PU.1. We performed an integrated analysis of our dual microarray data from PUER cells, the complementary HSC PU.1 KD microarray dataset[22] and a genome-wide analysis of macrophage promoters bound by PU.1 using ChIP-Chip.[17] 33 genes were induced by OHT in PUER cells, repressed by PU.1 knockdown, and *in vivo* bound by PU.1 (**Supplementary Table S1**). Interestingly, none of the genes down-regulated by PU.1 induction fulfilled these criteria, indicating that PU.1 binding to response elements elicits mainly positive transcriptional activity on target genes. These data are in accordance with the overall transcript pattern of PU.1 knockdown in HSC with 225 down-regulated genes and only 97 up-regulated genes.[22]

Having identified direct transcriptional targets for PU.1, our next aim was to construct a transcriptional regulatory network orchestrated by PU.1. The Genomatix BiblioSphere tool was used to combined gene co-citations and binding site prediction in promoter regions. To include additional transcription factors in the analysis we specifically screened for nuclear proteins with a significant differential expression in OHT-treated PUER cells. Nine transcription factors were identified, including the strongly regulated genes *early growth reponse* 2 (EGR2) and *growth*

factor independent 1 (Gfi1) (**Supplementary Table S2**). A three level regulatory network could be constructed (**Fig. 2**). In the first level, PU.1 orchestrates several direct target genes reflected by the presence of enriched ChIP-Chip signals (**Fig. 2**, asterisks). In the second level, PU.1 activates EGR2 transcription, which in turn leads to up-regulation of several macrophage genes. In the third level, EGR2 represses the counter-balancing transcription factor Gfi1, which otherwise blocks myeloid and promotes neutrophil differentiation. Our regulatory network confirms earlier findings showing a cooperation of PU.1 with EGR2 and Gfi1 in macrophage cell priming.[16] In addition, our network provides a novel and precise insight into the relationship of PU.1 target genes.

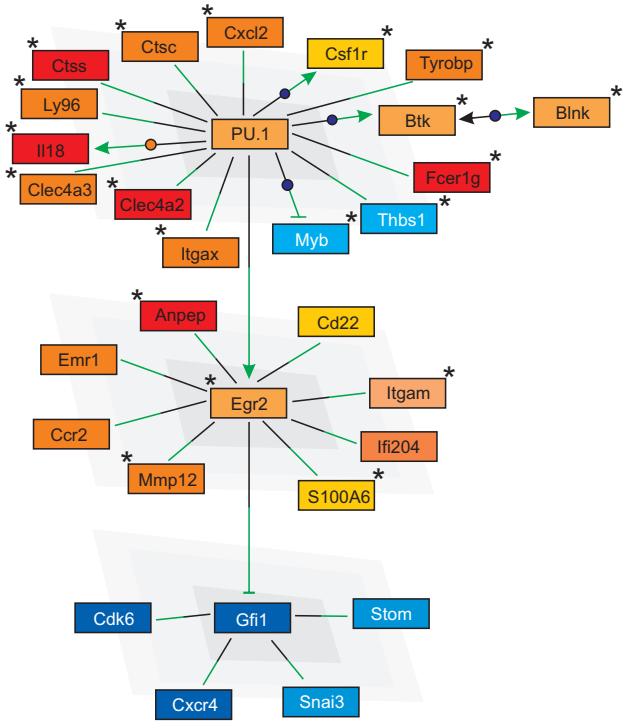


Fig.2. *Regulatory network of OHT-stimulated PUER cells with selected PU.1, EGR2 and Gfi1 target genes. Bibliographic relationships for differentially expressed gene profiles analyzed with the Genomatix Bibliosphere software tool. Arrowheads at the ends of a connecting line symbolize the type of functional relationship between the connected genes. If a gene encoding for a transcription factor is connected to a gene with binding site for this transcription factor in its promoter, the connecting line is colored green over half of its length. Experimentally verified gene-gene relationships are indicated by a circle in the centre of the connection line. Red color indicates upregulated genes and blue color indicates downregulated genes during 24 h OHT stimulation of PUER cells. Asterisks denote a positive binding in ChIP-Chip assays.*

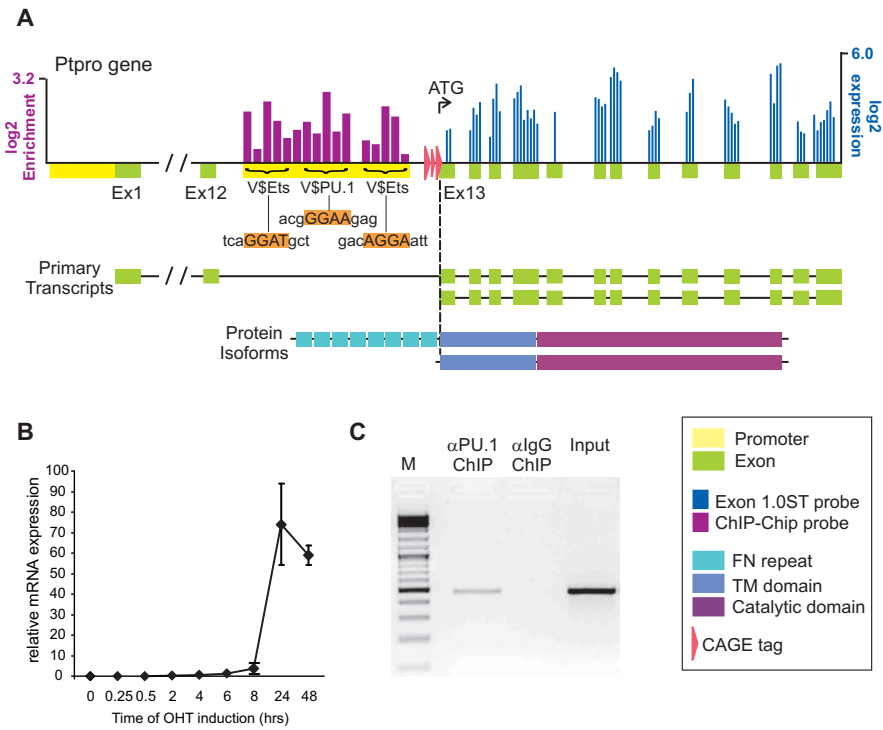


Fig.3. *PU.1 activates an alternative Ptpro transcript via an intronic promoter region. (A) Genomic structure of the Ptpro gene and log2 enrichment/expression values of tiled probes spanning the alternative promoter and an alternative transcript starting at exon 13. Significant probes are shown in purple (promoter array) and blue (exon array) above the genomic region. The primary transcripts and protein isoforms are depicted. (B) Time kinetics of Ptpro transcript expression after OHT treatment of PUER cells. The real-time qRT-PCR assay detects the alternative truncated isoform. A specific assay for the 5´-region of the long transcript form did not yield a result. Error bars indicate the standard deviation of duplicate measurements from two independent experiments. (C) Chromatin immunoprecipitation analysis of PU.1 binding to the alternative intron 12 promoter region of Ptpro. Agarose gel analysis of macrophage DNA after immunoprecipitation with anti-PU.1 or anti-IgG-control antibodies. Input DNA was used as positive control in the PCR reaction.*

PU.1 activates the transcription of Ptpro via an alternative promoter

In contrast to standard expression arrays, Exon 1.0 ST arrays allow the identification of alternative transcripts by comparing the expression signals from individual exons. Using the Genomatix ChipInspector and RegionMiner programs, we identified up-regulation of an alternative transcript of Ptpro in OHT-stimulated PUER cells (**Fig. 3**). This transcript starting at exon 13 was covered by several CAGE-tags and its expression was stimulated by PU.1 binding to the alternative promoter in intron 12 (**Fig. 3A**). The selective induction of this short Ptpro transcript form

lacking fibronectin domains was verified with independent RNAs from a time series of OHT-treated PUER cells by real-time qRT-PCR (**Fig. 3B**). In addition, the *in vivo* binding of PU.1 to the predicted ETS/PU.1 recognition sites could be validated by ChIP analysis (**Fig. 3C**). The short Ptpro isoform containing only the conserved transmembrane region and a single catalytic domain has been recently identified to promote osteoclast differentiation.[23, 24] Since PU.1 is involved in both the induction of osteoclast and macrophage differentiation, our data suggest that PU.1 regulates the promoter in intron 12 of the PTPRO gene to mediate the differentiation of macrophages and osteoclasts.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (FOR1075 Project 4) and the ProRetina Foundation. We thank Prof. Harinder Singh for providing the PUER cells and Dr. Andreas Keck and Dr. Matthias Prucha from Affymetrix UK Ltd. for providing exon arrays and Dr. Christoph Moehle and Dr. Thomas Stempfl for their support in data analysis.

LITERATURE

1. Rosenbauer F, Tenen DG. Transcription factors in myeloid development: balancing differentiation with transformation. *Nat Rev Immunol* 2007; **7**(2)**:** 105-117.
2. McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H *et al.* Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *The EMBO journal* 1996; **15**(20)**:** 5647-5658.
3. Scott EW, Fisher RC, Olson MC, Kehrli EW, Simon MC, Singh H. PU.1 functions in a cell-autonomous manner to control the differentiation of multipotential lymphoid-myeloid progenitors. *Immunity* 1997; **6**(4)**:** 437-447.
4. DeKoter RP, Singh H. Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science (New York, NY* 2000; **288**(5470)**:** 1439-1441.
5. DeKoter RP, Walsh JC, Singh H. PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors. *The EMBO journal* 1998; **17**(15)**:** 4456-4468.
6. Mueller BU, Pabst T, Osato M, Asou N, Johansen LM, Minden MD *et al.* Heterozygous PU.1 mutations are associated with acute myeloid leukemia. *Blood* 2002; **100**(3)**:** 998-1007.
7. Rosenbauer F, Wagner K, Kutok JL, Iwasaki H, Le Beau MM, Okuno Y *et al.* Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. *Nature genetics* 2004; **36**(6)**:** 624-630.
8. Ross IL, Yue X, Ostrowski MC, Hume DA. Interaction between PU.1 and another Ets family transcription factor promotes macrophage-specific Basal transcription initiation. *J Biol Chem* 1998; **273**(12)**:** 6662-6669.
9. Hume DA, Yue X, Ross IL, Favot P, Lichanska A, Ostrowski MC. Regulation of CSF-1 receptor expression. *Molecular reproduction and development* 1997; **46**(1)**:** 46-52; discussion 52-43.
10. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 2005; **5**(12)**:** 953-964.
11. Ravasi T, Wells CA, Hume DA. Systems biology of transcription control in macrophages. *Bioessays* 2007; **29**(12)**:** 1215-1226.
12. Weigelt K, Ernst W, Walczak Y, Ebert S, Loenhardt T, Klug M *et al.* Dap12 expression in activated microglia from retinoschisin-deficient retina and its PU.1-dependent promoter regulation. *J Leukoc Biol* 2007; **82**(6)**:** 1564-1574.
13. Walton MR, Gibbons H, MacGibbon GA, Sirimanne E, Saura J, Gluckman PD *et al.* PU.1 expression in microglia. *Journal of neuroimmunology* 2000; **104**(2)**:** 109-115.
14. Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science (New York, NY* 1994; **265**(5178)**:** 1573-1577.
15. Walsh JC, DeKoter RP, Lee HJ, Smith ED, Lancki DW, Gurish MF *et al.* Cooperative and antagonistic interplay between PU.1 and GATA-2 in the specification of myeloid cell fates. *Immunity* 2002; **17**(5)**:** 665-676.
16. Laslo P, Spooner CJ, Warmflash A, Lancki DW, Lee HJ, Sciammas R *et al.* Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell* 2006; **126**(4)**:** 755-766.
17. Weigelt K, Moehle C, Stempfl T, Weber B, Langmann T. An integrated workflow for analysis of ChIP-chip data. *Biotechniques* 2008; **45**(2)**:** 131-132, 134, 136 passim.
18. Ebert S, Schoeberl T, Walczak Y, Stoecker K, Stempfl T, Moehle C *et al.* Chondroitin sulfate disaccharide stimulates microglia to adopt a novel regulatory phenotype. *J Leukoc Biol* 2008; **84**(3)**:** 736-740.
19. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C *et al.* Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nature genetics* 2001; **29**(4)**:** 365-371.
20. Eichler GS, Huang S, Ingber DE. Gene Expression Dynamics Inspector (GEDI): for integrative analysis of expression profiles. *Bioinformatics (Oxford, England)* 2003; **19**(17)**:** 2321-2322.

21. Dennis G, Jr., Sherman BT, Hosack DA, Yang J, Gao W, Lane HC *et al.* DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome biology* 2003; **4**(5)**:** P3.
22. Steidl U, Rosenbauer F, Verhaak RG, Gu X, Ebralidze A, Otu HH *et al.* Essential role of Jun family transcription factors in PU.1 knockdown-induced leukemic stem cells. *Nature genetics* 2006; **38**(11)**:** 1269-1277.
23. Yang JH, Amoui M, Lau KH. Targeted deletion of the osteoclast protein-tyrosine phosphatase (PTP-oc) promoter prevents RANKL-mediated osteoclastic differentiation of RAW264.7 cells. *FEBS letters* 2007; **581**(13)**:** 2503-2508.
24. Yang JH, Amoui M, Strong DD, Lau KH. Characterization and comparison of the intronic promoter of murine osteoclastic protein-tyrosine phosphatase, PTP-oc, with the human PTP-oc promoter. *Arch Biochem Biophys* 2007; **465**(1)**:** 72-81.

Supplementary Table S1: PU.1 target genes identified with integrated microarray analysis.

Gene Symbol	Gene Name	FC Exon[1]	FC 3´-IVT[1]	FC KD[2]	FC ChIP[3]
Asahl	N-acylsphingosine amidohydrolase-like	20.0	24.3	-1.6	5.9
Blnk	B-cell linker protein	6.5	10.5	-2.5	4.2
Btk	Bruton agammaglobulinemia tyrosine kinase	6.6	4.2	-2.3	4.7
Cd14	CD14 antigen	4.6	4.2	-2.2	3.9
Cfp	Complement factor properdin	12.6	20.5	-2.6	4.7
Clec4a2	C-type lectin domain family 4, member a2	34.1	42.2	-6.0	6.8
Csf1r	Colony stimulating factor 1 receptor	4.5	4.2	-1.2	4.5
Cst3	Cystatin C	4.8	4.6	-1.5	5.6
Ctsc	Cathepsin C	21.3	41.9	-1.1	3.6
Ctss	Cathepsin S	12.9	10.6	-2.5	4.2
Cttnbp2nl	CTTNBP2 N-terminal like	10.4	6.5	-1.2	3.4
Cxcl2	Chemokine (C-X-C motif) ligand 2	8.9	9.9	-2.3	4.2
D1Ertd622e	DNA segment, Chr 1, ERATO Doi 622, expressed	7.3	7.3	-1.2	3.3
Emr4	EGF-like module containing, mucin-like, hormone receptor-like sequence 4	5.8	9.0	-1.3	3.0
Epsti1	Epithelial stromal interaction 1	4.3	4.5	-1.5	3.1
Evi2a	Ecotropic viral integration site 2a	5.0	5.8	-1.3	5.3
Igsf6	Immunoglobulin superfamily member 6	4.7	6.0	-3.1	4.9
Il18	Interleukin 18	25.8	108.4	-2.0	4.0
Itgax	Integrin alpha X	10.5	5.6	-1.2	4.2
Lpxn	Leupaxin	4.5	5.5	-1.0	5.0
Ly96	Lymphocyte antigen 96	6.6	4.4	-2.3	5.0
Ms4a6d	Membrane-spanning 4-domains, subfamily A, member 6D	4.3	6.3	-2.0	4.4
Pdxk	Pyridoxal kinase	4.4	4.1	-1.3	11.8
Prkcd	Protein kinase C delta	4.6	4.9	-1.9	4.5
Rgs18	Regulator of G-protein signaling 18	8.3	13.5	-1.2	3.4
Serpinb12	Serine (or cysteine) peptidase inhibitor, clade B (ovalbumin), member 12	16.9	47.5	-1.3	5.6
Skap2	Src family associated phosphoprotein 2	6.4	5.8	-1.6	3.2
Sla	Src-like adaptor	19.0	12.8	-2.2	4.3
Snx20	Sorting nexin 20	7.7	4.0	-1.2	5.9
Tlr3	Toll-like receptor 3	9.5	5.3	-1.2	3.9
Tmem106a	Transmembrane protein 106A	4.7	4.0	-1.2	3.1
Tyrobp	TYRO protein tyrosine kinase binding protein	21.3	53.5	-1.8	3.6
Wdfy2	WD repeat and FYVE domain containing 2	6.5	5.2	-1.4	3.9

Gene expression omnibus (GEO) datasets: [1]GSE13125, [2]GSE5654, [3]GSE9011
Abrreviations: FC, fold change; Exon, Affymetrix mouse Exon 1.0 ST array; 3´-IVT, Affymetrix mouse expression 430 2.0 array; KD, knock-down; ChIP, Chromatin immunoprecipitation / Affymetrix mouse promoter 1.0R array

Supplementary Table S2: Transcription factors identified by BiblioSphere analysis

Gene Symbol	Gene name	FC (Exon array)
Fos	FBJ osteosarcoma oncogene	14.6
Egr2	Early growth response 2	9.7
Jun	Jun oncogene	7.0
Egr1	Early growth response 1	5.4
Nfyb	Nuclear transcription factor-Y beta	4.3
Pparg	Peroxisome proliferator activated receptor gamma	-4.3
Snai3	Snail homolog 3 (Drosophila)	-5.8
Myb	Myeloblastosis oncogene	-8.3
Gfi1	Growth factor independent 1	-10.0

Abbreviations: FC, fold change

CHAPTER 4

TREM-1 AND DAP12 EXPRESSION IN MONOCYTES OF PATIENTS WITH SEVERE PSYCHIATRIC DISORDERS. EGR3, ATF3 AND PU.1 AS IMPORTANT TRANSCRIPTION FACTORS.

Karin Weigelt, Livia A. Carvalho, Roos C. Drexhage, Annemarie Wijkhuijs, Harm de Wit, Nico J.M. van Beveren, Tom K. Birkenhäger, Veerle Bergink, and Hemmo A. Drexhage

Brain, Behavior, and Immunity, 2011 Aug;25(6):1162-9

ABSTRACT:

Introduction: Immune activation is a characteristic of schizophrenia (SCZ), bipolar disorder (BD) and unipolar major depressive disorder (MDD). The triggering receptor expressed on myeloid cells 1 (TREM-1), its' adaptor molecule DAP12 and their transcription factor (TF) PU.1 are important key genes in inflammation and expressed in activated monocytes and microglia.

Aim: To test 1) if the expressions of TREM-1, DAP12 and PU.1 are increased in monocytes of patients with severe psychiatric disorders and 2) If PU.1 and the TFs ATF3 and EGR3 (which have been found as prominent increased monocyte genes in previous studies) are involved in the regulation of TREM-1 and DAP12 expression.

Methods: Using Q-PCR, we studied the gene expression of TREM-1, DAP12, PU.1, ATF3 and EGR3 in the monocytes of 73 patients with severe psychiatric disorders (27 recent onset SCZ patients, 22 BD patients and 24 MDD patients) and of 79 healthy controls (HC). Using *in silico* TF binding site prediction and *in vivo* chromatin immunoprecipitation (ChIP), we studied the actual binding of EGR3, ATF3 and PU.1 to the promoter regions of TREM-1 and DAP12.

Results:

1. TREM-1 gene expression was increased in the monocytes of SCZ and BD patients and tended to be increased in the monocytes of MDD patients.
2. DAP12 expression levels were neither increased in the monocytes of SCZ, BD, nor MDD patients.
3. PU.1 expression levels were increased in the monocytes of MDD patients, but not in those of SCZ and BD patients
4. TREM-1 expression levels correlated in particular to ATF3 and EGR3 expression levels, DAP12 expression levels correlated in particular to PU.1 expression levels.
5. We found using binding site prediction and ChIP assays that the TFs EGR3 and ATF3 indeed bound to the TREM-1 promoter, PU.1 bound to both the TREM-1 and DAP12 promoter.

Conclusion: In this study, we provide evidence that TREM-1 gene expression is significantly increased in monocytes of SCZ and BD patients and that the TREM-1 gene is a target gene of the TFs ATF3 and EGR3. In MDD patients, PU.1 gene expression was increased with a tendency for TREM-1 gene over expression. Our observations support the concept that monocytes are in a pro-inflammatory state in severe psychiatric conditions and suggest differences in monocyte inflammatory set points between SCZ, BD and MDD.

INTRODUCTION

Mounting evidence indicates that chronic deregulation of the monocyte-macrophage system in schizophrenia (SCZ), bipolar disorder (BD) and major depressive disorder (MDD) leads to a deregulated production of cytokines and inflammatory compounds destabilizing brain development and function via an altered activation state of the microglia, the resident macrophages of the nervous system.[1] The immune-induced aberrant brain development and function would allow various environmental factors (e.g. stress) to precipitate major psychiatric episodes. This concept was originally introduced as the "Macrophage or cytokine theory of depression and schizophrenia".[2, 3]

Raised levels of macrophage cytokines (such as IL-1β, IL-6 and TNF) have indeed been reported in serum, plasma and cerebral spine fluid of SCZ, BD and MDD patients[4, 5] and there are studies showing that administration of pro-inflammatory cytokines can activate "sickness behaviors"[6] and depressive symptoms,[7] which can be partially abrogated by pre-treatment with antidepressant medications. Furthermore, IFN-α and IL-2 treatment are able to elicit depressive symptoms and mania[8] and injecting endotoxin or vaccination with *S. typhi* showed that such inflammatory challenges induce in humans feelings of social disconnection and cognitive disturbances in addition to depressed mood.[9-11] Also in animal models cytokines like IL-1β and TNF are capable of inducing distinct behavioural changes that are reminiscent of the symptoms of mood disorder and psychosis.[12]
The concept that microglia is involved in the regulation of behaviour was recently supported by Chen et al. showing that the transplantation of wild type (WT) bone marrow cures Hox8b mutant mice of pathological grooming.[13] Since the Hox8b lineage contributes to bone marrow-derived microglia in the brain, these findings indeed support intriguing connections between monocyte-macrophage-microglia dysfunction and neuropsychiatric disorders. The concept of neuro-immune deregulation in psychiatric disorders is further supported by the finding in PET-scan of activated microglia in the hippocampus of SCZ patients.[14]

Triggering receptor expressed on myeloid cells 1 (TREM-1), its *adaptor protein DNAX-activation protein of 12 kDA* (DAP12) and the transcription factor *Purine-rich Box-1* (PU.1) - a regulator of DAP12 and TREM-1 expression – are genes that are expressed in both activated monocytes[15] and microglia.[16, 17] TREM-1, DAP12 and PU.1 have been described as key genes in inflammatory processes.[18-22] Activation of primary microglia with Borrelia burgdorferi induced profound changes in the TREM-1 pathway.[16] Increased DAP12 expression was found in the brains of SCZ patients with a long duration of illness.[23]

We therefore here investigate using Q-PCR the expression levels of TREM-1, DAP12 and PU.1 in monocytes of 73 patients with psychiatric disorders (27 SCZ, 22 BD and 24 MDD patients) and 79 age/gender matched healthy controls (HC). The actual binding ability of PU.1 to the TREM-1 and DAP12 promoter was studied using *in silico* binding site prediction software and *in vivo* chromatin immunoprecipitation (ChIP) assays.

In a previous study on gene expression levels in monocytes of patients with SCZ or BD [24, 25] we found the transcription factors/regulators ATF3, EGR3, MAFF, MXD1 and NAB2 increased. The increase of these transcription factors/regulators correlated to the increased gene expression of important pro-inflammatory cytokines such as IL-1B, TNF and IL-6 in the monocytes, suggesting a key role of these transcription factors/regulators in the inflammatory state of patient monocytes. We therefore additionally investigated the putative binding of ATF3, EGR3, MAFF, MXD1, NAB2 to the TREM-1 and DAP12 promoter using *in silico* binding site prediction software and *in vivo* ChIP.

MATERIALS AND METHODS

Patients

The study protocol was approved by the institutional review boards of the University Medical Center Rotterdam and Utrecht. All patients provided written informed consent before participation. All patients were diagnosed according to the DSM-IV criteria. For patients with bipolar disorder and major depressive disorder the Structural Clinical Interview for DSM-IV (SCID)[26] was used; for patients with schizophrenia the Comprehensive Assessment of Symptoms and History (CASH) interview.[27] Patients did not suffer from another severe medical illness, verified with a medical history assessment and routinely laboratory testing (Hb, Ht, leukocyte count, blood smear and kidney/liver function). Demographics and medication use of all 73 patients are summarized in **Table 1**.

Patients with schizophrenia (n=27) were recruited at the department of Psychiatry of the Erasmus Medical Center in Rotterdam. All patients were inpatients and psychotic at inclusion. The majority of patients with schizophrenia were recent onset cases and had a median duration of illness of 0.3 yrs. In those patients with symptoms less than 6 months, a final diagnosis was made after 6 months to comply with the DSM-IV criteria. All but one of the patients received antipsychotic medication at the time of blood withdrawal; none of the patients were drug naïve. Patients with major depression (n=24) were also recruited at the inpatient department of Psychiatry of the Erasmus Medical Center in Rotterdam. All patients suffered from severe depression without psychotic features. In these patients

medication was tapered of before participation in a randomized control treatment trial, thus all were medication free for at least one week before blood withdrawal (except for Lorazepam max 3 mg).

Patients with DSM-IV bipolar I or II disorder (n=22) were recruited from the Dutch site of the former Stanley Foundation Bipolar Network (Utrecht), an international multicenter research program described elsewhere in detail.[28] Patients were all outpatients with a median disease episode of 14 years. All patients were euthymic and on medication.

Age- and gender-matched healthy controls (HC) for each patient group 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 and an absent history of these disorders in first-degree family members. HC had to be in self-proclaimed good health and free of medical illness for at least two weeks prior to the blood withdrawal, including acute infections and allergic reactions.

