Chapter 1

General introduction

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Chapter 1

Index

1. Epidemiology of multiple sclerosis (MS)
2. Etiology
3. Clinical (sub) types of MS and disease course
4. Monitoring of MS and clinical outcome measurement
   4.1 Magnetic Resonance Imaging (MRI)
   4.2 Expanded Disability Status Scale (EDSS)
   4.3 Biomarker in MS
5. Immunopathology of MS and the role of monocyte and monocyte-derived macrophages
   5.1 Adhesion and extravasation of monocytes
   5.2 Monocytes differentiation into macrophages and role of macrophage in MS
6. Transglutaminase
7. Tissue Transglutaminase (TG2)
   7.1 Alternative splicing of TG2
   7.2 TG2 functions in monocyte and inflammatory macrophages
      7.2.1 TG2 in cell adhesion and extravasation
      7.2.2 TG2 in the differentiation of monocytes into macrophages
      7.2.3 TG2 in efferocytosis
   7.3 TG2 in inflammation
      7.3.1 Inflammatory mediators
   7.4 TG2 in MS
8. Aims and outline of the thesis
1. Epidemiology of multiple sclerosis (MS)

It was in 1868 when Jean-Martin Charcot observed for the first time accumulation of inflammatory cells in the perivascular space in post-mortem brain of patients who had suffered from periodic episodes of neurological dysfunction. This pathological condition was termed “sclérose en plaques” or Multiple Sclerosis (MS). Nowadays, MS is described as a chronic inflammatory neurodegenerative disease of the central nervous system (CNS). It is considered the leading cause of non-traumatic disability among young adults affecting more than 2.5 million people worldwide. The disease has a peak onset between the age of 20 and 40 years and it affects women more than men. MS is characterized by a variety of symptoms such as fatigue, disturbed vision, sensory problems, paresis, spasticity and cognitive dysfunction which reflect the complexity of the disease. The heterogeneous clinical manifestation highly depends on spatial dissemination of the pathological lesions in the CNS.

These lesions are a feature of MS and are primarily characterized by immune cell infiltration across the inflamed blood brain barrier (BBB) into brain parenchyma which ultimately leads to the formation of demyelinated areas. Indeed, during MS one of the target of the immune system is myelin, a lipid/protein-based substance that is enwrapped around axons to increase the speed of electrical communication between neurons.

2. Etiology

Although the causal factors of MS are still not elucidated, both environmental and genetic factors have been proposed to play a role in MS onset. In particular, the risk of developing MS is increased in patient’s relatives compared to non-related individuals, suggesting that genetic factors may play a role in the pathogenesis of the disease. Recently, genome wide association studies (GWAS) have become one of the most powerful tools to search for new genes involved in the predisposition to MS. Genes of the major histocompatibility complex class II (MHC-II) were shown to have the strongest association with MS. In particular, the HLA-DRB1*1501 allele has been consistently associated with elevated risk for MS, displaying a six-fold risk increase in homozygous carriers. Moreover, 110 polymorphisms in 103 discrete loci have been identified outside the MHC complex. Most of those alleles are associated with immune functions such as the interleukin (IL)-7 receptor and IL-2 receptor. Interestingly, genetic variation accounts only for 30% of the overall disease risk pointing toward other risk factors such as epigenetic regulation and environmental factors. For example, increased risk of developing MS is associated with living at increasing latitude (e.g. Northern Europe) that points toward a role for low sun exposure, and most likely vitamin D in MS pathogenesis. In fact, lower blood levels of vitamin D were observed in MS patients. Furthermore, it has been demonstrated that exposure to some infectious agents could also contribute to the onset of MS. In particular, infection with the Epstein-Barr virus (EBV) in late adolescence has been associated with increased risk of
developing the disease. An explanation for this observation could be similarity of the EBV nuclear antigen to myelin surface proteins. While some infections may increase the risk for MS, others have been shown to be protective; in fact it has been demonstrated that helminth parasite infection promotes an anti-inflammatory environment and ameliorates the clinical course of the disease. In addition, according to the “hygiene hypothesis”, individuals not exposed to certain infections early in life (due to an improvement of the hygienic conditions) may develop an hyperalert immune system, favoring the occurrence of autoimmune diseases.

3. Clinical (sub) types of MS and disease course

From a clinical perspective, 3 different disease courses have been characterized according to development, remission and progression of symptoms (Fig. 1). In about 85% of the patients, the disease starts with a Relapsing-Remitting (RR) course in which episodes of neurological dysfunction are followed by periods of complete or incomplete recovery. After several years, the disease progresses into a secondary progressive phase (SP) characterized by steadily worsening disability. Some patients may experience progressive disability from the onset of the disease (primary progressive (PP) MS). MRI measures of the disease include gadolinium-enhancing lesions (represent damage to the blood brain barrier and infiltration of immune cell into the brain parenchyma), accumulation of T2 burden of the disease and decrease brain volume. Reprinted and adapted from Neuron, volume 97, Baecher-Allan C. et al., Multiple Sclerosis: Mechanisms and Immunotherapy, p742–768, Copyright (2019), with permission from Elsevier.
dysfunction are followed by periods of complete or incomplete recovery of symptoms\textsuperscript{28}. Relapses coincide with focal CNS inflammation and demyelination and can be monitored by magnetic resonance imaging (MRI). After one or two decades, in the majority of RR patients the disease progresses into a secondary progressive (SP) phase which is characterized by progressive neurological decline, brain atrophy and absence of remission\textsuperscript{29}. Approximately 10% of MS patients are diagnosed with primary progressive MS (PP) in which neurological decline is progressive from onset of the disease\textsuperscript{30,31}.

