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CD169 is a marker for highly pathogenic phagocytes in multiple sclerosis

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Abstract

Background: Phagocytes, such as macrophages and microglia, are key effector cells in the pathophysiology of multiple sclerosis (MS). It is widely accepted that they instigate and promote neuroinflammatory and neurodegenerative events in MS. An increasing amount of studies indicate that Siglec-1, also known CD169, is a marker for activated phagocytes in inflammatory disorders.

Objective: In this study, we set out to define how CD169+ phagocytes contribute to neuroinflammation in MS.

Methods: CD169-diphteria toxin receptor (DTR) mice, which express human DTR under control of the CD169 promoter, were used to define the impact of CD169+ cells on neuroinflammation. Flow cytometry and immunohistochemistry were utilized to determine the expression and distribution of CD169.

Results: We show that CD169 is highly expressed by lesional and circulating phagocytes in MS and experimental autoimmune encephalomyelitis (EAE). Our data further indicate that CD169 represents a selective marker for early activated microglia in MS and EAE lesions. Depletion of CD169+ cells markedly reduced neuroinflammation and ameliorated disease symptoms in EAE-affected mice.

Conclusion: Our findings indicate that CD169+ cells promote neuroinflammation. Furthermore, they suggest that CD169+ phagocytes play a key role in the pathophysiology of MS. Hence, targeting CD169+ phagocytes cells may hold therapeutic value for MS.
Introduction

Multiple sclerosis (MS) is one of the most prevalent autoimmune diseases in the Western world. Phagocytes, such as monocyte-derived macrophages and microglia, play a key role in the pathophysiology of MS. The general consensus is that they display a disease-promoting phenotype in the initial stages of the disease. However, increasing evidence supports immunosuppressive and reparative features of phagocytes in MS. Driving inflammatory phagocytes towards an anti-inflammatory, regenerative phenotype or depleting them represent promising therapeutic options. Markers that allow for selective identification of early disease-promoting inflammatory phagocytes represent excellent candidates for specific targeting of these cells. However, to date, no markers that selectively identify these cells exist.

Siglec-1, also known as sialoadhesin or CD169, is a sialic acid receptor that is primarily expressed by macrophage subsets in secondary lymphoid organs. However, CD169-expressing macrophages also reside within the CNS in the meninges and choroid plexus. Interestingly, a growing number of studies show that CD169 expression is strongly enhanced on activated phagocytes and that the number of CD169+ phagocytes is increased in pathological conditions. CD169 expression is increased on circulating monocytes of MS patients and synovial membrane macrophages of rheumatoid arthritis patients. Likewise, monocytes of individuals with HIV-1, systemic sclerosis, and systemic lupus erythematosus show increased expression of CD169. The elevated expression of CD169 by monocytes in some of these disorders is reported to be controlled by type 1 interferon (IFN) signaling. Functionally, CD169 is implicated in the uptake of sialylated bacteria and viruses, leukocyte interactions, and antigen presentation. In autoimmune disorders, the presence of CD169+ phagocytes in the circulation and affected tissues often closely correlates with disease severity. In contrast, CD169+ marginal zone macrophages in the spleen are also crucial in maintaining tolerance and suppressing autoimmunity.
In this study, we set out to define the distribution of CD169+ phagocytes in MS patients and an experimental MS animal model, experimental autoimmune encephalomyelitis (EAE). Moreover, we sought to determine if temporal depletion of CD169+ cells affects EAE disease severity. We show that CD169 is selectively expressed by perilesional (early) activated microglia in MS and EAE lesions. Furthermore, we observed a substantial increase in the percentage of CD169+ phagocytes in the circulation and lesions of MS patients and EAE animals. Finally, we demonstrate that temporal depletion of CD169+ cells reduces EAE disease symptoms. Collectively, our data strongly suggest that CD169 is a marker for pathogenic phagocytes in MS.
Material and Methods

Animals and EAE induction

Mice expressing the human diphtheria toxin receptor (DTR) under the control of the CD169 gene promoter (CD169-DTR mice) were kindly provided by Kenji Kohno, Ph.D. and Masato Tanaka, Ph.D. CD169-DTR were immunized and evaluated for neurological signs of the disease as described previously. CD169-DTR mice were treated intraperitoneal with PBS (Lonza) or DT (10 µg/kg, Sigma-Aldrich) every other 5 days starting 5 days post immunization (dpi). Experiments were conducted in accordance with institutional guidelines and approved by the local Ethical Committee for Animal Experiments.

