Chapter

3

Local tissue Transglutaminase activity directs experimental Multiple Sclerosis pathology

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Abstract

A critical pathogenic aspect of multiple sclerosis (MS) is the influx of immunomodulatory cells into the central nervous system (CNS) leading to neurological deficits. We here provide evidence that tissue Transglutaminase (TG2) is an attractive therapeutic target for MS that, in contrast to other MS therapies, is acting locally. During MS and its animal model chronic-relapsing EAE (cr-EAE), TG2 levels are significantly increased and reduction of TG2 activity in cr-EAE animals dramatically attenuated clinical deficits. The mechanism underlying the clinical beneficial effects points toward a reduction in monocyte migration into the CNS due to attenuated monocytic cytoskeletal flexibility and RhoA GTPase activity. We conclude that reduction of TG2 activity opens new avenues for therapeutic intervention in MS.

Introduction

Multiple Sclerosis (MS) is a chronic neuro-inflammatory disease affecting mainly young adults, and clinically resulting in major motor and sensory deficits amongst other symptoms. A critical pathogenic aspect of MS is the influx of immunomodulatory cells into the central nervous system (CNS), which induce inflammation, demyelination and axonal damage[11, 203] causing neurological deficits. During MS, the entry of lymphocytes and monocytes from the bloodstream into the CNS across the blood brain barrier is stimulated by chemokines interacting with their corresponding receptors[139] and by integrins (e.g. $\alpha 4\beta 1$) binding to adhesion molecules (e.g. VCAM-I and ICAM-I)[355] resulting in firm adhesion of immunomodulatory cells onto the vascular endothelium.[416] Following endothelial transmigration, cells traverse the endothelial basement membrane and glia limitans and subsequently migrate into brain parenchyma, a process that is facilitated by local tissue destruction via activation of matrix metalloproteases (MMPs).[405] Infiltrated activated macrophages are considered to be a source of inflammatory mediators e.g. tumor necrosis factor α (TNFα) and reactive oxygen species, including nitric oxide (NO) that stimulate myelin phagocytosis resulting in subsequent demyelination.[102, 384] Current therapies for MS reduce cell infiltration or induce immunosuppression but additionally disturb the immune balance,[98, 321, 334] illustrating the need for a locally acting drug to treat MS.

Tissue Transglutaminase or Transglutaminase type 2 (TG2, EC:2.3.2.13) is a 78-kD multifunctional enzyme that belongs to a multigene family of Ca^{2+}-dependent protein cross-linking enzymes[222] localized in the cytoplasm, on the cell surface and in the extracellular matrix (ECM), where it plays an important role in cell-matrix interactions.[106, 222] Importantly, TG2 activity is enhanced during inflammatory processes,[144, 201, 255] but TG2 itself also plays a modulatory role in various immune processes. TG2 is known to act as a co-receptor for $\beta 1$ and $\beta 3$-integrin subunits,[10] TG2 can regulate MMP2 and MMP9 activity[8] and is involved in cytoskeletal remodeling.[341] Furthermore it has been shown that TG2 activity is involved in adhesion and migration of monocytes onto the extracellular matrix protein fibronectin.[9] Since TG2 is involved in numerous immune-regulatory processes, we hypothesized that TG2 is crucial for adhesion and migration of immune cells into the CNS during MS and thereby contributes to its pathogenesis.

Results

TG2 immunoreactivity in perivascular space-located cells in human MS lesions

When investigating the presence of TG2 immunoreactivity in active subcortical white matter MS lesions and in non-affected subcortical white matter of control subjects, clear TG2 immunopositive blood vessels (Fig. 1A) could be observed in both groups without a clear difference in localization or intensity. In addition, in active MS lesions, TG2 positive cells appeared within parenchymal tissue located near blood vessels (Fig. 1A), but most often in the perivascular space (Fig. 1A,B). By double immunofluorescence these cells could partially be identified as MHC class II positive inflammatory cells (Fig. 1D-F). These cells very likely constitute activated monocytes. Preadsorption of the anti-TG2 antibody with TG2 resulted in a dramatic reduction in TG2 immunoreactivity in these macrophage-

like cells (Fig. 1B,C), indicating its specificity.

TG2 mRNA and protein levels are upregulated during chronic relapsing EAE in rats

Also during chronic-relapsing experimental autoimmune encephalomyelitis (cr-EAE), TG2 immunoreactivity was, in addition to blood vessels, localized at perivascular infiltrating cells in the rat spinal cord (Fig. 2A,C). In IFA immunized (control) animals, TG2 immunoreactivity was observed in blood vessels only (Fig. 2B). These TG2 immunoreactive infiltrating cells were identified as activated monocytes by double labeling with the calcium binding peptide Iba-1 (Fig. 2C,D). Notably, during the course of cr-EAE, TG2 mRNA and protein levels in spinal cord increased up to 2-3 times the levels present in control animals (Fig. 2E,F).

TG2 inhibition attenuates the clinical symptoms of cr-EAE

To understand the function of TG2 in the disease process, cr-EAE animals were treated intraperitoneally (i.p.) with KCC009, an irreversible inhibitor of TG2 activity. Daily treatment of the cr-EAE animals with KCC009 did neither result in additional weight loss nor in gross anatomical abnormalities in the gut (data not shown). Inhibition of TG2 activity from the onset of clinical symptoms (Fig. 3A, encircled arrow 1) or from later stages of ongoing disease (Fig. 3A, encircled arrows 2 and 3) caused an immediate and marked reduction in clinical deficits. Importantly, administration of KCC009 from the peak of clinical symptoms onwards (Fig. 3A, encircled arrow 2) prevented relapsing disease.

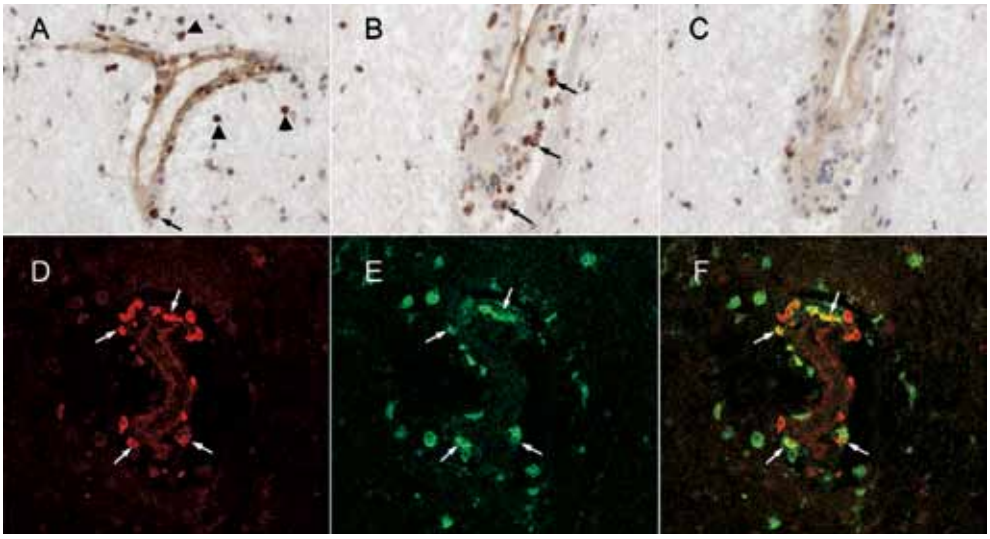


Figure 1: TG2 immunoreactivity in human active subcortical MS lesions. **(A,B)** TG2 in perivascular space-located cells and in tissue parenchyma which is almost absent after **(C)** preadsorption of the anti-TG2 antiserum with guinea pig TG2. **(D)** TG2 positive cells can partly be identified as **(E)** MHC class II expressing cells and thus likely represent activated monocytes (arrows in **F**). Magnification 60x.