Table 1. *Characteristics of patients with schizophrenia (SCZ), bipolar disorder (BD) and unipolar major depressive disorder (MDD) and their respective matched healthy controls (HC).*

	SCZ	HC	BD	HC	MDD	HC
Group size	27	34	22	24	24	21
Mean age, yrs (range)	30 (22-63)	29 (24-46)	43 (34-61)	42 (24-58)	54 (34-67)	49 (33-62)
Male gender	22 (81 %)	28 (82.3 %)	8 (36%)	6 (25%)	13 (54%)	10 (47%)
Duration of illness, yrs (range)	0.3 (0-3)	-	14 (4.1-34)	-	0.9 (0-9)	-
Medication						
Antipsychotics	25	0	7	0	0	0
Lithium	0	0	15	0	0	0
Antiepileptics	0	0	3	0	0	0
Antidepressants	0	0	6	0	0	0
None	2	0	0	0	24	0
Current psychiatric status	psychotic inpatients	-	euthymic outpatients	-	depressed inpatients	-

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 by low-density gradient centrifugation in the early afternoon, as described in detail before [29], within maximum 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.[30]

RNA isolation and quantitative PCR (qPCR)

RNA was isolated from purified monocytes using RNAeasy columns according to the manufacturer's instructions (Qiagen, USA) and as previously described.[31] To obtain cDNA for Q-PCR, 1 μg RNA was reversed-transcribed using High Capacity cDNA Kit (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands) for 120 min at 42°C. Q-PCR was performed with Taqman Universal PCR Mastermix (Applied Biosystems, USA). All Taqman probes and consensus primers were pre-formulated and designed by Applied Biosystems (Assays on Demand). 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 haematopoetic cells.[32] The calculation of the data is described in detail in the legend of Table 2.

We used this collection procedure for monocyte RNA isolation successfully in our lab in previous studies.[24, 25, 33, 34]

Chromatin immunoprecipitation (ChIP)

Human buffycoat monocytes from healthy controls were cross-linked for 10 min with formaldehyde, nuclei were lysed and the chromatin was prepared by sonication. The lysates were precleared with 5 μg salmon sperm DNA/Sepharose CL-4B beads and precipitated with 2.5 μg polyclonal ATF3, EGR3, PU.1 antibody or IgG rabbit isotype control (Santa Cruz Biotechnology, Sata Cruz, CA, USA). Complexes were recovered with protein A sepharose beads and cross-linking was reversed. The DNA was purified with Qiaquick PCR purification columns (Qiagen, Hilden, Germany) and enrichment was analyzed by standard PCR with primers amplifying the corresponding promoter regions (**Supplementary Table S1**).

Data analysis

Statistical analysis was performed using the SPSS 15.0 package for Windows. Correlations were determined via Spearman rank correlation coefficients.

RESULTS

TREM-1, DAP12 gene and PU.1 expression in monocytes of patients with severe psychiatric disorders

Table 2 shows that the level of TREM-1 mRNA expression was significantly higher in monocytes of patients with severe psychiatric disorders. TREM-1 was statistically significantly increased in the monocytes of SCZ and BD patients (1.56 and 1.46 fold respectively), but only 1.21 fold in the monocytes of MDD patients with at best marginal significance (p=0.08).

DAP12, the TREM-1 associated adapter molecule, was not increased in patient monocytes, neither in the SCZ, BD nor MDD group (**Table 2**).

Table 2. *Q-PCR analysis of TREM-1, DAP12, ATF3, EGR3, MAFF, MXD1, NAB2 and PU.1 expression in all patients with psychiatric disorders (n=73; SCZ n=27; BD n=22; MDD n=24) as compared to healthy controls (HC) (n=79) values, set at 1 fold. The quantitative value obtained from Q-PCR is a cycle threshold (C_T). The fold change values between different groups were determined from normalized C_T values (C_T gene- C_T reference gene Abl), by the $\Delta\Delta C_T$ 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 U test. For the expression levels of the genes in monocytes of patients with severe psychiatric disorder the results of the Q-PCR assay on single genes of Applied Biosystems were used which has also been extensively reported and described in Drexhage et al [24]. Transcription factors are highlighted in grey. FC, Fold change SCZ, Schizophrenia; BD, Bipolar disease; MDD, Unipolar Disease.*

	All patients		SCZ		BD		MDD	
	FC (median)	p-value	FC (median)	p-value	FC (median)	p-value	FC (median)	p-value
TREM-1	**1.44**	<0.001	**1.56**	0.002	**1.46**	0.035	1.21	0.083
DAP12	0.98	0.593	1.04	0.323	0.96	0.200	0.96	0.554
PU.1	**1.09**	0.036	1.09	0.248	1.08	0.452	**1.41**	0.003
ATF3	**2.29**	<0.001	**3.50**	0.000	**2.42**	0.001	**2.23**	0.014
EGR3	**5.11**	<0.001	**5.36**	0.000	**3.90**	0.028	**3.60**	0.015
MXD1	**1.55**	<0.001	**1.49**	<0.001	**1.46**	0.037	**1.56**	0.003
MAFF	**5.84**	<0.001	**5.10**	<0.001	**4.07**	0.012	**7.89**	0.001
NAB2	1.00	0.362	0.76	0.21	**1.95**	0.026	1.02	0.682

With regard to their transcription factor PU.1, this transcription factor was significantly increased in the monocytes of patients with severe psychiatric disorders (**Table 2**), but this was mainly due to a clear increase of 1.41 fold in the monocytes of MDD patients. PU.1 was not increased in the monocytes of SCZ and BD patients (**Table 2**).
Table 2 also shows the data of the ATF3, EGR3, MXD1, MAFF and NAB2 expression in the patient groups (in part published in Drexhage et al 2010). The expression levels of the TFs ATF3, EGR3, MAFF and MXD1 were all clearly and significantly increased in the monocytes of patients with SCZ, BD and MDD. NAB2 was only over expressed 1.95 fold in the monocytes of BD patients (**Table 2**).

Relation of TREM-1, DAP12 and PU.1 expression to medication use
Since medication could have been responsible for the differences found between BD, SCZ and MDD we carried out correlation studies for TREM-1, DAP12, PU.1 expression and the usage of lithium and anti-psychotics, since these were the drugs most commonly used in the SCZ and BD group. To test for the influence of lithium and antipsychotics, we turned to the group of BD patients, since almost all patients with SZ were on antipsychotics; only one was not, but had used an antipsychotic in the past. MDD patients were drug free at the time of testing. Of the 22 BD patients, 15 were on lithium, 7 were on antipsychotics, 5 used both. Use of lithium and antipsychotics either alone or in combination did not result in a significant relation to the expression levels of TREM-1, DAP12 or PU.1 (**Table 3**).

Table 3. *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.*

		BD patients		
Medication	**Genes**	p-value	B	95% CI
Lithium		0.980	-0.025	-1.947 to 1.897
Antipsychotics	**TREM-1**	0.767	0.425	-2.389 to 3.239
Both		0.955	-0.095	-3.424 to 3.234
Lithium		0.876	-0.045	-0612 to 0.522
Antipsychotics	**DAP12**	0.768	-0.125	-0.955 to 0.705
Both		0.583	0.275	-0.707 to 1.257
Lithium		0.872	-0.026	-0.350 to 0.297
Antipsychotics	**PU.1**	0.329	0.236	-0.238 to 0.709
Both		0.367	-0.258	-0.818 to 0.142

The correlation of TREM-1 and DAP12 expression levels to the expression levels of the transcription factors/regulators PU.1, ATF3, EGR3, MAFF, MXD1 and NAB2

In a next step we assessed the mutual correlation between the expression levels of TREM-1, DAP12 and the transcription factors/regulators PU.1, ATF3, EGR3, MAFF, MXD1 and NAB2 and calculated the Spearman rank correlations (**Table 4**).

Table 4 firstly shows the results for the correlations of TREM-1; the 3 patient groups were taken together, since a separate analysis of the data for the 3 disease entities did not yield noteworthy differences between groups. It is clear that TREM-1 expression correlated the strongest to the expression levels of the transcription factors EGR3 and MXD1 (0.662 and 0.543 respectively), but also to a somewhat lesser extent to ATF3, MAFF and NAB2. TREM-1 expression did not correlate to PU.1 expression.

We also correlated the expression levels of DAP12 to the expression levels of the afore-mentioned transcription factors/regulators. Though DAP12 was not differentially expressed in patients with severe psychiatric diseases, its expression correlated significantly to the expression of PU.1 (r=0.346) and also MXD1 (r=0.282) (**Table 4**).

Table 4. *Correlation analysis of the TREM-1 and DAP12 expression level compared to expression levels of TREM-1, DAP12, PU.1, ATF3, EGR3, MAFF, MXD1 and NAB2 in monocytes of all patients with a severe psychiatric disorder. Data show the Spearman's correlation coefficients and significance levels. Transcription factors (TFs) are highlighted in grey.*

	TREM1 correlates to (all patients)		**DAP12 correlates to (all patients)**	
	Spearman's rho	Sig. (2-tailed)	Spearman's rho	Sig. (2-tailed)
TREM-1	1	-	**0.293**	**0.012**
DAP12	**0.293**	**0.012**	1	-
PU.1	0.160	0.176	**0.346**	**<0,005**
ATF3	**0.446**	**<0,005**	0.128	0.284
EGR3	**0.662**	**<0,005**	-0.018	0.881
MXD1	**0,543**	**<0,005**	**0.282**	**0.016**
MAFF	**0.491**	**<0,005**	0.226	0.055
NAB2	**0.368**	**<0,005**	0.066	0.584

EGR3, ATF3 and PU.1 interaction with the proximal TREM-1 and DAP12 promoters

In macrophages and microglia cell lines the *in vivo* regulation of the TREM-1 and the DAP12 promoter by PU.1 has been described previously by us and others.[17, 35]

To characterize the potential regulation of TREM-1 and DAP12 expression in monocytes by the transcription factors/regulators ATF3, EGR3, MAFF, MXD1 and NAB2 we screened *in silico* 1kb of the upstream regulatory region of the TREM-1 and the DAP12 promoter for possible binding sites for ATF3, EGR 1-4, MXD1, MAFF and NAB2 (due to reported redundancy in regulation of transcription between EGRs 1-4,[36-38] the promoter fragments were analyzed for binding sites to these 4 transcription factors).
For MAFF and MXD1 we could not find binding sites in the TREM-1 or the DAP12 promoter region. These factors only function either as obligatory heterodimeric partner molecule[39-41] or in case of MAFF also as homodimeric transcriptional repressor.[40] MXD1 does not form homodimers.[42] NAB2 also does not contain a DNA binding domain and acts as a co-activator or co-repressor of EGR-mediated gene transcription.[43] Based on these notions we excluded MAFF, MXD1 and NAB2 from our further ChIP experiments.

Table 5. *ATF3, EGR1-3 and PU.1 target gene regions identified in the TREM-1 and Dap12 promoter used for chromatin immunoprecipitation (ChIP). TSS, transcription start site.*

		Binding Site prediction						
Gene	**Region[2]**	**Sequence + Binding site[1]**	**Position relative to main TSS**	**Transcription factor**				
				ATF3	Egr1	Egr2	Egr3	PU.1
TREM-1								
Fragment 1	-346/+1	taggaaggGGAAcagagggcc	-269					x
		cttagattttGTAAggttgaa	-205	x				
		ttgtgtTGATgtcaggagtca	-101	x				
		tggtgaTGAAacagaacccaa	-73	x				
Fragment 2	-20/+261	aaggatgaGGAAgaccaggct	+70					x
		tttgtctcagGTAAgacaagg	+113	x				
		agcaggggAGGCgtttg	+140		x			
Dap12								
Fragment 1	-529/-199	aactccTGACctcaggtgatc	-478	x				
		aggtcaTGACactgaccctcc	-218	x				
Fragment 2	-199/+9	gcaactaGACGtgaagggcaa	-134	x				
		gggccaTGCCacaggccccca	-110	x				
		gtgaaggaGGAAgtctgaggc	-10					x

[1]ATF3, EGR1-3 or PU.1 binding site or binding region[2] relative to the main transcription start site.

Table 5 displays the number of *in silico* identified binding sites for the analysed transcription factors PU.1, ATF3 and the EGRs and their position relative to the main

transcription start site (TSS) given by the Gene2Promoter software (Genomatix Software GmbH Muenchen). For ATF3 many binding sites were predicted in both the TREM-1 and DAP12 promoter region, an EGR binding site was only found *in silico* in the TREM-1 promoter and not in the DAP12 promoter. PU-1 binding was predicted for both the TREM-1 and DAP12 promoter.
For validation of actual *in vivo* binding of PU.1, ATF3 and EGR3 to the regulatory regions of TREM-1 and DAP12 ChIP was carried out with human $CD14^+$ monocytes. After preparation of the chromatin and reversal of cross-linking, PCR was performed on the ChIP–DNA using primers flanking the predicted EGR, ATF3 and PU.1 sites in the TREM-1 or the DAP12 proximal promoter.

As shown in **Fig. 1**, the TREM-1 core promoter region was strongly and specifically immune precipitated by anti-EGR3, anti-ATF3 and anti-PU.1 antibody, whereas the IgG isotype control shows only background binding. The ChIP using an antibody against PU.1 significantly enriched the proximal promoter of DAP12, verifying our earlier observations that PU.1 is indeed a TF for DAP12 (data not shown). The bio-informatically predicted ATF3 sites in the regulatory region of DAP12 could not be verified in ChIP due to the high GC content of the promoter fragment. The ChIP data are thus consistent with a view that transcriptional regulation of TREM-1 is under the control of the transcription factors EGR3, ATF3 and PU.1, whereas DAP12 expression would at least be regulated by PU.1.

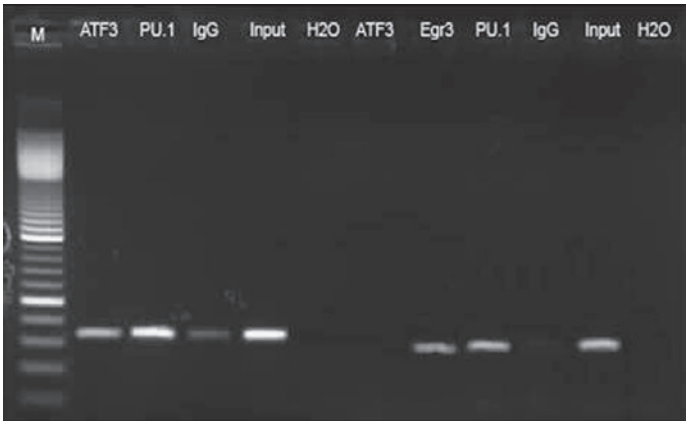


Fig. 1. *ATF3, EGR3 and PU.1 are associated with the TREM-1 promoter region. ChIP assays using human $CD14^+$ monocytes from healthy controls with antibodies against ATF3, EGR3 and PU.1. PU.1 antibody and Input DNA served as positive control. IP with rabbit IgG antibody served as negative control. DNA fragments were analyzed by PCR for two different TREM-1 promoter fragments. The TREM-1 promoter fragment -111/-111 displayed ChIP-PCR positive signals for ATF3 and PU.1 whereas the TREM-1 promoter fragment -111/-111 showed specific binding for EGR3 and PU.1 but not for ATF3.*

DISCUSSION

The main finding of this study is that TREM-1 gene expression is significantly increased in the monocytes of patients with SCZ and BD (and given the power limitations of the study perhaps also in patients with MDD).

The ligand for the TREM-1 receptor (an immunoglobulin-family receptor) is still unknown but induction of receptor cross-linking with an agonistic antibody leads to an association with an Immuno-receptor Tyrosine-based Activation Motif (ITAM) of DAP12 and downstream signalling results in an increase in intracellular calcium and pro-inflammatory cytokine secretion.[20] TREM-1 signalling has also been reported to result in increased nuclear levels of the Nuclear Factor-kappa B (NF-κB) transcription factor,[44] which plays a crucial role in the transcription of inflammatory response genes such as TNF-α, IL-2, IL-12p40 and Il-1β.[45] Since TREM-1 is in addition known to amplify the acute inflammatory response to noxes,[21] the here presented data on the increase of the TREM-1 gene in monocytes of patients with SCZ and BD (and perhaps MDD) can be interpreted as further strengthening the involvement of an inflamed monocyte/macrophage system in severe psychiatric disease (a view earlier expressed by us, Drexhage et al, 2010).

Interestingly a study[46] observed a disproportional increase of TREM-1 expression in disease tissue of rheumatoid arthritis patients and the investigators suggested a potential modulating role for TREM-1 in (autoimmune) inflammatory diseases. The same study showed that TREM-1 activation induced INHBA and TNFSF15 expression which are both genes implicated in autoimmune pathologies,[47-51] but that – similar to our studies - elevated mRNA levels of DAP12 and PU.1 were not involved.

The here reported study in addition suggests that the TFs ATF3 and EGR3 (which are also increased in the monocytes of SCZ and BD patients) are important transcription regulators for TREM-1 (a finding that has not been reported in the literature thus far). Based on the correlation data between TREM-1 expression and the expression levels of ATF3 and EGR3 and additional *in silico* promoter prediction algorithms, we predicted that ATF3 and EGR3 would bind the TREM-1 promoter region in monocytes, indeed the *in vivo* ChIP binding of EGR3 and ATF3 to the TREM-1 promoter indeed confirmed this view.

A transcription regulating role of EGR3 for TREM-1 has been suggested before: Increased mRNA levels of TREM-1 and EGR3 co-appeared in the severe immune deregulated Nrf2$^{-/-}$ mice,[52] which are characterized by a proneness for inflammation,[53] including that of the brain.[54-57]

The involvement of EGR3 in the pathology of major psychiatric diseases is also clearly shown in EGR3$^{-/-}$ mice. EGR3$^{-/-}$ mice display increased aggression in response to the

stressful social stimulus of a foreign intruder. Chronic administration of clozapine significantly inhibits the observed aggressive behaviour in these animals, though they are more resistant to the side effects (sedating activity) of clozapine compared to their wild type littermates.[58] This decreased susceptibility to the side effects of antipsychotic medications is also found in SCZ patients. In fact, a broad range of characteristics of EGR3$^{-/-}$ mice is consistent with animal models of schizophrenia. These include e.g. hyperactivity, an accentuated response and abnormal adaptation to novelty and stress.[59] It is thus not surprising that EGR3 was recently identified as a susceptibility gene for schizophrenia and other neuropsychiatric disorders in humans,[60-63] that EGR3 was highlighted as a key regulatory gene in a recent miRNA-transcription factor network study on SCZ genes,[64] and that EGR3 was also shown to play an essential role in neuronal development[65] as well as in learning and memory.[66] Interestingly, increased expression of EGR3 and pro-inflammatory molecules such as IL-1B and COX2 co-appeared in human leukocytes from individuals that experienced social isolation for at least 3 years.[67] Taking our data and those from the literature together, it is plausible that EGR3 has a critical role in both the immune system and brain abnormalities in severe psychiatric disease.
Our data also indicate that the transcription factor ATF3 binds to the TREM-1 promoter. It is known that ATF3 and TREM-1 mRNA levels are rapidly induced after LPS-stimulation.[20, 68-70] However, ATF3 is on itself not pro-inflammatory, but it negatively regulates immune responses to prevent uncontrolled inflammatory disease.[68] This indicates that the increase of ATF3 in patient monocytes might earlier serve as a control mechanism of inflammation than as an aggravating factor. Suffice to say that further knock out experiments are needed to establish such dampening role.

We found DAP12 expression not differently expressed in the monocytes of all three tested patient groups. In contrast to the restricted pro-inflammatory role of TREM-1, DAP12 associates with many receptors. DAP12-paired receptors can have activating or inhibitory effects in innate immune response[71, 72] and a recent study showed that DAP12 is important for the proliferation and survival of macrophages.[73]

Last, but not least we found that circulating monocytes of MDD patients did over express the transcription factor PU.1, while monocytes of SCZ and BD patients did not.
One of us[17] reported previously on high PU.1 levels in activated retinal microglia of retinoschisin-deficient mice,[17] strong microglial PU.1 expression was also detected after hypoxic-ischemia brain injury.[74] The transcription factor PU.1 belongs to the

ETS family of transcription factors which possesses the ETS-Domain as a DNA-binding region. PU.1 is involved in the development and specific gene regulation of the myeloid- and lymphoid-lineages, including macrophages, dendritic cells (DC), neutrophils, mast cells (MC) and lymphoid cells.[75-81] Transcriptomic profiling of the PU.1-regulatory network in macrophages[82] revealed that a large number of linked genes are associated with immune responsiveness.[82] An involvement of PU.1 in immune reactivity was further indicated in studies showing that binding sites for PU.1 coexist with those for ubiquitous stress-inducible inflammatory transcription factors such as NF-κB, IRF and AP-1.[83]

The overexpression of PU.1 in monocytes of MDD patients, and not in those of SCZ and BD patients, suggests an inflammatory state of monocytes of MDD patients distinct from that of the monocytes of SCZ and BD patients (the monocyte inflammatory state of the latter two groups differed with regard to NAB2 expression, see also Drexhage et al, 2010). A putative molecular difference between the inflammatory states of monocytes of SCZ, BD and MDD needs however to be studied in more depth and in investigations involving more MDD, SCZ and BD patients and larger panels of inflammatory genes before firm conclusions can be drawn (see also later in limitations of our studies). It must also be noted that with regard to the over expression of the transcription factors/regulators ATF3, EGR3, MXD1 and MAFF there was overlap in the inflammatory monocyte states of SCZ, BD and MDD patients

Our study has limitations. Firstly, although the series of patients numbered in total 73 patients, numbers in the three separate disease entities ranged from 22 to 27 patients. All patients were naturalistically treated and were on a number of medications (see **Table 1**). Outcomes of our studies should thus be viewed as influenced by power limitations and it is also not clear whether the relationship between the here reported TREM-1 and transcription factor gene expression in monocytes of patients with a severe psychiatric disorder indeed stems from an intrinsic abnormality of these inflammatory compounds in psychiatric disease or is in fact due to medications given.[6] It is known that various drugs used in psychiatry have immune effects, though they are in general anti-inflammatory/immune suppressive. Virtually all our BD and SCZ patients were on medication, the MDD patients were not at the time of testing. Our correlation studies in BD patients show that neither lithium, nor anti-psychotics influenced the expression levels of TREM-1, DAP12 or PU.1, yet the series of patients tested was relatively small. At an earlier occasion we reported on anti-inflammatory effects of lithium and anti-psychotics

on the expression level of another inflammatory gene in the monocytes: PDE4B, yet this study included 56 BD patients.[24] Suffice to say that larger groups of BD, SCZ and MDD patients (including larger numbers of patients with no or minimal treatment) need to be tested before firm conclusions on effects of medication and intrinsic gene expression differences between the disease entities can be drawn.
Secondly, although the correlation analysis of the co-expression of TREM-1 and the TFs ATF3 and EGR3 and the ChIP assay suggest that there is a functional relationship between these genes and that ATF3 and EGR3 act as key transcription regulators for TREM-1 in the monocytes of psychiatric patients, neither ATF3 nor EGR3 expression has been experimentally manipulated in the monocytes to formerly prove this. Moreover ChIP analysis was carried out on monocytes of healthy controls (since considerable numbers of cells are needed in the assay and since we have no ethical permission to obtain such quantities of blood from our patients). Suffice to say that experimental manipulation of the expression of these genes is required in patient monocytes to definitely establish causation.

Taken together, this gene expression study further strengthens the concept that monocytes are in a pro-inflammatory state in severe psychiatric illnesses, which point of view has been expressed by us before testing an array of other pro-inflammatory genes in circulating monocytes of SCZ and BD patients and reviewing the literature.[24, 25] The present work suggests altered inflammatory set points of the monocyte/macrophage system in severe psychiatric disease and represents the first steps in unravelling the molecular transcription regulatory networks behind this abnormal inflammatory state of patient monocytes.

Support
This work was supported by the EU (Health FP7, nr 222963, acronym MOODINFLAME), the Dutch Organisation for Research, Medical Sciences (NWO-MW-TOP 08425) and an ECNP Young Investigator Award to Livia A. Carvalho

Acknowledgements
We thank Harm de Wit and Annemarie Wijkhuijs for their excellent technical assistance and Caspar Looman for statistical advice.

Statement of interest:
H.A. Drexhage has received grants from the Netherlands Organisation for Health Research and Development, the European Union, the Stanley Medical Research Institute, the Dutch Diabetic Foundation and the JDRF; he has served in advisory boards of the Netherlands Organisation for Health Research and Development, the European Union and the JDRF.
All other authors declare that they have no conflicts of interest.

