

***Summarizing discussion***



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Multiple Sclerosis (MS) is a well known chronic inflammatory disease of the central nervous system (CNS) that mainly affects young adults.<sup>75</sup> Much research is performed to understand and develop treatment of MS which has led to the introduction of several medications for MS.<sup>398</sup> However, these treatments do not cure MS and are troubled by side effects. The failure of current forms of therapy to stop progression of the disease and their lack of specificity therefore provides strong impetus to continue the search for more effective and less toxic therapies. During the initial phase of relapsing-remitting MS, the infiltration and migration of leukocytes into the CNS is a major pathological process.<sup>50</sup> When MS progresses, there are also other neuropathological features including astrogliosis and failure of remyelination, resulting in neurodegeneration and long-lasting neurological deficits.<sup>409</sup> Therefore, it is essential to characterize molecular and cellular events that underlie the different neuropathological processes occurring during MS. This may ultimately lead to the identification of novel therapeutic targets to combat the various neuropathological processes contributing to MS.

The studies described in this thesis were aimed to investigate the contribution of tissue Transglutaminase (TG2) to various neuropathological processes underlying MS. TG2 is a multifunctional enzyme that is ubiquitously expressed in the human body. It is a calcium dependent enzyme which can catalyze post-translational modification of proteins by cross-linking via  $\gamma(\epsilon\text{-glutamyl})$  lysine bonds.<sup>113, 136</sup> Moreover, TG2 has been shown to be a GTP-binding protein and has GTPase activity.<sup>6</sup> TG2 is localized in the cytoplasm, on the cell surface and in the extracellular matrix (ECM), where it plays an important role in cell-matrix interactions.<sup>106, 222</sup> Of the transglutaminase family, TG1, TG2 and TG3 have been shown to be expressed in the healthy human CNS.<sup>187</sup> TG2 has been implicated in various (patho)physiological processes, including cell adhesion and migration, extracellular matrix formation, cell apoptosis and survival, and cell differentiation.<sup>106</sup> As described in chapter 1, MS is characterized by infiltration of immune cells into the CNS, production of inflammatory mediators, demyelination, astrogliosis, failure of remyelination, and subsequent axonal damage. Since TG2 is expressed in the human CNS,<sup>227</sup> and has been shown to be involved in a number of cellular processes, including cell adhesion and migration, apoptosis, cell differentiation and inflammation, which contribute to MS pathology, we hypothesized that TG2 plays a crucial role in neuropathological processes underlying MS including; 1) monocyte infiltration and migration into the CNS (chapter 3), 2) glial scar formation (chapters 4 and 5), and 3) differentiation of oligodendrocyte precursor cells (OPCs) and subsequent (re)myelination (chapter 6). Although the results are discussed separately in each chapter, I will summarize them here, and put the most important observations in perspective to draw conclusions about their importance for future development of improved therapy.

### ***TG2 expression in human MS lesions and in the CNS of cr-EAE animals***

To be able to quantitatively measure TG2 protein levels in human and rodent tissue and cells, a TG2 specific sandwich enzyme-linked immunosorbent assay (ELISA) was developed as described in chapter 2. This study was the first to describe a quantitative TG2 protein measurement across species and across matrices containing TG2 protein. This assay is

of considerable interest for the research described within this thesis, but also for other research groups working on TG2.

We started our studies on the role of TG2 in MS by determining alterations in TG2 immunoreactivity in human post-mortem material of MS patients and in fresh frozen material from the CNS of animals suffering from experimental MS. As described in chapter 3, TG2 immunoreactivity appeared in infiltrating monocytes/macrophages in active and chronic active MS lesions, and in the spinal cord of rats suffering from chronic relapsing experimental autoimmune encephalomyelitis (cr-EAE), a widely used animal model for MS.<sup>124</sup> Expression levels of other transglutaminase family members that have been shown to be present in the human brain, i.e. TG1 and TG3, were unaltered in human active MS lesions and in the spinal cord of cr-EAE animals compared to healthy controls (data not shown).

Thus, as described in chapter 3, TG2 is present in infiltrating monocytes around blood vessels in active MS lesions and in the spinal cord of cr-EAE rats. Furthermore, in chapter 4, it was shown that TG2 also colocalizes with glial fibrillary acidic protein (GFAP), a marker for reactive astrocytes, in active and chronic active MS lesions. Interestingly, in contrast to MS lesions, in the spinal cord of cr-EAE rats, TG2 is mainly expressed in infiltrating monocytes, but only observed occasionally in GFAP positive astrocytes (chapter 3). This might be due to species differences or staining problems since we show in chapter 5 that TG2 expression and activity is increased in primary rat astrocytes upon cytokine treatment *in vitro*.

