OXFORD

ORIGINAL ARTICLE

Tissue Transglutaminase Appears in Monocytes and Macrophages but Not in Lymphocytes in White Matter Multiple Sclerosis Lesions

Navina L. Chrobok, MSc, John G.J.M. Bol, BASc, Micha M.M. Wilhelmus, PhD,
Benjamin Drukarch, MD, PhD, and Anne-Marie van Dam, PhD

Abstract

Leukocyte infiltration is an important pathological hallmark of multiple sclerosis (MS) and is therefore targeted by current MS therapies. The enzyme tissue transglutaminase (TG2) contributes to monocyte/macrophage migration and is present in MS lesions and could be a potential therapeutic target. We examined the cellular identity of TG2-expressing cells by immunohistochemistry in white matter lesions of 13 MS patients; 9 active and chronic active lesions from 4 patients were analyzed in detail. In these active MS lesions, TG2 is predominantly expressed in leukocytes (CD45^{+}) but not in cells of the lymphocyte lineage, that is, T cells (CD3^{+}) and B cells (CD20^{+}). In general, cells of the monocyte/macrophage lineage (CD11b^{+} or CD68^{+}) are TG2^{+} but no further distinction could be made regarding pro- or anti-inflammatory macrophage subtypes. In conclusion, TG2 is abundantly present in cells of the monocyte/macrophage lineage in active white matter MS lesions. We consider that TG2 can play a role in MS as it is associated with macrophage infiltration into the CNS. As such, TG2 potentially presents a novel target for therapeutic intervention that can support available MS therapies targeting lymphocyte infiltration.

Key Words: Leukocytes, Macrophages, Monocytes, Multiple sclerosis, Tissue transglutaminase.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative disease that is the most common cause of neurological disabilities in young adults [1]. MS clinical features can be diverse but include motor and sensory deficits and cognitive impairment [2]. MS pathology is characterized by infiltration of leukocytes into the central nervous system (CNS) that results in inflammatory lesion formation concomitant with demyelination and axonal damage [3–6]. The inflammatory active lesions in the CNS white matter consist mainly of leukocytes infiltrated from the blood and of resident CNS cells such as microglia and astrocytes that are activated by the local inflammatory response [7]. Cellular infiltration of the CNS is highly regulated and involves a complex adhesion and migration cascade. This cascade is modulated by many factors, including chemokines and adhesion molecules, which are upregulated during inflammation [5, 8, 9]. It is thought that autoreactive T cells enter the CNS during MS pathogenesis. This is then followed by recruitment and influx of other leukocyte cell types including B cells and monocytes. B cells play a pathogenic role in MS development by prolonging and supporting inflammation by antibody and cytokine secretion as well as stimulating T cells [10–13]. Infiltrating monocytes can differentiate into macrophages upon entering the CNS. Locally, they can diverge into macrophages exerting damage and promoting further inflammation or having anti-inflammatory properties and induce axonal regeneration and repair [14, 15].

Novel treatments for MS patients target mainly lymphocyte infiltration into the CNS [16, 17], which can be achieved either by reduction in the number of circulating lymphocytes [18, 19] or by interference with mechanisms associated with cellular infiltration [20–22]. Because MS pathology is highly heterogeneous between lesions and patients [23], it is important to focus also on other cell types as potential (additional) targets to combat the disease. In this respect, we propose that monocytes and macrophages, whose detrimental roles have been established in the pathogenesis of MS and MS animal models [3, 24, 25], are of interest as potential targets.

The enzyme tissue transglutaminase (TG2) is involved in adhesion and migration of several cell types, including monocytes and macrophages [26, 27], which is observed in MS pathology [28]. The precise role of TG2 in MS has not been delineated yet, but its expression is confirmed in human leukocyte antigen-D related (HLA-DR)-positive cells in active white matter MS lesions [29]. Furthermore, TG2 is involved in inflammation and other MS-associated processes such as cell adhesion, migration and efferocytosis as previously reviewed [30]. Additional data from MS rodent and primate models showed that TG2 is expressed in monocytes and

macrophages and contributes to the development of MS-like disease symptoms (29, 31).

If monocyte and macrophage-derived TG2 contributes to MS pathology as indicated by animal model experiments, TG2 could hold promise as a potential target to reduce monocyte and macrophage infiltration and thus as add-on therapy in MS. Considering the remaining uncertainty as to the cellular localization of TG2 in MS lesions and its potential impact on therapeutic approaches using modulation of TG2 activity, in the present study we questioned whether TG2 is expressed by monocytes and macrophages or by lymphocytes present in (chronic) active white matter MS lesions. In addition, we studied if a macrophage subtype expressing TG2 can be established in these lesions.

MATERIALS AND METHODS

Brain Tissue

Human postmortem tissue from MS patients and nonneurological control subjects was obtained from the Pathology department of the Amsterdam University Medical Center and the Netherlands Brain Bank (Amsterdam, The Netherlands). The subcortical white matter tissue contained active or chronic active MS lesions as classified by the presence of HLA-DR-positive cells and loss of myelin (32, 33). We studied postmortem material of 13 clinically diagnosed and pathologically verified MS patients. All tissue samples contained TG2-immunopositive cells, although with a high variation in the number of immunoreactive cells. To be able to better characterize TG2 expressing cell type(s), we selected 4 of the 13 MS cases (Table 1; #1, 2, 3, 7) with a total of 9 active/chronic active lesions and a relatively high number of TG2-immunopositive cells. In addition to these selected MS patients (average age 47 ± 5.7 years), 6 nonneurological control subjects (Table 1; average age 58.8 ± 2.9 years) were included. All subjects were matched for postmortem delay. The clinicopathological data of the patients and control donors are summarized in Table 1. Informed consent was given by the donors for brain autopsy and use of brain tissue for research purposes. Use of tissue in combination with the clinical information for scientific research was in compliance with the local ethical and legal guidelines.

Tissue Processing

The snap-frozen brain tissue was cut into 10-μm-thick cryosections. Sections were mounted on positively charged glass slides (SuperFrost Plus slides, Fisher Scientific, Pittsburgh, PA). After drying for 30 minutes at 37°C, the dry slides were stored until use at −80°C.

Lesion Classification and Selection

White matter MS lesions were classified by standard immunohistochemical staining for myelin-specific proteolipid protein (PLP) and the major histocompatibility complex class II receptor HLA-DR, a marker for inflammatory cells, as described previously (34). Active and chronic active lesions with ongoing demyelination were selected by the presence of foamy macrophages and neutral lipids by Oil Red O staining.