Study subjects

Blood samples were collected from 19 healthy controls and 57 MS patients. MS patients were either untreated (n=19) or received treatment with interferon β (IFNβ Avonex, Rebif, Betaferon, n=22), alemtuzumab (Mabcampath, n=6) or natalizumab (n=10, Tysabri). Healthy controls and treated MS patients were age and gender matched. This study was approved by the local Medical Ethical Committee and informed consent was obtained from all study subjects. Details of our study population are depicted in table 1.

Cell lines, isolation, and culture

Human and mouse peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-paque (GE Healthcare Life Sciences, Buckinghamshire, UK) and histopaque (Sigma-Aldrich) density gradient centrifugation, respectively. RAW 264.7 cells were cultured in RPMI 1640 medium (Lonza) enriched with 10% FCS (Hyclone, Erenbodegen, Belgium), 50 U/ml penicillin, and 50 U/ml streptomycin (P/S, Invitrogen, Merelbeke, Belgium). RAW 264.7 cells were exposed to 100 ng/ml LPS (Sigma-Aldrich), 500 U/ml interferon γ (IFNγ,

**Immunohistochemistry**

Brain tissue samples were obtained from the Netherlands Brain Bank (Amsterdam, The Netherlands). Details of frozen MS brain tissue are depicted in table 2. Animals were transcardially perfused with ringer solution containing heparin, after which spinal cord (cervical and thoracic region) and spleen tissue was isolated and snap-frozen in liquid nitrogen. Immunohistochemical stainings were performed as described previously 22, 23. Antibodies used; mouse anti-human CD169 (Abcam, Cambridge, UK), rat anti-mouse CD169 (MOMA-1, Abd Serotec), rabbit anti-Iba1 (Wako, Neuss, Germany), rabbit anti-CD11b/c (Novus, Cambridge, UK) rabbit anti-PLP (Abcam), goat anti-mouse Alexa 555, goat anti-rat Alexa 555, swine anti-rabbit biotin, and/or streptavidin Alexa 488 (Invitrogen).

**Flow cytometry**

For flow-cytometric analysis of PBMCs, the following antibodies were used; rat-anti-mouse CD169 FITC (Abd Serotec), rat-anti mouse CD14 PE (eBioscience, San Diego, CA), mouse anti-human CD14 PerCP (BD Biosciences, Erembodegem, Belgium), mouse anti-human CD56 PE-Cy7 (BD biosciences), mouse anti-human CD169 FITC (Abd Serotec), and mouse anti-human CD7 PE (BD biosciences). The following isotype controls were used to establish proper gating strategies; rat IgG2a FITC (AbD Serotec), rat IgG2b PE (eBioscience), mouse IgG2b PerCP (Biolegend, London UK), mouse IgG1κ PE-Cy7 (BD biosciences), Mouse IgG1 FITC (Abd Serotec), and Mouse IgG1 PE (BD Biosciences).
Statistics

Data were analyzed using GraphPad Prism and are reported as mean ± SEM. An ANOVA (post-hoc; Tukey) or two-tailed unpaired Student t-test was used for normally distributed data sets. The Kruskal-Wallis (Dunns post hoc comparison) or Mann-Whitney analysis was used for data sets which did not pass normality. An overall effect of treatment in the EAE model was assessed by measuring the AUC. *P < 0.05, **P < 0.01 and ***P < 0.001.
Results

**CD169**+ phagocytes are increased in the circulation and CNS of EAE animals

An increasing amount of studies indicate that CD169 is a marker for activated phagocytes in inflammatory disorders. Here, we defined the expression of CD169 on phagocytes within the CNS and circulation of EAE-affected animals. Our data indicate that CD169 is highly expressed by Iba-1+ and CD11b/c+ macrophages and microglia within spinal cord lesions of EAE-affected mice (Figure 1a-b). Occasionally, perilesional cells showing a clear microglial morphology expressed CD169 (Figure 1c). Quantitative analysis showed that CD169 was exclusively expressed by Iba1+ and CD11b/c+ cells (data not shown), and that amount of CD169+Iba1+ and CD169+CD11b/c+ phagocytes in spinal cord increased substantially upon EAE induction (Figure 1d). While in control mice CD169 was barely expressed by Iba1+ microglia in the CNS, approximately 50% of the Iba1+ phagocytes expressed CD169 at the acute and chronic phase of EAE (Figure 1e). Within the circulation, both the percentage of CD14+ monocytes that expressed CD169 and the level of CD169 expression on these cells were increased in the acute and chronic phase of EAE (Figure 1f-g). No difference in the percentage of CD14+CD169+ monocytes as well CD169 expression on these cells was observed between the acute and chronic phase of EAE. Similar to a previous study, we found that type I IFNs (IFNα and IFNβ) markedly increased the percentage of CD169-expressing phagocytes *in vitro* (Supplementary figure 1). LPS and IFNγ also increased the number of CD169-expressing cells, albeit to a lesser extent than IFNα and IFNβ (Supplementary figure 1). Collectively, these findings indicate that CD169 is abundantly expressed by circulating, CNS-resident, and infiltrated phagocytes in EAE animals.
Transient depletion of CD169-expressing cells reduces EAE severity