### **TG2 determines the clinical outcome of experimental MS**

To study if TG2 plays a clinically relevant role, we reduced TG2 activity during cr-EAE using the highly specific small molecule inhibitor KCC009.<sup>421</sup> Inhibition of TG2 activity resulted in an immediate and dramatic reduction of clinical scores, even when treatment with KCC009 was started only during ongoing disease. Also use of cystamine, a less specific TG2 inhibitor,<sup>86</sup> from first signs of cr-EAE onwards, induced less pronounced, but significant clinical improvement. These observations suggest that TG2 activity is of clinical importance, and is a potential novel therapeutic target for the treatment of MS.

### **TG2 facilitates monocyte infiltration into the CNS**

Previous studies showed that TG2 expression is present in monocytes and TG2 activity is involved in monocyte differentiation into macrophages.<sup>9</sup> Moreover, upon binding of monocytes to endothelial cells, TG2 gene expression was increased suggesting that TG2 also is involved in subsequent monocyte migration across endothelial cell layers.<sup>368</sup> Furthermore, it is thought that TG2 plays a major role in cell adhesion and migration processes. In fact, TG2 expression on the surface of monocytes serves as an integrin-associated co-receptor for adhesion to and migration on the ECM protein fibronectin.<sup>9</sup> Besides the direct evidence that TG2 is involved in adhesion and migration of cells, also observations indirectly related to cell migration have been presented. For example, TG2 can regulate the activity of matrix metalloproteases (MMPs),<sup>8</sup> which are proteins that are involved in cell migration. Furthermore, TG2 was shown to be involved in cytoskeletal remodeling,<sup>341</sup> which is essential for cell adhesion and migration. Thus, the presence of TG2 in infiltrating monocytes/macrophages, as shown in chapter 3, suggests that TG2 is involved in monocyte/macrophage adhesion onto endothelial cells, and subsequent

infiltration and migration into the CNS.

The data described in chapter 3 of this thesis are the first to illustrate that TG2 is indeed involved in monocyte adhesion to and migration across endothelial cells in the spinal cord of cr-EAE rats *in vivo* and were confirmed by using several dedicated *in vitro* cell models. To determine the role of TG2 in monocyte infiltration, we inhibited TG2 activity *in vivo* and *in vitro* using the previously mentioned TG2 activity inhibitor KCC009. Upon inhibition of TG2 activity from different stages of cr-EAE onwards, monocyte adhesion onto brain endothelium appeared to be increased whereas monocyte migration into the CNS was significantly reduced indicating that TG2 is particularly important for monocyte migration across endothelial cells. Interestingly, KCC009 treatment of cr-EAE animals reduced TG activity selectively in spinal cord and not in cervical lymph nodes or spleen although recruitment of activated monocytes to these organs has been observed in MS and EAE.<sup>83</sup> These data suggest that treatment with KCC009 locally inhibits TG2 activity at a site where it is most pronounced during cr-EAE, i.e. at the interface between the blood and the CNS. Although MS and EAE are considered to be T-cell mediated diseases,<sup>241</sup> no TG2 activity was detected in T-cells isolated from cr-EAE animals, and no effect on T-cell migration into the spinal cord of cr-EAE animals was observed after KCC009 treatment, suggesting that TG2 is not involved in T-cell infiltration.

The data presented in chapter 3 demonstrate that TG2 activity, exhibited by activated monocytes but not by T-cells, is a key element in regulating monocyte adhesion and migration into the CNS during cr-EAE, thereby determining the clinical outcome. This is in line with previous studies where it was shown that activated monocytes and macrophages play a crucial role in the neuropathology and clinical outcome of EAE.<sup>149, 158</sup>

Neuropathological studies of the spinal cord of KCC009 treated cr-EAE animals showed that activated monocytes accumulated within the lumen of blood vessels and in the perivascular space with far less cells migrating into the CNS parenchyma compared to vehicle-treated cr-EAE animals. These findings were confirmed by subsequent *in vitro* studies using monocytes adhering onto and migrating over a brain endothelial cell layer. As shown in chapter 3, TG2 regulates monocyte adhesion and migration via activation of RhoA, thereby inducing cytoskeletal rearrangements. RhoA belongs to the Rho family of small G-proteins and is crucial in regulation of cell morphology, cell differentiation and cytoskeletal rearrangement.<sup>38, 226, 313</sup> RhoA is a substrate for TG2. In fact, activation of TG2 leads to transamidation of RhoA<sup>340</sup> and after transamidation, RhoA is activated and binds/activates RhoA-associated kinase-2 (ROCK-2), a downstream target of GTP-bound Rho. This RhoA-ROCK-2 complex is transported to the cell surface to increase formation of stress fibers and focal adhesion complexes.<sup>341</sup>