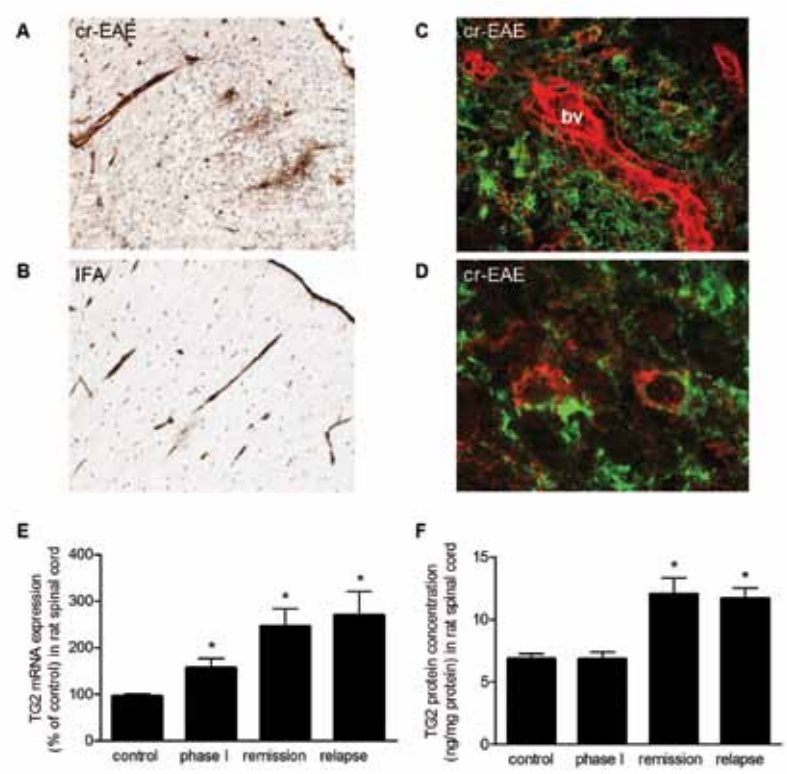


Figure 2: Enhanced TG2 expression in rat spinal cord during cr-EAE compared to IFA immunized controls. **(A)** TG2 immunoreactivity in the cervical spinal cord during the relapse phase of cr-EAE compared to **(B)** IFA control; magnification 20x **(A,B)**. **(C,D)** Immunofluorescent double labeling of Iba-1 positive monocytes and TG2 positive cells in spinal cord of cr-EAE animals during relapse; bv = blood vessel, magnification 80x and **(D)** 250x. **(E)** TG2 mRNA and **(F)** TG2 protein levels in the spinal cord during the different clinical phases of cr-EAE compared to IFA immunized controls. *P<0.05 vs. control.

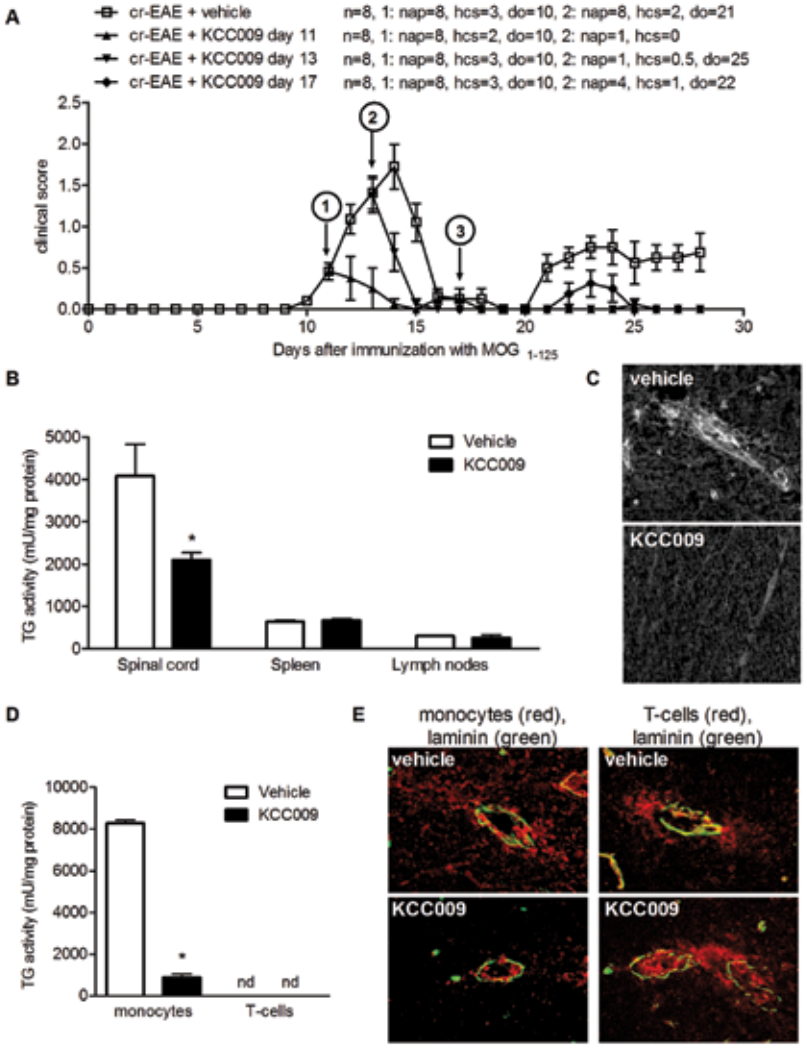
KCC009 reduces TG activity in and infiltration of monocytes into the CNS

Interestingly, KCC009 treatment of cr-EAE animals selectively reduced TG activity in the spinal cord, and not in cervical lymph nodes or the spleen (Fig. 3B). Within the spinal cord of vehicle-treated cr-EAE animals, TG activity is mainly present in infiltrated cells as illustrated by cellular incorporation of the fluorescently-labeled TG substrate cadaverine. This TG activity is attenuated in KCC009-treated cr-EAE (Fig. 3C). Moreover, particularly monocytes, and not T-lymphocytes, isolated from cr-EAE animals showed TG activity that was reduced by KCC009 treatment of the animals (Fig. 3D). Additionally, TG2 inhibition resulted in a reduced presence of infiltrated monocytes, but did not clearly affect T-cell infiltration in the spinal cord of cr-EAE animals (Fig. 3E).

TG2 is involved in monocyte adhesion and/or migration during cr-EAE

To examine more closely the effects of TG2 inhibition on monocyte infiltration and

migration during the course of cr-EAE, we studied post-mortem tissues of KCC009-treated and vehicle-treated cr-EAE animals obtained at different time points during disease. In vehicle-treated cr-EAE animals MHC class II positive monocytes have passed the endothelial cell layer and migrated into the spinal cord parenchyma (Fig. 4A). After treatment of cr-EAE animals with KCC009 from first clinical signs (day 11) onwards, monocytes remained to a large extent in the blood vessel lumen and very little cells entered the perivascular space (Fig. 4B). When cr-EAE animals were treated from first peak of clinical symptoms (day 13) onwards, numerous monocytes were present in the blood vessel lumen, but also to some extent in the perivascular space (Fig. 4C), and when cr-EAE animals were treated with KCC009 from remission (day 17) onwards, monocytes migrated into the spinal cord parenchyma, whereas still quite some cells were detected within the



perivascular space, but hardly in the blood vessel lumen (Fig. 4D). After quantification, we observed a reduction in the spinal cord area containing infiltrated and migrated MHC class II positive macrophages in KCC009-treated versus vehicle-treated cr-EAE animals. This occurred at all time points measured, but is exemplified for cr-EAE animals treated with KCC009 from first clinical signs (day 11) onwards, resulting in the most dramatic reduction (Fig. 4G). Interestingly, in contrast to the observations in the spinal cord, MHC class II positive monocytes did not accumulate within blood vessels in the spleen of KCC009-treated cr-EAE animals (Fig. 4E) but had a similar localization as in vehicle-treated cr-EAE animals (Fig. 4F). These data suggest a role for TG2 in monocyte adhesion and migration within the CNS. As β1-integrins clearly contribute to monocyte adhesion, its expression was studied and found to be increased by 44% in spinal cord of cr-EAE animals treated with KCC009 compared to vehicle-treated animals (Fig. 4H). Furthermore, the amount of active MMP9, involved in monocyte migration, was reduced by 50% in the spinal cord of KCC009-treated cr-EAE animals (Fig. 4H).