**4. Monitoring of MS and clinical outcome measurement**

Disease activity and progression of MS are currently monitored by the use of MRI which can detect brain and spinal cord lesions and their dissemination in both time and space. MRI represents an important tool as it can detect appearance of new lesions, increase in size of established lesions and reduction in brain volume. Furthermore, measurement of patients’ disability by the Expanded Disability Status Scale (EDSS) and detection of CSF/blood-derived biomarkers play a very important role in supporting the monitoring of disease activity and progression.

**4.1 Magnetic resonance imaging (MRI)**

MRI is an imaging-based technique widely used to visualize MS lesions in the brain in patients in vivo. Common MRI measures used to define MS lesions are: 1) T2-hyperintense lesions, 2) contrast enhanced T1 lesions, 3) T1 hypointense lesions or “black holes”. They all make use of magnetic fields and radio waves to measure the relative water content in the brain. Since the layer of myelin that protects neurons is fat-based, it will repulse water molecules. Brain areas in which inflammation occurs and the myelin has been damaged and/or disrupted by MS (called demyelinating lesions) will contain more water, which will be visualized on MRI scan as either a bright white spot (T2) or a darkened area (T1) depending on the type of scans. In clinically definite MS, T2-hyperintense lesions (also called T2 lesions) are observed in 90-95% of the patients and provide information about disease burden or lesion load. MS activity can be evaluated over time either by counting the number of new and enlarged lesions\textsuperscript{32} or by assessing changes in lesion volume\textsuperscript{33}. Contrast enhanced T1 lesions supply information about current disease activity by highlighting areas of active inflammation. They are obtained by the use of contrast agents that are intravenously injected in the patient. The most frequently used contrast agent is Gadolinium (Gad, Gd).

T1-weighted Gd-enhancing lesions represent presence of active lesions; indeed, Gadolinium extravasation into CNS correlates with dysfunction of the BBB and therefore, with active inflammation (especially with the presence of active macrophages)\textsuperscript{34,36}. Gadolinium enhancement is a transient phenomenon that lasts around two to six weeks\textsuperscript{37}; it generally precedes or accompanies the appearance of new lesions in T2-weighted images suggesting that BBB breakdown is a hallmark of the onset of new lesions\textsuperscript{38}. T1 hypointense lesions or black
holes are associated with severe tissue damage e.g. both axonal loss and demyelination. Indeed, it has been shown that T1 lesions load strongly correlates with chronic disability.

4.2 Expanded Disability Status Scale (EDSS)

EDSS is the most commonly used MS clinical outcome measurement and it is widely used to provide an index of functional disability (e.g. motor impairment) in MS patients. This scale is divided into 10 steps (0-10) with a unit step of 0.5. It is based on assessment of several neurological systems e.g. visual, pyramidal (motor), and brainstem. It is generally accepted that EDSS 0-3.5 is indicative of patients with a good functional status. In the middle part of the range (4.0-5.5), it is mainly an evaluation of ambulation ability, while EDSS 6.0-9.5 reflects the need of patients for assistance in performing daily activities. EDSS 10 represents death due to MS. Despite being the most widely used outcome measure for disability status and progression in MS, there are many limitations in EDSS measurement. First, EDSS scores are highly variable because of the subjective nature of the neurological examination. Secondly, the non-linearity of the EDSS score is another limitation; in fact, changes between steps (e.g. 1.0 to 2.0 and 4.0 to 5.0) are unequal. To circumvent this issue, it has been suggested that an increase of 1.0 or more is clinically significant if the EDSS at baseline was 0 to 5.5 while an increase of 0.5 or more is clinically relevant if the baseline EDSS was higher than 5.5. Finally, some argue that, from scale 4.0 upwards, the EDSS measurement focuses too much on patient’s walking ability and that does not provide an accurate measurement of other important determinants in MS such as cognitive functions.