In addition to the effects of KCC009 on monocyte adhesion and migration, we observed also that inhibition of TG2 activity by KCC009 resulted in reduced demyelination in the spinal cord of cr-EAE animals. This is certainly of clinical interest, and may be due to the fact that iNOS and TNF $\alpha$  expression was reduced in the spinal cord. In MS, macrophages are the effector cells in active demyelination, primarily by production of iNOS and TNF $\alpha$ .<sup>102, 322, 382</sup> Again, this effect of TG2 inhibition seemed to be restricted to the spinal cord, as the expression levels of iNOS and TNF $\alpha$  in the spleen of cr-EAE animals were not affected. By treatment with KCC009, however, the expression levels of other inflammatory mediators, including pro- and anti-inflammatory cytokines and chemokines, remained unaltered in

both spinal cord and spleen of KCC009 treated animals versus vehicle treated EAE animals, suggesting that infiltrating macrophages are not the primary source of those mediators. Of additional interest is that the activation status of local microglial cells, as measured by CD68 mRNA expression, a marker for activated microglia and macrophages, was not different between KCC009 treated or vehicle treated cr-EAE animals, perhaps accounting for at least part of the production of inflammatory mediators.<sup>365</sup>

### ***Astrocyte-derived TG2 interacts with fibronectin***

In the active phase of MS, astrocytes become activated, migrate, and contribute to local tissue remodeling that ultimately results in the so-called astroglial scar. This reactivity of astrocytes is accompanied by changes in expression of e.g. adhesion molecules, cytokines, proteoglycans and proteases thereby creating an environment which may impede tissue repair.<sup>95, 312</sup> Therefore, astroglial scar formation is considered as a major mechanical impediment to remyelination and axonal regeneration in MS. Likely, the formation of the astroglial scar is facilitated by deposition of extracellular matrix proteins, including fibronectin. Indeed, an increased expression of various ECM proteins at the lesion sites in MS has been described.<sup>93, 119, 348, 349, 390</sup>

An example of TG2's role in tissue remodeling has been shown in glioblastomas. Reduction of TG2 activity by KCC009 disrupts the assembly of fibronectin fibrils in the extracellular matrix in glioblastomas.<sup>422</sup> This results in increased apoptosis of tumor cells after chemotherapy. Furthermore, TG2 has been implicated in adhesion and migration of fibroblasts and monocytic cells onto extracellular matrix proteins, in particular fibronectin.<sup>9, 22</sup>

In this thesis, in chapters 4 and 5, we showed that TG2 can mediate the interaction between astrocytes and fibronectin. At first we observed that TG2 immunoreactivity appears in reactive astrocytes in and at the rim of human MS lesions (chapter 4). This TG2 at least partly colocalized with intracellular or extracellular fibronectin. We subsequently studied the role of TG2 in adhesion and migration of astrocytes onto fibronectin. Indeed, downregulation of TG2 by siRNA or inhibition of its activity by KCC009 reduced astrocyte adhesion and migration, suggesting a role for TG2 in regulation of astrocyte motility (chapter 4). An additional *in vitro* study further extended these observations. Thus, as shown in chapter 5, TG2 expression, inside cells and present at the surface of astrocytes, is regulated by inflammatory cytokines. Moreover, these *in vitro* experiments showed that cell surface TG2 is clearly involved in the interaction of astrocytes with the extracellular matrix protein fibronectin and thereby modulates astrocyte adhesion to fibronectin.

Similar to monocytes/macrophages, we show that TG2 is an essential factor in regulating RhoA GTPase activity in astrocytes to stimulate focal adhesion formation necessary for interaction of astrocytes with the ECM and astrocyte migration.<sup>313</sup> These focal adhesions link the extracellular matrix to the cytoskeleton via integrin receptors and adapter proteins such as vinculin.<sup>406</sup> Focal adhesions can transmit signals into the cell via activation of focal adhesion kinase (FAK).<sup>285</sup> In astrocytes, TG2 is involved in focal adhesion formation and this is mediated by phosphorylation of FAK and expression of vinculin. Therefore, we conclude that TG2 activity at the surface of astrocytes is involved in binding to fibronectin. This might result in fibronectin remodeling that leads to stabilization of the ECM to facilitate glial scar formation. Since it has been shown in EAE mice that migration of OPCs remained

restricted to the margins of the lesions<sup>23</sup> and in chronic MS lesions a reduced number of OPCs were present compared to non-lesioned areas,<sup>64</sup> I suggest that astrocyte-ECM interactions, mediated at least partially by TG2, could impair oligodendrocyte migration and thereby remyelination.