REFERENCES

1. Bessis A, Bechade C, Bernard D, Roumier A. Microglial control of neuronal death and synaptic properties. *Glia* 2007; **55**(3)**:** 233-238.
2. Smith RS. A comprehensive macrophage-T-lymphocyte theory of schizophrenia. *Med Hypotheses* 1992; **39**(3)**:** 248-257.
3. Smith RS, Maes M. The macrophage-T-lymphocyte theory of schizophrenia: additional evidence. *Med Hypotheses* 1995; **45**(2)**:** 135-141.
4. Schuld A, Hinze-Selch D, Pollmacher T. [Cytokine network in patients with schizophrenia and its significance for the pathophysiology of the illness]

   Zytokinnetzwerke bei Patienten mit Schizophrenie und ihre Bedeutung fur die Pathophysiologie der Erkrankung. *Nervenarzt* 2004; **75**(3)**:** 215-226.
5. Potvin S, Stip E, Sepehry AA, Gendron A, Bah R, Kouassi E. Inflammatory cytokine alterations in schizophrenia: a systematic quantitative review. *Biol Psychiatry* 2008; **63**(8)**:** 801-808.
6. Dantzer R. Cytokine-induced sickness behavior: mechanisms and implications. *Ann N Y Acad Sci* 2001; **933:** 222-234.
7. Miller AH, Maletic V, Raison CL. Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. *Biol Psychiatry* 2009; **65**(9)**:** 732-741.
8. Greenberg DB, Jonasch E, Gadd MA, Ryan BF, Everett JR, Sober AJ *et al.* Adjuvant therapy of melanoma with interferon-alpha-2b is associated with mania and bipolar syndromes. *Cancer* 2000; **89**(2)**:** 356-362.
9. Eisenberger NI, Inagaki TK, Mashal NM, Irwin MR. Inflammation and social experience: an inflammatory challenge induces feelings of social disconnection in addition to depressed mood. *Brain, behavior, and immunity* 2010; **24**(4)**:** 558-563.
10. Reichenberg A, Yirmiya R, Schuld A, Kraus T, Haack M, Morag A *et al.* Cytokine-associated emotional and cognitive disturbances in humans. *Arch Gen Psychiatry* 2001; **58**(5)**:** 445-452.
11. Wright CE, Strike PC, Brydon L, Steptoe A. Acute inflammation and negative mood: mediation by cytokine activation. *Brain, behavior, and immunity* 2005; **19**(4)**:** 345-350.
12. Dunn AJ, Swiergiel AH, de Beaurepaire R. Cytokines as mediators of depression: what can we learn from animal studies? *Neurosci Biobehav Rev* 2005; **29**(4-5)**:** 891-909.
13. Chen SK, Tvrdik P, Peden E, Cho S, Wu S, Spangrude G *et al.* Hematopoietic origin of pathological grooming in Hoxb8 mutant mice. *Cell* 2010; **141**(5)**:** 775-785.
14. Doorduin J, de Vries EF, Willemsen AT, de Groot JC, Dierckx RA, Klein HC. Neuroinflammation in schizophrenia-related psychosis: a PET study. *J Nucl Med* 2009; **50**(11)**:** 1801-1807.
15. Klesney-Tait J, Colonna M. Uncovering the TREM-1-TLR connection. *Am J Physiol Lung Cell Mol Physiol* 2007; **293**(6)**:** L1374-1376.
16. Myers TA, Kaushal D, Philipp MT. Microglia are mediators of Borrelia burgdorferi-induced apoptosis in SH-SY5Y neuronal cells. *PLoS pathogens* 2009; **5**(11)**:** e1000659.
17. Weigelt K, Ernst W, Walczak Y, Ebert S, Loenhardt T, Klug M *et al.* Dap12 expression in activated microglia from retinoschisin-deficient retina and its PU.1-dependent promoter regulation. *J Leukoc Biol* 2007; **82**(6)**:** 1564-1574.
18. Tessarz AS, Cerwenka A. The TREM-1/DAP12 pathway. *Immunol Lett* 2008; **116**(2)**:** 111-116.
19. del Fresno C, Gomez-Pina V, Lores V, Soares-Schanoski A, Fernandez-Ruiz I, Rojo B *et al.* Monocytes from cystic fibrosis patients are locked in an LPS tolerance state: down-regulation of TREM-1 as putative underlying mechanism. *PLoS One* 2008; **3**(7)**:** e2667.
20. Bouchon A, Dietrich J, Colonna M. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J Immunol* 2000; **164**(10)**:** 4991-4995.
21. Bouchon A, Facchetti F, Weigand MA, Colonna M. TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature* 2001; **410**(6832)**:** 1103-1107.
22. Gallant S, Gilkeson G. ETS transcription factors and regulation of immunity. *Arch Immunol Ther Exp (Warsz)* 2006; **54**(3)**:** 149-163.

23. Narayan S, Tang B, Head SR, Gilmartin TJ, Sutcliffe JG, Dean B *et al.* Molecular profiles of schizophrenia in the CNS at different stages of illness. *Brain research* 2008; **1239:** 235-248.
24. Drexhage RC, van der Heul-Nieuwenhuijsen L, Padmos RC, van Beveren N, Cohen D, Versnel MA *et al.* Inflammatory gene expression in monocytes of patients with schizophrenia: overlap and difference with bipolar disorder. A study in naturalistically treated patients. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP)* 2010**:** 1-13.
25. Padmos RC, Hillegers MH, Knijff EM, Vonk R, Bouvy A, Staal FJ *et al.* A discriminating messenger RNA signature for bipolar disorder formed by an aberrant expression of inflammatory genes in monocytes. *Arch Gen Psychiatry* 2008; **65**(4)**:** 395-407.
26. First MB SR, Gibbon M, Williams JBW, ed. Structured Clinical Interview for DSM IV Axis I Disorders, Patient Edition (Nederlandse Versie). *Swets & Zeitlinger, BV: Lisse, Nederland* 1999.
27. Andreasen NC, Flaum M, Arndt S. The Comprehensive Assessment of Symptoms and History (CASH). An instrument for assessing diagnosis and psychopathology. *Arch Gen Psychiatry* 1992; **49**(8)**:** 615-623.
28. Post RM, Nolen WA, Kupka RW, Denicoff KD, Leverich GS, Keck PE, Jr. *et al.* The Stanley Foundation Bipolar Network. I. Rationale and methods. *The British journal of psychiatry* 2001; **41:** s169-176.
29. Knijff EM, Breunis MN, van Geest MC, Kupka RW, Ruwhof C, de Wit HJ *et al.* A relative resistance of T cells to dexamethasone in bipolar disorder. *Bipolar disorders* 2006; **8**(6)**:** 740-750.
30. Lyons PA, Koukoulaki M, Hatton A, Doggett K, Woffendin HB, Chaudhry AN *et al.* Microarray analysis of human leucocyte subsets: the advantages of positive selection and rapid purification. *BMC genomics* 2007; **8:** 64.
31. Staal FJ, Weerkamp F, Baert MR, van den Burg CM, van Noort M, de Haas EF *et al.* Wnt target genes identified by DNA microarrays in immature CD34+ thymocytes regulate proliferation and cell adhesion. *J Immunol* 2004; **172**(2)**:** 1099-1108.
32. Beillard E, Pallisgaard N, van der Velden VH, Bi W, Dee R, van der Schoot E *et al.* Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - a Europe against cancer program. *Leukemia* 2003; **17**(12)**:** 2474-2486.
33. Beyan H, Drexhage RC, van der Heul Nieuwenhuijsen L, de Wit H, Padmos RC, Schloot NC *et al.* Monocyte gene-expression profiles associated with childhood-onset type 1 diabetes and disease risk: a study of identical twins. *Diabetes* 2010; **59**(7)**:** 1751-1755.
34. van der Heul-Nieuwenhuijsen L, Padmos RC, Drexhage RC, de Wit H, Berghout A, Drexhage HA. An inflammatory gene-expression fingerprint in monocytes of autoimmune thyroid disease patients. *The Journal of clinical endocrinology and metabolism* 2010; **95**(4)**:** 1962-1971.
35. Zeng H, Ornatowska M, Joo MS, Sadikot RT. TREM-1 expression in macrophages is regulated at transcriptional level by NF-kappaB and PU.1. *Eur J Immunol* 2007; **37**(8)**:** 2300-2308.
36. Tourtellotte WG, Nagarajan R, Bartke A, Milbrandt J. Functional compensation by Egr4 in Egr1-dependent luteinizing hormone regulation and Leydig cell steroidogenesis. *Mol Cell Biol* 2000; **20**(14)**:** 5261-5268.
37. Laslo P, Spooner CJ, Warmflash A, Lancki DW, Lee HJ, Sciammas R *et al.* Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell* 2006; **126**(4)**:** 755-766.
38. Carter JH, Tourtellotte WG. Early growth response transcriptional regulators are dispensable for macrophage differentiation. *J Immunol* 2007; **178**(5)**:** 3038-3047.
39. Motohashi H, Katsuoka F, Engel JD, Yamamoto M. Small Maf proteins serve as transcriptional cofactors for keratinocyte differentiation in the Keap1-Nrf2 regulatory pathway. *Proc Natl Acad Sci U S A* 2004; **101**(17)**:** 6379-6384.
40. Motohashi H, Shavit JA, Igarashi K, Yamamoto M, Engel JD. The world according to Maf. *Nucleic Acids Res* 1997; **25**(15)**:** 2953-2959.
41. Ye X, Li Y, Huang Q, Yu Y, Yuan H, Wang P *et al.* The novel human gene MIP functions as a co-activator of hMafF. *Arch Biochem Biophys* 2006; **449**(1-2)**:** 87-93.
42. Grinberg AV, Hu CD, Kerppola TK. Visualization of Myc/Max/Mad family dimers and the competition for dimerization in living cells. *Mol Cell Biol* 2004; **24**(10)**:** 4294-4308.

43. Svaren J, Sevetson BR, Golda T, Stanton JJ, Swirnoff AH, Milbrandt J. Novel mutants of NAB corepressors enhance activation by Egr transactivators. *The EMBO journal* 1998; **17**(20)**:** 6010-6019.
44. Gibot S, Kolopp-Sarda MN, Bene MC, Bollaert PE, Lozniewski A, Mory F *et al.* A soluble form of the triggering receptor expressed on myeloid cells-1 modulates the inflammatory response in murine sepsis. *J Exp Med* 2004; **200**(11)**:** 1419-1426.
45. Sharif O, Bolshakov VN, Raines S, Newham P, Perkins ND. Transcriptional profiling of the LPS induced NF-kappaB response in macrophages. *BMC Immunol* 2007; **8:** 1.
46. Dower K, Ellis DK, Saraf K, Jelinsky SA, Lin LL. Innate immune responses to TREM-1 activation: overlap, divergence, and positive and negative cross-talk with bacterial lipopolysaccharide. *J Immunol* 2008; **180**(5)**:** 3520-3534.
47. Eramaa M, Hurme M, Stenman UH, Ritvos O. Activin A/erythroid differentiation factor is induced during human monocyte activation. *J Exp Med* 1992; **176**(5)**:** 1449-1452.
48. Gribi R, Tanaka T, Harper-Summers R, Yu J. Expression of activin A in inflammatory arthropathies. *Mol Cell Endocrinol* 2001; **180**(1-2)**:** 163-167.
49. Hubner G, Brauchle M, Gregor M, Werner S. Activin A: a novel player and inflammatory marker in inflammatory bowel disease? *Lab Invest* 1997; **77**(4)**:** 311-318.
50. Yu EW, Dolter KE, Shao LE, Yu J. Suppression of IL-6 biological activities by activin A and implications for inflammatory arthropathies. *Clin Exp Immunol* 1998; **112**(1)**:** 126-132.
51. Bamias G, Martin C, 3rd, Marini M, Hoang S, Mishina M, Ross WG *et al.* Expression, localization, and functional activity of TL1A, a novel Th1-polarizing cytokine in inflammatory bowel disease. *J Immunol* 2003; **171**(9)**:** 4868-4874.
52. Thimmulappa RK, Lee H, Rangasamy T, Reddy SP, Yamamoto M, Kensler TW *et al.* Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. *J Clin Invest* 2006; **116**(4)**:** 984-995.
53. Kong X, Thimmulappa R, Kombairaju P, Biswal S. NADPH oxidase-dependent reactive oxygen species mediate amplified TLR4 signaling and sepsis-induced mortality in Nrf2-deficient mice. *J Immunol* 2010; **185**(1)**:** 569-577.
54. Johnson DA, Amirahmadi S, Ward C, Fabry Z, Johnson JA. The absence of the pro-antioxidant transcription factor Nrf2 exacerbates experimental autoimmune encephalomyelitis. *Toxicol Sci* 2010; **114**(2)**:** 237-246.
55. Innamorato NG, Lastres-Becker I, Cuadrado A. Role of microglial redox balance in modulation of neuroinflammation. *Current opinion in neurology* 2009; **22**(3)**:** 308-314.
56. Innamorato NG, Rojo AI, Garcia-Yague AJ, Yamamoto M, de Ceballos ML, Cuadrado A. The transcription factor Nrf2 is a therapeutic target against brain inflammation. *J Immunol* 2008; **181**(1)**:** 680-689.
57. Rojo AI, Innamorato NG, Martin-Moreno AM, De Ceballos ML, Yamamoto M, Cuadrado A. Nrf2 regulates microglial dynamics and neuroinflammation in experimental Parkinson's disease. *Glia* 2010; **58**(5)**:** 588-598.
58. Gallitano-Mendel A, Wozniak DF, Pehek EA, Milbrandt J. Mice lacking the immediate early gene Egr3 respond to the anti-aggressive effects of clozapine yet are relatively resistant to its sedating effects. *Neuropsychopharmacology* 2008; **33**(6)**:** 1266-1275.
59. Gallitano-Mendel A, Izumi Y, Tokuda K, Zorumski CF, Howell MP, Muglia LJ *et al.* The immediate early gene early growth response gene 3 mediates adaptation to stress and novelty. *Neuroscience* 2007; **148**(3)**:** 633-643.
60. Yamada K, Gerber DJ, Iwayama Y, Ohnishi T, Ohba H, Toyota T *et al.* Genetic analysis of the calcineurin pathway identifies members of the EGR gene family, specifically EGR3, as potential susceptibility candidates in schizophrenia. *Proc Natl Acad Sci U S A* 2007; **104**(8)**:** 2815-2820.
61. Kim SH, Song JY, Joo EJ, Lee KY, Ahn YM, Kim YS. EGR3 as a potential susceptibility gene for schizophrenia in Korea. *Am J Med Genet B Neuropsychiatr Genet*; **153B**(7)**:** 1355-1360.
62. Kim SH, Song JY, Joo EJ, Lee KY, Ahn YM, Kim YS. EGR3 as a potential susceptibility gene for schizophrenia in Korea. *Am J Med Genet B Neuropsychiatr Genet* 2010; **153B**(7)**:** 1355-1360.
63. Tabares-Seisdedos R, Rubenstein JL. Chromosome 8p as a potential hub for developmental neuropsychiatric disorders: implications for schizophrenia, autism and cancer. *Molecular psychiatry* 2009; **14**(6)**:** 563-589.

64. Guo AY, Sun J, Jia P, Zhao Z. A novel microRNA and transcription factor mediated regulatory network in schizophrenia. *BMC systems biology* 2010; **4:** 10.

65. Eldredge LC, Gao XM, Quach DH, Li L, Han X, Lomasney J *et al.* Abnormal sympathetic nervous system development and physiological dysautonomia in Egr3-deficient mice. *Development* 2008; **135**(17)**:** 2949-2957.

66. Li L, Yun SH, Keblesh J, Trommer BL, Xiong H, Radulovic J *et al.* Egr3, a synaptic activity regulated transcription factor that is essential for learning and memory. *Mol Cell Neurosci* 2007; **35**(1)**:** 76-88.

67. Cole SW, Hawkley LC, Arevalo JM, Sung CY, Rose RM, Cacioppo JT. Social regulation of gene expression in human leukocytes. *Genome biology* 2007; **8**(9)**:** R189.

68. Gilchrist M, Thorsson V, Li B, Rust AG, Korb M, Roach JC *et al.* Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature* 2006; **441**(7090)**:** 173-178.

69. Knapp S, Gibot S, de Vos A, Versteeg HH, Colonna M, van der Poll T. Cutting edge: expression patterns of surface and soluble triggering receptor expressed on myeloid cells-1 in human endotoxemia. *J Immunol* 2004; **173**(12)**:** 7131-7134.

70. Gomez-Pina V, Soares-Schanoski A, Rodriguez-Rojas A, Del Fresno C, Garcia F, Vallejo-Cremades MT *et al.* Metalloproteinases shed TREM-1 ectodomain from lipopolysaccharide-stimulated human monocytes. *J Immunol* 2007; **179**(6)**:** 4065-4073.

71. Turnbull IR, Colonna M. Activating and inhibitory functions of DAP12. *Nat Rev Immunol* 2007; **7**(2)**:** 155-161.

72. Hamerman JA, Lanier LL. Inhibition of immune responses by ITAM-bearing receptors. *Sci STKE* 2006; **2006**(320)**:** re1.

73. Otero K, Turnbull IR, Poliani PL, Vermi W, Cerutti E, Aoshi T *et al.* Macrophage colony-stimulating factor induces the proliferation and survival of macrophages via a pathway involving DAP12 and beta-catenin. *Nat Immunol* 2009; **10**(7)**:** 734-743.

74. Walton MR, Gibbons H, MacGibbon GA, Sirimanne E, Saura J, Gluckman PD *et al.* PU.1 expression in microglia. *Journal of neuroimmunology* 2000; **104**(2)**:** 109-115.

75. Lloberas J, Soler C, Celada A. The key role of PU.1/SPI-1 in B cells, myeloid cells and macrophages. *Immunology today* 1999; **20**(4)**:** 184-189.

76. Guerriero A, Langmuir PB, Spain LM, Scott EW. PU.1 is required for myeloid-derived but not lymphoid-derived dendritic cells. *Blood* 2000; **95**(3)**:** 879-885.

77. Scott EW, Fisher RC, Olson MC, Kehrli EW, Simon MC, Singh H. PU.1 functions in a cell-autonomous manner to control the differentiation of multipotential lymphoid-myeloid progenitors. *Immunity* 1997; **6**(4)**:** 437-447.

78. Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science (New York, NY* 1994; **265**(5178)**:** 1573-1577.

79. McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H *et al.* Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *The EMBO journal* 1996; **15**(20)**:** 5647-5658.

80. Anderson KL, Perkin H, Surh CD, Venturini S, Maki RA, Torbett BE. Transcription factor PU.1 is necessary for development of thymic and myeloid progenitor-derived dendritic cells. *J Immunol* 2000; **164**(4)**:** 1855-1861.

81. Walsh JC, DeKoter RP, Lee HJ, Smith ED, Lancki DW, Gurish MF *et al.* Cooperative and antagonistic interplay between PU.1 and GATA-2 in the specification of myeloid cell fates. *Immunity* 2002; **17**(5)**:** 665-676.

82. Weigelt K, Lichtinger M, Rehli M, Langmann T. Transcriptomic profiling identifies a PU.1 regulatory network in macrophages. *Biochem Biophys Res Commun* 2009; **380**(2)**:** 308-312.

83. Ghisletti S, Barozzi I, Mietton F, Polletti S, De Santa F, Venturini E *et al.* Identification and characterization of enhancers controlling the inflammatory gene expression program in macrophages. *Immunity* 2010; **32**(3)**:** 317-328.

Supplementary Table 1. ATF3, Egr1, Egr2, Egr3 and PU.1 target gene regions and primer sequences used for chromatin immunoprecipitation (ChIP).

Gene	Locus ID[a]	Forward Primer (5'-3')	Reverse Primer (5'-3')	Region[b]
TREM1	GXP_48560	TGGGCCTGACTCTCTTCACT	TGACCTAGAGGCTTCGGAAA	-346/+1
TREM1	GXP_48560	TTTCCGAAGCCTCTAGGTCA	CCCAATTCTGGGTAGAGCAG	-20/+261
DAP12	GXP_221106	GGCTCACACTTGCAATCCTA	GTCAGGGGTCAGACATCGTT	-529/-199
DAP12	GXP_221106	GTCAGGGGTCAGACATCGTT	CAGGCAAGTGAAGGAGGAAG	-199/+9

[a] Genomatix Locus ID (www.genomatix.de)
[b] ATF3, Egr1, Egr2, Egr3 and PU.1 binding site region relative to the main transcription start site

CHAPTER 5

DOWNREGULATION OF AUTOINFLAMMATION-PROTECTIVE MICRORNAS 146A AND 212 IN MONOCYTES OF PATIENTS WITH POSTPARTUM PSYCHOSIS

Karin Weigelt, Veerle Bergink, Karin Burgerhout, Mario Pescatori, Annemarie Wijkhuijs and Hemmo A. Drexhage

Submitted for publication

ABSTRACT:

Background: Postpartum psychosis is thought to belong to the bipolar spectrum. Recently we described an immune activation signature in monocytes of patients with bipolar disorder using gene expression profiling. Immune activation genes are regulated by microRNAs (miRNAs), a newly discovered class of gene expression regulators. We profiled the expression of miRNAs in monocytes of postpartum psychosis (PP) patients to identify differentially expressed miRNAs between post partum psychosis and the healthy state.

Methods: In a profiling study we carried out miRNA profiling using TaqMan array human microRNA A cards v2.0 and monocytes of 8 PP patients. Data were analyzed against monocytes of healthy postpartum women (CP). Nine miRNAs were selected and tested using individual Q-PCR in a larger validation study on monocytes of 20 PP patients, 20 CP and 20 healthy non-postpartum women (HC).

Results: In the validation study miR-146a expression was significantly downregulated in the monocytes of the PP patients as compared to CP and HC; miR-212 expression was also significantly downregulated in PP as compared to HC (but not to CP). *In silico* miR-146a targeted 4 genes of the previously described monocyte activation signature in bipolar disorder; miR-212 targeted 2 of such genes.

Conclusions: This study identified changes in miR-146a and miR-212 monocyte expression in PP. Since there is ample evidence that these miRNAs are linked to auto-inflammatory conditions, this study further strengthens the view that PP is an auto-inflammation like condition.

INTRODUCTION

Postpartum psychosis is an acute psychiatric emergency and considered the most severe postpartum mood disorder. The incidence has been estimated at 1 or 2 of 1,000 deliveries and it has been described as the abrupt onset of manic or psychotic symptoms within four weeks postpartum.[1] In many cases postpartum psychosis is the incipient onset of a bipolar mood disorder.[2] Genetic studies have confirmed the strong etiological link between severe postpartum episodes and vulnerability for bipolar disorder.[3] This relationship is further evidenced by high occurrence of mania and psychosis immediately postpartum in women for whom a diagnosis of bipolar disorder was made already prior to pregnancy.[1 4]

Little is known about the biological mechanism underlying postpartum psychosis, but we recently described an immune activation of monocytes in patients bipolar disorder.[5-7] In these studies we used expression profiling of inflammation-related genes to determine the immune activation state of the circulating cells. The overexpression of inflammation-related genes in the monocytes of patients was in agreement with accumulating evidence pointing to an abnormal activation of the immune system as a central phenomenon in the pathogenesis of mood disorders.[6, 8, 9] It is also noteworthy that the postpartum period is considered as a period of immune activation and many (auto-)inflammatory diseases like rheumatoid arthritis[10, 11] and multiple sclerosis[12] flare up or have their first episode in this period, e.g. autoimmune thyroiditis often starts in the postpartum period.[13, 14]

Gene expression is regulated by the recently discovered microRNAs (miRNAs). MiRNAs mediate their regulatory effects by binding to the 3'–untranslated sequences of particular mRNAs through partially complementary sequences and prevent the mRNAs from being translated into proteins.[15, 16] Various miRNAs play key roles in pathways orchestrating immune responses and chronic inflammatory processes,[17] and there is an increasing amount of data connecting miRNAs to the development of various auto-inflammatory conditions such as rheumatoid arthritis,[18-20] Sjögren's syndrome,[21] multiple sclerosis,[22-24] systemic lupus erythematosus,[25] inflammatory bowel disease[26] and Alzheimer's disease.[27]

In our study, we aimed at investigating the involvement of miRNAs in the activation status of inflammation-related genes in monocytes of postpartum psychosis patients. Therefore, we performed miRNA expression profiling employing TaqMan array cards containing a panel of 377 miRNAs. We used the monocytes of a limited series of 8 postpartum psychosis patients. Data were analyzed against monocytes

of healthy non-postpartum women and healthy postpartum women given that previous studies reported immune activation during the healthy postpartum period. We selected 9 monocyte miRNAs being the most discriminative between postpartum psychosis and the healthy (postpartum) state for further validation. These selected miRNAs were tested in a larger validation study on the monocytes of 20 postpartum psychosis patients, 20 postpartum controls and 20 healthy non-postpartum women using individual Q-PCR.

MATERIALS AND METHODS

Subjects

Postpartum Psychosis Patients: A total of 28 Patients with postpartum psychosis (PP) diagnosed according to the DSM-IV-TR criteria[28] were recruited from the Mother-Baby Inpatient Unit of the Department of Psychiatry of the Erasmus University Medical Center in Rotterdam between 2005 and 2011. Diagnosis was made by means of the Structured Clinical Interview for Disease (SCID – 1/P research version). The clinical characteristics of this cohort have been described in detail previously.[7, 29] We included 17 patients with first-onset postpartum psychosis and 11 patients with postpartum psychosis and a previous diagnosis of bipolar disorder. All but four of the patients (n=28) received medication at the time of blood withdrawal; one of the patients was drug naïve. The demographics and drug usage of all patients are summarized in **Table 1**.

Table 1. *Characteristics of patients with postpartum psychosis (PP), postpartum controls (CP) and age-matched non-postpartum healthy controls (HC).*

	Postpartum psychosis patients	Healthy controls postpartum	Healthy controls non-postpartum
Group size	**28**	**28**	**28**
Mean age, yrs (range)	**33 (25-41)**	**33 (25-42)**	**32 (22-44)**
Medication			
Benzodiazepines	**23**	**-**	**-**
Antipsychotics	**17**	**-**	**-**
Lithium	**5**	**-**	**-**
Psychiatric history			
PP only postpartum period	**17**	**-**	**-**
Bipolar disorder	**11**	**-**	**-**

Briefly, we have included patients with any of the following diagnoses, including the specifier "onset postpartum": depressive disorder with psychotic features, mania with psychotic features, mixed episode with psychotic features, or brief psychotic disorder. Importantly, the specifier "onset postpartum" requires that the onset of symptoms must occur within 4 weeks postpartum. Physical examination and routine laboratory screening were performed at the time of study enrolment to confirm the absence of infection.
Controls Postpartum: Twenty eight age-matched healthy postpartum women (CP) were recruited through the Department of Obstetrics & Gynaecology (Erasmus MC, Rotterdam) and 28 healthy age-matched non-postpartum women were recruited from the Rotterdam general population between 2005 and 2011. The inclusion criteria for these women were an absence of any medical, neurologic, psychiatric, or autoimmune disorder, including acute infections.