TG2 modulates monocyte adhesion and migration through cytoskeletal rearrangements

To further explore the mechanism of KCC009 action in monocyte adhesion and migration processes, we performed *in vitro* experiments using monocyte adhesion onto and migration through monolayers of brain endothelial cells. Treatment of monocytes with KCC009 to inhibit TG2 activity resulted in a significant enhancement of adhesion of monocytes onto the brain endothelial cell layer by 60% (Fig. 5A), which was not detected upon KCC009 treatment of the brain endothelial cells alone (data not shown). In addition, a marked reduction of 75% in transendothelial migration of KCC009-treated monocytes was observed (Fig. 5B). Cytoskeletal rearrangements could possibly contribute to these effects on monocytes by KCC009. Indeed, cellular staining for F-actin, using rhodamine phalloidin, showed a clear difference in the appearance of cellular extensions between vehicle-treated and KCC009-treated monocytes. Cellular ramifications were extensively present in vehicle-treated cells, whereas in KCC009-treated monocytes these ramifications were retracted (Fig. 5C). Furthermore, KCC009 treatment attenuated the amount of active GTP-bound RhoA in the monocytes by 79% whereas the amount of total RhoA remained unaffected (Fig. 5D).

◀— **Figure 3**: Beneficial effect of KCC009 treatment on clinical outcome of cr-EAE and effects on cellular TG activity. **(A)** Clinical scores of cr-EAE rats, treated i.p. daily with vehicle or KCC009 from first clinical signs onwards (day 11, arrow 1), from peak symptoms onwards (day 13, arrow 2) or from remission onwards (day 17, arrow 3); 1: first peak, 2: relapse, nap: number of animals paralyzed, hcs: highest clinical score, do: day of onset. Data represent mean + S.E.M. **(B)** TG activity in the rat spinal cord, spleen and cervical lymph nodes. Data represent mean + S.E.M. **(C)** TG2 activity in the cervical spinal cord of vehicle and KCC009-treated rats during the relapse phase of cr-EAE, visualized using fluorescein cadaverine. Magnification 33x. **(D)** TG activity in primary isolated monocytes and T-cells (collected at day 12 and treated with KCC009 from day 11). Data represent mean + S.E.M., *P<0.01, nd=not detectable. **(E)** Immunofluorescent staining for MHC class II positive macrophages/monocytes (OX6) and CD5 positive T-cells (OX19) together with laminin (to stain the basal lamina) after treatment of cr-EAE animals with vehicle or KCC009. Magnification 60x. Material was collected from the same animals that were used for monocyte and T-cell isolation.

TG2 inhibition affects expression levels of specific genes in the CNS but not in the spleen

During cr-EAE the expression levels of various (inflammatory) genes involved in the pathological process are regulated. We determined whether KCC009 treatment affected gene expression levels in the CNS and spleen of cr-EAE treated animals.

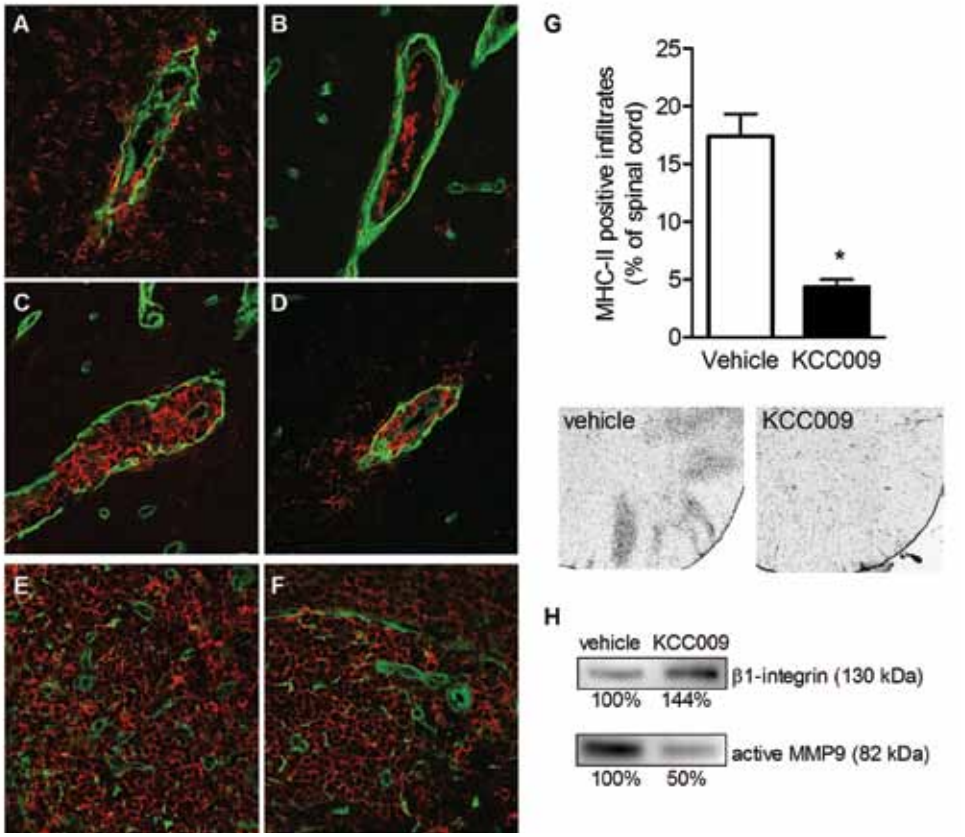


Figure 4: Fluorescent double labeling of MHC class-II positive monocytes/ macrophages (red) and laminin (green) in the cervical part of the rat spinal cord of cr-EAE animals treated with **(A)** vehicle from day 11 onwards or with KCC009 from **(B)** day 11, **(C)** day 13 or **(D)** day 17 onwards. Animals were sacrificed at day 27. MHC class-II positive cells do not accumulate in the blood vessel lumen of the spleen of cr-EAE animals after treatment with **(E)** KCC009 or **(F)** vehicle; magnification 80x **(A-F)**. **(G)** Quantification of the spinal cord area containing MHC class-II positive cells in four representative animals from vehicle-treated and from KCC009-treated cr-EAE animals. Data represent mean + S.E.M., *P< 0.05. **(H)** Protein expression of β1-Integrin and the active form of MMP9 in spinal cord of vehicle and KCC009 treated rats. Bands represent pooled protein from 6 animals/group. Semi-quantitative data represent % expression compared to vehicle-treated cr-EAE animals and are corrected for β-actin.

In the spinal cord of cr-EAE animals treated with KCC009 from the initial clinical symptoms (day 11) onwards, KCC009 treatment increased the mRNA levels of the integrins β1- and β3 by 25% and 31%, and reduced the expression of iNOS and TNFα by 80% and 46%. Expression of other inflammation-related genes was unaltered (Fig. 6A). In the spleen of these animals the expression levels of all measured relevant genes remained unaffected (Fig. 6B).

TG2 inhibition reduces demyelination

In vehicle-treated cr-EAE animals that were sacrificed at day 27, perivascular luxol fast blue (LFB) staining was decreased, indicating loss of myelin in the spinal cord. This decrease in LFB staining was prevented when cr-EAE animals were treated with KCC009 from day 11 onwards (Fig. 6C).