**TG2 is involved in differentiation of oligodendrocyte precursor cells and remyelination**

As an alternative to the astroglial scar theory, it has been proposed that remyelination failure in MS is due to a lack of factors stimulating differentiation of OPCs into myelinating oligodendrocytes.<sup>118</sup> Simultaneous to demyelination, remyelination occurs in MS lesions. This remyelination, however, is often not fully successful. Despite partial, spontaneous myelin repair in MS giving rise to so-called shadow plaques,<sup>24, 49</sup> most OPCs remain in an undifferentiated state resulting in a failure to generate mature myelinating oligodendrocytes.<sup>119</sup>

TG2 is involved in different cellular functions including cell differentiation and apoptosis.<sup>112, 222</sup> With regard to cellular differentiation, it has been shown that TG2 can enhance neuronal differentiation via activation of RhoA GTPase activity.<sup>377</sup> As shown in chapter 6, TG2 contributes to differentiation of OPCs into myelin-producing oligodendrocytes. TG2 expression is present in OPCs and reduced when these cells differentiated into more mature and myelinating oligodendrocytes. Interestingly, TG2 is also present in PDGFR $\alpha$  positive OPCs in the developing human cerebellum at gestational week 28 when OPCs start to differentiate. Inhibition of TG2 activity *in vitro* reduced the differentiation of OPCs into mature oligodendrocytes. Furthermore, *in vivo* experiments using TG2 knockout and wild-type mice in the cuprizone model for de- and remyelination showed that TG2 is involved in remyelination since myelin formation lagged behind in TG2 knockout mice. This impaired remyelination in TG2 knockout mice had negative consequences for the recovery of motor-performance of these animals. Reduced remyelination in TG2 knockout mice correlated with impaired motor-performance, which is in line with previous studies where it was shown that demyelination in the corpus callosum correlated with motor function of rodents.<sup>150</sup>

Investigation of the underlying mechanism(s) points to a role for RhoA GTPase activity in TG2-mediated OPC differentiation. It is known that Rho-GTPases are expressed by OLGs<sup>99</sup> and their activity is essential for promoting process extension during OPC differentiation.<sup>214</sup> Since TG2 is clearly present in OPCs, as shown in chapter 6, and TG2 can transamidate RhoA GTPase thereby increasing its activity,<sup>341</sup> we expected that inhibition of TG2 activity would attenuate RhoA activity. Indeed, RhoA activity was reduced in OPCs upon KCC009 treatment. Our data suggest that, in OPCs, TG2 plays an intracellular role. During OPC differentiation into more mature oligodendrocytes, intracellular calcium levels increase,<sup>352</sup> which can result in activation of TG2. Upon transamidation of RhoA by TG2, activated RhoA GTPase enhances the activity of the mitogen-activated protein kinase (MAPK) pathway, which in its turn is involved in expression of myelin specific genes<sup>341</sup> that regulate OLG elongation and branching.<sup>420</sup> In this manner, TG2 may play an important role in OPC differentiation and probably acts by upregulating RhoA activity which subsequently activates intracellular pathways, e.g. the MAPK pathway, thereby promoting differentiation of OPCs into myelin forming OLGs.

## General conclusions

The data described in this thesis support our original hypothesis and provide the first evidence that TG2 has a multifaceted role in the neuropathology of MS. These various proposed levels of involvement of TG2 in the neuropathology of MS are illustrated in Fig. 1.