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

Blood collection and preparation
Blood (drawn in the morning hours) was collected 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, as described in detail before,[9, 30] 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, Bergisch Gladbach, Germany) Viability and purity of monocytes were both >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 [31].

Total RNA isolation from monocytes
Total RNA was isolated from purified monocytes using the mirVana miRNA Isolation Kit by (Ambion, Austin, Texas, USA) as described by the manufacturer's manual. Purity and integrity of the RNA were assessed on the Agilent 2100 bioanalyzer with

the RNA 6000 Nano LabChip® reagent set (Agilent Technologies, Santa Clara, CA, USA) and the RNA was then stored at -80 °C.

Real time quantitative PCR (qRT PCR)

TaqMan array cards

The TaqMan array human microRNA A cards v2.0 by Applied Biosystems (ABI, Nieuwerkerk a/d IJssel, The Netherlands) contain 384 features per card, enabling the quantitation of 377 human microRNAs. The card includes three endogenous controls (*RNU44*, *RNU48* and *MammU6* in quadruplicate) for data normalization. One assay unrelated to any mammalian species *ath-miR-159a* is included as a negative control. Simultaneous synthesis of cDNA for mature miRNAs was performed using Megaplex Reverse Transcription Human Pool A (ABI) which is a set of pre-defined pools of 380 stem-looped reverse transcription primers. Pre-amplification was performed using the Megaplex PreAmp Primer Pools, Human Pools A v2.1 (ABI). Real time quantitative PCR was performed using TaqMan® Universal Master Mix, NoAmpErase®UNG, with an ABI 7900 HT real-time PCR machine.

Individual microRNA qRT-PCR assays/Validation

Total RNA was isolated from monocytes as described above. Simultaneous synthesis of cDNA for mature miRNAs was performed using Megaplex Reverse Transcription Human Pool A (ABI) and the TaqMan® MicroRNA Reverse Transcription Kit (ABI). QRT-PCR was performed using pre-designed TaqMan® microRNA assays with an ABI 7900 HT real-time PCR machine.

TaqMan® array and individual assay data processing

The SDS software (ABI) was used to collect the data and the RQ Manager program (ABI) was used to assign, check, and standardize C_T values. The Data Assist software (ABI) and the BRB Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html) were used to normalize the data to the small RNAs *RNU44* and *RNU48*. For threshold cycles below 40, the corresponding miRNA was considered detected, higher cycle numbers were not included in calculations. The results were quantified using the ΔΔCT method ($2^{-\Delta\Delta CT}$, User Bulletin 2, ABI, see **Table 2, 3** and **5**).

Data analysis

qRT-PCR data were analyzed using the SDS software, the RQ manager, the Data Assist software (Applied Biosystems) and BRB Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html). Statistical analysis was performed using the SPSS 17.0 package for Windows and using Prism 5 software, version 5.0 (GraphPad Software,

San Diego, CA). The nonparametric Mann-Whitney U test was used to draw comparisons between groups, with the exception that an unpaired *t*-test was used to compare reporter gene activity. Spearman's test was used for correlation studies. *P* values (2-tailed) less than 0.05 were considered statistically significant.

RESULTS

MicroRNA expression profiling in monocytes of patients with postpartum psychosis.

We profiled a panel of 377 miRNAs in monocytes of postpartum psychosis patients (n=8), age-matched postpartum and non-postpartum controls (CP and HC respectively; each n=8) using the TaqMan Array Human MicroRNA A Card v2.0 (Applied Biosystems). On average 169 miRNAs were detected in each sample, 166 in CP, 172 in PP and 168 in HC, but none of these miRNAs was found to be uniquely expressed in postpartum psychosis patients or the control groups. We filtered our dataset for those miRNAs that were expressed in all samples for further analysis. To identify differentially expressed miRNAs, we used two analysis programs (BRB array tools (http://linus.nci.nih.gov/BRB-ArrayTools.html) and DataAssist (ABI). Relative expression was calculated using the comparative cycle treshold (Ct) method (2-ddCt). The samples were normalized to *RNU44* and *RNU48*, but not to snRNA *MammU6,* since large inter-sample variation of the *MammU6* levels made this snRNA an inappropriate normalization control for monocytes (similar unstable expression of *MammU6* has also been reported in other studies[32, 33]). We identified a list of differently expressed miRNAs and all miRNAs with a significance level below p< 0.20 (compared to CP) were selected to be included in the validation study on a larger series of patients (see below). Table 2 displays the 9 differently expressed miRNAs (3 up and 6 down) found in monocytes of postpartum psychosis patients compared to CP and normalized to *RNU44 (*for normalization to *RNU48* see Suppl. Table 1). Only levels of miR-125a-5p (up), miR-618 (down) and miR-296-5p (down) were significant at the p<0.05 level. MiR-212 expression was down in monocytes of PP patients and only reached significance at the p<0.05 level in the class comparison analysis with BRB Array Tools in combination with the reference *RNU48* (Suppl. Table 2). The profiling data of the 8 healthy non-postpartum controls showed that the differences in the expression levels of the 9 selected miRNAs were not the consequence of pregnancy (**Table 2**).

Table 2. *Relative microRNA expression in monocytes of PP patients (n=8) as compared to CP (n=7) and HC (n=8) values, with HC set at 1 fold. The expression all microRNAs was normalized to* RNU44. *One array containing a CP sample was excluded from the analysis because of its average CT values ranging far below those of the other samples.*

MicroRNA Expression Profiling							
		Endogenous Control RNU44					
		Postpartum psychosis to controls postpartum		**Postpartum psychosis to controls non-postpartum**		**Controls postpartum to controls non-postpartum**	
Assay		**FC**	**p-value**	**FC**	**p-value**	**FC**	**p-value**
1	**miR-125a-5p**	**3.22**	**(0.01)**	**14.73**	**(0.01)**	1.54	(0.82)
2	**miR-618**	**0.56**	**(0.02)**	0.68	(0.09)	1.20	(0.47)
3	**miR-296-5p**	**0.49**	**(0.02)**	**0.59**	**(0.03)**	1.21	(0.53)
4	miR-212	0.44	(0.07)	0.60	(0.11)	1.35	(0.50)
5	miR-92a	0.73	(0.11)	0.84	(0.23)	1.16	(0.42)
6	miR-708	0.57	(0.13)	0.60	(0.13)	1.05	(0.91)
7	miR-146a	0.62	(0.15)	0.61	(0.11)	1.00	(0.99)
8	miR-494	1.65	(0.16)	1.49	(0.22)	0.91	(0.77)
9	miR-184	3.50	(0.19)	2.63	(0.13)	0.75	(0.78)

PP, Postpartum Psychosis; CP, Controls Postpartum; HC, Healthy Controls.

Validation of miRNA arrays by qRT-PCR

In a next step, we used individual quantitative stem-loop real-time PCR for the 9 selected miRNAs; these were tested in an additional series of 20 postpartum psychosis patients and their corresponding controls (20 CP; 20 HC). To control for technical problems, we included 6 miRNAs that had been found not to be expressed in monocytes at all (miR-139-3p, miR-149, miR-518b, miR-615-3p, miR-655, miR-758) in the validation. Indeed none of these 6 miRNAs was expressed in the samples used for validation supporting the technical validity of the assay.

Table 3 shows that in this series only the level of miR-146a expression was significantly decreased in monocytes of postpartum psychosis patients as compared to CP and HC monocytes. MiR-212 and miR-92a expression were significantly decreased in monocytes of PP patients compared to HC but not to CP (**Table 3**). MiR-212 expression was slightly and not significantly decreased in CP versus HC, indicating that this microRNA is probably partly linked to the disease state of postpartum psychosis and partly to the postpartum state itself. MicroRNA-92a expression was also significantly decreased in CP compared to HC, indicating that the linkage found with postpartum psychosis is earlier the consequence of the

postpartum state than the consequence of the psychotic state. MicroRNA-296-5p was significantly increased in CP compared to HC, linking this microRNA also to the postpartum state (**Table 3** and Suppl. Table 3). Normalization with *RNU44* and *RNU48* gave comparable results (Suppl. Table 3). The other selected miRNAs did not reach statistical significance in this verification study and miR-184 was found not to be expressed in all samples (data not shown).

Table 3. *Single Assay qRT-PCR analysis of miR-125a-5p, miR-146a, miRNA-269-5p, miR-212, miR-618, miR-708, and miR-494 expression in PP patients (n=20) as compared to CP (n=20) or HC (n=20) values, set at 1 fold. The expression of these microRNAs was normalized to* RNU44.

Validation TaqMan Single Assays							
		Endogenous Control RNU44					
		Postpartum psychosis compared to controls postpartum		**Postpartum psychosis compared to controls non-postpartum**		**Controls postpartum compared to controls non-postpartum**	
Assay		**FC**	**p-value**	**FC**	**p-value**	**FC**	**p-value**
1	**miR-146a**	**0.71**	**(0.03)**	**0.54**	**(0.000)**	0.75	(0.07)
2	**miR-296-5p**	0.75	(0.12)	1.11	(0.55)	**1.47**	**(0.03)**
3	miR-125a-5p	1.10	(0.68)	1.02	(0.93)	0.93	(0.68)
4	miR-618	2.55	(0.27)	2.61	(0.26)	1.02	(0.88)
5	**miR-212**	0.76	(0.12)	**0.63**	**(0.024)**	0.83	(0.27)
6	miR-708	1.21	(0.75)	0.45	(0.15)	0.37	(0.16)
7	**miR-92a**	0.77	(0.15)	**0.51**	**(0.001)**	**0.65**	**(0.02)**
8	miR-494	1.06	(0.76)	0.98	(0.91)	0.93	(0.69)

PP, Postpartum Psychosis; CP, Controls Postpartum; HC, Non-postpartum Healthy Controls.

Relation of microRNA-146a and microRNA-212 expression levels to medication use, diagnosis of bipolar disorder and the presence of thyroid autoimmune disease in patients.

Of the 20 postpartum psychosis patients included in the validation study 4 were treated with lithium, 12 with antipsychotics, 17 with benzodiazepines, and 2 patients were medication-free on the date of blood collection. No correlations were found between the miRNA expression and medication use, but patients were relatively short on medication (less than on average 8 days) and the group of non-medication users was in fact too small (n=4) for a reliable analysis; we can therefore not completely exclude that the changes observed in miRNA expression levels are a consequence of the short medication.

Eleven of 20 patients included in the validation were diagnosed with bipolar

disorder prior to their postpartum psychosis. **Table 4** shows the division into subgroups according to their psychiatric history. As can be seen from this table miR-146a expression was in particular decreased in the monocytes of first-onset PP patients, in the patients with a previous history of bipolar disorder (PP-BD) miR-146a was also decreased but only significantly as compared to HC (not to CP). MiR-212 expression was significantly decreased in monocytes of PP-BD patients compared to HC and CP, but not in monocytes of first-onset PP patients (**Table 4**). MicroRNA-92a expression was also significantly decreased in monocytes of PP-BD patients compared to HC and CP, but also in CP compared to HC (**Table 3**), indicating that this decrease is not a consequence of the psychiatric but the postpartum state.
The levels of miRNA-146a expression have been shown to be altered in different autoimmune diseases.[34, 35] Thyroid autoimmune disease was present in 3 of the 20 patients included in the validation study. Since this number is too small for a reliable correlation analysis we did not carry out such analysis.

Table 4. *Single Assay qRT-PCR analysis of miR-125a-5p, miR-146a, miRNA-269-5p, miR-212, miR-618, miR-708, and miR-494 expression in the two subgroups (with an earlier diagnosis of bipolar disorder n=11 or first-onset postpartum psychosis n=9) of PP patients (n=20) as compared to CP (n=20) or HC (n=20) values, set at 1 fold. The expression of these microRNAs was normalized to* RNU44.

Validation TaqMan Single Assays									
		Endogenous Control RNU44							
		Postpartum psychosis+bipolar disorder compared to controls postpartum		**Postpartum psychosis+bipolar disorder compared to controls non-postpartum**		**First-onset postpartum psychosis compared to controls postpartum**		**First-onset postpartum psychosis compared to controls non-postpartum)**	
Assay		**FC**	**p-value**	**FC**	**p-value**	**FC**	**p-value**	**FC**	**p-value**
1	**miR-146a**	0.75	(0.18)	**0.56**	**(0.01)**	**0.67**	**(0.05)**	**0.50**	**(0.00)**
2	miR-296-5p	0.68	(0.06)	1.00	(0.99)	0.85	(0.52)	1.25	(0.36)
3	miR-125-5p	1.12	(0.62)	1.04	(0.85)	1.07	(0.86)	0.99	(0.98)
4	miR-618	1.21	(0.22)	1.23	(0.13)	1.04	(0.79)	1.07	(0.65)
5	**miR-212**	**0.65**	**(0.01)**	**0.54**	**(0.00)**	0.90	(0.74)	0.75	(0.38)
6	miR-708	1.02	(0.97)	0.38	(0.11)	1.49	(0.57)	0.55	(0.37)
7	**miR-92a**	**0.63**	**(0.00)**	**0.42**	**(0.00)**	0.98	(0.95)	0.64	(0.16)
8	miR-494	0.98	(0.92)	0.91	(0.64)	1.18	(0.50)	1.09	(0.71)

PP, Postpartum Psychosis; CP, Controls Postpartum; HC, Non-postpartum Healthy Controls.

Target prediction for miR-146a and miR-212 in patient monocytes.

We next asked if there are *in silico* indications linking miRNA-146a and miR-212 expression to the regulation of the expression of the 34 inflammation-related genes of the monocyte gene signature we previously described in psychiatric and autoimmune patients.[5, 6, 9, 36] We used miRecords which is a resource for miRNA-target interactions and integrates predicted miRNA targets produced by 11 established miRNA target prediction programs (DIANA-microT, MicroInspector, miRanda, MirTarget2, miTarget, NBmiRTar, PicTar, PITA, RNA22, RNAhybrid and TargetScan/TargetScanS, available at http://www.mirecords.bioled.org).

We performed prediction analysis for miR-146a and miR-212 with a minimal target gene prediction coverage of three algorithms. No restriction on the target gene coverage resulted in 31544 hits for miR-146a, whereas filtering to a minimum coverage of three algorithms resulted in 2829 hits (and 2135 hits for miR-212).

Table 5. *MiR-146a and miR-212 binding prediction to the 3'UTR of the fingerprint genes[7] and four known targets* IRAK1, TRAF6, MeCP2 *and* SPRED1[38-41] *by 6 available algorithms integrated by miRecords.*

MicroRNA-146a binding prediction using algorithms integrated by miRecords						
	miRanda	MirTarget2	PicTar	PITA	RNAhybrid	TargetScan
IRAK1	**x**	**x**	**x**	**x**	**x**	**x**
TRAF6		**x**	**x**	**x**	**x**	**x**
ADAM17	**x**			**x**	**x**	
EGR3	**x**		**x**	**x**	**x**	**x**
IRAK2	**x**			**x**	**x**	
PTGS2	**x**	**x**		**x**	**x**	
MicroRNA-212 binding prediction using algorithms integrated by miRecords						
MECP2	**x**		**x**	**x**	**x**	**x**
SPRED1			**x**	**x**	**x**	**x**
CXCL2	**x**			**x**	**x**	
PTGS2	**x**	**x**		**x**	**x**	

*Predicted interactions are shown as "**x**".*

Table 5 shows that some of the previously identified genes in bipolar patients, i.e. *ADAM17, EGR3, IRAK2* and *PTGS2,*[6, 37] are predicted as targets of miR-146a at least in three and maximally in five algorithms, while *CXCL2* and *PTGS2* are predicted as targets of miRNA-212 in at least three and maximally five algorithms. Interestingly, all our predicted targets derived from the same 6 (miRanda, Mir Target2, PicTar,

PITA, RNAhybrid and Target Scan) of 11 algorithms integrated by miRecords and these are in good overlap with the few known bona fide targets of miR-146a and miR-212 (**Table 5**) Therefore, the algorithms predictive for the signature genes all belong to the group of algorithms which had been of value in the prediction of the known and established genes for the miRNAs.

DISCUSSION

The main finding of this study is that miR-146a and miR-212 expression are decreased in monocytes of patients with postpartum psychosis. MiR-146a was in particular decreased in monocytes of patients with first onset postpartum psychosis and also (but to a lesser extent) in those with a previous history of bipolar disorder. MiR-212 expression was decreased in monocytes of postpartum psychosis patients with a history of bipolar disorder only, and not in first onset postpartum psychosis patients.
Two microRNAs were found linked to the postpartum state itself: MiR-92a was significantly decreased and miR-296-5p significantly increased in monocytes of healthy postpartum women versus healthy non-postpartum women.

Long-term use of mood stabilizers has been reported to selectively influence miRNA expression in rat hippocampal cells.[42] Another study showed that drug-treated patients with bipolar mania increased their plasma miR-134 levels after 4 weeks of treatment, the plasma levels were significantly reduced in bipolar mania before treatment.[43] The authors hypothesized that a decrease in miR-134 plasma levels may be a state marker of a manic episode and that plasma miR-134 levels may associate with successful mood stabilizer treatment.[43] We did not test this miRNA since it was not detected as a discriminating miRNA in our profiling study. Our detected and for postpartum psychosis discriminating miR-146a and miR-212 were not influenced by medication or lifestyle factors (alcohol use, smoking), however we cannot completely rule out an effect of (short-term) medication, since all but 4 of our patients were on short term medication.

An abnormal miR-146a expression has been found in various auto-inflammatory diseases.[20, 35] In addition a recent study identified a downregulated miR-146a expression in peripheral blood mononuclear cells (PBMCs) as part of a schizophrenia-associated miRNA signature[44] and downregulated miR-146a expression has also been reported in the prefrontal cortex of depressed suicide objects.[45] Interestingly, Boldin et al[38] reported that *miR-146a-null* mice die prematurely of a pathological

condition that has all classical signs of an auto-inflammatory state, namely splenomegaly, lymphadenopathy, multi-organ inflammation with tissue damage, a high titer of auto-antibodies, and elevated levels of pro-inflammatory cytokines. The phenotype of *miR-146a-null* mice thus suggests that an absence of or a strong reduction in miR-146a contributes to auto-inflammation. These notions thus strengthen our view expressed in the introduction that an auto-inflammatory monocyte/macrophage component is part of the abnormalities of the disorders of the bipolar spectrum including postpartum psychosis.

What is known about the regulation of the innate immune response by miRNA-146a? The *miR-146a*-null mice systematically overproduce pro-inflammatory cytokines (such as TNF, IL-6 and IL-1β) in response to injection with a sublethal LPS dose; bone-marrow-derived macrophages are the primary source of this enhanced pro-inflammatory cytokine production. This implicates miRNA-146a in attenuating macrophage (auto-)inflammatory responses.[38] In agreement with these results, two more studies show that induction of miR-146a expression in cell lines negatively regulates the inflammatory response.[46, 47]
Recently, Saba et al., transfected a miR-146a mimic into microglial cells and reported that the mRNA expression of CCL20, ADM, IL-1A, IL-1B, IL6 and PTGS2 was downregulated following the transfection of this miR-146a mimic in LPS stimulated EOC 13.31 microglia cells.[48] A transfection with an anti-miR in both resting and LPS-stimulated microglial cells had an opposite effect and resulted in an upregulation of these inflammation-related genes. Interestingly the same genes as reported by Saba et al (*CCL20, ADM, IL-1A, IL-1B, IL6* and *PTGS2*) are part of the inflammation-related gene signature in monocytes of psychiatric and autoimmune patients reported by us previously.[5, 6, 9, 36]
Using miRecords which is a resource for miRNA-target interactions and integrates miRNA targets predicted by 11 established miRNA target prediction algorithms, we identified (*in silico) ADAM17, EGR3, IRAK2* and *PTGS2* as predicted direct targets of miR-146a in patient monocytes. Suffice to say that besides the differential expression of these genes in combination with the corresponding miR-146a levels experimental identification of direct miR-146a targets is necessary to establish causation. The lack of a global profiling of miR-146a targets is a limitation of our study and in fact of almost all studies investigating the role of miR-146a. Up to date, only *TRAF6* and *IRAK1* are established as bona fide targets of miR-146a.[38, 47]

Our data suggest a role miR-212 in the immune activation of monocytes of patients with postpartum psychosis and a history of previous bipolar disorder, and not

in first onset postpartum psychosis. Interestingly, a recent review highlighted the involvement of miR-212 in the neuronal context, immune processes and importantly in the cross-talk between neural and immune functions.[49] Deregulation of miR-212 and miR-132 was found in the prefrontal cortex of individuals affected by schizophrenia and bipolar disorders.[50, 51] These two microRNAs share similar mature sequences and the same seed region, however several studies have outlined that one or the other of these miRNAs was more strongly induced and/or more expressed than the other one in different contexts.[49] Increased expression of miR-132 together with increased expression of miR-155, miR-16 and miR-146a was found in the monocyte/macrophage population of patients with rheumatoid arthritis.[35] However, miR-212 expression and therefore its potential role in autoimmune diseases, was not assessed in this study. Our study indicates that decreased miR-212 expression is, in contrast to miR-146a, a potential marker for immune activation of monocytes of postpartum psychosis patients with an earlier diagnosis of bipolar disorder. Whether this indicates that post partum psychosis in the course of bipolar disorder is intrinsically different from post partum psychosis never occurring in conjunction with bipolar disorder, needs further study, e.g. via follow-up studies of patients and profiling their miRNAs during the course of their illnesses. Also larger groups of PP patients (including larger numbers of patients with no or minimal treatment) need to be tested before firm conclusions on miRNA expression differences between postpartum psychosis, their psychiatric history and postpartum controls can be drawn.

Acknowledgements
We are grateful to the women who participated in this research endeavor.

Statement of interest:
H.A. Drexhage has received grants from the Netherlands Organisation for Health Research and Development, the European Union, the Stanley Medical Research Institute, the Dutch Diabetic Foundation and the JDRF; he has served in advisory boards of the Netherlands Organisation for Health Research and Development, the European Union and the JDRF.
All other authors declare that they have no conflicts of interest.

REFERENCES

1. Spinelli MG. Postpartum psychosis: detection of risk and management. *The American journal of psychiatry* 2009; **166**(4)**:** 405-408.
2. Jones I, Craddock N. Familiality of the puerperal trigger in bipolar disorder: results of a family study. *The American journal of psychiatry* 2001; **158**(6)**:** 913-917.
3. Chaudron LH, Pies RW. The relationship between postpartum psychosis and bipolar disorder: a review. *J Clin Psychiatry* 2003; **64**(11)**:** 1284-1292.
4. Oates M. Perinatal psychiatric disorders: a leading cause of maternal morbidity and mortality. *British medical bulletin* 2003; **67:** 219-229.
5. Padmos RC, Hillegers MH, Knijff EM, Vonk R, Bouvy A, Staal FJ *et al.* A discriminating messenger RNA signature for bipolar disorder formed by an aberrant expression of inflammatory genes in monocytes. *Archives of general psychiatry* 2008; **65**(4)**:** 395-407.
6. Drexhage RC, van der Heul-Nieuwenhuijsen L, Padmos RC, van Beveren N, Cohen D, Versnel MA *et al.* Inflammatory gene expression in monocytes of patients with schizophrenia: overlap and difference with bipolar disorder. A study in naturalistically treated patients. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP)* 2010; **13**(10)**:** 1369-1381.
7. Bergink V, Burgerhout K, Weigelt K, Pop V, de Wit H, Drexhage RC *et al.* submitted.
8. Drexhage RC, Knijff EM, Padmos RC, Heul-Nieuwenhuijzen L, Beumer W, Versnel MA *et al.* The mononuclear phagocyte system and its cytokine inflammatory networks in schizophrenia and bipolar disorder. *Expert Rev Neurother* 2010; **10**(1)**:** 59-76.
9. Weigelt K, Carvalho LA, Drexhage RC, Wijkhuijs A, de Wit H, van Beveren NJ *et al.* TREM-1 and DAP12 expression in monocytes of patients with severe psychiatric disorders. EGR3, ATF3 and PU.1 as important transcription factors. *Brain, behavior, and immunity* 2011; **25**(6)**:** 1162-1169.
10. Wallenius M, Skomsvoll JF, Irgens LM, Salvesen KA, Koldingsnes W, Mikkelsen K *et al.* Postpartum onset of rheumatoid arthritis and other chronic arthritides: results from a patient register linked to a medical birth registry. *Annals of the rheumatic diseases* 2010; **69**(2)**:** 332-336.
11. Haupl T, Ostensen M, Grutzkau A, Radbruch A, Burmester GR, Villiger PM. Reactivation of rheumatoid arthritis after pregnancy: increased phagocyte and recurring lymphocyte gene activity. *Arthritis and rheumatism* 2008; **58**(10)**:** 2981-2992.
12. Hellwig K, Beste C, Schimrigk S, Chan A. Immunomodulation and postpartum relapses in patients with multiple sclerosis. *Therapeutic advances in neurological disorders* 2009; **2**(1)**:** 7-11.
13. Nicholson WK, Robinson KA, Smallridge RC, Ladenson PW, Powe NR. Prevalence of postpartum thyroid dysfunction: a quantitative review. *Thyroid* 2006; **16**(6)**:** 573-582.
14. Bergink V, Kushner SA, Pop V, Kuijpens H, Lambregtse-van den Berg MP, Drexhage RC *et al.* Prevalence of autoimmune thyroid dysfunction in postpartum psychosis. *Br J Psychiatry* 2011; **198**(4)**:** 264-268.
15. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nature reviews* 2008; **9**(2)**:** 102-114.
16. Novina CD, Sharp PA. The RNAi revolution. *Nature* 2004; **430**(6996)**:** 161-164.
17. Tsitsiou E, Lindsay MA. microRNAs and the immune response. *Current opinion in pharmacology* 2009; **9**(4)**:** 514-520.
18. Murata K, Yoshitomi H, Tanida S, Ishikawa M, Nishitani K, Ito H *et al.* Plasma and synovial fluid microRNAs as potential biomarkers of rheumatoid arthritis and osteoarthritis. *Arthritis research & therapy* 2010; **12**(3)**:** R86.
19. Niimoto T, Nakasa T, Ishikawa M, Okuhara A, Izumi B, Deie M *et al.* MicroRNA-146a expresses in interleukin-17 producing T cells in rheumatoid arthritis patients. *BMC musculoskeletal disorders* 2010; **11:** 209.
20. Pauley KM, Cha S. miRNA-146a in rheumatoid arthritis: a new therapeutic strategy. *Immunotherapy* 2011; **3**(7)**:** 829-831.