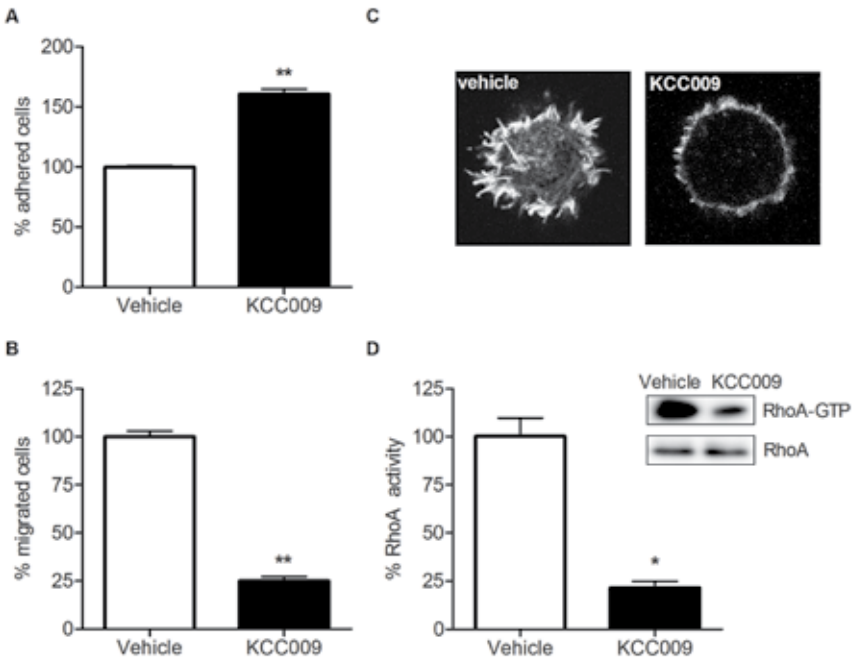


Figure 5: Effect of TG2 inhibition on monocyte adhesion and transendothelial migration **(A)** Monocyte adhesion to monolayers of brain endothelial cells (ECs). Data represent mean (n=6 wells) + S.E.M., **P<0.001. **(B)** Monocyte migration across confluent monolayers of brain ECs. Data represent mean (n=4 wells) + S.E.M., **P<0.001. **(C)** Cytoskeletal rearrangements of monocytes visualized with rhodamine phalloidin staining **(D)** Western blot illustrating representative RhoA GTPase activity in monocytes. Western blot quantification represents mean of 5 different active RhoA isolation experiments + S.E.M., *P<0.001.

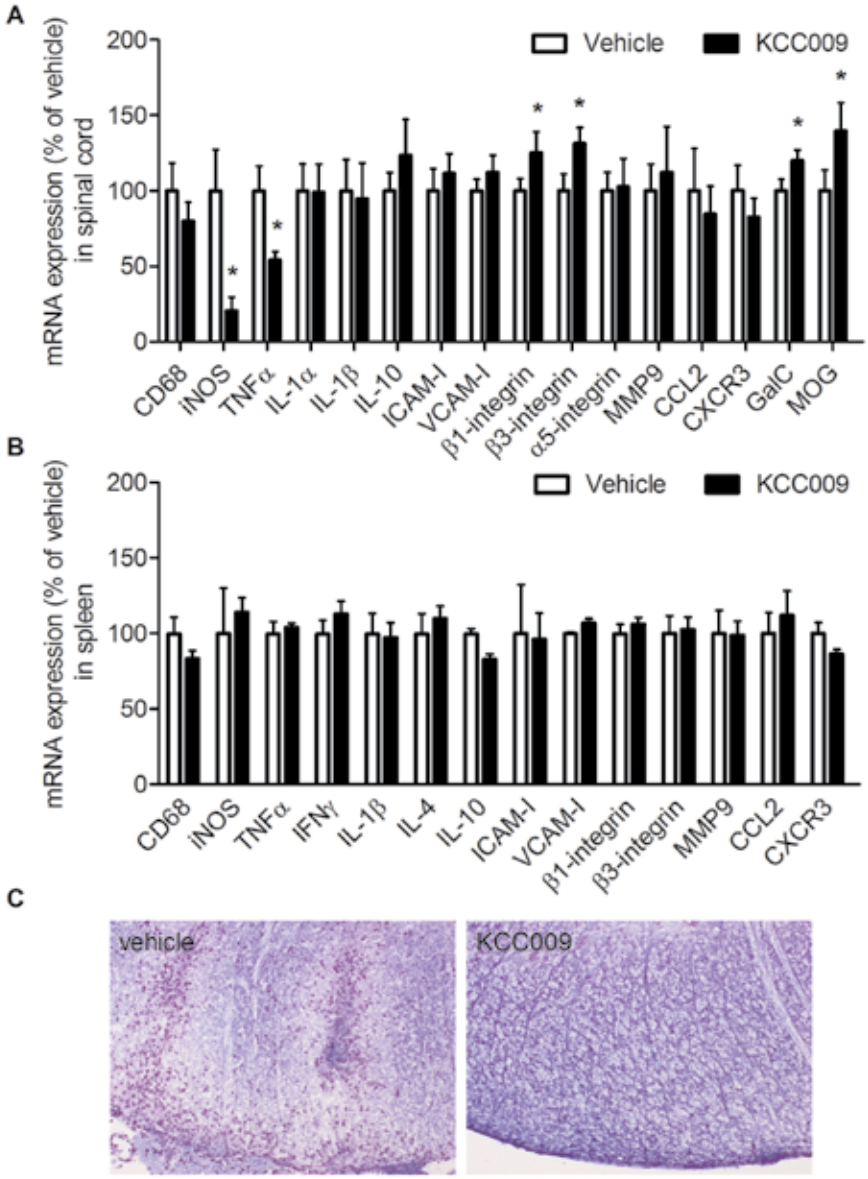


Figure 6: The effect of TG2 inhibition on mRNA expression levels of various genes involved in cr-EAE in rat spinal cord and spleen and demyelination in the rat spinal cord. **(A)** mRNA expression levels of CD68, various cytokines and genes involved in cell migration and adhesion related processes in the rat spinal cord, and **(B)** in the rat spleen, of cr-EAE animals after treatment with KCC009 or vehicle *in vivo*. Data represent mean + S.E.M., *P < 0.01. **(C)** Demyelination was determined by LFB staining in the rat spinal cord after treatment of cr-EAE animals with vehicle or KCC009. Representative spinal cord sections are shown. Magnification 33x.

Discussion

The present study clearly demonstrates a crucial role for TG2, locally regulating the pathological process underlying cr-EAE, and possibly MS. This is supported by the presence of TG2 in infiltrated monocytes in human post-mortem material of MS patients, the effects of inhibition of TG2 activity on the clinical and local pathological outcome of cr-EAE, a model mimicking relapsing-remitting MS, and our *in vitro* data demonstrating that TG2 is involved in monocytic cytoskeletal rearrangements resulting in reduced transendothelial migration.

The observed presence of TG2 immunoreactivity in endothelial cells in both healthy and diseased human brain, and in rat brain material is in line with previous studies.[128, 284] Interestingly, in active MS lesions and during cr-EAE, TG2 immunoreactivity appeared in MHC class-II positive, infiltrating monocytes. Also at the mRNA and protein levels, an increase in TG2 was found in spinal cord of cr-EAE animals. Although monocytes have been described to produce TG,[29, 15] this is the first time that TG2 production by infiltrated monocytes in MS brain and in cr-EAE is shown. As TG2 can actively contribute to cell adhesion and migration processes,[9] our data supports the idea that TG2, present in or at the surface of infiltrating monocytes during active MS and cr-EAE, is important in monocyte adhesion and/or migration processes.