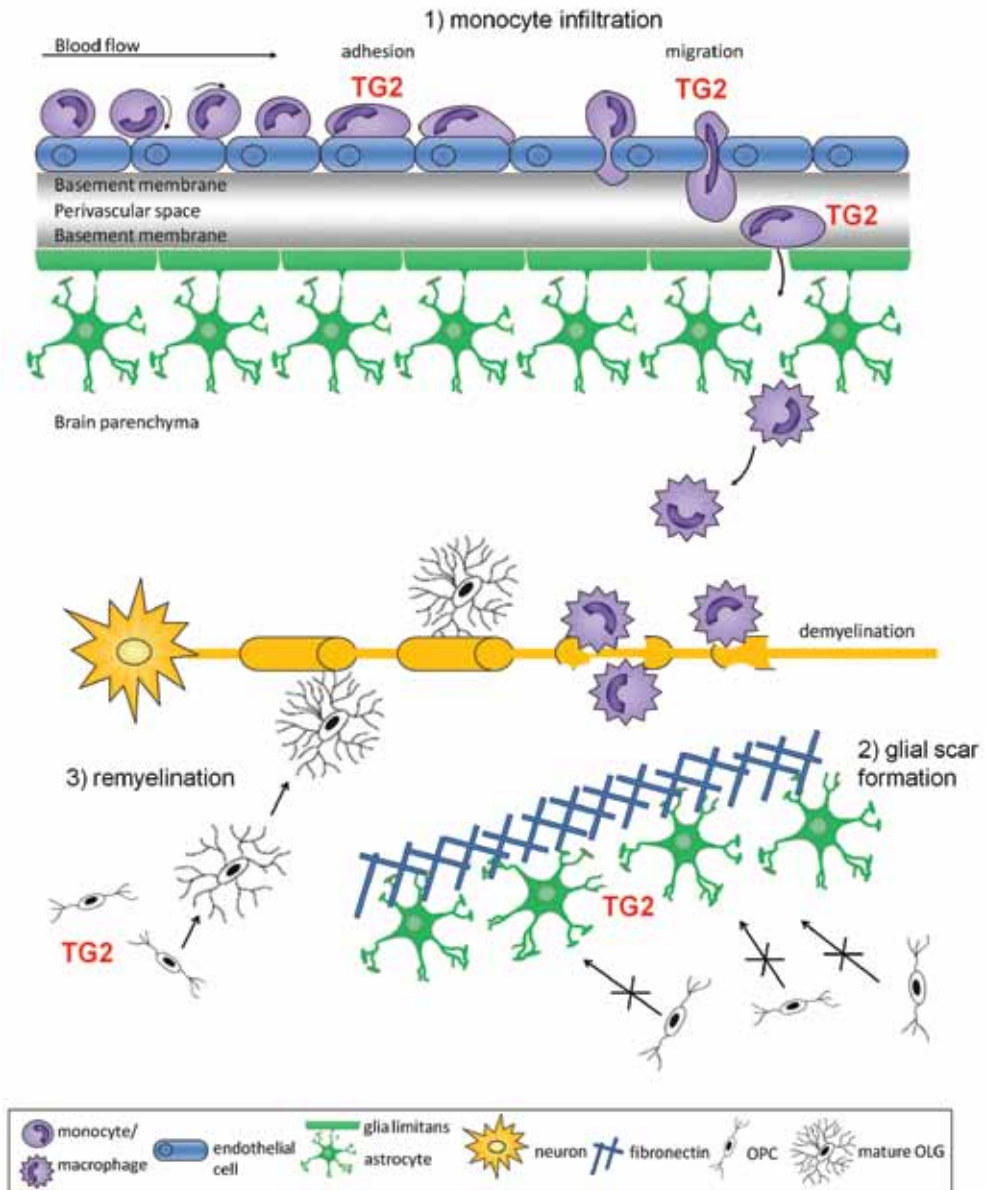
In active human MS lesions and in the spinal cord of cr-EAE animals, TG2 expression is induced and expressed by monocytes/macrophages. *In vivo* analysis and subsequent *in vitro* studies showed that TG2 is essential for monocyte infiltration into the CNS. This will result in the presence of macrophages in the CNS which express pro-inflammatory factors such as iNOS and TNF $\alpha$ , leading to demyelination. Thus, due to its role in monocyte/macrophage infiltration, TG2 is indirectly involved in demyelination.

In active and chronic active MS lesions, TG2 is expressed by astrocytes. Subsequent *in vitro* studies showed that TG2 is present on the surface of astrocytes and increased after treatment with pro-inflammatory cytokines. The data described in this thesis suggests that TG2 can contribute to astrogliosis by mediating the adherence between (reactive) astrocytes and fibronectin, an ECM protein upregulated in MS lesions. Based on previous findings, e.g. that tumor cell derived TG2 is involved in remodeling of fibronectin in the ECM,<sup>422</sup> I suggest that in MS lesions TG2 is active in ECM remodeling and glial scar formation. These actions of TG2 likely impair remyelination.