4.3 Biomarkers in MS

Biomarkers or biological markers, refer to CSF or blood-derived characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathogenic or pathological processes or of responses to a therapeutic intervention. In MS, biomarkers can be categorized in different groups: 1) markers for clinically isolated syndrome (CIS)-to-MS conversion and diagnosis, 2) markers for MS activity/progression and 3) markers for (adverse) response to treatment. The first category includes biomarkers that are able to predict conversion from CIS into clinically definite MS such as IgM oligoclonal bands in CSF and Chitinase-3-like protein 1 (CHI3L1, chitin-binding proteins homologous to chitinase but lacking their capacity of chitin hydrolysis) in CSF. The second category includes biomarkers that correlate with disease activity or progression. Among those, neurofilaments are emerging as some of the most promising new biomarkers, offering exciting potential to monitor ongoing axonal injury and neurodegeneration. Neurofilament (Nf, neuron-specific intermediate filaments) levels in biological fluids, specifically in the CSF, are suggested to reflect the degree of axonal damage based on their release into the extracellular space during axonal injury. Nf-Light chain is considered to reflect early, acute, inflammatory-mediated axonal damage due to its correlation with inflammatory disease and correlates
Upon activation in the circulation, myelin-specific T cells can cross the blood brain barrier (BBB) and enter the central nervous system (CNS) where they are reactivated by antigen presenting cells. Subsequently, the will differentiate into different T-cell subsets. Among those, Th1 and Th17 will secrete pro-inflammatory cytokines which in turn will contribute to activation of microglia and macrophages. This ultimately leads to axonal damage and demyelination. Reprinted and adapted from Neurobiology of Brain Disorders, Schaeffer J. et al., Multiple Sclerosis, p497-520, Copyright (2019), with permission from Elsevier.
less accurately with disability progression\textsuperscript{50}. Conversely, Nf-Heavy chain levels best correlate with disease progression and thus are thought to reflect ongoing neurodegenerative axonal damage\textsuperscript{50}.

For the third category, antibody formation to interferon (IFN)-\textbeta\textsuperscript{51,52} or natalizumab\textsuperscript{53-56} upon prolonged treatment with those drugs indicate reduced therapeutic efficiency. More challenging and urgent is the identification of biomarkers for PP-MS and SP-MS. As to date, there is a lack of biomarkers that can provide objective assessments of neurologic worsening such as ongoing grey and white matter demyelination and remyelination.

5. Immunopathology of MS and the role of monocyte and monocyte-derived macrophages

Although the exact pathophysiology of MS is still debated, an early pathological observation is the infiltration into the CNS of peripheral blood mononuclear cells (PBMC) which consists of lymphocytes (T cells, B cells) natural killer cells and monocytes. In particular, peripheral myelin-specific autoreactive T-cells are primed by professional antigen presenting cells (APC) in peripheral organs and therefore, become able to enter the CNS by crossing the BBB. Once in the CNS, they get reactivated upon interaction with myelin-presenting perivascular APC, such as dendritic cells and macrophages\textsuperscript{57-59}, thus promoting demyelination and axonal loss. Although the reason for T-cell activation is still unclear, it is known that upon reactivation, inflammatory mediators are released leading to microglial and astrocyte activation and recruitment of more immune cells from the periphery. Besides T-cells and B-cells, also monocytes will be drawn to the CNS, generating monocyte-derived macrophages and thereby contributing to neuroinflammation (Fig. 2).

5.1 Adhesion and extravasation of monocytes

Two crucial processes in the early phases of inflammation mediated by monocytes are adhesion and extravasation. Monocytes are drawn to sites of inflammation by the chemotactic gradient of inflammatory factors released at the inflamed site. This facilitates their adherence to the activated vascular endothelium, followed by extravasation and migration through the tissue\textsuperscript{60}. In more details, monocytes in the bloodstream first roll on the activated and/or inflamed endothelial lumen, before they strengthen their adherence and crawl along the lumen to find an extravasation site into the inflamed tissue\textsuperscript{60,61}. Monocyte adhesion is dependent on a plethora of cell surface molecules\textsuperscript{60}. A well-known group of molecules that is involved in these processes is the family of integrins. Integrin heterodimers expressed on monocytes are involved in the arrest and initial adhesion of these cells to the vascular endothelium by binding to endothelial cell adhesion molecules (CAM) like intracellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1)\textsuperscript{51,62}. This process facilitates the extravasation of these cells from the bloodstream into the tissue (Fig. 3).
5.2 Monocyte differentiation into macrophages and role of macrophages in MS

Blood monocytes recruited to inflammatory sites differentiate into inflammatory macrophages with distinct functions and act as key players in the innate immune response. Macrophages can adopt diverse activation states in response to inflammatory stimuli.