Active (inflammatory) lesions were identified by massive presence of HLA-DR and Oil Red O-positive cells throughout the lesion area. Chronic active lesions were characterized as lesions that had a broad active rim of HLA-DR and Oil Red O-positive cells combined with reduced cell numbers in the center of the lesion.

Immunohistochemistry

Sections were thawed, air-dried and fixed in acetone for 10 minutes (60 minutes for delipidization when used for PLP staining). In addition, sections were rinsed for 5 minutes with 50 mM Tris-buffered saline ([TBS]; pH 7.4), followed by incubation for 20 minutes with 0.3% H_2O_2 in TBS including 0.1% sodium azide to quench endogenous peroxidases. Nonspecific binding of antibodies was blocked for 30 minutes with 3% bovine serum albumin ([BSA] Sigma-Aldrich, St. Louis, MO) in TBS containing 0.5% Triton X-100 ([TBS-T] Sigma-Aldrich).

Single Antigen Detection

Single stainings were performed for HLA-DR, PLP, and TG2 (Table 2). Sections were incubated with primary antibodies overnight at 4°C in 3% BSA in TBS-T. After washing with TBS, a 2-hour incubation at room temperature followed with donkey antigoat or goat antimouse biotinylated IgGs (dilution 1:400, 3% BSA in TBS-T [Jackson ImmunoResearch Laboratories Inc., West Grove, PA]). After washing using TBS, the sections were incubated for 1 hour at room temperature with horseradish peroxidase-labeled avidin-biotin complex ([ABC] 1:400 dilution; Vector Laboratories, Burlingame, CA). Detection of immunoreactivity was performed by adding chromogen 3, 3-diaminobenzidine ([DAB] Sigma-Aldrich) and 0.01% hydrogen peroxide in Tris-HCl buffer (pH 7.6) to the sections. Nuclear counterstaining with hematoxylin was applied. Sections were dehydrated in graded ethanol solutions and cleared in xylene before cover-slipping with Entellan mounting medium (Merck Millipore, Darmstadt, Germany). To ensure TG2 antibody specificity, antiTG2 antibody was pre-adsorbed with excess recombinant TG2 protein as follows. Recombinant guinea pig TG2 (2 units, Sigma-Aldrich) was mixed with 1.5 mL of a 1:10 000 dilution of the TG2 antibody and incubated at 4°C for 6 hours. Subsequent tissue staining was performed as described above, using this mixture as primary antibody.

Colabeling of Antigens

To identify the cell type immunopositive for TG2, we colabeled TG2 with immune markers for general leukocytes (CD45), pan-T cells (CD3), pan-B cells (CD20), and macrophages (CD11b, CD68, CD163, CD206, CX3CR1; Table 2). The fixed and pretreated sections were incubated with primary antibody directed to one of the immune markers, diluted in TBS-T with 3% BSA. Incubation was performed for 1 hour at room temperature or overnight at 4°C (no differences observed in protocol used). After washes in TBS, ImmPRESS alkaline phosphatase reagent directed to mouse or rabbit IgGs (Vector Laboratories) was added to the sections for 30 minutes. Subsequently, slides were rinsed with TBS and

TABLE 1. Clinicopathological Characteristics of MS Patients and Control Subjects

#	Sex	Age	Type of Tissue	MS Disease Duration (Years)	PMD (h:min)	Cause of Death
1	m	41	MS	14	7:20	Pneumonia, urosepsis
2	m	44	MS	22	10:15	General deterioration/infection
3	m	49	MS	25	8:00	Pneumonia
4	f	50	MS	17	7:35	Euthanasia
5	f	50	MS	12	9:05	Euthanasia
6	m	51	MS	>20	11:00	Unknown, infection 2 days prior to death
7	f	54	MS	13	9:25	Unclear, most likely respiratory failure
8	m	54	MS	21	8:15	Euthanasia
9	f	60	MS	7	10:40	Euthanasia
10	m	64	MS	35	7:30	Euthanasia
11	f	66	MS	22	6:00	Unknown
12	f	66	MS	23	9:35	Euthanasia
13	f	66	MS	17	10:45	Pulmonary hypertension
14	m	55	Control	–	7:30	Euthanasia with oesophageal cancer
15	f	57	Control	–	7:40	Euthanasia with metastatic urothelial cancer
16	m	57	Control	–	9:45	Multisystem atrophy
17	f	60	Control	–	7:30	Infection, fever of Unknown origin
18	f	62	Control	–	7:55	Unknown
19	m	62	Control	–	7:20	Unknown

PMD, postmortem delay.

TABLE 2. Primary Antibodies Used in Immunohistochemistry

Target	Origin	Dilution	Supplier	Article Code
HLA-DR (LN3)	Mouse	1:1000	Pierce (Rockford, IL)	MA5-11966
PLP	Mouse	1:1000	AbD Serotec (Oxford, UK)	MCA839G
TG2	Goat	1:10 000	Upstate (via Merck Millipore, Darmstadt, Germany)	06-471
CD3	Rabbit	1:3000	Dako (Glostrup, Denmark)	A0452
CD11b	Mouse	1:500	Abcam (Cambridge, UK)	ab34216
CD14	Mouse	1:400	Dako	M0825
CD20	Mouse	1:2000	Dako	M0755
CD45	Mouse	1:2000	Dako	M0701
CD68	Mouse	1:20 000	Dako	M0718
CD163	Mouse	1:8000	Gift, Dept. Molecular Cell Biology and Immunology, VUmc (Amsterdam, The Netherlands)	–
CD206	Mouse	1:8000	Abcam	ab8918
CX3CR1	Rabbit	1:3000	Abcam	ab8020

Red O staining. An Oil Red O stock solution of 0.5 mg Oil Red O (Sigma-Aldrich) in 100 mL isopropanol was prepared. After 3:2 dilution with distilled water and filtration, unfixed tissue sections were incubated for 10 minutes at room temperature. This was followed by a rinse with water, nuclear hematoxylin counterstaining and cover slipping with aquatex (Merck Millipore).