On the other hand, TG2 is present in OPCs and stimulates differentiation of these precursor cells into mature, myelinating oligodendrocytes, thereby positively contributing to (re) myelination.

The exact role of TG2 in MS pathology is thus dependent on the cell type which is expressing TG2, the activation state of this cell type and the intracellular, cell surface or extracellular localization of TG2.



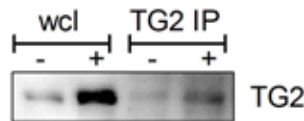


**Figure 1:** Schematic model of the possible role of TG2 in the neuropathology of MS. 1) TG2 that is expressed by monocytes is involved in monocyte infiltration into the CNS. 2) TG2 activity present on the surface of astrocytes interacts with the ECM molecule fibronectin. Thereby, TG2 facilitates ECM remodeling and glial scar formation. 3) TG2 expressed by OPCs is crucial in the differentiation of these precursor cells into myelinating oligodendrocytes explaining the role of TG2 in (re) myelination. (TG2: tissue Transglutaminase, OLG: oligodendrocyte, OPC: oligodendrocyte precursor cell)

## Future research and therapeutic perspectives

### **The role of TG2 in monocyte/macrophage infiltration during MS**

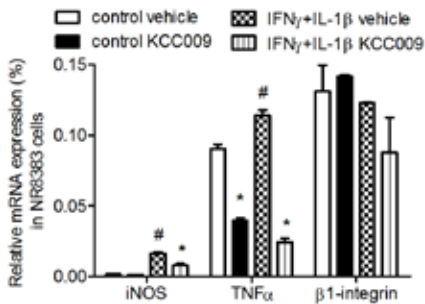
As shown in this thesis, TG2 is present in infiltrating monocytes in human MS lesions and in the spinal cord of cr-EAE animals where TG2 plays a crucial role in monocyte/macrophage infiltration. Therefore, TG2 is a very interesting therapeutic target for the treatment of MS patients. However, more research needs to be performed to study the exact role of TG2 expressed by monocytes. For instance, it was not determined whether TG2 is present on the cell surface of monocytes or expressed inside the cell. More knowledge on the cellular location of TG2 expression would give more insight about the function of TG2 in monocytes in MS and cr-EAE. Preliminary data in monocytes *in vitro* showed that TG2 is present on the surface of monocytes and increased after treatment with cytokines (Fig. 2). TG2 that is expressed on the cell surface of monocytes can bind to different substrates that are present in the extracellular matrix, on the surface of monocytes or on other cells, such as endothelial cells. On the surface of various cells, TG2 forms stable non-covalent complexes with  $\beta 1$  and  $\beta 3$  integrins.<sup>10</sup> Thereby, TG2 can affect integrin signaling by promoting their clustering and increasing activation of FAK and RhoA.<sup>10, 168</sup> To date, only evidence is presented on the role of binding of TG2 to  $\beta$ -integrins that are allegedly present on the surface of the same cell.<sup>10</sup> As shown in chapter 3, mRNA and protein expression levels of  $\beta$ -integrins were increased in the spinal cord of cr-EAE animals upon inhibition of TG2 activity. Preliminary data in monocytes *in vitro* showed that upon treatment with KCC009, mRNA expression of iNOS and TNF $\alpha$  were decreased (Fig. 3), which is in line with what was found in cr-EAE *in vivo*. Interestingly, however, mRNA expression levels of  $\beta 1$ -integrin were unaltered (Fig. 2), in contrast to the increased levels that were found *in vivo*. This data suggest that the increased level of  $\beta$ -integrins *in vivo* is not monocyte derived, but might be present on the surface of endothelial cells, suggesting that TG2 interacts with  $\beta$ -integrins on another cell type. More knowledge on the protein expression pattern of TG2 in or on the cell surface of monocytes after treatment with KCC009 and the possible interaction with  $\beta$ -integrins might provide more mechanistic insight to reduce migration of monocytes *in vivo*. Furthermore, more information on the possible interaction of TG2 with  $\beta$ -integrins on the surface of other cell types, also in a non MS-related setting, is interesting since there is no evidence on the role of TG2 present in one cell in binding to  $\beta$ -integrins on another cell.