The two extremes of the polarization spectrum are nowadays referred to as classically activated (M1) macrophages and alternatively activated (M2) macrophages (Fig. 3). In general, the M1 phenotype is regarded to represent a pro-inflammatory activation state with cytotoxic properties, whereas alternatively activated macrophages promote tissue repair by secreting anti-inflammatory cytokines (e.g. IL-10, IL-13, and Transforming growth factor (TGF)-β). Additionally, the latter can be divided in various functionally different subsets depending on the stimuli encountered, such as IL-4 or IL-10. Classically and alternatively activated macrophages can be characterized and distinguished by a panel of functional markers such as IL-1β and tumor necrosis factor (TNF)-α for classically activated macrophages and the Mannose Receptor (MR or CD206) for the alternatively activated macrophages. Although both phenotypes can also be identified in vivo, the majority of
these cells exhibit intermediate polarization states. Nevertheless, it is widely accepted that macrophages play a dual role in MS as they contribute to myelin destruction and lesion formation, but also support removal of myelin debris thereby inhibiting inflammation and promoting tissue repair. In particular, IL-4 activated macrophages (called M(IL-4)) are more efficient in removing myelin debris through phagocytosis compared to classically activated macrophages. Reactive macrophages, together with locally activated microglia, can therefore mediate cell damage or neuroprotective effects in MS, and they can contribute to the relapsing-remitting feature of MS. Based on studies in animal models for MS, it is known that during the acute phase, the number of classically activated microglia and macrophages is relatively high. Conversely, the alternatively activated macrophages and microglia (anti-inflammatory) undergo a gradual increase during the process of inflammation until the peak of disease, whereas the number of pro-inflammatory cells is decreased. During the later phase of the disease, anti-inflammatory cells are predominant in the CNS, where they can release a variety of anti-inflammatory mediators such as IL-4, IL-10 known to suppress inflammation. These anti-inflammatory mediators are also implicated in the resolution of inflammation, tissue repair and remodelling.

6. Transglutaminase

The term transglutaminase (TGase, TG) was introduced for the first time in 1957 to describe a liver enzyme able to incorporate amines into proteins. Nowadays, TGases are defined as a widely distributed family of Ca²⁺-dependent enzymes that catalyze post-translational modifications of proteins by formation of isopeptide bonds. The human genome encodes for 9 different members of the TG family: FXIIIa (plasma TGase) has a crucial role in the blood clotting cascade as well as in wound healing and it is highly expressed in blood cells such as monocytes. TG1 (keratinocytes TGase), TG3 and TG5 are mainly involved in the terminal differentiation of keratinocytes. TG4 (prostatic secretory TGase) is essential for fertility in rodents. TG6 and TG7 are still largely uncharacterized. TG2 (tissue transglutaminase, tTG) is widely distributed and the best characterized member of the TG family. Besides one of the transglutaminase family members, named Band 4.2, which lacks enzymatic activity, the catalytic mechanism of all the other transglutaminases is highly conserved and results in the formation of a covalent bond between the γ-carboxamide group of a peptide bound glutamine residue and a primary amine group. The resulting isopeptide bond is very resistant to proteolytic degradation and it has been demonstrated that cross-linked products accumulate in pathological conditions.

7. Tissue Transglutaminase (TG2)

Tissue transglutaminase (TG2) is the most ubiquitous and multifunctional TG member. In addition to its best known transamidating activity, it binds and hydrolyzes GTP. It has extensively been demonstrated that Ca²⁺ and GTP/GDP inversely regulate the transamidating activity of
TG2 by inducing a conformational change; TG2 is active as a transglutaminase when bound to Ca\(^{2+}\) and then in an open conformation. On the contrary, it is enzymatically inactive when bound to GTP/GDP and in a closed conformation\(^8\). Nevertheless, in the closed conformation, TG2 is able to regulate signal transduction; in fact, alike other members of the family, TG2 has various additional enzymatic functions, i.e. disulfide isomerase function\(^8\), G protein (GTPase) function during which TG2 is also known as Gha\(^8,9\) and protein kinase activity\(^8\). The molecular structure of TG2 consists of four domains, which are involved in distinct functions: the N terminal β-sandwich (binds fibronectin and integrins), the catalytic core (transamidating activity) and two C terminal β-barrels of which the latter includes a phospholipase C binding sequence\(^8\).

As various as its alleged functions are the reported (sub)cellular locations of TG2. Although predominantly present in the cytoplasm, TG2 has also been found in the nucleus, mitochondria, endoplasmatic reticulum and on the cell surface\(^9-14\). Furthermore, TG2 can be secreted from the cell into the extracellular matrix (ECM)\(^8,15,16\). TG2 is constitutively expressed in many tissues and cell-types, amongst others in macrophages, and is up-regulated in a cell type-dependent manner by several physiological and pathological stimuli. Increased TG2 expression and transamidation activity are often observed under inflammatory conditions in which cytokines and growth factors, released by injured cells, regulate TG2 expression and activity\(^97\). With its highly diverse functions and locations, TG2 is in an ideal position to contribute to a variety of cellular events, including cell adhesion and migration\(^98\), cell differentiation\(^99\) and efferocytosis\(^100\) (Fig. 4).

### 7.1 Alternative splicing of TG2

Full length TG2 or TGM2_v1, reported for the first time by Gentile et al., has a predicted molecular mass of 78 kDa\(^101\). In addition, four alternative splice variants of TG2 mRNA have been described in literature, all producing shorter variants with predicted different properties/functions compared to full-length TG2. Because different abbreviations have been assigned to these splice variants, the classification used by Phatak et al., will be followed in this thesis\(^102\). The common feature of all the short variants is that they all lose, to a certain extent, their C-terminus, which has been shown to contain a GTP binding domain thus altering the response of TG2 to Ca\(^{2+}\) activation. The loss of amino acids at the C-terminus could reduce GTP binding and allow the short variants to escape GTP regulation and exhibit TGase activity when there is a transient increase in Ca\(^{2+}\) levels in comparison to that required for activation of full length TG2.