21. Zilahi E, Tarr T, Papp G, Griger Z, Sipka S, Zeher M. Increased microRNA-146a/b, TRAF6 gene and decreased IRAK1 gene expressions in the peripheral mononuclear cells of patients with Sjogren's syndrome. *Immunology letters* 2012; **141**(2)**:** 165-168.
22. Paraboschi EM, Solda G, Gemmati D, Orioli E, Zeri G, Benedetti MD *et al.* Genetic association and altered gene expression of mir-155 in multiple sclerosis patients. *International journal of molecular sciences* 2012; **12**(12)**:** 8695-8712.
23. Lescher J, Paap F, Schultz V, Redenbach L, Scheidt U, Rosewich H *et al.* MicroRNA regulation in experimental autoimmune encephalomyelitis in mice and marmosets resembles regulation in human multiple sclerosis lesions. *Journal of neuroimmunology* 2012; **246**(1-2)**:** 27-33.
24. Paraboschi EM, Solda G, Gemmati D, Orioli E, Zeri G, Benedetti MD *et al.* Genetic association and altered gene expression of mir-155 in multiple sclerosis patients. *International journal of molecular sciences* 2011; **12**(12)**:** 8695-8712.
25. Lofgren SE, Frostegard J, Truedsson L, Pons-Estel BA, D'Alfonso S, Witte T *et al.* Genetic association of miRNA-146a with systemic lupus erythematosus in Europeans through decreased expression of the gene. *Genes and immunity* 2012; **13**(3)**:** 268-274.
26. Archanioti P, Gazouli M, Theodoropoulos G, Vaiopoulou A, Nikiteas N. Micro-RNAs as regulators and possible diagnostic bio-markers in inflammatory bowel disease. *Journal of Crohn's & colitis* 2011; **5**(6)**:** 520-524.
27. Lukiw WJ, Surjyadipta B, Dua P, Alexandrov PN. Common micro RNAs (miRNAs) target complement factor H (CFH) regulation in Alzheimer's disease (AD) and in age-related macular degeneration (AMD). *International journal of biochemistry and molecular biology* 2012; **3**(1)**:** 105-116.
28. First MB SR, Gibbon M, Williams JBW, ed. Structured Clinical Interview for DSM IV Axis I Disorders, Patient Edition (Nederlandse Versie). *Swets & Zeitlinger, BV: Lisse, Nederland* 1999.
29. Bergink V, Lambregtse-van den Berg MP, Koorengevel KM, Kupka R, Kushner SA. First-onset psychosis occurring in the postpartum period: a prospective cohort study. *The Journal of clinical psychiatry* 2011; **72**(11)**:** 1531-1537.
30. Knijff EM, Breunis MN, van Geest MC, Kupka RW, Ruwhof C, de Wit HJ *et al.* A relative resistance of T cells to dexamethasone in bipolar disorder. *Bipolar disorders* 2006; **8**(6)**:** 740-750.
31. Lyons PA, Koukoulaki M, Hatton A, Doggett K, Woffendin HB, Chaudhry AN *et al.* Microarray analysis of human leucocyte subsets: the advantages of positive selection and rapid purification. *BMC genomics* 2007; **8:** 64.
32. Chang KH, Mestdagh P, Vandesompele J, Kerin MJ, Miller N. MicroRNA expression profiling to identify and validate reference genes for relative quantification in colorectal cancer. *BMC cancer* 2010; **10:** 173.
33. Witwer KW, Sarbanes SL, Liu J, Clements JE. A plasma microRNA signature of acute lentiviral infection: biomarkers of central nervous system disease. *AIDS (London, England)* 2011; **25**(17)**:** 2057-2067.
34. Li L, Chen XP, Li YJ. MicroRNA-146a and human disease. *Scandinavian journal of immunology* 2010; **71**(4)**:** 227-231.
35. Pauley KM, Stewart CM, Gauna AE, Dupre LC, Kuklani R, Chan AL *et al.* Altered miR-146a expression in Sjogren's syndrome and its functional role in innate immunity. *European journal of immunology* 2011; **41**(7)**:** 2029-2039.
36. van der Heul-Nieuwenhuijsen L, Padmos RC, Drexhage RC, de Wit H, Berghout A, Drexhage HA. An inflammatory gene-expression fingerprint in monocytes of autoimmune thyroid disease patients. *The Journal of clinical endocrinology and metabolism* 2010; **95**(4)**:** 1962-1971.
37. Padmos RC, Bekris L, Knijff EM, Tiemeier H, Kupka RW, Cohen D *et al.* A high prevalence of organ-specific autoimmunity in patients with bipolar disorder. *Biological psychiatry* 2004; **56**(7)**:** 476-482.
38. Boldin MP, Taganov KD, Rao DS, Yang L, Zhao JL, Kalwani M *et al.* miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *The Journal of experimental medicine* 2011; **208**(6)**:** 1189-1201.
39. Bonauer A, Carmona G, Iwasaki M, Mione M, Koyanagi M, Fischer A *et al.* MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. *Science (New York, NY* 2009; **324**(5935)**:** 1710-1713.

40. Hollander JA, Im HI, Amelio AL, Kocerha J, Bali P, Lu Q *et al.* Striatal microRNA controls cocaine intake through CREB signalling. *Nature* 2010; **466**(7303)**:** 197-202.

41. Wada R, Akiyama Y, Hashimoto Y, Fukamachi H, Yuasa Y. miR-212 is downregulated and suppresses methyl-CpG-binding protein MeCP2 in human gastric cancer. *International journal of cancer* 2009; **127**(5)**:** 1106-1114.

42. Zhou R, Yuan P, Wang Y, Hunsberger JG, Elkahloun A, Wei Y *et al.* Evidence for selective microRNAs and their effectors as common long-term targets for the actions of mood stabilizers. *Neuropsychopharmacology* 2009; **34**(6)**:** 1395-1405.

43. Rong H, Liu TB, Yang KJ, Yang HC, Wu DH, Liao CP *et al.* MicroRNA-134 plasma levels before and after treatment for bipolar mania. *Journal of psychiatric research* 2011; **45**(1)**:** 92-95.

44. Gardiner E, Beveridge NJ, Wu JQ, Carr V, Scott RJ, Tooney PA *et al.* Imprinted DLK1-DIO3 region of 14q32 defines a schizophrenia-associated miRNA signature in peripheral blood mononuclear cells. *Molecular psychiatry* 2011.

45. Smalheiser NR, Lugli G, Rizavi HS, Torvik VI, Turecki G, Dwivedi Y. MicroRNA expression is down-regulated and reorganized in prefrontal cortex of depressed suicide subjects. *PloS one* 2012; **7**(3)**:** e33201.

46. Perry MM, Moschos SA, Williams AE, Shepherd NJ, Larner-Svensson HM, Lindsay MA. Rapid changes in microRNA-146a expression negatively regulate the IL-1beta-induced inflammatory response in human lung alveolar epithelial cells. *J Immunol* 2008; **180**(8)**:** 5689-5698.

47. Nahid MA, Pauley KM, Satoh M, Chan EK. miR-146a is critical for endotoxin-induced tolerance: IMPLICATION IN INNATE IMMUNITY. *The Journal of biological chemistry* 2009; **284**(50)**:** 34590-34599.

48. Saba R, Gushue S, Huzarewich RL, Manguiat K, Medina S, Robertson C *et al.* MicroRNA 146a (miR-146a) Is Over-Expressed during Prion Disease and Modulates the Innate Immune Response and the Microglial Activation State. *PloS one* 2012; **7**(2)**:** e30832.

49. Wanet A, Tacheny A, Arnould T, Renard P. miR-212/132 expression and functions: within and beyond the neuronal compartment. *Nucleic acids research* 2012.

50. Perkins DO, Jeffries CD, Jarskog LF, Thomson JM, Woods K, Newman MA *et al.* microRNA expression in the prefrontal cortex of individuals with schizophrenia and schizoaffective disorder. *Genome biology* 2007; **8**(2)**:** R27.

51. Kim AH, Reimers M, Maher B, Williamson V, McMichael O, McClay JL *et al.* MicroRNA expression profiling in the prefrontal cortex of individuals affected with schizophrenia and bipolar disorders. *Schizophrenia research* 2010; **124**(1-3)**:** 183-191.

SUPPLEMENTARY INFORMATION

To date, no housekeeping miRNA has been established and normalization of miRNA expression data remains a major challenge. To confirm stability of our results, data analysis was performed with two independent references (*RNU44* and *RNU48*, Suppl. Table 1 and 3) and an alternative software (BRB Array Tools, Suppl. Table 2).

Supplemental Table 1. Relative microRNA expression in monocytes of PP patients (n=8) as compared to CP (n=7) and HC (n=8) values, set at 1 fold. The expression all microRNAs was normalized to *RNU48*.

MicroRNA Expression Profiling							
		Endogenous Control RNU48					
		Postpartum psychosis compared to controls postpartum		**Postpartum psychosis compared to controls non-postpartum**		**Controls postpartum compared to controls non-postpartum**	
Assay		**FC**	**p-value**	**FC**	**p-value**	**FC**	**p-value**
1	**miR-125a-5p**	3.24	**(0.01)**	**2.74**	**(0.01)**	0.85	(0.67)
2	**miR-618**	0.57	**(0.02)**	**0.65**	**(0.04)**	1.15	(0.54)
3	**miR-296-5p**	0.49	**(0.05)**	0.57	(0.07)	1.16	(0.64)
4	miR-212	0.45	(0.06)	**0.58**	**(0.04)**	1.30	(0.53)
5	miR-146a	0.62	(0.13)	0.57	(0.08)	0.96	(0.89)
6	miR-708	0.57	(0.13)	0.58	(0.21)	1.01	(0.99)
7	miR-184	3.51	(0.19)	2.54	(0.11)	0.72	(0.74)
8	miR-494	1.65	(0.17)	1.44	(0.26)	0.86	(0.71)
9	miR-92a	0.73	(0.18)	0.81	(0.24)	1.11	(0.58)

PP, Postpartum Psychosis; CP, Controls Postpartum; HC, Healthy Controls

Supplemental Table 2. Relative microRNA expression in monocytes of PP patients (n=8) as compared to CP (n=7) values, set at 1 fold. Using BRB Array Tools the expression all microRNAs was normalized to *RNU44* or *RNU48*.

MicroRNA Expression Profiling BRB Array Tools							
Endogenous Control		RNU44				RNU48	
Postpartum psychosis compared to controls postpartum				Postpartum psychosis compared to controls postpartum			
Assay		**FC**	**p-value**	**Assay**		**FC**	**p-value**
1	**miR-618**	0.56	**(0.01)**	1	**miR-125a-5p**	3.24	**(0.01)**
2	**mir-125a-5p**	3.22	**(0.01)**	2	**miR-618**	0.57	**(0.01)**
3	**miR-296-5p**	0.49	**(0.01)**	3	**miR-212**	0.45	**(0.04)**
4	miR-212	0.44	(0.06)	4	**miR-296-5p**	0.49	**(0.05)**
5	miR-92a	0.73	(0.10)	5	miR-146a	0.62	(0.12)
6	miR-708	0.57	(0.11)	6	miR-708	0.57	(0.13)
7	miR-184	3.50	(0.14)	7	miR-184	3.51	(0.14)
8	miR-494	1.65	(0.15)	8	miR-494	1.65	(0.16)

PP, Postpartum Psychosis; CP, Controls Postpartum.

Supplemental Table 3. Single Assay qRT-PCR analysis of miR-125a-5p, miR-146a, miRNA-269-5p, miR-212, miR-618, miR-708, and miR-494 expression in PP patients (n=20) as compared to CP (n=20) or HC (n=20) values, set at 1 fold. The expression of these microRNAs was normalized to *RNU48*.

Validation TaqMan Single Assays							
		Endogenous Control RNU48					
		Postpartum psychosis compared to controls postpartum		**Postpartum psychosis to controls non-postpartum**		**Controls postpartum compared to controls non-postpartum**	
Assay		**FC**	**p-value**	**FC**	**p-value**	**FC**	**p-value**
1	**miR-146a**	**0.73**	**(0.05)**	**0.58**	**(0.003)**	0.79	(0.13)
2	**miR-296-5p**	0.77	(0.19)	1.19	(0.37)	**1.54**	**(0.05)**
3	miR-125-5p	1.12	(0.61)	1.09	(0.66)	0.97	(0.88)
4	miR-618	2.62	(0.26)	2.80	(0.23)	1.07	(0.68)
5	**miR-212**	0.77	(0.18)	**0.67**	**(0.036)**	0.87	(0.41)
6	miR-708	1.24	(0.72)	0.48	(0.18)	0.39	(0.18)
7	**miR-92a**	0.79	(0.24)	**0.54**	**(0.002)**	**0.68**	**(0.04)**
8	miR-494	1.07	(0.76)	1.04	(0.83)	0.97	(0.88)

PP, Postpartum Psychosis; CP, Controls Postpartum; HC, Non-postpartum Healthy Controls

CHAPTER 6

GENERAL DISCUSSION

Activated MPS cells are clinically significant in responses to acute infections as well as chronic inflammation, but also in inherited and complex diseases. The research described in this thesis aimed at identifying core elements of transcriptional networks in MPS cells activated by chronic autoinflammatory processes. Disentangling transcriptional networks to identify core elements is important to get new insights into the common mechanisms underlying their activation.

By studying retinal *ex vivo* microglia from Rs1h$^{-/Y}$ mice and an *in vitro* model for microglia activation, increased DAP12 expression in the dystrophic retina of Rs1h$^{-/Y}$ mice could be assigned to activated microglia cells (**Chapter 2**). Furthermore, the transcription factor PU.1 was established as a major regulator of DAP12 expression in macrophages (**Chapter 2**). An expansion of these studies led to genome-wide identification of PU.1 target genes by integrating complementary microarray datasets and the identification of the biological pathways and networks build by these target genes (**Chapter 3**). Activation of MPS cells is a mutual event under a vast number of pathological conditions and in case of retinal dystrophies such as X-linked juvenile retinoschisis this activation represents a starting point for loss of retinal neurons and impairment of physiological properties.

In the context of complex autoinflammatory diseases, such as schizophrenia, bipolar disorder and major depressive disorder, we identified increased TREM-1 expression in patient monocytes and showed that the TREM-1 promoter is bound by the transcription factors ATF3, EGR3 and PU.1 in human monocytes (**Chapter 4**). Another part of the RNA components of a transcription regulatory network are the miRNAs which represent a newly discovered class of gene expression regulators and have demonstrated a role in controlling transcriptional networks. By comparing monocytes from patients with postpartum psychosis and non-postpartum controls, it was shown that miR-146 and miR-212 expression is decreased in monocytes of patients and that genes of the previously described monocyte activation signature in bipolar disorder might be targets of these miRNAs (**Chapter 5**).

Regulation of the DAP12 Promoter by PU.1

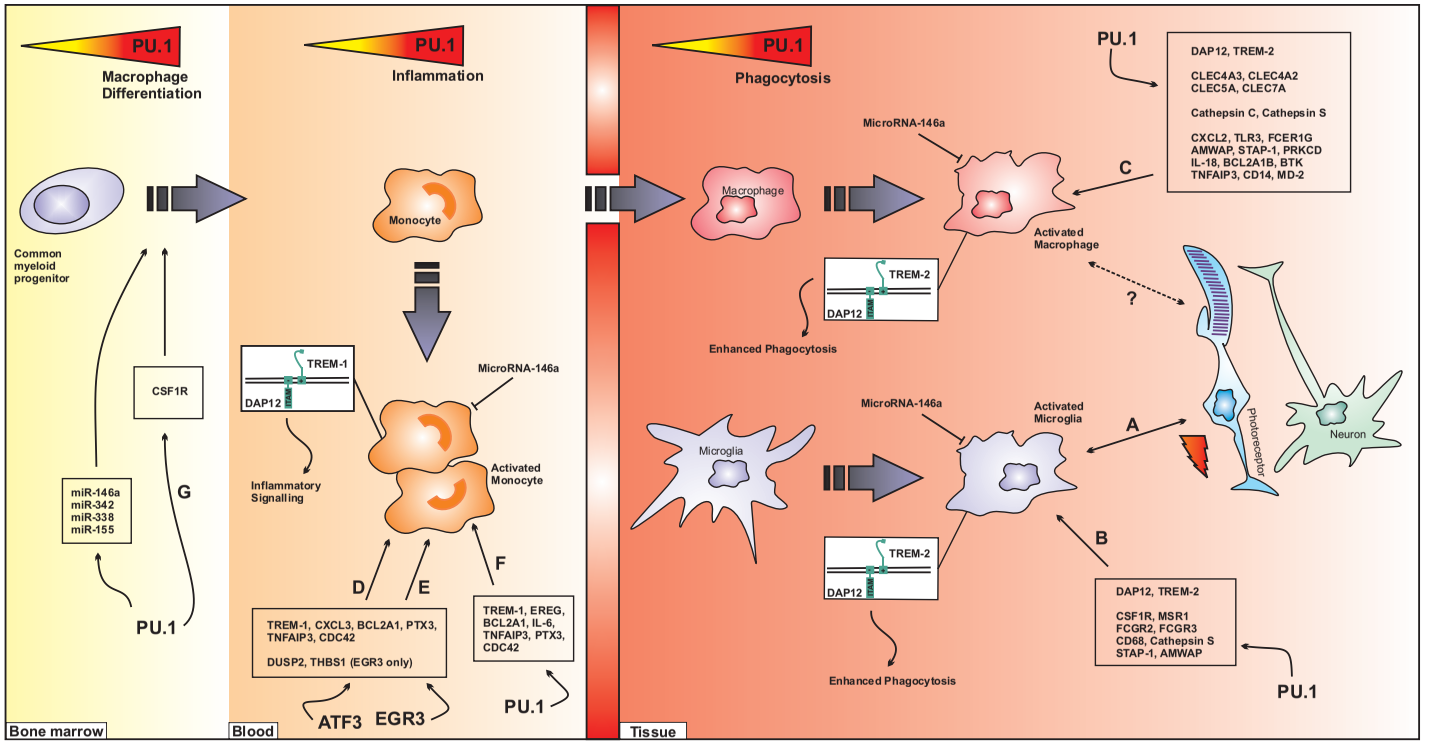
The Rs1h$^{-/Y}$ mouse has been described as a prototypic model for rapid retinal apoptosis and degeneration,[1] but only 5 years later genome-wide expression profiling of the degenerating retina of the Rs1h$^{-/Y}$ mouse suggested that activated microglia might contribute to photoreceptor loss in this model (**Fig. 1A**).[2] Many transcripts implicated in microglia/glia activity and inflammatory responses showed altered expression levels, including the strong expression of the adaptor protein DAP12.[2] Interestingly, the activation of microglia was detected before the onset of photoreceptor degeneration, indicating a causal relationship between these

events. By profiling primary retinal microglia from wild type and Rs1h$^{-/Y}$ mice,[3] we showed in **Chapter 2** that *ex vivo* microglia - devoid of loosing their activated state in culture - express high levels of DAP12 and also the transcription factor PU.1. *In vitro* stimulation of the microglia cell line BV-2 confirmed the activation-dependent induction of DAP12. In a next step, we characterized the DAP12 upstream gene regulatory region and showed that the activity of the murine DAP12 promoter is confined to myeloid cells. The DAP12 core promoter activity depends on conserved PU.1/ETS DNA segments in the proximal region, and PU.1 binds to these sites *in vitro* and *in vivo*. Finally, downregulation of PU.1 expression using siRNA and PU.1 re-expression and activation in deficient progenitor cells revealed direct regulation of endogenous DAP12 transcription by PU.1-dependent transactivation mechanisms. Therefore we propose that PU.1 is critical for the myeloid-specific transcriptional regulation of the murine DAP12 gene (**Fig. 1B+C**).

Microglial phenotype(s) in the retina

These results provided the first report of whole genome transcript profiling in primary microglia from a murine retinal degeneration model. Importantly, a significant overlap of transcript patterns was found in the cultured microglia comparted to the in situ situation in the retina, implicating excellent comparability of these two situations.[2] Our DNA microarray analysis also revealed increased TREM-2 mRNA levels in activated microglia.[3] However, whether DAP12 and TREM-2 have an anti-inflammatory and microglia-controlling function in the retina remains to be determined. Recent studies employing DNA microarrays to analyze retinal degeneration also identified activation of microglia during retinal degeneration but no detailed characterization of the microglial phenotype(s) and M1 versus M2 polarization.[5-8] Interestingly, the latest study by Sharma et al suggests that microglial activation may contribute a protective role to photoreceptor cell viability in the *rds* (*retinal degeneration slow*) model.[8] Since negative feedback mechanisms are part of macrophage/microglia activation to prevent excessive responses, it is not surprising that the molecules of our expression profiles of retinal Rs1h$^{-/Y}$ microglia can be assigned to both pro- and antiinflammatory macrophage activation (**Chapter 2** and **3**; **Fig. 1**) and might represent a mixed phenotype.[9] In a follow-up study, we crossed Rs1h$^{-/Y}$ mice with MacGreen reporter mice to better understand phentypic changes during microglia activation.[10] In these mice, a rapid transformation of ramified microglia into bloated phagocytes could be detected in retinal sections and flatmounts.[10] Such a study will however also not enable the researcher to estimate a relative M1/M2 distribution *in vivo* and mixed or unique phenotypes have been reported in a number of pathological conditions.[11]

Fig. 1. *Involvement of ATF3, EGR3, miR-146a and PU.1 in the activation of MPS cells. MiR-146a inhibits activation of MPS cells.*



*(**A**) PU.1 expression is increased in activated retinal microglia that are detrimental to photoreceptors, mainly via enhanced phagocytosis (**Chapter 2**). The activation of this microglia phenotype is associated with the induction of the TREM-2-DAP12 pathway. (**B+C**) PU.1 is essential for macrophage and microglia gene expression and induces activated phenotypes of macrophages and microglia that are detrimental to other cells, mainly via enhanced phagocytosis. Activation of these macrophages and microglia phenotypes is associated with the induction of the TREM-2-DAP12 pathway. (**D-F**) ATF3, EGR3 and PU.1 are important transcriptional regulators in activated patient monocytes and the activation of monocytes is associated with the induction of the TREM1/DAP12 pathway and enhanced inflammatory signaling (**Chapter 4**). (**G**) PU.1 plays an important role for the differentiation of myeloid progenitors by regulating a miR-cluster including miR-146a[4] and CSF1R expression (**Chapter 3**).*

Regarding the phenotypic spectrum of macrophages and microglia described in the introduction, it should also be doubted that a few single markers will be sufficient to describe different activation phenotypes, particularly if the activation phenotypes under investigation are not extremely polarized. In contrast to that, transcriptomic profiling results in a comprehensive view by dissecting the molecular networks orchestrating these mixed phenotypes on a case-by-case basis and at same time allows for the identification of common as well as distinct gene clusters within differentially activated phenotypes.

A PU.1 regulatory network in activated macrophages and microglia

Our transcript profiling approach provided first evidence of a regulatory network, consisting of 16 genes, under the control of the transcription factor PU.1 in a microglia population isolated from dystrophic retinas (**Fig. 1B**).[3] Relatively little data are available on the role of PU.1 in microglia. To gain new insights into the molecular mechanisms of microglia activation in degenerative disorders, we decided to further characterize the PU.1 regulatory network in microglia cells (**Chapter 3**).[12] We successfully performed genome-wide discovery of PU.1 target genes in RAW264.7 macrophages using ChIP Chip.[13] In the context of this project we also provided an integrated workflow using the Genomatix ChipInspector software to enable comprehensive and straightforward analysis of ChIP Chip datasets.[13] In total, we identified 1202 PU.1 target genes. For example, Clec5a and the human orthologous of BCL2A1 (also by us in human monocytes, discussed later) were confirmed as PU.1 transcriptional targets identified by us (**Fig. 1C**).[14, 15] Btk - a positive regulator in the TREM-1/DAP12 signaling pathway[16] - is a known target gene of PU.1[17] and was confirmed by us (**Fig. 1C**).

In a second dataset, PU.1$^{-/-}$ progenitors and PUER cells with restored PU.1 activity were analyzed with exon-specific microarrays to identify PU.1-regulated genes (**Chapter 3**). Gene ontology terms identified with the upregulated gene clusters were all associated with immune response, whereas the few downregulated genes were related to cell cycle, implicating that the activation of a PU.1-related transcriptome promotes macrophage differentiation and activation, and also cell cycle arrest. These results are in good agreement with main functions of PU.1 described in literature (**Introduction**; **Fig. 1**).[18] Interestingly, also PU.1-coordinated microRNA expression was recently shown to be essential for macrophage development (**Fig. 1**).[4]

Subsequent analysis of our data sets also identified high mRNA levels of STAP1 and AMWAP which had not previously been linked to microglia activation and we could show that these genes are also target genes of PU.1 (**Fig. 1B+C**).[19, 20] The adaptor protein STAP1 was shown to promote neurotoxic activation of microglia,[19] whereas

the novel activated microglia/macrophage WAP domain protein, AMWAP, acts a counter-regulator of proinflammatory response by reducing pro-inflammatory cytokine expression and induction of markers for alternative macrophage activation.[20] Therefore AMWAP may support alternative activation of microglial cells. An overview over important PU.1 targets and their function is given in **Table 1** and depicted in **Figure 1** (**Fig. 1C, G**).