To study the role of TG2 in MS and cr-EAE and its possible therapeutic potential, rats were treated with KCC009 to inhibit TG2 activity. We found that inhibition of TG2 activity starting at different phases of cr-EAE resulted in an immediate and dramatic reduction in clinical scores, indicating that inhibition of TG2 activity has therapeutic potential. This is supported by a similar clinical improvement observed after i.p. treatment of cr-EAE animals with cystamine (Supplementary Fig. 1). Cysteamine is the reduced form of cystamine, a competitive substrate for TG2, and approved by the FDA (US Food and Drug Administration) and EMEA (European Medicines Agency) for treating nephropatic cystinosis.[125] Furthermore, the clinical relevance of TG2 in EAE has been substantiated across species by the observation that another competitive substrate for TG, monodansylcadaverine (MDC),[421] administered i.p. to mice suffering from EAE clearly reduced clinical symptoms (Supplementary Fig. 2). Our data indicate that KCC009 given daily more effectively attenuates clinical symptoms of cr-EAE compared to cystamine, given daily, or a single MDC treatment. Although we cannot exclude that pharmacokinetic characteristics of KCC009 favour its potent effect, it may more likely be due to its pharmacodynamic properties i.e. that KCC009 is an irreversible inhibitor of TG2 activity, whereas cystamine and MDC are both competitive substrates for TG and not TG2 specific. Neuropathological studies of the spinal cord and spleen of cr-EAE animals treated with KCC009 showed that activated monocytes accumulate 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. No difference in monocyte localization within the spleen of vehicle- versus KCC009-treated cr-EAE animals was observed. Moreover, KCC009 treatment of cr-EAE animals selectively reduced TG activity in spinal cord and not in cervical lymph nodes or spleen although recruitment of activated monocytes to these organs has been observed in EAE and MS.[83] Thus, it appears that inhibition of TG2 activity in monocytes results in CNS-specific increased adherence of these cells to the brain

endothelium, but impairing their migration capacity into the spinal cord parenchyma. In addition, an increased expression of β1- and β3-integrins, relevant for cell adhesion[69] and a reduced level of active MMP9, important for cell migration[405] is found in the spinal cord of KCC009-treated cr-EAE animals. It is worth noting that TG activity in T-cells of cr-EAE animals is undetectable, and no effect on T-cell migration into the spinal cord can be observed after KCC009 treatment. This is of interest as MS and EAE are classically considered T-cell mediated diseases.[241] However, activated monocytes and macrophages have been shown to play an important role in the neuropathology and clinical outcome of EAE.[149, 158] The present study demonstrates that TG2 activity exhibited by activated monocytes, and not by T-cells, is a key element in regulating monocyte adhesion and migration in the CNS during cr-EAE, thereby determining the clinical outcome.

The subsequent *in vitro* studies demonstrate that inhibition of TG2 activity in monocytes attenuates profoundly the transendothelial migration, but enhances adhesion capacity of monocytes. This explains the clear presence of monocytes adhering onto CNS endothelium in combination with dramatically reduced migration of monocytes into the CNS of KCC009-treated cr-EAE animals. Thus, the observed *in vitro* effects are in complete agreement with our *in vivo* data, indicating that TG2 facilitates monocytes to pass the brain endothelium and stimulates migration into the tissue parenchyma by activating the extracellular matrix protein MMP9. While studying the effects of TG2 activity inhibition on monocyte adhesion and migration, we observed that KCC009 treatment reduced the movement of monocytes (data not shown). This observation suggests that inhibition of TG2 activity influences the cytoskeletal flexibility of monocytes. To understand the down-stream mechanism(s) underlying the remarkable effects of KCC009 on monocyte adhesion and migration, we thus examined polymerization of the cytoskeletal protein F-actin in KCC009-treated monocytes since it has been shown that monocyte migration over the blood-brain barrier requires a coordinated remodeling of the actin cytoskeleton.[143] We determined a clear difference in the appearance of cellular extensions being present in vehicle-treated and retracted in KCC009-treated monocytes. This reduction in F-actin containing cellular extensions of monocytes after treatment with KCC009 is indicative for reduced cytoskeletal rearrangements in monocytes.[156] It is reported that RhoA GTPase activity is required for transendothelial migration of monocytes but down-regulates integrin-mediated adhesion processes in monocytes.[413] Furthermore, it is shown that TG2 can activate RhoA GTPase.[341] Therefore, we suspected that TG2 inhibition would negatively affect RhoA GTPase activity in monocytes. Indeed, reduced RhoA activity was measured in monocytes upon KCC009 treatment, while leaving total RhoA levels unaffected. Thus TG2 is an essential factor in activating RhoA GTPase in monocytes to stimulate cytoskeletal rearrangements necessary for transendothelial migration.

Of particular clinical interest is our observation that KCC009 treatment did not affect relevant cytokine, chemokine or adhesion molecule expression levels in the spleen of cr-EAE animals. These findings indicate that, in contrast to other MS drugs currently used in patients (e.g. beta-interferon, natalizumab),[98, 236, 321] KCC009 leaves these immune-relevant responses intact probably resulting in less side effects. However, within the spinal cord, the mRNA levels of specifically iNOS and TNFα were reduced in the spinal cord after treatment with KCC009. This suggests that infiltrated, activated monocytes contribute significantly to the local enhanced expression of iNOS and TNFα, whereas resident cells within the CNS

are important producers of the other measured factors. Indeed, iNOS and TNFα have been shown to be merely produced by infiltrated monocytes in animal models for MS.[322, 382] Furthermore, the extent of perivascular demyelination was reduced after treatment of cr-EAE animals with KCC009. This beneficial effect of inhibiting TG2 activity may be due to the reduced presence of activated monocytes within the CNS and consequently less production of iNOS and TNFα. It has previously been shown that TNFα and excess NO, induced by iNOS, in infiltrating monocytes and microglia contribute to myelin damage and subsequent impaired axonal conductivity.[102, 322, 382]
Overall, we conclude that TG2 is an important newly discovered player in the pathogenesis of MS. Inhibition of TG2 activity resulting in clinical improvement together with selective blockade of monocyte migration into the CNS and diminished demyelination opens new avenues for therapeutic intervention in MS, which are seemingly not complicated by disturbance of the peripheral cytokine response.

Materials and methods

Human brain material

Cortical brain tissue from 14 patients with clinically diagnosed and neuropathologically confirmed MS was obtained at rapid autopsy and immediately frozen in liquid nitrogen (in collaboration with The Netherlands Brain Bank, coordinator Dr. I. Huitinga). Five subjects without neurological disease were incorporated as controls. White matter MS tissue samples were selected using postmortem magnetic resonance imaging as published previously.[43, 82] Additional relevant information was retrieved from the medical records and is summarized in Supplementary Table 1. All patients and control subjects had given informed consent for autopsy and use of their brain tissue for research purposes.

Immunohistochemistry

Cryosections (6 μm) were air-dried and fixed in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.6) for 10 minutes (min) (TG2). Subsequently, sections were preincubated for 15 min with 2% normal donkey serum in Tris-buffered saline (TBS) containing 0.5% Triton X-100. Thereafter, sections were incubated for 60 min at room temperature (RT) with goat anti-TG2 (Upstate, Charlottesville, USA; 1:3,000). After washes in TBS, the sections were incubated for 2 hours (h) at RT with biotinylated donkey anti goat IgG (Jackson ImmunoResearch, Suffolk, UK; 1:400). Following washes in TBS, the sections were incubated for 60 min at RT with ABC-HRP complex (Vectastain; Vector Laboratories, Burlingame, USA). Peroxidase activity was visualized by 3,3-diaminobenzidine (DAB, Sigma, St. Louis, USA). Finally, the sections were counterstained with haematoxylin. All antibodies were diluted in TBS containing 0.5% Triton X-100 and 2% normal donkey serum. Omittance of the primary antibodies for TG2 served as a negative control.
Specificity of the anti-TG2 antibodies was determined by preadsorption of the anti-TG2 antibody derived from Upstate with at least 100-fold (w/w) excess of guinea pig TG2 (Sigma-Aldrich, St. Louis, MO, USA). After 6 h of preadsorption, adjacent tissue sections were incubated with the preadsorbed or non-adsorbed antibodies and further treated as a regular immunohistochemical staining.

Immunofluorescent double labeling on human MS material
Colocalization of TG2 with MHC class II positive cells was determined by co-incubation of 4% paraformaldehyde fixed cryosections with the monoclonal antibody LN3 (Labvision; 1:100) and with goat anti-TG2 (Upstate; 1:4,000). Subsequently, sections were co-incubated with donkey anti-mouse Alexa-488 and donkey anti-goat Alexa-594 (Invitrogen, Carlsbad, USA; 1:400). Incubation procedures were performed as described above. Fluorescent double immunolabeling was identified by confocal laser scanning microscopy (Leica TCS-SP2-AOBS; Leica Microsystems, Wetzlar, Germany).