TGM2_v2 (also called TGH, Tgase S or TG2-S) and TG2M2_v3 (also called TGH2) variants were identified in retinoic acid-treated human erythroleukemia cells. Through a mechanism of intron retention (incorporation of intron X and VI) they generate a 62 kDa (TGM2_v2) and a 38 kDa (TG2M2_v3) protein\(^103-106\). Interestingly, expression of TGM2_v2 was found to be increased in the brain of Alzheimer disease patients\(^107\).
In addition, opposite cellular functions have been attributed to this splice variants of TG2 as it was found to induce cell differentiation in neuroblastoma cells as well as cell death in NIH3T3 cells alike TGM2_v1, which acted as repressor of differentiation in neuroblastoma cells and growth-inducer in NIH3T3 cells\textsuperscript{103,106}. Noteworthy is that the expression TGM2_v3 has been observed to be increased in leukocytes of coeliac disease patients compared to healthy controls\textsuperscript{108}.

More recently, two other alternative splice variants of TG2 have been identified and characterized in human umbilical vein endothelial cells (HUVEC), vascular smooth muscle cells and leukocytes namely TGM2_v4a (also called tTGv1) and TGM2_v4b (also called tTGv2)\textsuperscript{109}. Alike the first two variants, these originate from an atypical splice event that generates proteins with an alternative C-termini but similar molecular mass to the full

![Fig. 4 Enzymatic and non-enzymatic activity of TG2](image)

When present in the cytosol (C), nucleus (N) and in the extracellular space (E), TG2 catalyze Ca\textsuperscript{2+}-dependent 1) incorporation of primary amine into protein, 2) covalent cross-linking of protein (main activity in vivo), 3) deamidation of specific glutamines (when water replaces the amine donor). TG2 also has 4) Ca\textsuperscript{2+}-dependent isopeptidase activity. TG2 interaction with fibronectin and integrin is important for its appearance on the cell surface, where TG2 promote cell-matrix interaction. At the cell membrane (M), following stimulation of specific receptor, TG2 mediates signal transduction by the activation of phospholipase C (PLC), through its GTPase activity. Reprinted from Trends in Biochemical Sciences, volume 27, Fesus L. and Piacentini M., Transglutaminase 2: an enigmatic enzyme with diverse functions, p534–539, Copyright (2019), with permission from Elsevier
length form (75 kDa and 70 kDa, respectively). In addition, it has been suggested that the C-terminus is important for stabilizing the half-life of full length TG2\(^{109}\). Interestingly, expression of TGM2\(_v4\)a and TGM2\(_v4\)b in leukocytes is higher compared to HUVEC and vascular smooth muscle cells which could indicate that they may have unique functions in leukocytes\(^{109}\).

7.2 TG2 function in monocytes and inflammatory macrophages

7.2.1 TG2 in cell adhesion and extravasation

Participation of TG2 in cell adhesion and extravasation was first shown for fibroblasts and later for several other cell types, including monocytes\(^{98,110}\). The protein fibronectin is a major constituent of the extra cellular matrix (ECM) and serves as a primary substrate for adhesion and migration of cells\(^{111}\). Integrins on the cell surface bridge the intracellular cytoskeleton with fibronectin in the ECM and thus facilitate adhesion of cells onto the ECM\(^{111}\). The contribution of TG2 to adhesion was first described as interaction of TG2 with fibronectin\(^{112}\). Accordingly, the fibronectin binding domain in the TG2 structure was defined and as a result, fibronectin is the best studied substrate for TG2 action in adhesion and migration\(^{113}\).

Reduction of TG2 production by TG2 knock-down or inhibition of TG2-fibronectin binding by function-blocking antibodies strongly reduced the cellular adhesion and migration capacities of macrophages\(^{98}\). Special emphasis was put on investigating the participation of FXIII\(_a\) in these processes, as it is present in monocytes. However, no contribution of FXIII\(_a\) to adhesion and/or migration processes could be shown, so that the above mentioned effects are probably due solely to TG2\(^{114-116}\). Moreover, when monocytes differentiated into macrophages upon adhesion, the cellular expression of TG2 increased, whereas FXIII\(_a\) expression decreased, making it unlikely that FXIII\(_a\) is of relevance in cell adhesion processes of monocytes and macrophages\(^{98,117}\).