Next to PU.1, also increased expression of the transcription factor EGR1 was identified in degenerating retinae from Rs1h$^{-/Y}$ mice.[2] EGR1 can induce the expression of inflammatory molecules, such as TNFα and CCL2.[21-23] Increased EGR1 expession was however also suggested to contribute to a M2 phenotype in degenerating retina of *rds* mice.[8] Re-expression of PU.1 in PU.1-deficient cells induces EGR2 and EGR1 (**Chapter 3**). Furthermore, there is redundancy between EGR1 and EGR2, since EGR2 takes over EGR1 function in EGR1-deficient cells.[24]

***Table 1**. PU.1 target genes identified with integrated microarray analysis (**Chapter 3**).*

Gene Symbol	Gene Name	Function/Process
AMWAP	Activated microglia/macrophage WAP domain protein	Counter-Regulator of proinflammatory macrophage activation/cytokine production
BCL2A1B	BCL2-related protein A1	Cell Survival / Autophagy in Macrohpahges
BTK	Bruton agammaglobulinemia tyrosine kinase	B cell and mast cell maturation
CD14	CD14 antigen	LPS receptor, together with TLR4 and Ly96r
CFP	Complement factor properdin	Regulation of the alternative pathway of the innate immune system
CLEC4A2	C-type lectin domain family 4, member a2	DC immunoreceptor/Antigen uptake
CLEC4A3	C-type lectin domain family 4, member a3	DC immunoreceptor/Antigen uptake
CLEC5A	C-type lectin domain family 5, member a	Myeloid Cell Activation
CLEC7a (Dectin-1)	C-type lectin domain family 7, member a	Cooperates with TLR2/Response to fungal pathogens
CSF1R	Colony stimulating factor 1 receptor	differentiation of macrophages
CTSC	Cathepsin C	Antigen presentation/processing
CTSS	Cathepsin S	Antigen presentation/processing
CXCL2	Chemokine (C-X-C motif) ligand 2	Chemotaxis
DAP12	DNAX-activating protein of 12 kDa	Adaptor Protein in immune cells
FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	Antibody response/Development of immune response

***Table 1**. PU.1 target genes identified with integrated microarray analysis (**Chapter 3**). (Continued)*

Gene Symbol	Gene Name	Function/Process
Il18	Interleukin 18	Pro-inflammatory cytokine
Itgax	Integrin alpha X	Adherence to endothelium cells/ Phagocytosis
Ly96 (MD-2)	Lymphocyte antigen 96	LPS receptor, together with TLR4 and CD14
PRKCD	Protein kinase C, delta	iNOS production in microglia
STAP-1	Signal-transducing adaptor protein 1	Docking protein involved in microglial activation
TLR3	Toll-like receptor 3	Receptor for dsRNA/Pathogen Recognition
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	Limits Inflammation by terminating TNF-induced NF-KB responses
TREM-2	Triggering Receptor expressed on myeloid cells 2	Phagocytosis

These findings further strengthen the idea that PU.1 induction is essential for macrophage/microglia activation, and might consequently but not necessarily be enhanced by EGR1 induction. In a subsequent study, we could show that the induction of EGR1 mediates microglia activation *in vitro* but is dispensable *in vivo*.[25] In conclusion, our approach of combined genome-wide datasets led to the identification of novel targets regulated by PU.1 involved in macrophage differentation and activation (**Table 1**, **Fig. 1A-C,F,G**). Therefore PU.1, might not only be essential for the differentiation but also for the activation of macrophages and microglia under pathological conditions.

In support of our data, high expression levels of PU.1 immunoreactivity were identified in the hemispheres of hypoxia-injured hippocampus of rats.[26] The authors however speculated that increased PU.1 protein levels in the infarcted cortex may more likely be the result of microglia proliferation, rather than activation. More recently, Ponomarev et al identified miR-124 as a key regulator of microglia quiescence in the CNS. Peripheral administration of miR-124 in EAE caused systemic deactivation of macrophages via the C/EBP-α-PU.1 pathway, resulting in marked suppression of disease.[27] Furthermore, PU.1 was shown to be crucial for the induction of CXCL9 by IFN-γ in microglia. PU.1 also determined the cell-specific expression of this chemokine by microglia and CXCL9 is implicated in the pathogenesis of T-cell-mediated immunity in the CNS.[28] In macrophages, functional PU.1 in macrophages has a pivotal role in NF-κB activation and neutrophilic lung inflammation during endotoxemia.[29] Moreover, grouping genes

activated by PU.1 into pathways based on function not only involved cytokines and cytokine receptors regulating leukocyte growth and development, but also cytokines and cytokine receptors regulating inflammation.[30] In favour of this idea Natoli et al report that PU.1 sets the stage for the activity of ubiquitous transcription factors activated by inflammatory stimuli, like NF-κB, AP-1, and IRFs.[31] Additionally, upregulation of inflammatory cytokine expression and ROS production by PU.1 was also found in adipocytes and demonstrated to cause insuline resistance.[32]
Taken together with our gene expression profiles from degenerating retinae and the target genes of PU.1 identified in this thesis, these results strongly suggest an important role for PU.1 in the regulation of macrophage and microglia activation under pathological conditions.

An abberant immune activation in monocytes of patients with severe psychiatric disorders

Also in mood disorders and schizophrenia, genome-wide expression analysis led to the identification of activated monocytes circulating in the blood of bipolar and schizophrenic patients.[33, 34] In **Chapter 4** and **Chapter 5**, we provide evidence that an increased expression of TREM-1 is part of this pro-inflammatory expression profile (**Chapter 4**) and that this pro-inflammatory expression profile is likely to be influenced by miRNA-146a and miR-212 (**Chapter 5**). TREM-1 gene expression is significantly increased in the monocytes of patients with SCZ and BD (and with regard to the power limitations maybe also in patients with MDD) (**Chapter 4, Fig. 1**). Dower et al. observed a disproportional increase of TREM-1 expression in disease tissue of rheumatoid arthritis patients and the investigators suggested a potential modulating role for TREM-1 in (autoimmune) inflammatory diseases.[35] The same study showed that TREM-1 activation induced INHBA and TNFSF15 expression which are both genes implicated in autoimmune pathologies.[36-40] DAP12 expression did not differ in the monocytes of all three tested patient groups. In macrophages of the lung DAP12 has been shown to be constitutively expressed at high levels, whereas TREM-1, and also TREM-2, are induced upon mycobacterial infection.[41] Therefore an induction the TREM-1/DAP12 pathway might occur without an increase in DAP12 expression levels. PU.1 is known to regulate TREM-1 expression and we provide evidence that also the transcription factors ATF3 and EGR3 might be important transcription regulators for TREM-1, since *in vivo* binding of EGR3 and ATF3 to the TREM-1 promoter was identified by ChIP (**Chapter 4**; **Fig. 1D+E**).

ATF3 and EGR3 in MPS cell activation and the patient's brain

Increased mRNA levels of TREM-1 and EGR3 co-appeared in the lungs of the severe immune deregulated Nrf2$^{-/-}$ mouse, suggesting a regulating role of EGR3 for TREM-1 expression. The Nrf2$^{-/-}$ mice[42] are characterized by a proneness for inflammation,[43] including that of the brain.[44-47] The involvement of EGR3 in the pathology of major psychiatric diseases is also clearly shown in EGR3$^{-/-}$ mice[48] and EGR3 was recently identified as a susceptibility gene for schizophrenia and other neuropsychiatric disorders in humans.[49-52] EGR3 was highlighted as a key regulatory gene in a recent miRNA-transcription factor network study on SCZ genes,[53] and that EGR3 was also shown to play an essential role in neuronal development[54] and in mediating sympathetic target tissue innervation as well as in learning and memory.[55] Also, increased expression of EGR3 and pro-inflammatory molecules such as IL-1β and COX2 co-appeared in human leukocytes from individuals that experienced social isolation for at least 3 years.[56] In line with a potential regulation of TREM-1, these results suggest a general role for EGR3 in the immune system, and also in brain abnormalities in severe psychiatric disease.

***Table 2**. ATF3, EGR3 and PU.1 are associated with the promoter regions of different fingerprint genes.[34] ChIP assays were performed using human buffy coat monocytes and antibodies against ATF3, EGR3, and PU.1.*

Gene	ATF3	EGR3	PU.1	Function/Process
TREM1	+	+	+	Receptor involved in inflammatory signalling
Dap12			+	Adaptor Protein in immune cells
Cluster 1				
CXCL3	+	+		Chemotaxis
BCL2A1	+	+	+	Cell Survival / Autophagy in Macrohpahges
EREG			+	Ligand for epidermal growth factor receptors
PTX3	+	+	+	soluble Pattern Recognition Receptor/Antigen Processing
IL1β			+	Inflammatory Cytokine
IL6			+	Inflammatory Cytokine
DUSP2		+		Kinase Inactivation
TNFAIP3	+	+	+	Limits Inflammation by terminating TNF-induced NF-KB responses
THBS		+		Cell Adhesion
Cluster 2				
CDC42	+	+	+	Cell Cycle Regulation

Promoter binding is shown as "+".

The transcription factor ATF3 also bound the TREM-1 promoter (**Chapter 4**; **Fig. 1D**). ATF3 and TREM-1 mRNA levels are rapidly induced after LPS-stimulation.[57-60] However, ATF3 is on itself not pro-inflammatory, but it negatively regulates immune responses to prevent uncontrolled inflammatory disease.[57] Similarly to the markers described for microglial activation described in **Chapter 2** and **3**, this shows that activated monocytes from patients with a severe psychiatric disease also express both pro- and anti-inflammatory molecules. Further results of ChIP analysis in monocytes are summarized in **Table 2** and depicted in **Fig. 1** (**Fig. 1D-F**). The functions of these molecules are also shown in **Table 2** and further strengthen the concept of an activated state of monocytes of patients with severe psychiatric disease.

MicroRNA-146a in activated monocytes of postpartum psychosis patients

In line with these observations, also the work presented in **Chapter 5** suggests altered activation of the monocyte/macrophage system in severe psychiatric disease. Some patients experience symptoms only during the postpartum period, but often postpartum psychosis will in retrospect be appreciated as the incipient presentation of bipolar disorder.[61] In monocytes of patients with postpartum psychosis we found decreased miR-146a and miR-212 expression (**Chapter 5**). MiR-146a was particularly decreased in monocytes of patients with first onset postpartum psychosis and to a lesser extent in those with a previous history of bipolar disorder, whereas miR-212 expression was decreased in monocytes of postpartum psychosis patients with a history of bipolar disorder only (**Chapter 5**). Abnormal miR-146a expression has been found in various auto-inflammatory diseases.[62, 63] Two recent studies identified downregulated miR-146a expression in peripheral blood mononuclear cells (PBMCs) as part of a schizophrenia-associated miRNA signature[64] and in the prefrontal cortex of depressed suicide objects.[65] Unfortunately, Smalheiser et al did not investigate whether dysregulated miR-146a expression can be assigned to microglia in these objects. Boldin et al.[66] reported that *miR-146a-null* mice die prematurely of a pathological condition that has all classical signs of an auto-inflammatory state, including elevated levels of pro-inflammatory cytokines. Interestingly, bone-marrow-derived macrophages were identified as the primary source of this enhanced pro-inflammatory cytokine production.[66] The phenotype of *miR-146a-null* mice thus suggests that an absence of or a strong reduction in miR-146a contributes to (macrophage-related) auto-inflammation. In agreement with these results, two more studies show that induction of miR-146a expression in cell lines negatively regulates the inflammatory response (**Fig. 1**).[67, 68] Saba et al. recently suggested that miR-146a is involved in the regulation of CCL20,

ADM, IL-1A, IL-1B, IL6 and PTGS2 expression in microglia (**Fig. 1**).[69] These genes are part of the inflammation-related gene signature in monocytes of psychiatric and autoimmune patients reported by us previously.[33, 70-72] *ADAM17, EGR3, IRAK2* and *PTGS2* are predicted by miRecords (www.miRecords.org) as direct targets of miR-146a in patient monocytes (**Chapter 5**). Also, miR-212 might play a role in the immune activation of monocytes of patients with postpartum psychosis. Deregulation of miR-212 and miR-132 was found in the prefrontal cortex of individuals affected by schizophrenia and bipolar disorders.[73, 74]
In conclusion, **Chapter 5** further strengthens the concept of an activated state in monocytes of patients with a severe psychiatric disease and suggests that increasing miR-146a expression might block the immune activation in patient monocytes (**Fig. 1**).

Limitations
The ChIP analysis of ATF3 and EGR3 presented in **Chapter 4** was clearly limited by the fact that due to the high GC-content found in the promoter regions of many of the fingerprint genes, not for all of them a PCR product could be generated. Consequently, we lack the full picture and tried to overcome this problem by performing ChIP-sequencing (ChIP-seq). ChIP-seq is a technique for genome-wide profiling of DNA-binding proteins, histone modifications or nucleosomes. Instead of being hybridized on an array (or being analyzed by PCR), the DNA fragments are sequenced directly. Based on the enormous progess in next-generation sequencing technology, ChIP-seq offers higher resolution, less noise and greater coverage than its array-based predecessor ChIP Chip.[75] Therefore we decided to perform ChIP-seq to identify the transcriptional targets of ATF3 and EGR3 on a genome-wide scale. The value of these ChIP-seq data however depends crucially on the quality of the antibody used. Regarding ATF3 and EGR3 the antibodies used were unfortunately not sensitive and specific enough to give a high level of enrichment compared with the background. Therefore, binding events were hardly detectable and ChIP identification of ATF3 and EGR3 target genes remains limited.
Also, although the co-expression of TREM-1 and ATF3 and EGR3 and the ChIP assay suggest that there is a functional relationship between these genes and that ATF3 and EGR3 act as key transcription regulators for TREM-1 in the monocytes of psychiatric patients, neither ATF3 nor EGR3 expression has been experimentally manipulated in the monocytes to formerly prove this and definitely establish causation.
Furthermore, the study in **Chapter 4** is limited by number of patients. The series of patients numbered in total 73 patients, but the numbers in the three separate disease entities ranged from 22 to 27 patients. Outcomes of our studies should

thus be viewed as influenced by power limitations and it is also not clear whether the relationship between the here reported TREM-1 and transcription factor gene expression in monocytes of patients with a severe psychiatric disorder is a consequence of an intrinsic abnormality or medications given.[76] Various drugs used in psychiatry elicit immune effects, in general they are anti-inflammatory/immune suppressive. Similarly, also the study in **Chapter 5** was performed in 28 patients and might therefore be influenced by power limitations. Within this group 11 patients were also diagnosed with bipolar disorder, whereas 17 were only diagnosed with postpartum psychosis. The outcome of this study might therefore be influenced by these different diagnoses. All 28 postpartum psychosis patients only were on short term medication (on average 8 days), however medications given might still have influencend miR epxression.

Furthermore, we performed target prediction for miR-146a and miR-212 in the study presented in **Chapter 5**. Experimental identification of direct miR-146a and miR-212 targets however is necessary to establish causation. The lack of a global profiling of miR-146a targets is a limitation of our study and in fact of almost all studies investigating the role of miR-146a, miR-212 (and most other known miRNAs). Up to date, only *TRAF6* and *IRAK1* are established as bona fide targets of miR-146a.[66, 68] Similar to transcription factors, it is essential to identify the mRNA targets that miRNAs regulate to understand their biological roles *in vivo*. Computational target prediction is often hampered by the low degree of complementarity between the miRNA and its target sequence. Therefore, biochemical methods are necessary to identify miRNA targets experimentally. For example, the availability of highly specific monoclonal antibodies against Argonaute (Ago) proteins enables the isolation of functional Ago-miRNA–mRNA complexes from different cell lines, tissues, or even patient samples. Consequently, miRNA target mRNAs from immunoprecipitated Ago protein complexes could be directly identified by PCR, microarray or sequencing.[77]

Both patient studies (**Chapter 4** and **5**) provide data for only one time point. No conclusions can be drawn on whether the immune activation observed in monocytes of patients with a severe psychiatric disease is prior to the onset of these diseases or a consequence of the disease states. Although difficult to perform with psychiatric patients, measurements at different time points might provide temporal relations between abnormalities in the immune system and the disease state.

The studies in **Chapter 2** and **3** provided comprehensive analysis of microglial activation in a mouse model for retinal degeneration, but clearly these results need to be transferred into a clinical context.

Concluding and integrating remarks:

The Rs1h$^{-/Y}$ mouse model and *in vitro* cell culture experiments were intensively exploited to study microglia activation and the associated markers during retinal degeneration (**Chapter 2** and **3**). Subsequent (genome-wide) analysis led to identification of novel microglia/macrophage markers and the characterization of their PU.1-dependent regulation. The second part of this thesis (**Chapter 4** and **5**) describes two patient studies and a similar approach to identify key molecules in monocyte activation of patients with severe psychiatric disorders.

The essence of this thesis is summarized and illustrated in a hypothetical model depicted in **Fig. 1**. The common understanding is that activation of MPS cells gives rise to a spectrum of different phenotypes. The M1 and M2 phenotypes are thought to represent the extremes of this spectrum. Microglia activated during retinal degeneration express high levels of DAP12 and TREM-2 which implicates that there is enhanced phagocytosis of damaged photoreceptor cells *in vivo* (**Fig. 1**). In contrast to that, actived monocytes from patients with a severe psychiatric disorder show increased expression of TREM-1 and other pro-inflammatory cytokines *in vivo* (**Fig. 5**), suggesting enhanced inflammatory signalling by these cells. Our data suggest that PU.1 plays an important role in the regulation of both, the TREM-1/DAP12- and TREM-2/DAP12-pathway, and therefore in inflammatory signalling and phagocytosis. The transcription factors ATF3 and EGR3 were also upregulated in both, patient monocytes and microglia (**Chapter 3**, own unpublished observations), and a potential regulation of the TREM-2 promoter by ATF3 and EGR3 has not been tested in this thesis. However, TREM-1 expression was not induced in activated retinal microglia, and TREM-2 expression was not changed in activated patient monocytes (own unpublished observations).

In sum, the comparison of these results suggests that either induction of the TREM-1/DAP12 pathway or the TREM-2/DAP12 pathway is present in different MPS cell phenotpyes. Furthermore, the induction of either one of these two pathways indicates a restricted phenotype, which can be characterized by either enhanced inflammatory signalling or enhanced phagocytosis. This idea is supported by the other molecules expressed in the different presented MPS cell phenotypes. The known functions of these molecules are summarized in **Table 1** and **Table 2**. Thereby, suffering from a severe psychiatric disease is clearly associated with inflammatory signalling of monocytes. However, it remains elusive which phenotype is present in the microglia of these patients. In contrast to that, microglia of Rs1h$^{-/Y}$ mice seem to present a phagocytic phenotype which engulfs apoptic photoreceptors to prevent an extension of the damage to other layers/cells in the retina, without being inflammatory activated.

Perspectives

The work presented in this thesis cleary shows that it is not sufficient to simply speak of activation with regard to different MPS cell phenotypes. Both, transcriptional phenotyping and a functional characterization are essential in order to understand the biological context and to develop therapeutic strategies which allow for switching between these phenotpyes. Induction of the TREM-1/DAP12 pathway vs. the TREM-2/DAP12 pathway might provide a useful tool to detect phenotical switches related to inflammation or phagocytosis.

The combination of a mouse model with comprehensively analyzed large scale genome data has proven to provide a good basis for the identification of core elements and mechanisms involved in microglia. Especially, since (retinal) microglia can only be obtained from patients *postmortem*, and this is not a limiting factor in mouse studies. Also, patients are often under (undefined) influence of medication. Therefore, besides identification of transcriptional networks, also the response of the genes within these networks to certain substances/pharmaceuticals can easily be tested in model organisms. Nethertheless, investigations of human microglia are essential to validate the findings from animal studies in a clinical context. In schizophrenic patients microglial activation was recently imaged with PET scans,[78] but regarding more detailed analysis, such as transcriptional phenotyping or a functional characterization, human monocyte-derived microglia-like cells could provide an important and also convenient cellular model for both the brain and the retina.[79] Furthermore, the NOD mouse which originally serves as a model for autoimmune diabetes and autoimmune thyroiditis, has recently been established as an animal model for psychiatric diseases in our lab. A characterization of monocyte and microglia functions and phenotypes might provide new insights in the mechisms underlying psychiatric diseases.

LITERATURE

1. Weber BH, Schrewe H, Molday LL, Gehrig A, White KL, Seeliger MW *et al.* Inactivation of the murine X-linked juvenile retinoschisis gene, Rs1h, suggests a role of retinoschisin in retinal cell layer organization and synaptic structure. *Proc Natl Acad Sci U S A* 2002; **99**(9)**:** 6222-6227.
2. Gehrig A, Langmann T, Horling F, Janssen A, Bonin M, Walter M *et al.* Genome-wide expression profiling of the retinoschisin-deficient retina in early postnatal mouse development. *Investigative ophthalmology & visual science* 2007; **48**(2)**:** 891-900.
3. Weigelt K, Ernst W, Walczak Y, Ebert S, Loenhardt T, Klug M *et al.* Dap12 expression in activated microglia from retinoschisin-deficient retina and its PU.1-dependent promoter regulation. *J Leukoc Biol* 2007; **82**(6)**:** 1564-1574.
4. Ghani S, Riemke P, Schonheit J, Lenze D, Stumm J, Hoogenkamp M *et al.* Macrophage development from HSCs requires PU.1-coordinated microRNA expression. *Blood* 2011; **118**(8)**:** 2275-2284.
5. Walsh MM, Yi H, Friedman J, Cho KI, Tserentsoodol N, McKinnon S *et al.* Gene and protein expression pilot profiling and biomarkers in an experimental mouse model of hypertensive glaucoma. *Experimental biology and medicine (Maywood, NJ* 2009; **234**(8)**:** 918-930.
6. Feng Y, Wang Y, Li L, Wu L, Hoffmann S, Gretz N *et al.* Gene expression profiling of vasoregression in the retina--involvement of microglial cells. *PLoS One* 2011; **6**(2)**:** e16865.
7. Howell GR, Macalinao DG, Sousa GL, Walden M, Soto I, Kneeland SC *et al.* Molecular clustering identifies complement and endothelin induction as early events in a mouse model of glaucoma. *J Clin Invest* 2011; **121**(4)**:** 1429-1444.
8. Sharma YV, Cojocaru RI, Ritter LM, Khattree N, Brooks M, Scott A *et al.* Protective gene expression changes elicited by an inherited defect in photoreceptor structure. *PLoS One* 2012; **7**(2)**:** e31371.
9. Karlstetter M, Ebert S, Langmann T. Microglia in the healthy and degenerating retina: insights from novel mouse models. *Immunobiology* 2010; **215**(9-10)**:** 685-691.
10. Ebert S, Weigelt K, Walczak Y, Drobnik W, Mauerer R, Hume DA *et al.* Docosahexaenoic acid attenuates microglial activation and delays early retinal degeneration. *J Neurochem* 2009; **110**(6)**:** 1863-1875.
11. Sica A, Mantovani A. Macrophage plasticity and polarization: *in vivo* veritas. *J Clin Invest* 2012; **122**(3)**:** 787-795.
12. Weigelt K, Lichtinger M, Rehli M, Langmann T. Transcriptomic profiling identifies a PU.1 regulatory network in macrophages. *Biochem Biophys Res Commun* 2009; **380**(2)**:** 308-312.
13. Weigelt K, Moehle C, Stempfl T, Weber B, Langmann T. An integrated workflow for analysis of ChIP-chip data. *Biotechniques* 2008; **45**(2)**:** 131-132, 134, 136 passim.
14. Batliner J, Mancarelli MM, Jenal M, Reddy VA, Fey MF, Torbett BE *et al.* CLEC5A (MDL-1) is a novel PU.1 transcriptional target during myeloid differentiation. *Molecular immunology* 2011; **48**(4)**:** 714-719.
15. Jenal M, Batliner J, Reddy VA, Haferlach T, Tobler A, Fey MF *et al.* The anti-apoptotic gene BCL2A1 is a novel transcriptional target of PU.1. *Leukemia* 2012; **24**(5)**:** 1073-1076.
16. Ormsby T, Schlecker E, Ferdin J, Tessarz AS, Angelisova P, Koprulu AD *et al.* Btk is a positive regulator in the TREM-1/DAP12 signaling pathway. *Blood* 2011; **118**(4)**:** 936-945.
17. Muller S, Maas A, Islam TC, Sideras P, Suske G, Philipsen S *et al.* Synergistic activation of the human Btk promoter by transcription factors Sp1/3 and PU.1. *Biochem Biophys Res Commun* 1999; **259**(2)**:** 364-369.
18. Rosenbauer F, Tenen DG. Transcription factors in myeloid development: balancing differentiation with transformation. *Nat Rev Immunol* 2007; **7**(2)**:** 105-117.
19. Stoecker K, Weigelt K, Ebert S, Karlstetter M, Walczak Y, Langmann T. Induction of STAP-1 promotes neurotoxic activation of microglia. *Biochem Biophys Res Commun* 2009; **379**(1)**:** 121-126.
20. Karlstetter M, Walczak Y, Weigelt K, Ebert S, Van den Brulle J, Schwer H *et al.* The novel activated microglia/macrophage WAP domain protein, AMWAP, acts as a counter-regulator of proinflammatory response. *J Immunol* 2010; **185**(6)**:** 3379-3390.
21. Pritchard MT, Nagy LE. Ethanol-induced liver injury: potential roles for egr-1. *Alcoholism, clinical and experimental research* 2005; **29**(11 Suppl)**:** 146S-150S.

22. Thiel G, Cibelli G. Regulation of life and death by the zinc finger transcription factor Egr-1. *Journal of cellular physiology* 2002; **193**(3)**:** 287-292.

23. Yao J, Mackman N, Edgington TS, Fan ST. Lipopolysaccharide induction of the tumor necrosis factor-alpha promoter in human monocytic cells. Regulation by Egr-1, c-Jun, and NF-kappaB transcription factors. *J Biol Chem* 1997; **272**(28)**:** 17795-17801.