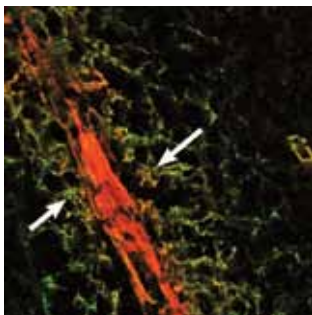
**Figure 2:** TG2 protein levels are increased in whole cell lysates and on the surface of monocytes after treatment with cytokines (IFN $\gamma$  + IL-1 $\beta$ , 50 ng/ml each) for 48 hours. Upon cytokine treatment, cells were surface biotinylated and lysed. Also whole cell lysates were prepared. Cell lysates were immunoprecipitated using a TG2 antibody (Ab3, Neomarkers), separated by SDS-PAGE and blots were stained with a TG2 antibody (Ab3, Neomarkers). (wcl: whole cell lysate, -: untreated, +: cytokine treated)

Secondly, it remains to be determined what factor triggers TG2 activation in monocytes/macrophages during MS and cr-EAE. It has been shown that upon monocyte adhesion to endothelial cells, TG2 mRNA expression in monocytes is increased.<sup>368</sup> Also, when monocytes differentiate into macrophages, TG2 protein expression, as well as TG2 activity increases.<sup>9</sup> These data, together with the findings that there was no effect of treatment with KCC009 on TG2 activity levels and monocyte migration in the liver and spleen (chapter 3), suggests that TG2 is only activated at the site of inflammation. Insight in the factors and mechanisms that influence TG2 activity is important to develop therapies for MS patients with reduced side effects compared to the therapies that are already used in the clinic.

Connected to this issue, more studies need to be performed on possible side effects of inhibition of TG2 activity. At this moment, it is not known whether inhibition of TG2 activity results in unwanted effects, e.g. whether inhibition of TG2 activity will result in a defective immune reaction when there is inflammation outside the CNS. TG2 activity is increased in monocyte derived dendritic cells.<sup>239</sup> Dendritic cells are important antigen presenting cells for initiation and regulation of the immune response<sup>220</sup> and it has been shown that TG2 plays an essential role in regulating the response of dendritic cells to stressful antigenic stimuli.<sup>239</sup> For this reason, inhibition of TG2 activity in dendritic cells has been suggested as an interesting therapeutic target for general inflammatory conditions such as sepsis,<sup>101, 239</sup> but could also give serious side effects during treatment of MS due to dendritic cell dysfunction.



**Figure 3:** mRNA levels of iNOS, TNF $\alpha$  and  $\beta$ 1-integrin in untreated and cytokine treated (48 hours) monocytes and subsequent TG2 inhibition using 0.5 mM KCC009 for 1 hour. Upon cytokine treatment, mRNA levels of iNOS and TNF $\alpha$  were increased. Expression of  $\beta$ 1-integrin was unaltered. Treatment with KCC009 significantly reduced the mRNA expression levels of iNOS and TNF $\alpha$ , whereas the mRNA levels of  $\beta$ 1-integrin remained unaltered. Data are expressed as mean + s.e.m., n=4. <sup>#</sup>P<0.05 compared to vehicle treated control (effect of cytokine treatment), <sup>\*</sup>P<0.05 compared to vehicle treated cells (effect of TG2 inhibition).



**Figure 4:** TG2 colocalizes with fibronectin, mainly around blood vessels (arrows), in the spinal cord of cr-EAE animals. Fresh frozen material from the cervical part of the spinal cord of cr-EAE rats (sacrificed at day 27) was immunofluorescently stained for TG2 (red) and fibronectin (green).

So far, however, our studies suggest that KCC009 specifically inhibits TG2 activity only at the site of inflammation, and thereby leaves immune-relevant responses intact resulting in fewer side effects.

### ***The role of TG2 in the formation of the astroglial scar***

If we consider TG2 as an interesting and novel therapeutic target of which the activity can be manipulated to improve the pathological and clinical outcome of MS, a better understanding of the role of TG2 in astrogliosis and demyelination is of importance. Since reactive astrogliosis is a complex and multifaceted process with many different functions, such as protection of neural cells, tissue and function, and restriction of the spread of inflammation and infection, therapeutic strategies need to be directed at specific aspects of reactive astrogliosis, without complete blockade of the different functions of astrogliosis.<sup>350</sup>