Cell adhesion onto other cells or the ECM includes integrin binding. TG2 on the cell surface of macrophages is complexed with integrins, i.e. β1/β3/β5-integrins\(^{98}\). Moreover, increased TG2 expression in macrophages results in a simultaneous increase in β-integrin expression and presence on the cell surface. These TG2-integrin complexes function as a bridge between integrins and fibronectin on cell surfaces and in the ECM, facilitating cell adhesion\(^{113}\). Additionally, the localization of cell surface TG2-integrin complexes in specialized adhesive structures in macrophages called podosomes, points toward a strong link between TG2-integrin complexes and adhesion and subsequent extravasation of the cell\(^{118,119}\). Interestingly, the interaction between TG2 and β-integrins does not require transamidation activity to increase adhesion, spreading and cell motility\(^{81,113,120-123}\). Thus, although the transamidation activity of TG2 is unlikely to play a role in adhesion and migration processes, the formation of the above-mentioned specific protein-complexes appears crucial.

In addition to its involvement in cell binding to ECM proteins, TG2 is also an important player in assembly, remodelling and stabilization of the ECM via crosslinking of several ECM...
substrates, in particular fibronectin, vitronectin, von Willebrand factor, proteoglycans and collagens\textsuperscript{95,124}. ECM stabilization by TG2-mediated transamidation increases the rigidity of the ECM. This rigidity can selectively trigger focal adhesion formation and hence may result in increased cell adhesion to the ECM\textsuperscript{125,126}. Moreover, cross-linking of ECM proteins increases the clustering of binding sites for cells to adhere to the ECM\textsuperscript{127,128}. The above described contribution of TG2 to adhesion and migration appears to be mediated by cell surface and/or extracellular TG2. Migration and especially diapedesis of cells through the blood vessel endothelium requires a high motility of the actin cytoskeleton to be able to change the shape of the cell in an efficient and fast manner. This reorganization of the cytoskeletal structure and rearrangement is mediated by focal adhesion kinase (FAK) and Rho-associated protein kinase (ROCK)\textsuperscript{129,130}. Both of these are stimulated by TG2 induced clustering of cell surface integrins, which leads to higher initiation of cell adhesion and cytoskeletal flexibility\textsuperscript{131}. In line with this, it has been reported that RhoA activity, an upstream stimulator of ROCK was reduced after inhibition of TG2 transamidating activity in rat macrophages which coincided with reduced F-actin cytoskeletal rearrangement\textsuperscript{132}. Interference of the specific protein-complex formation of TG2 with integrins and fibronectin in the process of adhesion, extravasation and migration could serve as a pharmacological target to modulate these cellular processes. Recently, such a manipulation has been described to prevent cancer metastasis by interfering with cell adhesion onto fibronectin and cellular migration\textsuperscript{133,134}.

7.2.2 TG2 in the differentiation of monocytes into macrophages

To execute their functions in inflamed tissue, circulating monocytes need to adhere, migrate and ultimately differentiate into mature tissue macrophages. The molecular mechanism(s) by which monocytes undergo this morphological and functional differentiation during inflammation is not fully characterized yet. However, it has been proposed that TG2 plays an important role in this process. This notion is based on the in vitro observations that TG2 expression and cross-linking activity are low in freshly isolated monocytes, but exponentially increase during the maturation process of monocytes, induced either chemically or by adherence to the cell culture dish\textsuperscript{135-137}. Moreover, in this context it may be of importance that the up-regulation of TG2 expression in monocytes represents a constitutive process which takes place under physiological conditions e.g. after monocyte adhesion onto endothelial cells\textsuperscript{138}. During adhesion onto naïve endothelial cells, the monocytes up-regulate genes required for the transendothelial migration and initiate the differentiation program into phagocytes. This suggests that TG2, as one of the highly expressed genes, can play a role in the maturation process\textsuperscript{138}. In addition, although the classification of tissue macrophages is still under debate, TG2 up-regulation seems to occur predominantly in alternatively activated (M2) macrophages\textsuperscript{139,140}. In fact, this observation recently led to the proposal of TG2 as a specific M2 macrophage marker\textsuperscript{139}. 

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Chapter 1
7.2.3 TG2 in efferocytosis