Image Acquisition and Semiquantitative Analysis of Immunopositive Cells

Immunohistochemical images of 4–12 regions of interest ([ROI] 690 μm by 665 μm) per patient were acquired with Leica DM5000B microscope (Leica Microsystems, The Hague, The Netherlands) equipped with a spectral imaging camera (Nuance, PerkinElmer, Waltham, MA). Semiquantification of colabeled cells was performed by assessment of the numbers of TG2-positive ($TG2^+$) cells that were also immunopositive for one of the immune cell markers. For this purpose, the slides were imaged according to the Nuance user manual (provided by PerkinElmer). In short, for each chromogen (that is, DAB, liquid permanent red, and hematoxylin), the specific emission spectrum was acquired and characterized with the Nuance software based on light absorbance/scattering. Acquired spectral images from the triple chromogen stainings were separated into the 3 individual channels using the predefined spectra. The manually counted number of $TG2^+$ cells per patient varied from 45 to 295 cells, depending on number of ROIs size and numbers of available tissue blocks per patient. To control for variation in cell numbers, we

Tris-HCl and then developed with liquid permanent red as substrate (according to manufacturer, Dako, Glostrup, Denmark). Subsequently, the sections were incubated with goat antiTG2 antibody for 1 hour at room temperature. Washing steps with TBS and Tris-HCl were followed by incubation with ImmPRESS horseradish peroxidase antigoat IgG reagent (Vector Laboratories) for 30 minutes at room temperature. The reagent was washed off with TBS and Tris-HCl and the signal developed with DAB (Sigma-Aldrich). Finally, slides were washed in Tris-HCl and tap water, counterstained with hematoxylin and mounted with an aqueous mounting medium containing mowiol (24 g glycerol, 24 mL distilled water, 48 mL 0.2 M Tris buffer, pH 8.5, 9.6 g mowiol 4-88 [Sigma-Aldrich]) and dried at 4°C.

For all immunostainings, omission of the primary antibodies on several sections served as negative controls for nonspecific IgG binding (data not shown).

Oil Red O Staining

Neutral lipids, present in macrophages and indicative of myelin phagocytosis and degradation, were detected with Oil

determined the number of TG2 cells colabeling with one of the immune cell markers as a percentage of total TG2$^+$ cells counted per patient. Images that were taken from brightfield microscopy are shown pseudocolored in the style of fluorescent staining to increase visual contrast.

RESULTS

The 9 analyzed MS lesions were classified as active (n = 1) or chronic active (n = 8) by immunohistochemistry using the above-described criteria. HLA-DR, the general marker for antigen-expressing cells demonstrating lesion activity, was abundantly present in all MS lesions either in the center (active lesion) or in a broad rim surrounding the lesion (chronic active lesion; Fig. 1A, B). Reduction in antiPLP antibody immunoreactivity indicated demyelination, which was partial or complete in all lesions (Fig. 1D, E). To further classify the activity state of the lesions, Oil Red O staining was performed. The intense red cellular staining specified myelin ingested by macrophages in all analyzed lesions, suggesting ongoing demyelination (Fig. 1G, H). Brain tissue obtained from control patients showed very little HLA-DR expression (Fig. 1C), no loss of antiPLP immunoreactivity (Fig. 1F), and no Oil Red O staining signal (Fig. 1I).

TG2 Immunoreactivity in MS Lesions

TG2 was commonly present in blood vessels in tissue from control subjects (Fig. 2A, arrowhead) and in MS normal appearing white matter (NAWM; Fig. 2B, arrowhead) and MS lesions (Fig. 2C, arrowhead). In inflammatory active and chronic active MS lesions, additional TG2$^+$ round cells were found in the perivascular cuff, the lesion area surrounding the blood vessels and in the parenchyma (Fig. 2C, arrows). This cellular TG2 immunoreactivity was not observed in inactive lesions (not shown), in NAWM of MS patients, or in control brain tissue, as shown previously by our group (29). Preadsorption of the TG2 antibody with recombinant TG2 protein resulted in absence of cellular TG2 staining in MS lesions with only a slight residual vascular staining remaining (Fig. 2D, arrowhead), indicating the specificity of the immunostaining.

Characterization of TG2$^+$ Cells in MS Lesions

For cell type characterization of TG2$^+$ cells in MS lesions, we first stained postmortem tissue with various immune cells markers, followed by colabeling with TG2-antibodies and subsequent analysis.

TG2 Immunoreactivity Is Present in Leukocytes

The majority of TG2$^+$ cells found in active MS lesions appeared as round cells, characteristic of infiltrated leukocytes. Therefore, we performed double labeling of the TG2 antibody (Fig. 3A) with the general leukocyte marker CD45 (Fig. 3B). We found numerous cells demonstrating both CD45 and TG2 immunoreactivity (Fig. 3C, arrow and inserts). After quantification of the amount of TG2$^+$ cells that are CD45$^+$,

we observed that ∼70% of the TG2$^+$ cells were leukocytes (Fig. 3D).

TG2 Immunoreactivity in Lymphocytes

To further characterize TG2$^+$ leukocytes, we used markers that stain specific leukocytes subsets. First, we studied lymphocytes, that is, T and B cells, using CD3 and CD20 as pan-markers, respectively. In the MS lesions, especially around larger blood vessels, where TG2$^+$ cells were also present (Fig. 4A, E), CD3$^+$ T cells (Fig. 4B) and CD20$^+$ B cells (Fig. 4E) were found. Although CD3 (Fig. 4A–D) and CD20 immunoreactivity (Fig. 4E, F) were present in numerous cells, these hardly colocalized with TG2 as shown in the overlay of both stainings (Fig. 4C, G). Quantification of the colabeled cells indicated that of the TG2$^+$ cells only 3.8% and 2.3% were identified as T- and B-cells, respectively (Fig. 4D, H).

TG2 Immunoreactivity in Macrophage Subtypes

Another subset of leukocytes found in MS lesions are of the monocyte and macrophage type. For their analysis as potential TG2 expressing cells, a broad panel of different cell markers was used. These markers are also known to be expressed by parenchymal microglia, depending on their activation status (35, 36). Analysis of the MS lesions for macrophages and TG2$^+$ cells showed many round cells representing monocytes and macrophages were present in these areas (Fig. 5A, E, I, M). First, we assessed the population of CD14$^+$ cells in MS lesions (Fig. 5B) and found numerous CD14$^+$ monocytes in close vicinity to blood vessels but also spread throughout the lesion. Most of them showed limited colocalization with TG2 staining (3.5% colabeling, Fig. 5C, D). The 2 macrophage markers used were CD11b, a pan macrophage marker and CD68, a lysosomal marker for phagocytic active macrophages (37). Both markers, that is, CD11b (Fig. 5F) and CD68 (Fig. 5J), were present in cells in the lesion areas. Their colabeling with TG2 staining indicated that 45% of TG2$^+$ cells were CD11b$^+$ macrophages (Fig. 5G, H) and 11% were positive for CD68 (Fig. 5K, L). Another cell surface macrophage marker, that is, CX3CR1 (38), is expressed in MS lesions but also only showed limited colocalization (5.3%) with TG2 staining (Fig. 5N, O, P).