Cr-EAE induction in rats
Chronic relapsing experimental autoimmune encephalomyelitis (cr-EAE), an experimental animal model mimicking relapsing-remitting MS,[359] was induced in adult male Dark Agouti (DA) rats (Harlan, Horst, The Netherlands), weighing 230-250 g. The rats were anaesthetized with isoflurane and immunized intradermally in the dorsal tail base with 75 μg of recombinant rat myelin oligodendrocyte glycoprotein ($rrMOG_{1-125}$) emulsified in incomplete Freund's adjuvant (IFA; Difco, Detroid, MI, USA) together with 10 mM NaAc (pH 3.0). Control rats received IFA and NaAc only. Rats were weighed and examined daily for neurological symptoms of EAE that were scored on the following scale: 0, no clinical disease; 0.5, partial loss of tail tone; 1, complete tail atony; 2, paresis, partial hind limb paralysis; 3, complete paralysis of the hind limbs and/or lower part of the body; 4, moribund or dead due to EAE. All experimental procedures were approved by the Animal Experiment Committee of the VU University Medical Center.

In vivo experiments
TG2 expression during the course of cr-EAE
cr-EAE rats (n=10/group) were sacrificed at different phases of disease, i.e. during the first phase of neurological symptoms (day 12-13 post immunization, p.i.), remission (~ day 15 p.i.) and relapse (~day 22 p.i.) to study TG2 expression at the mRNA and protein level.

Effect of TG2 inhibition on the clinical course and pathology of cr-EAE
Cr-EAE rats were injected i.p. daily from initial clinical signs (day 11), from abundant presence of clinical signs during the first phase of disease (day 13) or from remission (day 17) of cr-EAE onwards with KCC009 (40 mg/kg, kind gift from Alvine Pharmaceuticals, San Carlos, USA) in PBS/DMSO (1:1) (n=8/group). KCC009 is a small peptidergic, irreversible inhibitor of TG2 activity.[68] Control animals were injected i.p daily with vehicle (PBS/DMSO 1:1) from initial clinical symptoms (day 11) onwards (n=8). Animals were sacrificed when vehicle-treated cr-EAE rats reached peak clinical symptoms during the relapse. Spleen, cervical lymph nodes and spinal cords were dissected and half of each tissue was frozen in liquid nitrogen and stored at -80^0C for quantitative real-time (RT)-PCR, protein and/or TG activity analysis. From the same animals, the other half of the spleen and spinal cord were embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), frozen in liquid nitrogen and stored at -80^0C for immunohistochemistry.

Cellular TG activity in cr-EAE animals
To investigate TG2 activity levels in leukocytes, cr-EAE animals were injected i.p. daily, for

2 days, with KCC009 (40 mg/kg, Alvine Pharmaceuticals) in PBS/DMSO (1:1) or with vehicle (PBS/DMSO 1:1) from initial clinical symptoms (day 11) onwards (n=6 per group). Two h after the second i.p. injection (day 12), animals were sacrificed and spinal cords were collected for immunohistochemistry to perform immunofluorescent stainings for monocytes and T-cells, and blood was collected for T-cell and monocyte isolation. Peripheral blood mononuclear cells (PBMCs) were isolated as the interphase on a Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient as described.[84] The population consisted of >90% viable monocytes (ED9-positive cells as determined by FACS analysis) and <10% lymphocytes. T-cells were collected by plating the beads (containing B and T-cells) in a 6-well plate (Labtek, Nalge Nunc International, Naperville, IL) in RPMI medium (Invitrogen, Carlsbad, CA, USA). Cells were incubated at 37^0C in 5% CO_2. After 24 h, B-cells are still attached to the beads, T-cells are detached and can be collected, resulting in a yield of about $1x10^6$ T-cells per rat.

Quantitative RT-PCR

To determine the mRNA transcript levels of different genes involved in MS and cr-EAE, spinal cord and spleen tissues were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was isolated and of 1 μg cDNA was synthesized using the Reverse Transcription System (Promega, Madison, WI, USA) with oligo-dT primers and AMV enzyme according to the manufacturer's instructions. For quantitative RT-PCR, the SYBR Green PCR Core reagents kit (Applied Biosystems, Foster City, CA, USA) was used. Amplification of cDNA was performed in MicroAmp Optical 96-well Reaction Plates (Applied Biosystems) on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The reaction mixture (20 μl) was composed of 1x SYBR Green buffer, 3 mM $MgCl_2$, 875 μM dNTP mix with dUTP, 0,3 U AmpliTaq gold, 0.12 U Amperase UNG, 12,5 ng cDNA and 15 pmol of each primer (Supplementary Table 2). The reaction conditions were an initial 2 min at 50^0C, followed by 10 min at 95^0C and 40 cycles of 15 sec at 95^0C and 1 min at 59^0C. The mRNA expression levels were quantified relatively to the level of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) using the following calculation: *2–(Threshold cycle of target mRNA – Threshold cycle of GAPDH) x 100*.

TG2 ELISA

TG2 protein levels were measured in tissue homogenates using an enzyme-linked immunosorbent assay (ELISA) specific for TG2 as described previously.[47] Briefly, tissue samples were homogenized in ice-cold lysis buffer containing 10 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 μM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml aprotinin. Homogenates were cleared by centrifugation (14,000 rpm for 30 min at 4^0C) and protein concentrations of supernatants were determined by the BCA method (Pierce Biotechnology, Perbio Science, Etten-Leur, NL). Of each sample, 10 μg of protein was loaded in the assay. An immunoaffinity-purified polyclonal goat anti-TG2 antibody (Upstate) was used as coating antibody and a monoclonal mouse anti-TG2 antibody (Ab-2, clone TG100, Labvision, Fremont CA, USA) was used as detecting antibody. Recombinant human TG2 (Zedira Biotec GmbH, Darmstadt, Germany) was used as the standard.

TG activity assay

To measure TG activity in cell extracts and tissue homogenates, T-cells, monocytes and tissue samples were homogenized in ice-cold lysis buffer as used for TG2 ELISA. Activity was measured by using the TG Covtest TCMA (Transglutaminase Colorimetric Microassay; Covalab, Villeurbanne, France) following the manufacturer's protocol.[323]

In addition, cellular TG activity was detected in fresh frozen rat spinal cord sections. The unfixed sections were washed in TBS and incubated with 0.5 mM of the TG substrate fluorescein-labeled cadaverine (5-((5-aminopentyl)thioureidyl)fluorescein, hydrobromide salt, Promega) in TBS/0.5% Tween-20 at 37^{0}C for 2 h. Subsequently, the sections were washed with TBS and fixed for 10 min with 100% methanol. Slides were examined under a fluorescent microscope (Vanox-T, Olympus, Zoeterwoude, The Netherlands).

Western blot

Tissue samples were homogenized in ice-cold lysis buffer as used for TG2 ELISA. Of each sample, 10 μg of protein was subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen). Membranes were incubated o/n with primary antibodies (rabbit anti-MMP9, Serotec Ltd., 1:2,000, rabbit anti-VCAM-I, Santa Cruz Biotechnology Inc., 1:1,000 and rabbit anti-integrin β1, Chemicon, Millipore, Amsterdam, The Netherlands, 1:1,000). For subsequent antigen detection, blots were incubated for 2 h with corresponding goat-anti mouse or goat-anti rabbit Immunoglobulins/HRP (Dako, Glostrup, Denmark, 1:10,000). Bands were visualized using the enhanced chemiluminescence (ECL) detection system SuperSignal West Dura (Pierce Biotechnology) and a Chemidoc image capture system (Bio-Rad, Veenendaal, The Netherlands). Signal intensity of the bands was semi-quantified using Quantity One software (Bio-Rad).