Clearance of apoptotic cells by efferocytosis is crucial for resolution of inflammation by preventing secondary necrosis and release of inflammatory mediators\textsuperscript{141,142}. The involvement of TG2 in efferocytosis was established in 1981 when TG2 was suggested to play a role in the efferocytic capacity of macrophages\textsuperscript{99,100,135,136,143}, which was generally lacking in undifferentiated monocytes with little TG2 expression\textsuperscript{136}. Therefore, TG2 in macrophages is considered as an important mediator of efferocytosis that limits inflammation by removal of apoptotic cells and additionally decreases pro-inflammatory cytokine secretion, both of which are impaired in the absence of TG2 or when TG2 activity is inhibited\textsuperscript{115,116,143-147}. Furthermore, defects in efferocytosis are also linked to autoimmunity\textsuperscript{148}. Indeed, TG2 knockout (TG2\textsuperscript{-/-}) mice demonstrate signs of autoimmunity due to insufficient efferocytosis with increasing age, supporting again TG2 as a mediator of efferocytosis\textsuperscript{144}. Of interest is that macrophages in TG2\textsuperscript{-/-} mice show specifically reduced efferocytosis of apoptotic leukocytes but not of phagocytosis per se, as shown for bacteria, yeast, opsonized non-apoptotic thymocytes or monosodium urate crystals\textsuperscript{116,144,145}. Efferocytosis is mediated by the formation of a phagocytic cup that tethers apoptotic cells to macrophages and facilitates their engulfment\textsuperscript{116}. It has been reported that a lack of TG2 in macrophages diminishes efferocytosis by reduced engulfment and does not abridge tethering of apoptotic cells\textsuperscript{115}. However, other data indicated that TG2 can be, indirectly, involved in the tethering of apoptotic cells\textsuperscript{116}. β3-integrin promotes the tethering of apoptotic cells to macrophages\textsuperscript{149} and TG2 induces β3-integrin clustering on the cell surface in a phagocytic cup\textsuperscript{116}. Therefore a lack of TG2 results in a less efficient formation of phagocytic portals which leads to reduced tethering and engulfment of apoptotic cells\textsuperscript{116}. TG2 mediating efferocytosis has so far been linked to extracellular or surface localization of the enzyme. This is supported by correction of in vitro efferocytosis in TG2\textsuperscript{-/-} macrophages after adding exogenous recombinant TG2, either adding wild type or the catalytically inactive enzyme\textsuperscript{145}. Moreover, the function of TG2 in efferocytosis is apparently independent of GTPase activity\textsuperscript{145}.

7.3 TG2 and inflammation

As described, TG2 contributes to various processes that occur in monocytes and/or macrophages during inflammation, including cell adhesion, extravasation and efferocytosis. The remaining question is whether TG2 itself, produced by macrophages is regulated by inflammation or inflammation-related mediators, e.g. cytokines.

7.3.1 Inflammatory mediators

During inflammation, various inflammatory mediators, including cytokines, are produced to modulate the cellular responses involved. To experimentally induce an inflammatory state, lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative
bacteria, is often used. It has been shown that LPS increases TG2 mRNA expression and activity in macrophages and BV-2 microglial cells. The induction of TG2 production was closely associated with enhanced phagocytic properties of microglia and nitric oxide production, mediated via LPS-induced activation of the NFκB pathway. The promoter region of TG2 contains certain cytokine and NFκB responsive elements, thus when inflammation occurs and the NFκB pathway is activated, this may up-regulate TG2 expression. Once induced, TG2 can in turn contribute to further NFκB activation by cross-linking the inhibitory molecule IκBα leading to a sustained expression of various target genes involved in inflammation, such as inducible nitric oxide synthase and TNF-α. This activation loop has also been demonstrated in a mouse macrophage cell line in which LPS treatment induced the production and activation of TG2, NFκB and metastatic tumour antigen 1 (MTA1, a master chromatin modifier). The latter interacts with NFκB to induce TG2 gene expression.

Besides LPS, the pro-inflammatory cytokine interferon gamma (IFN-γ) is an inducer of TG2 production in monocytes. Although two other pro-inflammatory mediators, TNF-α and IL-1, are able to induce TG2 expression in different cell types, including liver cells, chondrocytes and astrocytes, no literature is available regarding the regulation of TG2 expression by these mediators in monocytes or macrophages.

In contrast to its onset, the resolution of inflammation is mediated by the secretion of anti-inflammatory cytokines such as interleukin-4 (IL-4) and TGF-β, which can induce tissue repair. Intriguingly, in apparent contrast to the above mentioned pro-inflammatory regulation of TG2, regulation of monocyte and macrophage-derived TG2 by anti-inflammatory mediators has been recently proposed as well. Indeed, IL-4 treated primary human macrophages up-regulate TG2 gene expression in vitro. In addition, using a proteomics approach, TG2 was identified as a novel M2 marker in human and mouse macrophages. Moreover, induction of an M2 phenotype in rat macrophages coincides with an upregulation of TG2 expression. In line with these findings, it has been reported that Glycine Tomentella Hayata, a herbal medicine with anti-inflammatory properties, up-regulates TG2 expression in RAW264.7 mouse macrophages and enhances the clearance of apoptotic cells. In addition to IL-4, TGF-β exerts pro-resolution activity during an inflammatory response. Although TGF-β-induced TG2 expression is very well established in various cell types, such as fibroblasts or human trabecular meshwork cells, thus far no studies report regulation of TG2 expression by TGF-β in monocytes and macrophages. In addition, TG2 has been described to mediate TGF-β production in these cells, clearly indicating a link between the two.