Because TG2 has been qualified as a marker for alternatively activated macrophages (M2) (39), we further characterized whether TG2$^+$ cells colabel with markers for alternatively activated macrophages, that is, CD163 and CD206 (40). In the area of TG2$^+$ cells in the lesions (Fig. 6A, E), both CD163 (Fig. 6B) and CD206 (Fig. 6F) were present. Colabeling of TG2 and the M2 markers (Fig. 6C, G), however, resulted in only small numbers of double-stained cells, that is, CD163 was expressed in 3.8% and CD206 in 5% of the TG2$^+$ cells in the MS lesions (Fig. 5P, T). The results of the semi-quantification of TG2$^+$ cells colabeling with immune cell markers in inflammatory MS lesions are summarized in Table 3.
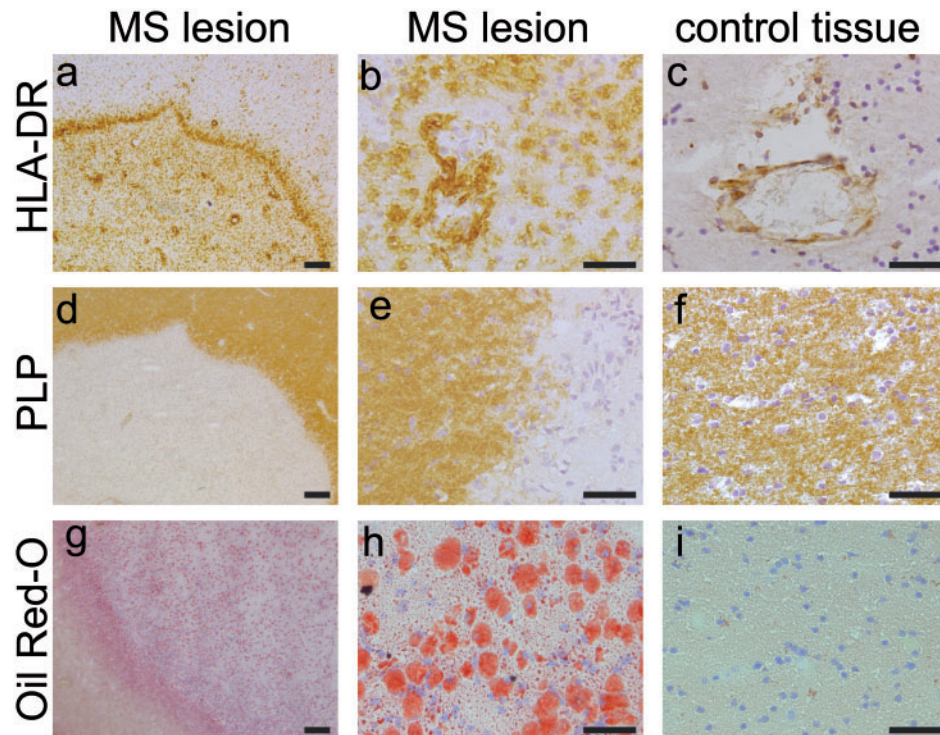
FIGURE 1. MS white matter lesion classification with PLP, HLA-DR, and ORO immunohistochemical staining. HLA-DR **(A–C)**, PLP **(D–F)**, and ORO **(G–I)** staining of postmortem MS lesion tissue (2 magnifications of 1 lesion each) and control tissue **(C, F, I)**. Scale bars: **B, C, E, F, H, I** = 50 μm; **A, D, G** = 200 μm. Abbreviations: HLA-DR, human leukocyte antigen-D related; PLP, myelin proteolipid protein; ORO, Oil Red O.
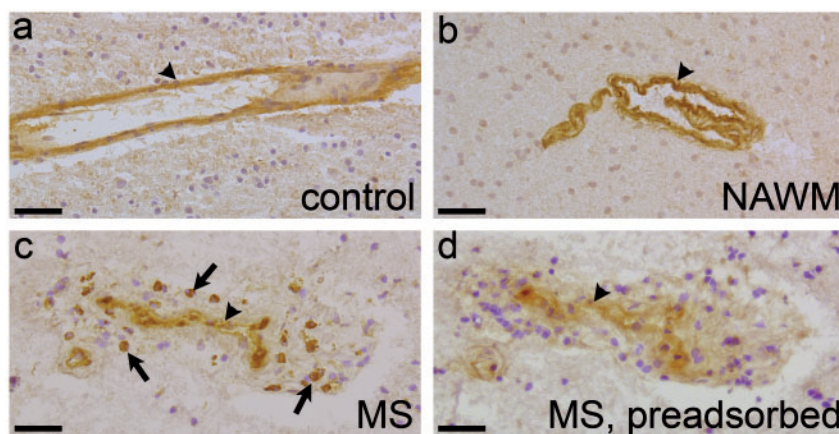

FIGURE 2. TG2 expression in white matter MS lesions and control tissue. **(A–C)** Vascular immunohistochemical TG2 staining (arrowhead) was observed in healthy control tissue **(A)**, normal appearing white matter (NAWM) tissue of MS patients **(B)**, and MS tissue **(C)**. Additionally, cellular TG2 staining outside the vasculature (arrow) was not observed in control tissue **(A)** or NAWM **(B)**, whereas in MS patient-derived tissue **(C)** some cells were strongly stained. **(D)** The antiTG2 immunoreactivity was strongly reduced after preadsorption of the TG2 antibody with recombinant TG2 protein. Scale bar: 50 μm.

DISCUSSION

Novel treatments of MS focus mainly on lymphocytes as a therapeutic target, but there is a heterogeneity between MS lesions and patients with regard to the pathological features. Therefore, other cell types, in particular monocytes and macrophages, may be of interest as a potential target. TG2

plays a role in monocyte/macrophage migration and the aim of the present study was to examine TG2 expressing cell types in MS lesions in detail.