Immunohistochemistry on rat material

Fresh frozen rat spinal cord sections were fixed for 10 min with acetone, washed in TBS, blocked in 5% milk in TBS with 0.5% Triton X-100 (TBS-T) and 0.03% H_2O_2 and incubated with the first antibody o/n at 4^{0}C. To detect TG2, a mouse monoclonal antibody raised against guinea pig TG2 was used (Ab2, Labvision, 1:1,000). A mouse monoclonal antibody raised against MHC-II was used (OX6, Serotec Ltd., 1:1,000) to detect macrophage/microglial immunoreactivity. Subsequently, sections were washed in TBS and incubated for 2 h at RT with appropriate secondary biotinylated IgGs (Jackson ImmunoResearch, 1:400), followed by washes in TBS and incubation for 1 h at RT with avidin–biotin–peroxidase complex (Vector Laboratories, 1:400). After washes in TBS and Tris–HCl, immunoreactivity was visualized using 0.5 mg/ml of DAB (Sigma-Aldrich) in Tris–HCl. Demyelination in the rat spinal cord was assessed using Luxol Fast Blue (LFB) staining.[204] Sections were stained in LFB at 56^{0}C for 16 h and counterstained with cresyl violet solution for 30-40 seconds. All sections were examined microscopically (Vanox-T, Olympus). The white matter area size containing MCH class II positive cells was quantified and related to the total white matter area of the spinal cord examined (AnalySIS software; Olympus). Per treatment group, 4 areas in the cervical and thoracic part of the spinal cord in 4 representative rats per group were examined.

Immunofluorescent double labeling on rat cr-EAE material

For immunofluorescent double stainings, fresh frozen rat spinal cord and spleen sections were fixed with acetone (OX6 or OX19 and laminin) or 2% paraformaldehyde (IbaI and TG2) for 20 min, washed in TBS, blocked in 5% milk in TBS with 0.5% Triton X-100 (TBS-T) and 0.03% H_2O_2 and incubated with the first antibodies o/n at 4^{0}C. Rabbit polyclonal anti laminin (Cappel, MP Biomedicals, Illkirch, France, 1:100) and mouse monoclonal OX6 (Serotec Ltd., 1:1,000) or mouse monoclonal OX19, an antibody raised against CD5$^+$ T cells (Serotec Ltd., 1:500) or mouse monoclonal anti-TG2 (Ab2, Labvision, 1:1,000) and goat polyclonal IbaI (Abcam Inc., Cambridge, MA, USA, 1:1000) were used followed by appropriate fluorescent secondary antibodies to detect laminin or IbaI (Alexa Fluor-488, Invitrogen, 1:400) and OX6, OX19 or TG2 (Alexa Fluor-594, Invitrogen, 1:400) immunoreactivity. Sections were embedded in Vectashield mounting medium (Vector Laboratories) and examined on a Leica confocal laser scanning microscope (Leica, Rijswijk, The Netherlands).

Monocyte adhesion assay

To study the role of TG2 in adhesion of monocytes onto brain endothelial cells (ECs), NR8383 cells (rat alveolar macrophage cell line, American Type Tissue Collection (ATCC), Manassas, CA, USA), resembling primary monocytes, were cultured as described previously[138] and fluorescently labeled with 1 μM 2′,7′-biscarboxyethyl-5(6) carboxyfluorescein acetoxymethylester (Molecular Probes, Invitrogen). The well-characterized immortalized rat brain EC cell line GP8/3.9[137] was used as an *in vitro* model for brain endothelium and was cultured on collagen-coated multiwell plates as described.[138, 385] Briefly, NR8383 cells were incubated with 0.5 mM KCC009 or 1% DMSO (control) for 1 h to inhibit TG2 activity. Then 1x10^5 NR8383 cells were added to cytokine-stimulated (50 ng/ml IL-1β + 50 ng/ml IFNγ, 48 h) EC monolayers/well, and allowed to adhere for 30 min in 10% FCS containing medium. After the incubation, nonadherent cells were removed by gently washing the monolayers with medium (37^{0}C), and the number of adherent cells was determined by lysing the cells with 0.1 M NaOH and measuring the fluorescence intensity in a fluorimeter (FLUOstar BMG Labtechnologies, Meeren, The Netherlands) (excitation wavelength, 485 nm; emission wavelength, 535 nm). The number of adhered monocytes was then calculated using a calibration curve with various cell concentrations ranging from 5x10^3 cells/ml to 1x10^6 cells/ml on monolayers of brain ECs.

Monocyte migration assay

The effect of KCC009 on the migratory capacity of NR8383 cells to cross a monolayer of brain ECs was assessed using time-lapse video microscopy as described previously.[138] Briefly, NR8383 cells (6x10^5 cells/well) were added to 96-well plates containing cytokine-stimulated (50 ng/ml IL-1β + 50 ng/ml IFNγ for 48 h) brain EC monolayers. Monocytes were preincubated with 0.5 mM KCC009 or 1% DMSO (control) for 1 h before starting the migration assay. Monocytes were allowed to adhere and migrate for a 4 h period. To monitor monocyte migration, cocultures were placed in an inverted phase-contrast microscope (Nikon Eclipse TE300) housed in a temperature-controlled (37^{0}C), 5% CO_2 gassed chamber. A microscopic field (220 x 220 μm) was randomly selected and recorded for 9 min at 50 times normal speed by using a color video 3CCD camera (Sony, with a CMAD2). Migrated monocytes (phase-dark) could be readily distinguished from those

remaining on the cell surface by their highly refractive (phase-bright) morphology. The level of migration was calculated as the percentage of migrated monocytes of the total monocytes within the microscopic field.

Cellular F-actin staining

The effect of KCC009 on cytoskeletal organization of the monocytes was visualized by detection of F-actin using rhodamine phalloidin as described previously.[156] In short, NR8383 cells were plated on fibronectin-coated 8-well chamber slides (Labtek, Nalge Nunc International) and left o/n at 37^{0}C in the presence of 10% FCS. The following day, the cells were pre-incubated with 0.5 mM KCC009 or 1% DMSO (control) for 1 h and subsequently fixed for 15 min with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS, blocked with 10% FCS in PBS, and stained for 1 h with rhodamine phalloidin in PBS (Molecular Probes, Invitrogen, 1:300). Slides were washed and embedded with Vectashield mounting medium (Vector Laboratories). Images were taken using a Leica Confocal microscope.

Rho activity assay

To determine the effect of KCC009 on RhoA activity in monocytes, 5x10^6 NR8383 cells were treated with IFNγ and IL-1β (50 ng/ml each) for 48 h followed by 1 h incubation with 0.5 mM KCC009 or 1% DMSO at 37^{0}C. Thereafter, cells were lysed with lysis buffer (50mM Tris, pH 7.6, 500mM NaCl, 0.1% SDS, 0.5% deoxycholate (DOC), 1% Triton X-100, 10mM $MgCl_2$, 100 μM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml aprotinin). Of the cleared lysates, 20 μl was stored to determine the total amount of RhoA (total cell lysate). Protein lysates were incubated with 60 μg bacterially produced GST-RBD (Rho Binding Domain of Rhotekin)[310] and bound to glutathione-agarose beads (Sigma-Aldrich). Beads were washed 4 times with lysis buffer, bound proteins were eluted in 30 μl SDS sample buffer and analyzed in parallel with 10 μl of the total cell lysate subjected to 15% SDS PAGE gel electrophoresis. Following blotting of the gel onto PVDF membrane, the active form of RhoA and total RhoA could be detected with a mouse monoclonal antibody (Santa Cruz Biotechnology, Inc. 1:250).

Statistics

Where appropriate, data were analyzed by one-way or two-way ANOVA, followed by a t-test for independent measurements (Fisher's LSD test). The statistical evaluation was carried out by using the NCSS 2007 statistical program (NCSS, East Kaysville, Utah, USA). Error bars represent standard error of the mean (S.E.M.).

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Supplementary data

Methods

In vivo role of TG2 in EAE in rats and mice

Cystamine treatment in cr-EAE rats

Similar to KCC009 treatment, cr-EAE rats were injected i.p. daily with 75 mg/kg cystamine (Sigma-Aldrich), a competitive substrate for TG activity, in PBS (n=12) or with PBS only (n=12, vehicle) starting from initial clinical symptoms (day 9) onwards. Animals were sacrificed when vehicle-treated cr-EAE animals reached the remission phase (day 16). Spinal cords were dissected, frozen in liquid nitrogen and stored at –80^{0}C for TG2 activity analysis. TG2 activity was measured with the TG Covtest TCMA (Covalab) as described in the materials and methods section.