IL-6 has been shown to positively regulate TG2 expression in macrophages. Treatment of human THP-1-derived macrophages with IL-6 induced almost a 2-fold up-regulation of TG2 mRNA expression paralleled by an increase in the production of anti-inflammatory cytokines and efferocytosis rate. All together, these observations indicate that TG2 production and/or activity can be regulated in macrophages during inflammatory processes, irrespective of the pro- or anti-inflammatory nature of the mediators involved.
7.4 TG2 in MS

The involvement of monocyte- and macrophage-derived TG2 in the pathogenesis of MS is an interesting and unexplored new field. Our group was the first to observe the appearance of immunoreactive TG2 in infiltrated macrophages in white matter lesions of MS patients\textsuperscript{132}. In addition, TG2 appeared in the CNS of marmosets suffering from Experimental Autoimmune Encephalomyelitis (EAE), a non-human primate model for MS\textsuperscript{170}. In active white matter (WM) lesions in these animals, TG2 immunoreactivity was observed in round shaped cells localized in proximity to the blood vessels. These cells co-localized with Iba1, a marker for macrophages and microglia. Furthermore, due to its co-localization with β1-integrin and the close association with extracellular fibronectin, we put forward that TG2 may play a prominent role in the adhesion and migration of infiltrating monocytes and macrophages during EAE. Noteworthy is the fact that TG2 seems to be differentially expressed at various stages of lesion activity. In particular, the number of TG2 positive cells reduced when the lesions lost activity. Conversely to the white matter lesions, in cortical grey matter (GM) lesions fibronectin expression is absent and TG2 seems to be predominately expressed by resident microglia.

The role of TG2 in the pathogenesis of MS was subsequently confirmed in a study in which we demonstrated that reduction of TG2 activity in a rat model for MS resulted in clinical improvement. The improvement coincided with reduced demyelination, reduced production of inflammatory mediators and less monocytes infiltrating into the CNS. These results thus support a role for TG2 in the adhesion, extravasation and migration of monocytes during EAE and possibly MS pathogenesis\textsuperscript{132}. Overall, our findings support a contributing role for TG2 to the pathogenesis of experimental models for MS resulting in monocyte infiltration into the CNS, which is a key factor in the development of clinical symptoms\textsuperscript{171}.

8. Aims and outline of the thesis

A well-known pathological hallmark of MS is the influx of circulating immune cells into the CNS which contributes to the secretion of inflammatory mediators, myelin damage and axonal loss. Although MS is mostly considered as a T-cell mediated disease, it is well established that also monocytes numerously infiltrate the CNS during lesion formation and that local macrophages play an important role in e.g. myelin phagocytosis.

TG2 is the most abundantly expressed enzyme of the Transglutaminase family. Through its diverse enzymatic and non-enzymatic activities, it is involved in many cellular functions including apoptosis, cell adhesion and migration. Alteration in TG2 expression and/or enzymatic activity has been associated with several diseases such as coeliac disease, cancer and neurodegenerative disorders.

Recent findings from our group showed TG2 in MHC-II positive cells in active MS lesions. Subsequent studies have pointed to a role for monocyte-derived TG2 in EAE, the most
common used animal model for MS. In particular, it has been demonstrated that inhibition of TG2 activity results in reduction of EAE clinical symptoms and reduced monocyte infiltration in the CNS in animals suffering from EAE.

Thus far, it is unexplored if TG2 is expressed in blood-derived immune cells of MS patients. Therefore, the aim of this thesis was to explore the expression and role of blood cell-derived TG2 in MS pathology. In particular, we aimed to:

1) Determine the expression and function of TG2 in human monocyte and macrophages
2) Explore TG2 as a novel biomarker for MS

The first aim is addressed in chapters 2 and 3. Particularly, in chapter 2 we studied the expression level of monocyte-derived TG2 mRNA in patients suffering from MS compared to healthy control subjects. As inflammation plays a dominant role in MS and TG2 is known to be upregulated during inflammation, we studied the inflammatory profile of TG2-expressing monocytes derived from the two groups. Furthermore, we explored which inflammatory factors could account for the upregulation of TG2 in human monocytes and possibly MS.

Infiltrating monocytes differentiate into macrophages upon entering tissue, e.g. the CNS, and they acquire potent phagocytic properties which can be beneficial in MS pathology. Recently, TG2 has been identified as a new marker for anti-inflammatory macrophages. This led us to investigate, in chapter 3, the regulation of TG2 expression by pro- and anti-inflammatory mediators, and the potential role of anti-inflammatory tuned macrophage-derived TG2 in the phagocytosis of human myelin debris.

The second aim of the thesis is the focus of chapters 4 and 5. In chapter 4 we sought to investigate the translational application of our previous findings. We studied TG2 expression level in PBMCs of patients with different subtypes of MS compared to healthy control subjects. Moreover, TG2 expression was correlated to various disease activity and disease progression measurement to explore its potential as a novel biomarker for MS.

Besides full length TG2, four shorter splice variants of TG2 have been described. These variants have different functional capacities and could have different or opposite roles compared to the full-length TG2 during disease. In chapter 5 we investigated expression levels of the four short splice variants and the ratio with full-length TG2 in PBMCs derived from primary progressive MS patients and healthy control subjects.

Finally, in chapter 6, the results of the previous chapters are summarized and discussed, together with their possible clinical implications and future perspective.
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Chapter 1


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