As the variation in the number of TG2$^+$ cells is high in different types of MS lesions, we included only active and chronic active lesions with significant HLA-DR staining in the
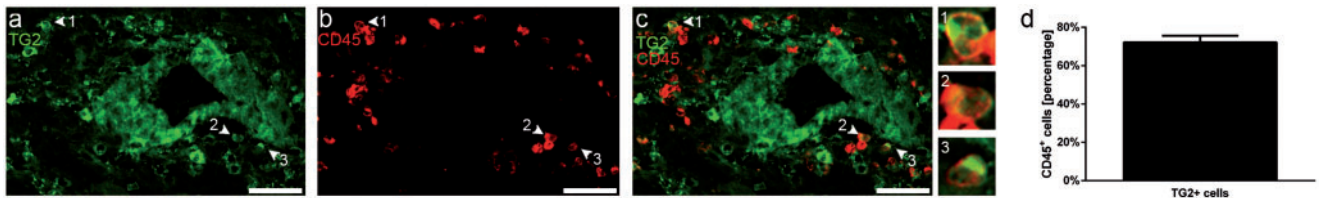
FIGURE 3. AntiTG2^{+} cells in MS lesions are positive for the leukocyte marker CD45. **(A)** TG2^{+} cells (arrowheads) surrounding a blood vessel in a MS lesion. **(B)** Cells immunoreactive for the antiCD45 antibody (arrowheads). **(C)** Double staining of TG2 and CD45 indicates immunoreactivity for antiTG2 antibody in antiCD45^{+} cells (arrowheads, magnified inserts). **(D)** Quantification of TG2^{+} cells immunoreactive for the antiCD45 antibody. Scale bar: 50 μm. Bar: standard error of the mean (SEM).
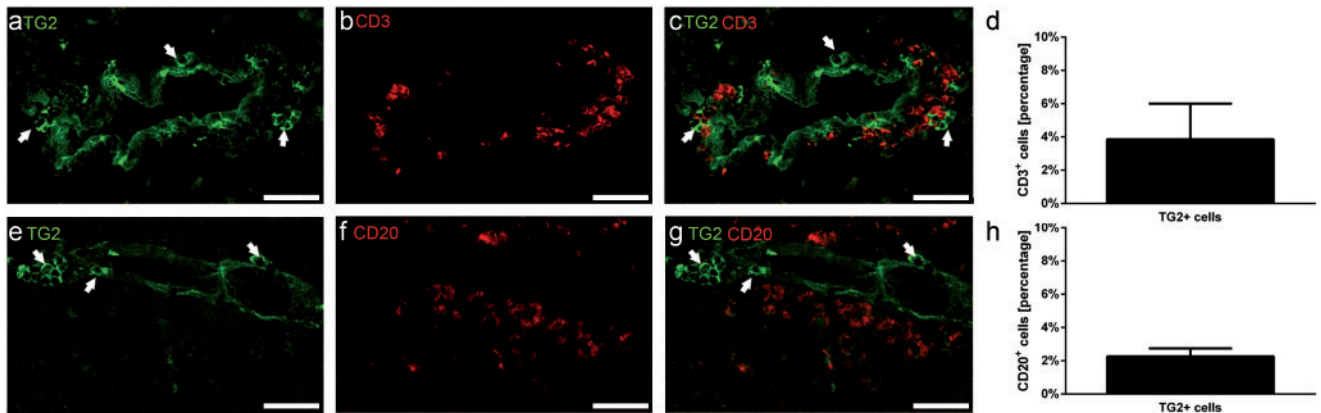

FIGURE 4. AntiTG2 antibody immunoreactivity in MS lesions was absent in lymphocytes. TG2^{+} cells (arrows) surrounding a blood vessel in a MS lesion **(A, E)**. **(B)** B cells present in MS lesions were detected using the B cell-specific marker CD20. **(C)** Double staining of antiCD20 antibody and antiTG2^{+} cells showed **(D)** very little overlap of these 2 markers (arrows) and therefore limited expression of TG2 in B cells. **(F)** CD3 expression indicated presence of T lymphocytes, but merge of antiTG2 and antiCD3 antibody staining **(G)** revealed that both markers were expressed in different cell types (arrows), as quantified in **(H)**. Scale bar: 50 μm. Bar: SEM.

rim and a relatively high number of TG2^{+} cells. The reason for the variation in TG2 expression between MS patients remains unsolved, but may potentially be related to disease duration and the clinical status of the patient. Since TG2 is upregulated under inflammatory conditions, it can be mostly seen in active or chronic active lesions with substantial leukocyte infiltration (30). With ongoing disease, the inflammation and cellular infiltration recedes, which in turn might reduce the expression of TG2 (41).

The main observation of our study is that the vast majority (~70%) of TG2^{+} cells is CD45^{+} and displays a round morphology, indicative of leukocytes infiltrated from the circulation. In our previous study, the TG2^{+}/HLA-DR-positive cells also had a round morphology and thus likely represent a (sub)population of infiltrated leukocytes (29). To further delineate the cell type(s) expressing TG2, we analyzed the 2 most abundant leukocyte cell types found in MS lesions, that is, lymphocytes and monocytes/macrophages. CD3^{+} T cells and CD20^{+} B cells were rarely associated with TG2^{+} cells, despite the fact that lymphocytes together with monocytes and macrophages migrate into the CNS as a part of MS pathology (13, 42). We therefore propose that the migration process of lymphocytes into the CNS in MS may be independent of TG2 in lymphocytes, which is in line with previous rodent MS models in which lymphocyte migration was not affected by TG2 inhibition (29, 43). In addition, we determined the presence of TG2 in monocytes and macrophages. CD14^{+} monocytes showed limited TG2 immunoreactivity. However, in circulating monocytes derived from MS patients, low but increased TG2 mRNA levels were present compared to control monocytes (44). Still, these mRNA levels may not result in sufficient levels of protein to be detected by immunohistochemistry. It is also possible that the TG2^{+} monocytes mostly remain in the circulation with a minority entering the CNS. TG2 immunoreactivity was clearly present in CD11b^{+} cells. Although CD11b can also be expressed by natural killer cells and granulocytes in addition to macrophages, these cell types are rather scarce in MS lesions compared to macrophages (45–47). Hence, we assume that the majority of the TG2^{+} round CD11b^{+} cells is of the macrophage lineage (27, 48).