By using CD169-DTR mice, we next determined the impact of depletion of CD169+ cells on EAE disease severity. The CD169-DTR model allows for transient and selective depletion of CD169+ cells upon DT administration, thereby excluding the involvement of developmental abnormalities on EAE disease outcome. To determine the efficacy of CD169 depletion and the time it takes for CD169 cells to repopulate upon depletion, healthy CD169-DTR mice and WT mice were sacrificed 2 or 8 days after DT administration. Two days post DT administration, spleens of CD169-DTR mice were completely deficient of CD169+ marginal zone macrophages (Figure 2a). A partial repopulation of CD169-expressing macrophages was observed 8 days post DT treatment (Figure 2a). Importantly, five days post DT administration, the CNS of EAE-affected animals was devoid of CD169+ phagocytes (Figure 2b). Hence, to define the impact of transient CD169 cell depletion on EAE disease severity, EAE-affected mice were treated every other 5 days with DT starting 5 days post-immunization. Diphtheria toxin treated CD169-DTR mice showed a delayed disease onset and a significantly reduced accumulative disease severity as compared to PBS treated CD169-DTR mice (Figure 2c-e). Notably, whilst DT treatment efficiently depleted CD169+ cells within the CNS of EAE-affected CD169-DTR mice in the chronic phase of EAE (Figure 2b), no difference in EAE disease severity was observed during this phase anymore. Collectively, these data indicate that CD169+ cells have a key role in driving EAE progression, in particular during the initial disease stages.

CD169-expressing monocytes cells are increased in the circulation of MS patients

Next, we sought to determine whether the number of CD169+ circulating monocytes, as well as CD169 abundance on these cells, is altered in MS patients compared to healthy controls. Similar to EAE animals, we found that the percentage of CD14+ monocytes expressing CD169 was increased in MS patients compared to healthy controls (Figure 3a). To determine
whether MS therapies changed the percentage of monocytes expressing CD169, MS patients were subdivided according to their treatment regime. None of the treatments (interferon beta, natalizumab, or alemtuzumab) affected the percentage of CD14+ cells expressing CD169 (Figure 3a). Similar, no differences were observed when MS patients were subdivided based on disease type (data not shown). Interestingly, while in healthy controls the percentage of CD14+ monocytes expressing CD169 positively correlated with age ($r^2 = 0.47$, $p = 0.007$), no signification correlation was observed in MS patients (data not shown). In addition, we show that CD14+CD169+ monocytes of MS patients have an increased expression (MFI) of CD169 compared to CD14+CD169+ monocytes of healthy controls (Figure 3b). Again, none of the treatments and MS disease types affected CD169 expression on CD14+ monocytes of MS patients (Figure 3b and data not shown). No significant correlation was found between CD169 abundance and age in both healthy controls and MS patients (data not shown).

CD169 is expressed on a variety of innate immune cells. Therefore, CD169 expression by CD56+CD7- monocyte/DC-like cells and CD56+CD7+ NK cells was assessed. We observed that the percentage of monocyte/DC-like cells and NK cells that expressed CD169, as well as the expression levels of CD169 on these cells, was increased in MS patients compared to healthy controls (Supplementary figure 2). Alemtuzumab and IFNβ treated MS patients showed a trend towards a decreased percentage of NK cells expressing CD169 (Supplementary figure 2c). MS patients that were treated with alemtuzumab demonstrated a decreased CD169 expression on NK cells (Supplementary figure 2d). None of the treatments significantly affected the percentage of monocyte/DC-like cells that expressed CD169 and CD169 abundance on these cells (Supplementary figure 2a-b).