24. Laslo P, Spooner CJ, Warmflash A, Lancki DW, Lee HJ, Sciammas R *et al.* Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell* 2006; **126**(4)**:** 755-766.

25. Langmann T, Ebert S, Walczak Y, Weigelt K, Ehrengruber MU, Stiewe T *et al.* Induction of early growth response-1 mediates microglia activation in vitro but is dispensable in vivo. *Neuromolecular Med* 2009; **11**(2)**:** 87-96.

26. Walton MR, Gibbons H, MacGibbon GA, Sirimanne E, Saura J, Gluckman PD *et al.* PU.1 expression in microglia. *Journal of neuroimmunology* 2000; **104**(2)**:** 109-115.

27. Ponomarev ED, Veremeyko T, Barteneva N, Krichevsky AM, Weiner HL. MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP-alpha-PU.1 pathway. *Nature medicine* 2011; **17**(1)**:** 64-70.

28. Ellis SL, Gysbers V, Manders PM, Li W, Hofer MJ, Muller M *et al.* The cell-specific induction of CXC chemokine ligand 9 mediated by IFN-gamma in microglia of the central nervous system is determined by the myeloid transcription factor PU.1. *J Immunol* 2010; **185**(3)**:** 1864-1877.

29. Karpurapu M, Wang X, Deng J, Park H, Xiao L, Sadikot RT *et al.* Functional PU.1 in macrophages has a pivotal role in NF-kappaB activation and neutrophilic lung inflammation during endotoxemia. *Blood* 2011; **118**(19)**:** 5255-5266.

30. Turkistany SA, DeKoter RP. The transcription factor PU.1 is a critical regulator of cellular communication in the immune system. *Arch Immunol Ther Exp (Warsz)* 2011; **59**(6)**:** 431-440.

31. Natoli G, Ghisletti S, Barozzi I. The genomic landscapes of inflammation. *Genes & development* 2011; **25**(2)**:** 101-106.

32. Lin L, Pang W, Chen K, Wang F, Gengler J, Sun Y *et al.* Adipocyte expression of PU.1 transcription factor causes insulin resistance through upregulation of inflammatory cytokine gene expression and ROS production. *American journal of physiology* 2012; **302**(12)**:** E1550-1559.

33. Padmos RC, Hillegers MH, Knijff EM, Vonk R, Bouvy A, Staal FJ *et al.* A discriminating messenger RNA signature for bipolar disorder formed by an aberrant expression of inflammatory genes in monocytes. *Arch Gen Psychiatry* 2008; **65**(4)**:** 395-407.

34. Drexhage RC, van der Heul-Nieuwenhuijsen L, Padmos RC, van Beveren N, Cohen D, Versnel MA *et al.* Inflammatory gene expression in monocytes of patients with schizophrenia: overlap and difference with bipolar disorder. A study in naturalistically treated patients. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP)* 2010**:** 1-13.

35. Dower K, Ellis DK, Saraf K, Jelinsky SA, Lin LL. Innate immune responses to TREM-1 activation: overlap, divergence, and positive and negative cross-talk with bacterial lipopolysaccharide. *J Immunol* 2008; **180**(5)**:** 3520-3534.

36. Bamias G, Martin C, 3rd, Marini M, Hoang S, Mishina M, Ross WG *et al.* Expression, localization, and functional activity of TL1A, a novel Th1-polarizing cytokine in inflammatory bowel disease. *J Immunol* 2003; **171**(9)**:** 4868-4874.

37. Eramaa M, Hurme M, Stenman UH, Ritvos O. Activin A/erythroid differentiation factor is induced during human monocyte activation. *J Exp Med* 1992; **176**(5)**:** 1449-1452.

38. Gribi R, Tanaka T, Harper-Summers R, Yu J. Expression of activin A in inflammatory arthropathies. *Mol Cell Endocrinol* 2001; **180**(1-2)**:** 163-167.

39. Hubner G, Brauchle M, Gregor M, Werner S. Activin A: a novel player and inflammatory marker in inflammatory bowel disease? *Lab Invest* 1997; **77**(4)**:** 311-318.

40. Yu EW, Dolter KE, Shao LE, Yu J. Suppression of IL-6 biological activities by activin A and implications for inflammatory arthropathies. *Clin Exp Immunol* 1998; **112**(1)**:** 126-132.

41. Aoki N, Zganiacz A, Margetts P, Xing Z. Differential regulation of DAP12 and molecules associated with DAP12 during host responses to mycobacterial infection. *Infect Immun* 2004; **72**(5)**:** 2477-2483.

42. Thimmulappa RK, Lee H, Rangasamy T, Reddy SP, Yamamoto M, Kensler TW *et al.* Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. *J Clin Invest* 2006; **116**(4)**:** 984-995.
43. Kong X, Thimmulappa R, Kombairaju P, Biswal S. NADPH oxidase-dependent reactive oxygen species mediate amplified TLR4 signaling and sepsis-induced mortality in Nrf2-deficient mice. *J Immunol* 2010; **185**(1)**:** 569-577.
44. Innamorato NG, Lastres-Becker I, Cuadrado A. Role of microglial redox balance in modulation of neuroinflammation. *Current opinion in neurology* 2009; **22**(3)**:** 308-314.
45. Innamorato NG, Rojo AI, Garcia-Yague AJ, Yamamoto M, de Ceballos ML, Cuadrado A. The transcription factor Nrf2 is a therapeutic target against brain inflammation. *J Immunol* 2008; **181**(1)**:** 680-689.
46. Rojo AI, Innamorato NG, Martin-Moreno AM, De Ceballos ML, Yamamoto M, Cuadrado A. Nrf2 regulates microglial dynamics and neuroinflammation in experimental Parkinson's disease. *Glia* 2010; **58**(5)**:** 588-598.
47. Johnson DA, Amirahmadi S, Ward C, Fabry Z, Johnson JA. The absence of the pro-antioxidant transcription factor Nrf2 exacerbates experimental autoimmune encephalomyelitis. *Toxicol Sci* 2010; **114**(2)**:** 237-246.
48. Gallitano-Mendel A, Izumi Y, Tokuda K, Zorumski CF, Howell MP, Muglia LJ *et al.* The immediate early gene early growth response gene 3 mediates adaptation to stress and novelty. *Neuroscience* 2007; **148**(3)**:** 633-643.
49. Yamada K, Gerber DJ, Iwayama Y, Ohnishi T, Ohba H, Toyota T *et al.* Genetic analysis of the calcineurin pathway identifies members of the EGR gene family, specifically EGR3, as potential susceptibility candidates in schizophrenia. *Proc Natl Acad Sci U S A* 2007; **104**(8)**:** 2815-2820.
50. Kim SH, Song JY, Joo EJ, Lee KY, Ahn YM, Kim YS. EGR3 as a potential susceptibility gene for schizophrenia in Korea. *Am J Med Genet B Neuropsychiatr Genet*; **153B**(7)**:** 1355-1360.
51. Kim SH, Song JY, Joo EJ, Lee KY, Ahn YM, Kim YS. EGR3 as a potential susceptibility gene for schizophrenia in Korea. *Am J Med Genet B Neuropsychiatr Genet* 2010; **153B**(7)**:** 1355-1360.
52. Tabares-Seisdedos R, Rubenstein JL. Chromosome 8p as a potential hub for developmental neuropsychiatric disorders: implications for schizophrenia, autism and cancer. *Molecular psychiatry* 2009; **14**(6)**:** 563-589.
53. Guo AY, Sun J, Jia P, Zhao Z. A novel microRNA and transcription factor mediated regulatory network in schizophrenia. *BMC systems biology* 2010; **4:** 10.
54. Eldredge LC, Gao XM, Quach DH, Li L, Han X, Lomasney J *et al.* Abnormal sympathetic nervous system development and physiological dysautonomia in Egr3-deficient mice. *Development* 2008; **135**(17)**:** 2949-2957.
55. Li L, Yun SH, Keblesh J, Trommer BL, Xiong H, Radulovic J *et al.* Egr3, a synaptic activity regulated transcription factor that is essential for learning and memory. *Mol Cell Neurosci* 2007; **35**(1)**:** 76-88.
56. Cole SW, Hawkley LC, Arevalo JM, Sung CY, Rose RM, Cacioppo JT. Social regulation of gene expression in human leukocytes. *Genome biology* 2007; **8**(9)**:** R189.
57. Gilchrist M, Thorsson V, Li B, Rust AG, Korb M, Roach JC *et al.* Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature* 2006; **441**(7090)**:** 173-178.
58. Bouchon A, Dietrich J, Colonna M. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J Immunol* 2000; **164**(10)**:** 4991-4995.
59. Knapp S, Gibot S, de Vos A, Versteeg HH, Colonna M, van der Poll T. Cutting edge: expression patterns of surface and soluble triggering receptor expressed on myeloid cells-1 in human endotoxemia. *J Immunol* 2004; **173**(12)**:** 7131-7134.
60. Gomez-Pina V, Soares-Schanoski A, Rodriguez-Rojas A, Del Fresno C, Garcia F, Vallejo-Cremades MT *et al.* Metalloproteinases shed TREM-1 ectodomain from lipopolysaccharide-stimulated human monocytes. *J Immunol* 2007; **179**(6)**:** 4065-4073.
61. Chaudron LH, Pies RW. The relationship between postpartum psychosis and bipolar disorder: a review. *J Clin Psychiatry* 2003; **64**(11)**:** 1284-1292.

62. Pauley KM, Stewart CM, Gauna AE, Dupre LC, Kuklani R, Chan AL *et al.* Altered miR-146a expression in Sjogren's syndrome and its functional role in innate immunity. *European journal of immunology* 2011; **41**(7)**:** 2029-2039.

63. Pauley KM, Cha S. miRNA-146a in rheumatoid arthritis: a new therapeutic strategy. *Immunotherapy* 2011; **3**(7)**:** 829-831.

64. Gardiner E, Beveridge NJ, Wu JQ, Carr V, Scott RJ, Tooney PA *et al.* Imprinted DLK1-DIO3 region of 14q32 defines a schizophrenia-associated miRNA signature in peripheral blood mononuclear cells. *Molecular psychiatry* 2011.

65. Smalheiser NR, Lugli G, Rizavi HS, Torvik VI, Turecki G, Dwivedi Y. MicroRNA expression is down-regulated and reorganized in prefrontal cortex of depressed suicide subjects. *PLoS One* 2012; **7**(3)**:** e33201.

66. Boldin MP, Taganov KD, Rao DS, Yang L, Zhao JL, Kalwani M *et al.* miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *The Journal of experimental medicine* 2011; **208**(6)**:** 1189-1201.

67. Perry MM, Moschos SA, Williams AE, Shepherd NJ, Larner-Svensson HM, Lindsay MA. Rapid changes in microRNA-146a expression negatively regulate the IL-1beta-induced inflammatory response in human lung alveolar epithelial cells. *J Immunol* 2008; **180**(8)**:** 5689-5698.

68. Nahid MA, Pauley KM, Satoh M, Chan EK. miR-146a is critical for endotoxin-induced tolerance: IMPLICATION IN INNATE IMMUNITY. *The Journal of biological chemistry* 2009; **284**(50)**:** 34590-34599.

69. Saba R, Gushue S, Huzarewich RL, Manguiat K, Medina S, Robertson C *et al.* MicroRNA 146a (miR-146a) Is Over-Expressed during Prion Disease and Modulates the Innate Immune Response and the Microglial Activation State. *PLoS One* 2012; **7**(2)**:** e30832.

70. Drexhage RC, van der Heul-Nieuwenhuijsen L, Padmos RC, van Beveren N, Cohen D, Versnel MA *et al.* Inflammatory gene expression in monocytes of patients with schizophrenia: overlap and difference with bipolar disorder. A study in naturalistically treated patients. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP)* 2010; **13**(10)**:** 1369-1381.

71. van der Heul-Nieuwenhuijsen L, Padmos RC, Drexhage RC, de Wit H, Berghout A, Drexhage HA. An inflammatory gene-expression fingerprint in monocytes of autoimmune thyroid disease patients. *The Journal of clinical endocrinology and metabolism* 2010; **95**(4)**:** 1962-1971.

72. Weigelt K, Carvalho LA, Drexhage RC, Wijkhuijs A, de Wit H, van Beveren NJ *et al.* TREM-1 and DAP12 expression in monocytes of patients with severe psychiatric disorders. EGR3, ATF3 and PU.1 as important transcription factors. *Brain, behavior, and immunity* 2011; **25**(6)**:** 1162-1169.

73. Perkins DO, Jeffries CD, Jarskog LF, Thomson JM, Woods K, Newman MA *et al.* microRNA expression in the prefrontal cortex of individuals with schizophrenia and schizoaffective disorder. *Genome biology* 2007; **8**(2)**:** R27.

74. Kim AH, Reimers M, Maher B, Williamson V, McMichael O, McClay JL *et al.* MicroRNA expression profiling in the prefrontal cortex of individuals affected with schizophrenia and bipolar disorders. *Schizophrenia research* 2010; **124**(1-3)**:** 183-191.

75. Park PJ. ChIP-seq: advantages and challenges of a maturing technology. *Nature reviews* 2009; **10**(10)**:** 669-680.

76. Dantzer R. Cytokine-induced sickness behavior: mechanisms and implications. *Ann N Y Acad Sci* 2001; **933:** 222-234.

77. Beitzinger M, Meister G. Experimental identification of microRNA targets by immunoprecipitation of Argonaute protein complexes. *Methods in molecular biology (Clifton, NJ* 2011; **732:** 153-167.

78. Doorduin J, de Vries EF, Willemsen AT, de Groot JC, Dierckx RA, Klein HC. Neuroinflammation in schizophrenia-related psychosis: a PET study. *J Nucl Med* 2009; **50**(11)**:** 1801-1807.

79. Leone C, Le Pavec G, Meme W, Porcheray F, Samah B, Dormont D *et al.* Characterization of human monocyte-derived microglia-like cells. *Glia* 2006; **54**(3)**:** 183-192.

ADDENDUM

SUMMARY

Host defense against invasive bacteria and viruses is composed of a complex system of mucosal barriers, humoral factors, and specialized cell types which constitute the immune system. The responses of the immune system can be divided into innate and adaptive immune responses. Generally, the recognition of viral and bacterial molecular patterns by immune cells of the innate immune system, in particular the DC, induces the release of inflammatory cytokines and activates effector cells of the adaptive immune system. B and T cells are the key players for adaptive immune responses. These cells help to clear the infection and to establish immunological memory to that specific infection.

Innate host defenses provide a first line of defense against invading pathogens and are therefore not as specific as adaptive immune responses. For that purpose, cascades of constitutive proteins such as the complement system and cellular responses involving phagocytes (monocytes, macrophages, microglia and neutrophils) or natural killer (NK) cells mediate the effects of the innate host defense. Together with their precursors in the bone marrow, monocytes, macrophages and microglia comprise a family of cells which has been defined as the mononuclear phagocyte system (MPS). The primary innate functions of monocytes, macrophages and microglia are phagocytosis and killing of microorganisms or lysis of virus-infected cells. However, these cells can also become activated to adopt one of many diverse phenotypes in complex and inherited diseases. Disturbances of tissue homeostasis, such as necrosis or hypoxia, can also activate cells of these cells and lead to an accelerated and/or aggravated progression of both inherited as well as complex diseases. In this thesis, we analyzed activation of monocytes from patients with a major psychiatric disorder which represent complex disease and activation of retinal microglia from Retinoschisin-deficient ($Rsh1^{-/Y}$) mice was assigned to a model for inherited disease.

Chapter 1 addresses the function and composition of the immune system with an emphasis on the cells of the MPS and the heterogenity and plasticity of these cells. In this context, the immunological aspects of psychiatric and eye diseases are explained. Additionally, TREM-1/DAP12 and TREM-2/DAP12 signalling are introduced together with the transcription factors ATF3, EGR3 and PU.1, and microRNAs as potential regulators of these pathways. All these molecules are associated with the activation of cells of the MPS and the molecular profiles of different activation phenotypes might provide a helpful tool to study and monitor switches of MPS cell phenotypes before and after application of potential therapeutics for the treatment of autoimmune-like diseases. In **Chapter 2** we provide the first report of whole genome transcript profiling in primary

microglia from a murine retina degeneration model. By studying retinal *ex vivo* microglia from retinoschisin-deficient ($Rs1h^{-/Y}$) mice and an *in vitro* model for microglia activation, high mRNA levels of the adaptor protein DAP12 and the transcription factor PU.1 were identified and assigned to activated microglia cells. Furthermore, PU.1 could be established as a major regulator of DAP12 expression in macrophages. Interestingly, we also found increased mRNA levels of TREM-2, a receptor which induces phagocytosis and inhibits inflammation. Therefore, phagocytosis of apoptotic photoreceptors might at the same time limit damage to the surrounding cells. However, whether TREM-2/DAP12 signalling has an anti-inflammatory and microglia-controlling function in the degenerating retina remains to be determined. In **Chapter 3** we extended our studies on the regulatory role of PU.1 in macrophage/microglia activation. This expansion led to genome-wide identification of novel PU.1 target genes by integrating complementary microarray datasets. In total we identified 1202 PU.1 target genes. The upregulated gene clusters were all associated with immune response. In contrast to that, the few downregulated genes were related to cell cycle, implicating that the activation of a PU.1-related transcriptome promotes macrophage differentiation and activation. In agreement with the results from **Chapter 2**, these results suggest that PU.1 is essential for the activation of macrophages and microglia.

In **Chapter 4** and **5** we investigated monocytes from patients with a severe psychiatric disease in order to examine the previously suggested activation of these cells. We provide evidence that the expression of TREM-1 is increased in monocytes of patients with bipolar (and maybe also unipolar) disorder and schizophrenia (**Chapter 4**), and miR-146a and miR-212 expression levels are decreased in monocytes of patients with a postpartum psychosis (**Chapter 5**). The TREM-1 promoter is bound by the transcription factors ATF3, EGR3 and PU.1 in human monocytes (**Chapter 4**) and TREM-1 signals via DAP12 to amplify inflammatory signaling and the production of cytokines. These observations are in line with the increased expression of other pro-inflammatory molecules in patient monocytes and also with decreased miR-146a expression. MiR-146a is an important negative regulator of the inflammatory response. In general, microRNAs are important regulatory molecules which inhibit the transcription of their target mRNAs. Therefore, these results suggest a key role for microRNA-146a the activation of monocytes involved the pathogenesis of major psychiatric disorders. Increasing the expression of this micoRNA might block the immune activation in patient monocytes. In this thesis, miR-146a expression has only been investigated in monocytes of patients with a postpartum psychosis, but remains to be analyzed in monocytes of patients with other major psychiatric disorders.

Finally, the results of the different chapters are integrated in **Chapter 6**. The cells of the MPS become activated to adopt different phenotypes in the degenerating retina of Rs1h$^{-/Y}$ mice and in patients with a severe psychiatric disease. Retinal microglia of Rs1h$^{-/Y}$ mice seem to present a phagocytic phenotype which is associated with the induction of the TREM-2/DAP12 pathway. In contrast to that, suffering from a severe psychiatric disorder is clearly associated with inflammatory signalling of monocytes and the induction of the TREM-1/DAP12 pathway. Thus, induction of either the TREM-1/DAP12 pathway or the TREM-2/DAP12 pathway seems to distinguish between these two different MPS cell phenotypes. Further characterization of the molecular profiles of these phenotypes is important to fully explore the functions of these cells depending on the disease and translate this basic knowledge into clinical applications in order to interfere with disease progression.

SAMENVATTING

De afweer tegen bacteriën en virussen bestaat uit een complex systeem van slijmvliezen, humorale factoren en gespecialiseerde cellen die samen het immuunsysteem vormen. De reacties van het immuunsysteem kunnen worden onderverdeeld in aangeboren en verworven reacties. In het algemeen leidt het herkennen van een viraal of bacterieel moleculair patroon door immuuncellen, met name dendritische cellen, tot het vrijkomen van inflammatoire cytokinen en het activeren van effectorcellen van het verworven immuunsysteem. B en T cellen vervullen een sleutelrol bij verworven immuunreacties. Deze cellen helpen een infectie te bestrijden en immunologisch geheugen voor de veroorzakers van de infectie op te bouwen.

De aangeboren afweer vormt een eerstelijnsafweer tegen pathogenen en is daarom minder specifiek dan de verworven immuunafweer. De spelers van de aangeboren afweer zijn cascades van verschillende proteïnen, zoals het complement systeem, en cellulaire reacties van fagocyten, zoals de monocyten, macrofagen, microglia en neutrophiele granulocyten en 'natural killer' (NK) cellen. Samen met hun voorlopercellen in het beenmerg vormen de monocyten, macrofagen en microglia een familie van cellen die het 'mononuclaire phagocyten systeem' (MPS) wordt genoemd. De primaire natuurlijke functies van cellen van het MPS en NK cellen zijn fagocytose en het doden van micro-organismen of lyseren van cellen die besmet zijn met een virus. De cellen kunnen echter ook door nog onbekende mechanismen worden geactiveerd tijdens ziekten die veroorzaakt worden door complexe gen-omgeving interacties ('complexe ziekte') en monogene erfelijke ziekten.

Verstoringen van de homeostase van weefels, zoals necrose of hypoxie, kunnen bijvoorbeeld de cellen activeren en leiden tot een sneller en/of ernstiger verloop van zowel complexe als erfelijke ziektes. In dit proefschrift heb ik de activatie van monocyten van patiënten met psychiatrische aandoeningen als een voorbeeld van een complexe ziekte onderzocht. Daarnaast heb ik de activatie van microglia uit de retina van Retinoschisin-deficiente ($Rsh1^{-/Y}$) muizen bestudeerd als model voor een erfelijke ziekte.

Hoofdstuk 1 geeft een overzicht over de functie en opbouw van het afweersysteem waarbij de nadruk wordt gelegd op cellen van het MPS en de heterogeniteit en plasticiteit van deze cellen. Daarnaast worden ook de immunologische aspecten van verschillende psychiatrische ziektebeelden beschreven en het muismodel voor retinoschisis geintroduceerd. Juveniele retinoschisis is een zeldzame, erfelijke aandoening van het netvlies waarbij door een oppervlakkige splijting (schisis) in het netvlies o.a. fotoreceptor cellen afsterven. Bovendien worden de TREM-1/DAP12 and TREM-2/DAP12 signaleringsroutes en de potentiële regulatie van deze routes

door de transcriptiefactoren ATF3, EGR3 en PU.1 beschreven. Daarnast worden de recent ontdekte microRNAs als regelaars van genexpressie geintroduceerd. Al deze moleculen zijn geassocieerd met afzonderlijke activatie stadia van MPS cellen. De moleculaire profielen van dergelijke verschillende activatie fenotypen zouden kunnen helpen om de diagnostiek en de prognose van therapie uitkomst te verbeteren.

In **hoofdstuk 2** hebben wij de genexpressie van het hele genoom van primaire microglia in het muis-retina-degeneratie-model geanalyseerd. Met hulp van deze analyse en een *in vitro* model voor microglia activatie, konden wij een verhoogde expressie van het adaptor proteïne DAP12 en de transcriptiefactor PU.1 identificeren passend bij de activatie van microgliacellen. Bovendien konden wij hiermee vaststellen dat PU.1 heel belangrijk is voor de regulatie van de DAP12 expressie in macrofagen. Interessant genoeg was ook de expressie van TREM-2 verhoogd. Deze receptor induceert fagocytose en remt ontsteking. Daarom zou de fagocytose van apoptotische fotoreceptor cellen tegelijkertijd niet een onsteking induceren maar beschermend kunnen werken voor de daaromheen liggende cellen. Maar of TREM-2/DAP12 signalering inderdaad een anti-inflammatoire rol en een functie in de controle van de microglia tijdens de retina degeneratie heeft, moet verder onderzocht worden.

In **hoofdstuk 3** hebben wij onze analyses van de regulator rol van PU.1 voor de activatie van macrofagen/microglia uitgebreid. Door de integratie van complementaire microarray datasets konden wij de doelgenen van PU.1 in het hele genoom onderzoeken en in totaal 1202 PU.1 doelgenen identificeren. De genen met een verhoogde expressie waren alle geassocieerd met immuunreacties. Daarentegen waren er enkele genen met een verlaagde expressie, deze waren geassocieerd met de celcyclus. Dit impliceert dat de activatie van het aan PU.1 gerelateerde transcriptoom de differentiatie en activatie van macrofagen stimuleert. Samen met de resultaten uit hoofdstuk 2 duiden deze resultaten erop dat PU.1 essentieel is voor de differentiatie en activatie van macrofagen en microglia in algemene zin.

In **hoofdstuk 4** hebben wij de monocyten van patiënten met ernstige psychiatrische aandoeningen onderzocht om meer over de eerder door ons beschreven activatie van MPS cellen te leren. We laten zien dat de expressie van TREM-1 in monocyten van patiënten met een ernstige stemmingstoornis of schizofrenie verhoogd is. In menselijke monocyten wordt de TREM-1 promoter gebonden door de transcriptiefactoren ATF3, EGR3 en PU.1. TREM-1 gebruikt DAP12 als adaptor molecuul om de inflammatoire signalering en de productie van cytokinen te versterken.

Hoofdstuk 5 toont dat de expressie van microRNA-146a en microRNA-212 in monocyten van patiënten met een postpartum psychose (een aandoening gerelateerd aan bipolaire stoornis) verlaagd is. MicroRNA-146 speelt een belangrijke rol voor de negatieve regulatie van ontsteking. MicroRNA-212 speelt een rol in de interactie tussen het neuronale systeem en het immuunsyteem. In het algemeen zijn microRNAs belangrijke regulator moleculen die de transcriptie van mRNAs blokkeren. Daarom duiden onze bevindingen op een cruciale rol van microRNA-146a en microRNA-212 bij de activatie van monocyten in de pathogenese van psychiatrische ziekten en zijn deze in overeenstemming met de verhoogde expressie van andere inflammatoire moleculen in monocyten van psychiatrische patiënten. Een toename van miRNA-146a expressie zou de immuunactivatie van de monocyten van patiënten dus kunnen blokkeren. In dit proefschrift, hebben wij miRNA-146a en microRNA-212 alleen maar in monocyten van patiënten met een postpartum psychose geanalyseerd en het is zeker belangrijk om dit fenomeen ook in monocyten van patiënten met andere psychiatrische aandoeningen te onderzoeken.