Macrophages are often subdivided into homeostatic (M0), classically activated (M1) and alternatively activated (M2) macrophages, based on a certain cell marker expression profile in vitro (49). Depending on their phenotype they might adopt different functions that can be either beneficial or detrimental. Our data of TG2 immunoreactivity in a number of CD68^{+} macrophages, that is, macrophages with lysosomal activity, may suggest that TG2 contributes to phagocytosis of,
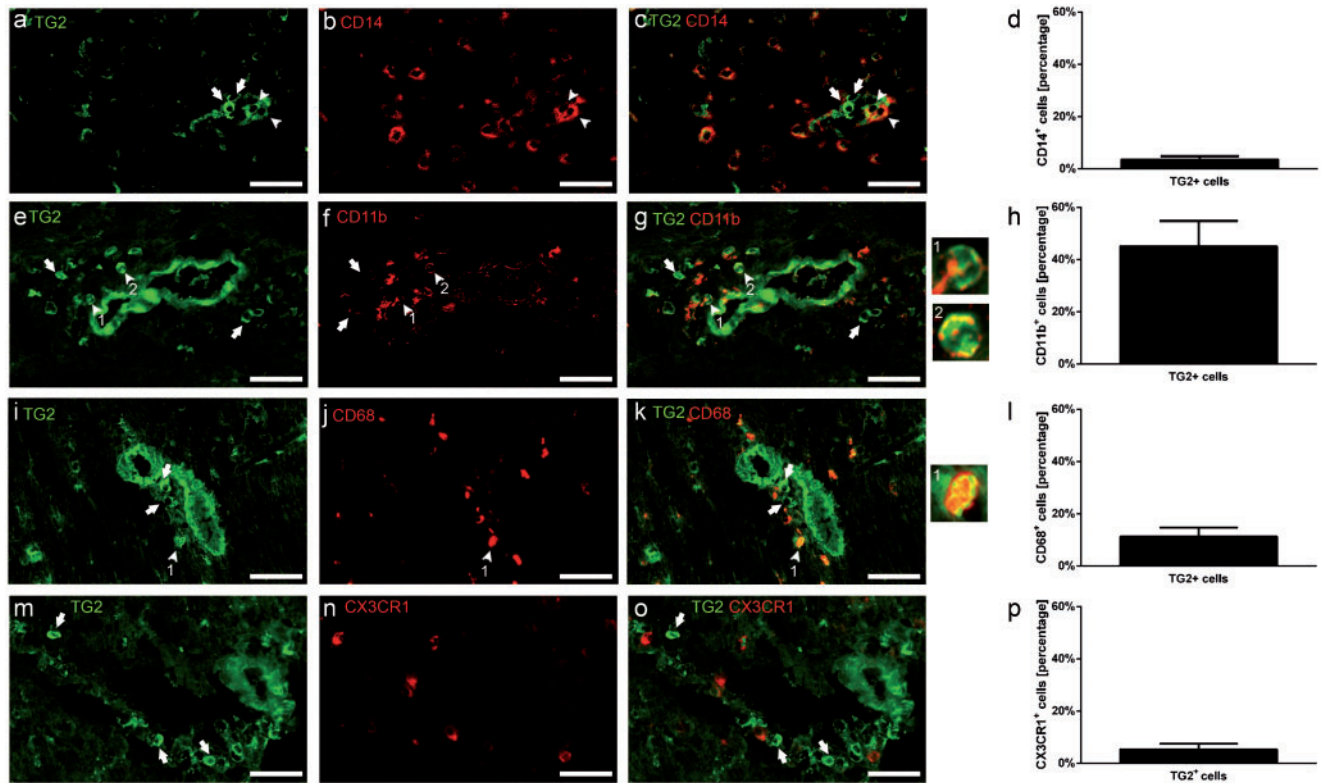
FIGURE 5. Abundant antiTG2 antibody immunoreactivity in monocyte/macrophages associated with MS lesions. **(A, E, I, M)** TG2$^+$ cells (arrows, arrowheads) surrounding blood vessels in MS lesions. **(B)** CD14$^+$-immunoreactive monocytes (arrowheads) were found in MS lesions. **(C, D)**; however, few of the antiCD14$^+$ cells expressed TG2 (arrowheads), whereas the majority did not show immunoreactivity for antiTG2 antibody (arrows). **(F)** CD11b expression was found in lesions (arrowhead) and **(G, H)** approximately half of the CD11b$^+$ cells expressed TG2$^+$ (arrowheads, magnified inserts). **(J)** CD68, a lysosomal macrophage marker, was present in MS lesions and **(K, L)** antiTG2 antibody immunoreactivity was present (arrowheads, magnified insert) in some but not all (arrows). **(N)**. Another cell surface macrophage marker, CX3CR1, was expressed in MS lesions but **(O, P)** little colocalization with the antiTG2 antibody immunoreactivity was found (arrows). Arrows indicate TG2$^+$ cells that did not correspond to one of the monocyte or macrophage markers. Arrowheads indicate monocytes or macrophages showing immunoreactivity for the antiTG2 antibody. Scale bar: 50 μm. Bar: SEM.
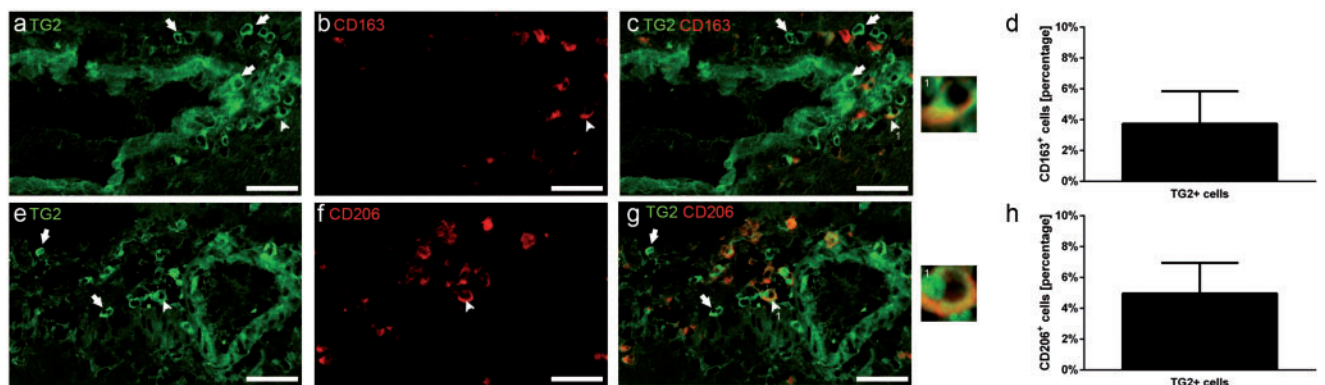

FIGURE 6. Expression of TG2 in alternatively activated (M2) type macrophages. **(A, E)** TG2$^+$ cells surrounding a blood vessel in a MS lesion (arrows, arrowheads). M2 macrophages were found in MS lesions as indicated by antiCD163 antibody **(B)** and antiCD206 antibody **(F)** immunoreactivity; however, only small proportions were found to be immunoreactive for the antiTG2 antibody in these M2 macrophages **(C, D/G, H,** arrowheads, magnified inserts) whereas TG2 staining was absent in the majority of M2 type cells (arrows). Scale bar: 50 μm. Bar: SEM.