Monodansylcadaverine treatment in EAE mice

6-8 weeks old female PLSJL/J mice (Jackson labs, Bar Harbor, USA, n=16) were anesthetized with isofluorane and immunized subcutaneously in the hind legs and tail with 200 μg of recombinant proteolipid protein peptide 139-151 ($PLP_{139-151}$), emulsified in complete Freund's adjuvant (CFA; Difco Laboratories, Detroid, MI, USA.). Pertussis toxin (PTX, Sigma-Aldrich, 0.2 μg/animal) was injected i.p. on days 0 and 3 after immunization. Monodansylcadaverine (MDC, Sigma-Aldrich, 17 mg/animal), a competitive substrate for TG activity, in PBS (n=8) or PBS only (n=8, vehicle) was injected i.p. on day 10 when initial clinical symptoms were present.

Time-lapse video microscopy

To visualize the real-time effect of KCC009 on monocyte migration over the brain endothelial cell layer, individual microscopically pictures were taken during the monocyte migration assay (as described in materials and methods section). These pictures were assembled using Cell^F imaging software (Olympus) to create videos. Each video contains 50 individual pictures that were taken in a 9 minute time interval.

Results

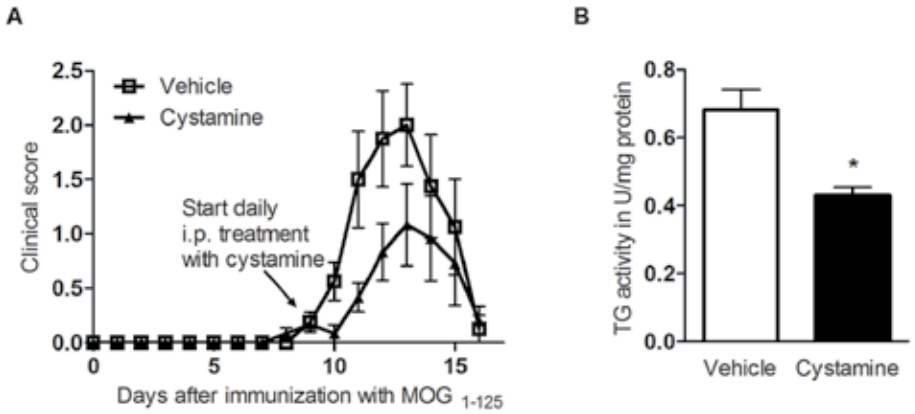
In vivo role of TG2 in EAE rats and mice

Cr-EAE rats were treated i.p. with cystamine, a competitive substrate inhibitor of TG2 activity, to study the clinical effect of TG2 inhibition using another inhibitor. Inhibition of TG2 activity from the onset of clinical symptoms (day 9) onwards (Supplementary Fig. 1A) caused an immediate reduction in clinical deficits. At the peak of clinical symptoms, average clinical score of cystamine treated animals was 1 compared to 2 for the vehicle-treated animals. TG2 activity in the rat spinal cord is reduced with 40% after treatment with cystamine compared to vehicle-treated cr-EAE animals (Supplementary Fig. 1B).

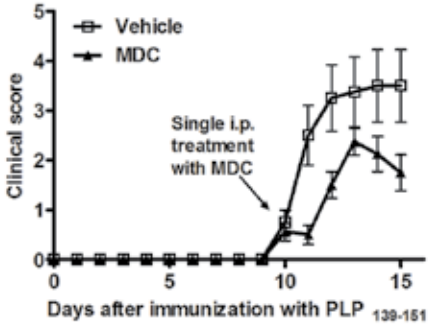
To study the clinical effect of TG2 inhibition across species, EAE mice were treated with MDC when first clinical signs were present (day 10). Clinical scores were reduced after treatment with MDC compared to vehicle-treated EAE animals. Average clinical scores during the peak of disease in mice treated with MDC was 2.4 compared to 3.5 for the

vehicle-treated EAE mice.

Movement of monocytes across brain endothelial cells in vitro
During the migration assay, movement of monocytes across brain endothelial cells was studied. Movement of the cells is clearly reduced after treatment with KCC009 compared to vehicle-treated cells (Supplementary videos 1A and B, not shown).



Supplementary Figure 1: Clinical scores of cr-EAE in rats and TG activity levels in the rat spinal cord after treatment with cystamine. **(A)** Clinical scores of cr-EAE after daily i.p. treatment with 75 mg/kg cystamine or vehicle started from first clinical symptoms onwards. Data represent mean ± S.E.M. **(B)** TG activity in the rat spinal cord of cr-EAE rats after treatment with cystamine or vehicle. Data are expressed as the mean of 12 animals per group + S.E.M., *P<0.01.



Supplementary Figure 2: Clinical scores of EAE in mice after treatment with monodansylcadaverine (MDC). Data represent mean of 8 animals per group ± S.E.M.

Tables

Supplementary Table 1: Summary of MS patient details

Age	Gender	Type of MS lesion	Disease duration (years)	PMD (hours)	Cause of death
78	female	control	NR	6:30	decompensatio cordis
82	male	control	NR	12:00	heart failure
81	male	control	NR	6:40	euthanasia
52	female	control	NR	6:00	leiomyosarcoma
88	female	control	NR	6:15	aging
52	female	active	13	8:25	respiratory insufficiency/pneumonia
53	female	active	27	8:30	euthanasia
70	male	active	unknown	6:25	unknown
69	female	active	26	13:20	viral infection
76	female	active	53	15:15	respiratory insufficiency/pneumonia

MS, multiple sclerosis; NR, not relevant; PMD, post-mortem delay

Supplementary Table 2: Oligonucleotide primers used for amplification of cDNAs[1]

rat cDNA	Sequence forward 5' to 3'	Sequence reverse 5' to 3'
GAPDH	TCAAGGGCATCCTGGGCTAC	CGTCAAGGTGGAGGAGTGG
CD68	CTCATCATTGGCCTGGTCCT	GTTGATTGTCGTCGTCTGCGGG
iNOS	AACTTGAGTGAGGAGCAGGTTGA	CGCACCGAAGATATCCTCATGA
TNFα	CCACACCGTCAGCCGATT	TCCTTAGGGCAAGGGCTCTT
TG2	AGAGGAGCGGCAGGAGTATG	AGGATCCCATCTTCAAACTGC
IL-1α	AGACAAGCCTGTGTG2CTGAAG	CAGAAGAAAATGAGGTCGGTCTC
IL-1β	AAAGAAGAAGATGGAAAAGCGGTT	GGAACTGTGCAGACTCAAACTC
IL-10	AAAGCAAGGCAGTGGAGCAG	TCAAACTCATTCATGGCCTTGT
IL-4	TGAGAAGCTGCACCGTGAATG	TCCCTCGTAGGATGCTTTTTAG
Integrin α5	TGTATCCTGCATCAACCTTAG	TCCAGTTGGAGTTCCACC
Integrin β1	GAATGTAGCACAGATGAAGTG	CACACTCTCCATTGTTACTG
Integrin β3	AGAACTGACGGATACTGG	GAGTCTTCATAGTACTGGAATC
MMP9	TGTATGGTCGTGGCTCTAAAC	GTGGGACACATAGTGGGAG
ICAM-I	GAGAAGACCTG2AGAATCTAC	CTTCACAGTTACTG2GTCC
VCAM-I	TCTACTCATTCCCTGAAGACC	ACATTAGGGACCGTGCAG
CCL2	ACGTGCTGTCTCAGCCAGATG	GACTCATTGGGATCATCTTGCC
CXCR3	GCCATGTACCTTGAGGTC	CCATAATCGTAGGGAGATG
GalC	ACGTTGAGTACCCATTATTTAG	TGCTCAAGGTCTTCGTTATTC
MOG	GCAGAAGTCGAGAATCTC	AACAGGGACAATAACAAAC

[1]Primers were designed using the Primer Express program (Applied Biosystems) and purchased from Eurogentec (Seraing, Belgium)