Currently, it is not clear how TG2 mediates the interaction of astrocytes with the ECM and if astrocyte derived TG2 is involved in glial scar formation. Furthermore, it is unclear if astrogliosis prevents oligodendrocyte migration and if TG2 is indirectly involved in inhibition of remyelination via deposition of fibronectin. Based on the study as presented in this thesis, it is known that astrocyte-derived TG2 is involved in astrocytic adhesion to fibronectin. However, it is not clear whether TG2 is involved in remodeling of fibronectin. Thus, more research on the contribution of TG2 to glial scar formation, matrix remodeling and oligodendrocyte migration should be performed. For this purpose, *in vitro* cell models using astrocytes and oligodendrocytes could be used and also *in vivo* models, including the cuprizone model for de- and remyelination as well as cr-EAE. It has been shown that astrogliosis is present in cr-EAE animals<sup>23</sup> and primary rat astrocytes *in vitro* showed increased TG2 protein activity upon cytokine treatment (chapter 5). However, in the present study, colocalization of TG2 with GFAP in astrocytes in the spinal cord of cr-EAE animals was hardly observed (data not shown). Interestingly, however, colocalization of TG2 and fibronectin was detected in the cervical part of the spinal cord of cr-EAE animals, mainly around blood vessels (Fig. 4). These data suggest that, also in the spinal cord of cr-EAE animals, extracellular TG2 is present in the ECM and interacts with fibronectin, which is one of the most prominent extracellular matrix proteins produced and deposited in MS lesions.<sup>390</sup> The differences in expression of TG2 in human MS lesions and primary cells *in vitro* compared to the findings in cr-EAE animals *in vivo* could be due to species differences. Furthermore, TG2 can be externalized by astrocytes in the CNS of cr-EAE animals. It has already been suggested that secreted TG2 from cells is involved in the assembly of fibronectin fibrils.<sup>127</sup> Moreover, it is conceivable that the TG2 immunoreactivity found in the ECM of cr-EAE animals is monocyte derived. Therefore, more research on the role of TG2 in ECM remodeling should be performed.

The role of TG2 in astrocytes adhesion to fibronectin could be of therapeutic interest for MS. The already available MS therapies are mainly used to treat the initial relapsing-remitting phase of MS, however, the secondary progressive phase remains untreatable. It has been shown that remyelination is most prominent in early MS lesions whereas the remyelination capacity deteriorates when the disease turns into a chronic phase.<sup>133</sup> Thus, stimulation of regeneration/remyelination in MS lesions is essential, especially in patients who have entered later stages of the disease. Reduction of TG2 activity on the surface

of astrocytes will reduce the interaction of astrocytes with the ECM protein fibronectin. I suggest that this will result in reduced ECM remodeling and astroglial scar formation, thereby reducing the barrier for OPCs to migrate to the lesion site.

### **TG2 in oligodendrocytes**

Since the data described in this thesis show that inhibition of TG2 activity is an interesting new therapeutic approach, more studies need to be performed on the role of TG2 in OPCs present in human MS lesions.

In chapter 6, it has been shown that TG2 is involved in differentiation of OPCs and subsequent (re)myelination. Inhibition of TG2 activity during cr-EAE and in MS could thus impair differentiation of OPCs and subsequent (re)myelination.

At this time, it is not known if TG2 is present in OPCs in and around MS lesions and if TG2 is involved in remyelination after demyelination in the CNS of cr-EAE animals and in human MS lesions. Therefore, performing more research to address these questions is necessary. Since enhancement of remyelination is an important field of therapeutic approaches in EAE and MS that will serve more attention in the future,<sup>119</sup> the role of TG2 in differentiation of OPCs is interesting for future therapy of MS.

A particularly interesting finding, shown in chapter 6, is that TG2 protein expression and activity is reduced when cells start to differentiate from OPC to more mature galactosylceramide (GalC) and 2'3'-cyclic nucleotide 3'-phosphohydrolase (CNP) positive cells and finally into mature myelinating oligodendrocytes. TG2 expression in more mature oligodendrocytes might thus negatively influence myelination. Furthermore, increased TG2 activity may induce cell death since over-expression of TG2 in neurons 'sensitizes' these cells to apoptosis.<sup>293</sup> In the present study, only inhibition of TG2 activity *in vitro* and the role of TG2 in myelination *in vivo* was studied. Therefore, the effect of over-expression of TG2 on differentiation and survival of OPCs and more mature oligodendrocytes needs to be studied to determine the possible therapeutic advantage of TG2 in OPC differentiation and improved myelination.

In summary, we show in this thesis that TG2 has multiple functions in the pathogenesis of MS. It is my hope and expectation that improved knowledge on the role of TG2 in MS pathogenesis will lead to novel therapeutic strategies to reduce cellular infiltration into the CNS, demyelination and astroglial scar formation and on the other hand, improve remyelination of demyelinated MS lesions.