TABLE 3. TG2 and Immune Cell Marker Colocalization in MS Lesions

Immune Marker	% of TG2 Cells Positive for Immune Marker	Standard Deviation (%)
CD45	72.16	10.50
CD3	3.85	6.46
CD20	2.27	1.45
CD14	3.52	3.97
CD11b	45.02	29.06
CD68	11.21	9.89
CX3CR1	5.29	6.77
CD163	3.74	6.32
CD206	4.96	5.95

for example, myelin debris. Also, a role for TG2 in the process of efferocytosis, that is, phagocytosis of apoptotic cells, has clearly been established (50–52). However, CX3CR1, an important factor in the clearance of myelin debris (53), and TG2 co-expression proved limited in CX3CR1$^+$ cells in MS lesions. Together, these data suggest that if macrophage-derived TG2 contributes to the clearance of myelin debris, its impact may be limited. Generally, lysosomal active macrophages have been shown to have an anti-inflammatory signature (54), and it has been shown that TG2 expression is increased in M2 macrophages in vitro (39, 55). In the MS lesions we studied, we could not confirm that TG2 is only expressed by anti-inflammatory M2 macrophages. Still, the low number of TG2$^+$ cells that colabel with M2 markers may represent macrophages involved in phagocytosis and/or efferocytosis. We cannot rule out that also macrophages of the M1 type can be TG2$^+$ representing another subpopulation of the CD11b$^+$ or CD68$^+$ macrophages. Altogether, it is of importance to note that macrophages present in MS lesions are predominantly of an intermediate phenotype (40), which may explain our finding of relative low numbers of TG2$^+$ anti-inflammatory macrophages in MS lesions.

With our analyses we determined that the majority of the TG2$^+$ cells was leukocytes of which the majority was of the monocyte/macrophage lineage. However, our analysis did not allow us to determine the lineage of the remaining 30% of cells. One possibility is that their expression of our analyzed markers was too low to be detected in our immunohistochemical assays. For example, CD45 expression in microglia might be below our detection limit (56). In addition, we cannot exclude that certain subtypes of lymphocytes that are neither CD3$^+$ nor CD20$^+$ might be expressing TG2. Additional studies are needed to enlighten the identity of these cells and their potential effects when considering TG2 as a potential target in the therapeutic treatment of MS patients.

Conclusions

On the basis of our observations, we conclude that TG2 is largely expressed by cells of the monocyte/macrophage lineage in MS lesions. The TG2$^+$ macrophage phenotype cannot be clearly classified as M1 or M2 and hence a potential dual role of macrophage-derived TG2 in MS lesion pathology is possible. Our observations in animal models of MS supported a role for monocyte/macrophage-derived TG2 in cell adhesion and migration contributing to the disease process (29). Because we observed that TG2 contributes to human monocyte adhesion and migration in vitro (44), this could also hold true for the in vivo disease. Therefore, monocyte/macrophage-derived TG2 could be a potential target for intervention during MS that adds to the available lymphocyte focused therapies and broadens the spectrum of cells targeted in the heterogeneous MS lesions.

REFERENCES

1. Pugliatti M, Sotgiu S, Rosati G. The worldwide prevalence of multiple sclerosis. Clin Neurol Neurosurg 2002;104:182–91
2. Compston A, Coles A. Multiple sclerosis. Lancet 2008;372:1502–17
3. Sospedra M, Martin R. Immunology of multiple sclerosis. Annu Rev Immunol 2005;23:683–747
4. Kutzelnigg A, Lassmann H. Pathology of multiple sclerosis and related inflammatory demyelinating diseases. Handb Clin Neurol 2014;122: 15–58
5. van der Valk P, De Groot CJ. Staging of multiple sclerosis (MS) lesions: Pathology of the time frame of MS. Neuropathol Appl Neurobiol 2000; 26:2–10
6. Cheng Y, Sun L, Xie Z. Diversity of immune cell types in multiple sclerosis and its animal model: Pathological and therapeutic implications. J Neurosci Res 2017;95:1973–83
7. Lucchinetti C, Bruck W, Parisi J, et al. Heterogeneity of multiple sclerosis lesions: Implications for the pathogenesis of demyelination. Ann Neurol 2000;47:707–17
8. Ley K, Laudanna C, Cybulsky MI, et al. Getting to the site of inflammation: The leukocyte adhesion cascade updated. Nat Rev Immunol 2007;7: 678–89
9. Kerfoot SM, Kubes P. Overlapping roles of P-selectin and alpha 4 integrin to recruit leukocytes to the central nervous system in experimental autoimmune encephalomyelitis. J Immunol 2002;169:1000–6
10. Bar-Or A, Fawaz L, Fan B, et al. Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS? Ann Neurol 2010;67:452–61
11. Barr TA, Shen P, Brown S, et al. B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. J Exp Med 2012;209:1001–10
12. Mauri C, Bosma A. Immune regulatory function of B cells. Annu Rev Immunol 2012;30:221–41
13. Wekerle H. B cells in multiple sclerosis. Autoimmunity 2017;50:57–60
14. Minogue AM. Role of infiltrating monocytes/macrophages in acute and chronic neuroinflammation: Effects on cognition, learning and affective behaviour. Prog Neuropsychopharmacol Biol Psychiatry 2017;79:15–8
15. Wlodarczyk A, Cedile O, Jensen KN, et al. Pathologic and protective roles for microglial subsets and bone marrow- and blood-derived myeloid cells in central nervous system inflammation. Front Immunol 2015; 6:463
16. Berkovich R. Treatment of acute relapses in multiple sclerosis. Neurotherapeutics 2013;10:97–105
17. Oh J, O'Connor PW. Established disease-modifying treatments in relapsing-remitting multiple sclerosis. Curr Opin Neurology 2015;28: 220–9
18. Moreno Torres I, Garcia-Merino A. Anti-CD20 monoclonal antibodies in multiple sclerosis. Expert Rev Neurother 2017;17:359–71
19. Willis MD, Robertson NP. Alemtuzumab for multiple sclerosis. Curr Neurol Neurosci Rep 2016;16:84

20. Blumenfeld S, Staun-Ram E, Miller A. Fingolimod therapy modulates circulating B cell composition, increases B regulatory subsets and production of IL-10 and TGFbeta in patients with Multiple Sclerosis. J Autoimmun 2016;70:40–51

21. Diebold M, Sievers C, Bantug G, et al. Dimethyl fumarate influences innate and adaptive immunity in multiple sclerosis. J Autoimmun 2018;86: 39–50

22. Delbue S, Comar M, Ferrante P. Natalizumab treatment of multiple sclerosis: New insights. Immunotherapy 2017;9:157–71