Tot slot worden de uitkomsten van het in dit proefschrift beschreven onderzoek geïntegreerd in **hoofdstuk 6**. De cellen van het MPS worden geactiveerd tijdens degeneratie van de retina van de Rs1h$^{-/Y}$ muis en tijdens psychiatrische aandoeningen, maar de fenotypen van deze activatie vormen verschillen erg en zijn afhankelijk van het ziektebeeld. Microglia uit de retina van Rs1h$^{-/Y}$ muizen blijken een fagocyten fenotype te hebben dat geassoceerd is met de inductie van de TREM-2/DAP12 route. Daarentegen zijn psychiatrische aandoeningen duidelijk geassocieerd met het ontstekingsfenotype van monocyten en de inductie van de TREM-1/DAP12 route. Wij veronderstellen dat de inductie van enerzijds TREM-1/DAP12 signalering of anderzijds TREM-2/DAP12 signalering onderscheidend is voor deze twee verschillende MPS fenotypen, namelijk fagocyterend/ ontstekingsremmend of ontstekingsbevorderend. Verdere karakterising van de moleculaire profielen van deze fenotypen is belangrijk om de functies van deze cellen in de context van ziekten te plaatsen en om uit deze basale kennis klinische behandelingen te ontwikkelen die het verloop van ziekten kunnen beïnvloeden.

LIST OF PUBLICATIONS

Drexhage RC, **Weigelt K**, van Beveren N, Cohen D, Versnel MA, Nolen WA, Drexhage HA. (2011). Immune and neuroimmune alterations in mood disorders and schizophrenia. Int Rev Neurobiol.;101:169-201. Review.

Weigelt K, Carvalho LA, Drexhage RC, Wijkhuijs A, Wit HD, van Beveren NJ, Birkenhäger TK, Bergink V, Drexhage HA. (2011). TREM-1 and DAP-12 expression in monocytes of patients with severe psychiatric disorders. EGR3, ATF3 and PU.1 as important transcription factors. Brain Behav Immun. *25*(6):1162-9

Karlstetter M, Walczak Y, **Weigelt K**, Ebert S, Van den Brulle J, Schwer H, Fuchshofer R, Langmann T. (2010). The novel activated microglia/macrophage WAP domain protein, AMWAP, acts as a counter-regulator of pro-inflammatory response. J Immunol. *185*(6):3379-90

Corbo JC, Lawrence KA, Karlstetter M, Myers CA, Abdelaziz M, Dirkes W, **Weigelt K**, Seifert M, Benes V, Fritsche LG, Weber BH, Langmann T. (2010). CRX ChIP-seq reveals the cis-regulatory architecture of mouse photoreceptors. Genome Res. *20*(11):1512-25

Ebert S*, **Weigelt K***, Walczak Y, Drobnik W, Mauerer R, Hume DA, Weber BH, Langmann T. (2009). Docosahexaenoic acid attenuates microglia activation and delays early retinal degeneration. J Neurochem. *110*(6):1863-75.
*These authors contributed equally to this work

Langmann T, Ebert S, Walczak Y, **Weigelt K**, Ehrengruber MU, Stiewe T, Weber BH. (2009). Induction of early growth response 1 mediates microglia activation *in vitro* but is dispensable *in vivo*. Neuromolecular Med. *11*(2):87-96.

Weigelt K, Lichtinger M, Rehli M, Langmann T. (2009). Transcriptomic profiling identifies a PU.1 regulatory network in macrophages. Biochem Biophys Res Commun. *380*(2):308-12.

Stoecker K, **Weigelt K**, Ebert S, Karlstetter M, Walczak Y, Langmann T. (2009). Induction of STAP-1 promotes neurotoxic activation of microglia. Biochem Biophys Res Commun. *379*(1):121-6.

Langmann T, Lai CC, **Weigelt K**, Tam BM, Warneke-Wittstock R, Moritz OL, Weber BH. (2008). CRX controls retinal expression of the X-linked juvenile retinoschisis (RS1) gene. Nucleic Acids Res. *36*(20):6523-34.

Langmann T, **Weigelt K**, Seifert M. (2008). ChIP-Chip: Identifizierung von Transkriptionsfaktor-Zielgenen. Laborwelt Nr. 5/2008 – 9. Jahrgang

Weigelt K, Moehle C, Stempfl T, Weber BH, Langmann T. (2008). An integrated workflow for analysis of ChIP-chip data. Biotechniques. *45*(2):131-2, 134, 136 passim.

Weigelt K*, Ernst W*, Walczak Y, Ebert S, Loenhardt T, Klug M, Rehli M, Weber BH, Langmann T. (2007). Dap12 expression in activated microglia from retinoschisin-deficient retina and its PU.1-dependent promoter regulation. J Leukoc Biol. *82*(6):1564-74.
* These authors contributed equally to this work

LIST OF ABBREVIATIONS

ADAM17	Tumor necrosis factor-α-converting enzyme
Ago	Argonaute
AMWAP	Activated Microglia/Macrophage WAP domain protein
AP-1	Acitvator protein-1
APC	Antigen presenting cell
ATF3	Activating transcription factor 3
ATP	Adenosin triphosphate
BBB	Blood brain barrier
BCL2A1	BCL2-related protein A1
BCR	B cell receptor
BCG	Bacillus Calmette Guerin
BD	Bipolar disorder
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
CAGE	Cap analysis of gene expression
CASH	Comprehensive Assessment of Symptoms and History
Casp11	Caspase 11
CCL2	Chemokine (C-C motif) ligand 2
CCL3	Chemokine (C-C motif) ligand 3
CCL4	Chemokine (C-C motif) ligand 4
CCL7	Chemokine (C-C motif) ligand 7
CCL20	Chemokine (C-C motif) ligand 20
CCR2	C-C chemokine receptor type 2
CD14	Cluster of differentiation 14
CD68	Cluster of differentiation 68
CDC42	Cell division control protein 42
cDNA	Copy DNA
C/EBP	CCAAT/enhancer binding protein, α
Chi3l3	Chitinase 3-like 3
ChIP	Chromatin immunoprecipitation
Clec5a	C-type lectin 5a
Clec7a	C-type lectin 7a
CLP	Common lymphoid progenitor cell
CMP	Common myeloid progenitor cell
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CP	Controls postpartum

cpm	Counts per minute
Cralbp	Cellular retinaldehyde-binding protein
CR3	Complement receptor 3
CREB	ATF/cyclic AMP responsive element binding-protein
cRNA	Copy RNA
Csf1r	Colony stimulating factor receptor 1
C_t	Cycle threshold
CXCL2	Chemokine (C-X-C motif) ligand 2
CXCL9	Chemokine (C-X-C motif) ligand 9
DAP12	DNAX-activating protein of 12 kDa
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DC	Dendritic cell
ΔΔCt	Delta delta Cycle threshold
DHA	Docosahexaenoic acid
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ds	Doublestranded
DSM	Diagnostic and Statistical Manual of Mental Disorders
DUSP2	Dual specificity protein phosphatase 2
EDTA	Ethylenediaminetetraacetic acid
EGR	Early growth response
EGR1	Early growth response-1
EGR2	Early growth response-2
EGR3	Early growth response-3
EGR4	Early growth response-4
EMP-1	Epithelial membrane protein 1
EMSA	Electrophoretic mobility shift assay
ETS	E-twenty six
FACS	Fluorescence-activated cell sorting
FC	Fold Change
FcγRI	Fc γ receptor I
FCS	Fetal calf serum
FDR	False discovery rate
Fig	Figure
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEDI	Gene Expression Dynamics Inspector
G-CFU	Granulocyte colony forming unit

G-CSF	Granulocyte colony stimulating factor
Gfap	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GM-CFU	Granulocytemacrophage colony-forming-unit
GM-CSF	Granulocytemacrophage colony stimulating factor
GMP	Granulocyte/macrophage progenitor
h	Hours
HC	Healthy controls
HDAC1	Histone deacetylase 1
HEPES	Hydroxyethyl piperazineethanesulfonic acid
Hox8b	Homeobox 8b
HSC	Hematopoietic stem cell
ICD	International classificaiton of diseases
IMDM	Iscove's Modified Dulbecco's
IDO	Indoleamine 2,3-dioxygenase
IFN-β	Interferon β
IFN-γ	Interferon γ
IgG	Immunoglobulin G
IL-1	Interleukine-1
IL-4	Interleukine-4
IL-6	Interleukine-6
IL-8	Interleukine-8
IL-10	Interleukine-10
IL-12	Interleukine-12
IL-13	Interleukine-13
IL-17	Interleukine-17
IL-18	Interleukine-18
IL-23	Interleukine-23
IL-25	Interleukine-25
IL-33	Interleukine-33
INHBA	Inhibin, beta A
ITAM	Immunotyrosine-based activation motif
IRAK1	Interleukin-1 receptor-associated kinase 1
IRF	Interferon regulatory factor
IVT	In vitro-translated
KD	Knockdown
Lmo7	Lim-domain only 7
LPS	Lipopolysaccheride

MAFF	V-maf musculoaponeurotic fibrosarcoma oncogene homolog F
M-CFU	Macrophage colony-forming unit
M-CSF	Macrophage colony-stimulating-factor
MDD	Major depressive disorder
MDP	Macrophage-DC progenitor
MEP	Megakaryocyte/erythroid progenitor
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
MIP1α	Macrophage inflammatory protein 1α
miR	MicroRNA
miRNA	MicroRNA
ml	Milliliter
MPS	Mononuclear phagocyte system
mRNA	Messenger RNA
MS	Multiple sclerose
MXD1	MAX dimerization protein 1
NAB2	NGFI-A-binding protein 2
NaCl	Natruim chloride
NF-κB	Nuclear Factor-kappa B
NGF	Nerve growth factor
ng	Nanogram
nM	Nanomolar
NK	Natural Killer
NLR	NOD-like receptor
NO	Nitric oxide
NOD mouse	Non-obese diabetic mouse
Nrf	Nuclear factor E2-related factor-2
OHT	Hydroxytamoxifen
P	Postnatal stage
p75NTR	p75 neurotrophin receptor
PAMP	Pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDE4B	cAMP-specific 3',5'-cyclic phosphodiesterase 4B
PET	Positron emission tomography

PMSF	Phenylmethylsulfonyl fluoride
PP	Postpartum psychosis
PPR	Pattern recognition receptor
PTGS2	Prostaglandin-endoperoxide synthase 2
PTPN7	Protein tyrosine phosphatase non-receptor type 7
PTPRO	Protein tyrosine phosphatase receptor type O
PTX3	Pentraxin-related protein
PU.1	Purine-rich box-1
RMA	Robust Multichip Analysis
RNA	Ribonucleic acid
RCS	Royal College of Surgeons
RLR	RIG-I-like receptors
RNU44	small nucleolar RNA, C/D box 44
RNU48	small nucleolar RNA, C/D box 48
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RPMI	Roswell Park Memorial Institute medium
RS	Retinoschisis
RT	Reverse Transcription
RXR	Retinoid X receptor
qRT-PCR	Quantitative real-time PCR
QUIN	Quinolinic acid
SAM	Significance Analysis of Microarray
SCID	Structured Clinical Interview for Disease
SCZ	Schizophrenia
SD	Standard deviation
seq	Sequencing
SFPI1	SFFV proviral integration 1
siRNA	Small interfering RNA
snRNA	small nuclear RNA
SOM	Self-organizing map
STAP-1	Signal-transducing adaptor protein 1
STX1a	Syntaxin-1A
TCR	T cell receptor
TF	Transcription factor
TLR	Toll-like receptor
TLR2	Toll-like receptor 2
TLR3	Toll-like receptor 3

TLR4	Toll-like receptor 4
TLR5	Toll-like receptor 5
TLR6	Toll-like receptor 6
TLR7	Toll-like receptor 7
TLR9	Toll-like receptor 9
TNFα	Tumor necrosis factor α
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15
TRAF6	TNF receptor associated factor 6
Treg	Regulatory T cell
TREM-1	Triggering receptor expressed on myeloid cells-1
TREM-2	Triggering receptor expressed on myeloid cells-2
TSS	Transcription start site
U	Unit
µg	Microgram
µm	Micrometer
µM	Micromolar
µl	Microliter
VDR	Vitamin D receptor
wt	Wild type

ACKNOWLEDGEMENTS

As this long and intense journey comes to an end, it is time to thank the people who travelled alongside with me, inspired me and helped me to keep on looking at the bright side of life.

Beste prof. dr. Rob Benner, ik wil u van harte bedanken voor de mogelijkheid om mijn promotieonderzoek op de afdeling Immunologie uit te kunnen voeren. Beste Hemmo, als mijn officiele promoter stond jouw deur altijd voor mij open. Dank je dat ik van jou de kans gekregen heb om mijn promotieonderzoek op jouw lab uit te kunnen voeren. Zonder jouw constructief commentaar en jouw steun tijdens dit werk, had ik dit proefschrift niet kunnen afmaken. De gesprekken met jou waren altijd leuk en leerzam en dat is o.a. omdat jij gewoon geweldig normaal en menselijk bent. Lieber Thomas, auch bei dir möchte ich herzlich dafür bedanken, dass du die wissenschaftliche Betreuung meiner Doktorarbeit übernommen hast. Durch deine Ideen, Erfahrung und hilfreichen Ratschläge hast du viel zum Gelingen dieser Doktorarbeit beigetragen und meine wissenschaftliche Entwicklung enorm gefördert. I am very grateful to the remaining members of my dissertation committee, Pieter Leenen, Fredrik Öberg, Stephan Claes, Steven Kushner and Sabine Bahn. Your academic support and input and personal cheering are greatly appreciated. Thank you.

Beste Erna, bedankt voor de hulp met mijn proefschrift, zonder jou was het met de formulieren niet goed gekomen.

Beste collega's van de Auto-Immun Unit, ik wil iederen graag bedanken voor jullie warm en open welkom. Jullie hebben er veel aan bijgedragen dat ik mij hier in Nederland zo op mijn gemak voel. Beste Pieter, het is altijd heel prettig om met jou samen te werken. Erg bedankt voor jouw commentaar op mijn manuscript. Ik stel het erg hoog op prijs dat je in mijn commissie wilt plaatsnemen. Beste Marjan, altijd relaxed en met een grote glimlach. Leuk dat jouw deur altijd op deze manier open stond. Lieve Zana, volgens mij moesten wij voor het eerst samen lachen over een bepald kapsel en gelukkig was dat niet het laatste melige moment met jou. Of het nou bij Westernpaviljoen is, op kantoor of bij jou op het balkon, met jou is filosoferen overal leuk. Je bent een super vriendin en ik ben heel blij dat je tijdens mijn verdedeging naast mij wil staan. Hvala ti na prijateljstvu! Lieve Angelique, je hebt mij al geholpen toen ik nog in Duitsland zat en dankzij jouw steun vielen veel van de "drempels" hier in het begin heel erg mee. Inmiddels zijn wij vrienden en ik ben heel trots dat je mijn paranimf wilt zijn. Thomas H., of het nu "silly walks" tijdens de dagelijkse wandeling naar Sophia zijn, biertjes na werk of af en toe een kleine musicale improvisatie op het lab, het is altijd gezellig met jou. Harm en Annemarie, dank jullie voor jullie hulp, gezelligheid en bijdrage aan dit proefschrift.

Het samenwerken met jullie op het lab was altijd ontzettend prettig. Roos, ook al ben je er niet meer, het was altijd gezellig met jou op kantoor en zeker ook tijdens borrels. Wouter, mijn Haagse OV-lotgenoot, "I hate my job, but I love the gossip". Volgens deze lijfspreuk hebben wij vaak lol samen in de trein en op kantoor en je bent altijd een grote hulp voor technische vragen. Naomi, danki duchi voor de Caribische Vibes op het lab. Beste A3, Jo, Joey, Lucy, Corine en Serena, jullie wil ik ook graag bedanken voor de leuke tijd op het lab en tijdens de labdagen.
Beste collega's van de afdeling immunologie van het ErasmusMC, hartstikke bedankt voor het warme welkom. Mati, baguettje, you definitely contributed a lot to the fun part of this thesis. Thank you for enjoying some really nice concerts and lots and lots of beers together. Chris, junger Mann, trotz des beinahe unüberbrückbaren Altersunterschiedes hatten wir viele lustige Abende zusammen und dank importierten Spätzlehobels und bayerischen Bieres konnten wir auch in Holland ein Stückchen Heimat zusammen genießen. Eddy en Renée, jullie zijn allebij hartstikke gezellig en een vrolijk glimlach in de ochtend deed soms gewoon goed. Junda, helaas ben je er op dit moment niet, maar ik kon je advies en ook je droge humor altijd waarderen. Ik wens je het beste voor je toekomst. Julia, falls du keine Lust mehr auf's Labor haben solltest, kannst du auch einfach ganz entspannt Zen-Meisterin werden. Deine Gelassenheit und dein Humor haben das Teaching auf jeden Fall um einiges erträglicher gemacht. Sonja, leider hast du unser Institut mittlerweile verlassen, aber auch dir (und Rebecca) vielen Dank für die lustigen Ausflüge und Grillabende in Amsterdam. Ilker, jammer dat je weg bent! Het was altijd lachen met jou. Jeroen B. en Monique, dankzij jullie voel ik mij in Den Haag een stukje meer te huis. Ik hoop dat wij ook in toekomst nog vaker samen de kroeg ingaan. Jan-Piet, op jou kon de "Boudewijn commissie" altijd rekenen. Dank je voor je gezelligheid. Patrick, sinds je leuke haar eraf is, kletsen wij niet meer zo vaak bij de liften. Waarschijnlijk omdat ik je niet meer herken, maar op feestjes is het altijd gezellig met jou. Gellof, je was altijd een grote hulp voor mij met alle administrative dingen en ook al praat je soms nog te snel voor mij, is het altijd gezellig om even bij jou langs te gaan. Winnie, ook jou wil ik erg bedanken voor je hulp met administrative dingen. Marie-Johan en Leo, jullie zijn hele leuke collega's, die altijd een luisterend oor voor mij hadden. Dank jullie wel. Beste Jack, ook jou wil ik graag bedanken. Door jouw vriendelijke hulp konden alle computer problemen heel soepel opgelost werden. Beste Menno en Ruth, jullie wil ik graag bedanken voor jullie advies tijdens het afronden van mijn promotie. Magda B., a trouble shared is a trouble halved. Good luck with your defense and no worries you will do great! Madga R., the last couple of months of my PhD I got to share an office with you and I really enjoyed your relaxed attitude.

Dear collaboratos of MoodInflame, it is always nice to meet you and discuss the latest results of this great project. The atmosphere at our meetings was always friendly and productive and I'm looking forward to continue working with some of you. Dear Livia, thank you very much for the pleasant and constructive cooperation while writing the TREM-1 article. Beste Veerle en Karin B., het was heel leuk om met jullie samen te werken. Bedankt voor jullie hulp met mijn artikel over postpartum psychose en dat ik bij jullie altijd terecht kon voor mijn klinische vragen. Furthermore I would like to thank the following persons for the contributions to the articles in thesis: Michael Rehli, Bernhard H. F. Weber, Monika Lichtinger, Nico J. M. van Beveren, Tom K. Birkenhäger and Mario Pescatori.

Natürlich gilt mein Dank auch allen ehemaligen Kollegen der AG Langmann und des Institutes für Humangenetik der Uni Regensburg. Yana, die Zusammenarbeit mit dir war super und unsere stundenlangen Gespräche in der Zellkultur waren auch der Beginn einer guten Freundschaft. Marcus, mit dir war's gemütlich und auch das ein oder andere gemeinsame Feierabendbier war immer a feine Sach'. Steffi, vielen Dank für die konstruktive Zusammenarbeit. Lars Fritsche und Frau Gellner, vielen Dank für die kleinen, aber feinen Pausen mit gutem Espresso.

Eva, Nina und Antonia, ihr steht am Ende der Kollegenliste, aber am Anfang der Freundesliste. Mit euch hat die Arbeit im Labor viel Spass gemacht und die wenigen Tage im Jahr, die wir uns jetzt sehen, sind immer schöne Tage. Hoffentlich hängen wir auch noch in 10 Jahren zusammen am Strand ab. Maria, da darfst du natürlich auch nicht fehlen! Manchmal hat stundenlanges Warten in Krankenhäusern auch sein Gutes. Jakob und Mike, die Schwibbögen werden dank euch immer unvergessen bleiben und ich bin froh, dass unsere genialen Revivals weder zeit- noch ortsabhängig sind. Doris, Lisa, Luci und Wolle, mittlerweile sind wir flügge geworden und jede von uns geht ihren eigenen Weg. Trotzdem ist, wenn wir uns sehen, irgendwie alles wie immer und genau das macht es so schön. Sebi, als Mitbewohner bist du nicht zu toppen und die Erinnerung an unsere unzähligen Rentnerausflüge als Helmut und Rosi sorgen noch oft für ein Lächeln auf meinem Gesicht. Betty, unsere Lebensplanung könnte unterschiedlicher nicht sein, aber trotzdem finden wir immer wieder den gemeinsamen Nenner. Josef, du warst definitiv der coolste Betreuer an der Uni und auch als Freund machst du 'ne extrem gute Figur. Sabine, wie had toen in 2001 gedacht, dat ik 8 jaar later in Den Haag zou komen wonen. Net zoals toen in Nieuw Zeeland is het ook hier weer heel gezellig met jou. Bovendien wil ik jou en Aafke graag voor jullie hulp met mijn verhuizing naar Den Haag, de Nederlandse belastingsaangifte en alle andere uitdagingen bedanken. Kim en Erik, jullie en jullie gezelligheid kreeg ik als kers op de taart bij mijn droomvrouw erbij en dat vind ik erg leuk. Bianka, Nicole und Hendrike,

die deutsche Connection mit euch in Den Haag hat schon viele fröhliche Abende hervorgebracht und das wird hoffentlich auch in Zukunft noch oft so sein.
Lieber Gerhard und Roland, große Brüder sind was Feines und solange ich denken kann, wart ihr immer für mich da. Auch wenn euch vielleicht nicht ganz klar ist, was ich die letzten Jahre so gemacht habe und warum, bin ich euch sehr dankbar für eurer Interesse, euren Rückhalt und eure Unterstützung.
Liebe Mama, lieber Papa ohne euch wäre es nicht möglich gewesen diese Doktorarbeit zu schreiben. Ihr steht immer hundertprozentig hinter mir und meinen Entscheidungen, habt mir mein Studium ermöglicht und mir während dieser Arbeit gegeben den nötigen Rückhalt gegeben. Danke für euer Vertrauen und dass immer für mich da seid, auch wenn die Entfernungen geographisch nun etwas größer sind als zu Beginn dieser Doktorarbeit.
Lieve Saskia, je hebt het laatste jaar van mijn promotie naast mij gestaan en enorm veel aan bijgedragen dat ik ook aan deze drukke periode met veel plezier terug zal kunnen denken. Dank je voor je steun en je liefde.

CURRICULUM VITAE

Karin Weigelt, MSc

Personal Details

Date of Birth:	November 5, 1981
Citizenship:	German
Gender:	female
Languages:	English, Dutch and German
Interests:	music, boxing, kite surfing, diving, reading

Education

04/2009-11/2012	**Ph.D. studies in immunology**, Department of Immunology, Erasmus Medical Center, Rotterdam, Netherlands
12/2007-03/2009	**Ph.D. studies in human genetics**, Institute of Human Genetics, University of Regensburg, Regensburg, Germany
10/2002-10/2007	**Diploma degree in biology**, University of Regensburg, Regensburg, Germany **Diploma thesis:** 01/2007 – 10/2007 PD. Dr. Langmann, Institute of Human Genetics, University of Regensburg Identification of target genes of the transcription factors Egr1 and PU.1 in microglia cells **Degree:** specialised in cell biology, developmental biology and biochemistry
10/2001–11/2001	**Diploma studies in Nanostructure Techniques**, University of Würzburg, Würzburg, Germany
09/1992 – 06/2001	**Abitur**, Ostendorfer Gymnasium, Neumarkt i. d. Opf., Germany

Presentations and Awards

09/2011	**Poster Presentation** at the 25th Annual Meeting of the European Macrophage and Dendritic Cell Society (EMDS) in Brussels, Belgium *Altered microRNA expression in monocytes of post partum patients*
04-05/2011	**Poster Presentation** at the 13th European Congress of Endocrinology (ECE) in Rotterdam, The Netherlands *A raised GRa/b expression ratio in monocytes of schizophrenic and bipolar patients*
09/2010	**Poster Presentation** at the 24th Annual Meeting of the European Macrophage and Dendritic Cell Society (EMDS) in Edinburgh, Scotland *A raised GRa/b expression ratio in monocytes of schizophrenic and bipolar patients*
08/2010	**ECNP Poster Award** at the 23rd European College of Neuropsychopharmacology (ECNP) Congress in Amsterdam, The Netherlands *Altered GRa/b expression in monocytes of schizophrenic and bipolar patients*
09/2009	**Oral Presentation** at the 23rd Annual Meeting of the European Macrophage and Dendritic Cell Society (EMDS) in Regensburg, Germany *Up-regulated TREM-1 expression in monocytes of schizophrenic patients*
05/2008 – 04/2009	**Scholarship** of the Pro Retina Stiftung Scholarship for Ph.D. students in research on retina degeneration
09/2008	**Poster Presentation** at the 22nd Annual Meeting of the European Macrophage and Dendritic Cell Society (EMDS) in Bresca, Italy *Signal-transducing adaptor protein 1 (STAP1) interacts with the M-CSF receptor and regulates microglia and macrophage activation*

Abroad Experience

12/2001 - 06/2002	Work & Travel in New Zealand