23. Rahmanzadeh R, Bruck W, Minagar A, et al. Multiple sclerosis pathogenesis: Missing pieces of an old puzzle. Rev Neurosci 2018;30:67–83

24. Huitinga I, van Rooijen N, de Groot CJ, et al. Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. J Exp Med 1990;172:1025–33

25. Duffy SS, Lees JG, Moalem-Taylor G. The contribution of immune and glial cell types in experimental autoimmune encephalomyelitis and multiple sclerosis. Mult Scler Int 2014;2014:285245

26. Leu RW, Herriott MJ, Moore PE, et al. Enhanced transglutaminase activity associated with macrophage activation. Possible role in Fc-mediated phagocytosis. Exp Cell Res 1982;141:191–9

27. Akimov SS, Belkin AM. Cell surface tissue transglutaminase is involved in adhesion and migration of monocytic cells on fibronectin. Blood 2001; 98:1567–76

28. Bogie JF, Stinissen P, Hendriks JJ. Macrophage subsets and microglia in multiple sclerosis. Acta Neuropathol 2014;128:191–213

29. van Strien ME, de Vries HE, Chrobok NL, et al. Tissue Transglutaminase contributes to experimental multiple sclerosis pathogenesis and clinical outcome by promoting macrophage migration. Brain Behav Immun 2015;50:141–54

30. Chrobok NL, Sestito C, Wilhelmus MM, et al. Is monocyte- and macrophage-derived tissue transglutaminase involved in inflammatory processes? Amino Acids 2017;49:441–52

31. Espitia Pinzon N, Stroo E, t Hart BA, et al. Tissue transglutaminase in marmoset experimental multiple sclerosis: Discrepancy between white and grey matter. PLoS One 2014;9:e100574

32. Bo L, Vedeler CA, Nyland H, et al. Intracortical multiple sclerosis lesions are not associated with increased lymphocyte infiltration. Mult Scler 2003;9:323–31

33. De Groot CJ, Bergers E, Kamphorst W, et al. Post-mortem MRI-guided sampling of multiple sclerosis brain lesions: Increased yield of active demyelinating and (p)reactive lesions. Brain 2001;124:1635–45

34. Hendrickx DAE, van Eden CG, Schuurman KG, et al. Staining of HLA-DR, Iba1 and CD68 in human microglia reveals partially overlapping expression depending on cellular morphology and pathology. J Neuroimmunol 2017;309:12–22

35. Walker DG, Lue LF. Immune phenotypes of microglia in human neurodegenerative disease: Challenges to detecting microglial polarization in human brains. Alzheimers Res Ther 2015;7:56

36. Hulshof S, van Haastert ES, Kuipers HF, et al. CX3CL1 and CX3CR1 expression in human brain tissue: Noninflammatory control versus multiple sclerosis. J Neuropathol Exp Neurol 2003;62:899–907

37. Holness CL, Simmons DL. Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. Blood 1993;81: 1607–13

38. Panek CA, Ramos MV, Mejias MP, et al. Differential expression of the fractalkine chemokine receptor (CX3CR1) in human monocytes during differentiation. Cell Mol Immunol 2015;12:669–80

39. Eligini S, Brioschi M, Fiorelli S, et al. Human monocyte-derived macrophages are heterogenous: Proteomic profile of different phenotypes. J Proteomics 2015;124:112–23

40. Vogel DY, Vereyken EJ, Glim JE, et al. Macrophages in inflammatory multiple sclerosis lesions have an intermediate activation status. J Neuroinflammation 2013;10:35 [Database]

41. Dutta R, Trapp BD. Relapsing and progressive forms of multiple sclerosis: Insights from pathology. Curr Opin Neurol 2014;27:271–8

42. Constantinescu CS, Gran B. The essential role of T cells in multiple sclerosis: A reappraisal. Biomed J 2014;37:34–40

43. Chrobok NL, Bol J, Jongenelen CA, et al. Characterization of Transglutaminase 2 activity inhibitors in monocytes in vitro and their effect in a mouse model for multiple sclerosis. PLoS One 2018;13:e0196433

44. Sestito C, Breve JJP, van Eggermond M, et al. Monocyte-derived tissue transglutaminase in multiple sclerosis patients: Reflecting an anti-inflammatory status and function of the cells? J Neuroinflammation 2017;14:257

45. Illes Z, Kondo T, Newcombe J, et al. Differential expression of NK T cell V alpha 24J alpha Q invariant TCR chain in the lesions of multiple sclerosis and chronic inflammatory demyelinating polyneuropathy. J Immunol 2000;164:4375–81

46. Durrenberger PF, Ettorre A, Kamel F, et al. Innate immunity in multiple sclerosis white matter lesions: Expression of natural cytotoxicity triggering receptor 1 (NCR1). J Neuroinflammation 2012;9:1

47. Rumble JM, Huber AK, Krishnamoorthy G, et al. Neutrophil-related factors as biomarkers in EAE and MS. J Exp Med 2015;212:23–35

48. Seiving B, Ohlsson K, Linder C, et al. Transglutaminase differentiation during maturation of human blood monocytes to macrophages. Eur J Haematol 1991;46:263–71

49. Murray PJ, Allen JE, Biswas SK, et al. Macrophage activation and polarization: Nomenclature and experimental guidelines. Immunity 2014;41: 14–20

50. Nadella V, Wang Z, Johnson TS, et al. Transglutaminase 2 interacts with syndecan-4 and CD44 at the surface of human macrophages to promote removal of apoptotic cells. Biochim Biophys Acta 2015;1853:201–12

51. Fesus L, Sandor M, Horvath LI, et al. Immune-complex-induced transglutaminase activation: Its role in the Fc-receptor-mediated transmembrane effect on peritoneal macrophages. Mol Immunol 1981;18:633–8

52. Murtaugh MP, Arend WP, Davies PJ. Induction of tissue transglutaminase in human peripheral blood monocytes. J Exp Med 1984;159:114–25

53. Lampron A, Larochelle A, Laflamme N, et al. Inefficient clearance of myelin debris by microglia impairs remyelinating processes. J Exp Med 2015;212:481–95

54. Boven LA, Van Meurs M, Van Zwam M, et al. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. Brain 2006;129:517–26

55. Martinez FO, Helming L, Milde R, et al. Genetic programs expressed in resting and IL-4 alternatively activated mouse and human macrophages: Similarities and differences. Blood 2013;121:e57–69

56. Jeong HK, Ji K, Min K, et al. Brain inflammation and microglia: Facts and misconceptions. Exp Neurobiol 2013;22:59–67