**CD169 is a marker for activated phagocytes in MS lesions**

Our results in EAE indicate that roughly half of the Iba-1+ phagocytes in the CNS of EAE animals express CD169. Here, we set out to define the relative distribution of CD169+ cells
within brain tissue of MS patients and non-neurological controls. In active and chronic active MS lesions, we found a substantial increase in the expression of CD169 compared to normal-appearing white matter (NAWM) of non-neurological controls, in which CD169 was solely expressed by perivascular macrophages (Figure 4a-c). A gradient of CD169 immunoreactivity was observed beginning perilesional and increasing towards the center of active and rim of chronic active MS lesions (Figure 4a-b). Higher magnification images revealed that CD169+ cells in perilesional areas displayed a microglial morphology (Figure 4d). Within active MS lesions, both the parenchyma and perivascular cuffs were packed with CD169-expressing cells resembling foamy macrophages and microglia (Figure 4e-4f). In chronic active MS lesions, CD169 was primarily expressed by cells in the lesion rim (Figure 4g) and only sporadically observed in the hypocellular demyelinated lesion center (Figure 4h). Double-staining showed that CD169 was primarily expressed by myelin (PLP)-containing Iba-1+ phagocytes within active MS lesions (Figure 5a and 5b). Quantification confirmed the increase in CD169+ phagocytes in active MS lesions as compared to NAWM of MS patients and non-demented controls (Figure 5c). In summary, we found a marked increase of CD169+ phagocytes in (chronic) active MS lesions. Strikingly, perilesional microglia also abundantly expressed CD169.
Discussion

In this study, we provide indications that CD169 is a marker for highly pathogenic phagocytes in MS lesions. We show that CD169 is abundantly expressed by perilesional microglia, suggesting that it is a selective marker for early activated microglia. Likewise, CD169 is highly expressed by lesional and circulating phagocytes in MS patients and EAE animals. Transient depletion of CD169+ cells markedly reduces early clinical symptoms in an experimental MS animal model. Collectively, our data strongly suggest that CD169+ phagocytes cells contribute to neuroinflammation and that specific targeting of these cells holds therapeutic value.

Our finding that phagocytes in the CNS and circulation of MS patients abundantly express CD169 is in line with other studies showing an increased expression of CD169 in inflammatory and autoimmune disorders. Even more, a recent study reported that circulating monocytes highly express CD169 in MS patients and that CD169 expression on these monocytes correlates with disease progression ⁹. We confirm the former finding by showing that CD169-expressing CD14+ monocytes and CD56+CD7+ monocyte/DC-like cells are increased in the circulation of MS patients. We further demonstrate that perivascular phagocytes highly express CD169 in both control brain tissue and MS lesions. Perivascular macrophages sustain neuroinflammation in MS by presenting CNS-derived antigens to infiltrating autoreactive T cells ²⁷. CD169 may represent a key receptor in this disease-promoting process as it tightly controls leukocyte interactions and antigen presentation ¹⁵-¹⁸. Interestingly, vascular resident CD169+ macrophages decrease the expression of intracellular adhesion molecule-1 (ICAM-1) on vascular endothelial cells ¹⁶. Perivascular macrophages might well utilize the same mechanism to prohibit the entrance of leukocytes into the healthy CNS. However, the latter mechanism is unlikely to play a significant role in our EAE experiment, as one would expect that an increased expression of ICAM-1 in mice lacking
CD169+ macrophages results in more leukocyte infiltration into the CNS and an increased EAE disease severity.

Clusters of activated microglia are scattered throughout the NAWM of MS patients, frequently in close proximity to active MS lesions. Here, we found that CD169 is a marker for early activated perilesional microglia in MS and EAE. This finding suggests that CD169 expression can be induced locally within the CNS during neuroinflammation and that CD169+ phagocytes in MS lesions do not originate from infiltrated CD169+ monocytes per se. Noteworthy, a previous study found that the expression of CD169 by microglia matches the temporal and spatial distribution of plasma leakage into the brain parenchyma after CNS injury. With respect to the latter, extravascular serum proteins are observed in NAWM of MS patients. Our data also indicate that type I interferons, LPS, and IFNγ increase the expression of CD169 on phagocytes \textit{in vitro}. IFNγ is a major cytokine found in the circulation and CNS of MS patients and serum levels of IFNβ are increased in a subset of MS patients. Hence, it is likely that IFNγ and type I IFNs, amongst other cytokines, control CD169 expression on CNS-resident and circulating phagocytes in MS patients. However, despite the capacity of IFNβ to increase CD169 expression on phagocytes \textit{in vitro}, IFNβ treated patients in our study population did not show an increased CD169 expression on circulating monocytes compared to untreated MS patients. Based on our findings, maximum CD169 expression levels may already have been induced by sustained exposure to inflammatory mediators, such as IFNγ and type I interferons, as witnessed by increased CD169 levels in untreated MS patients compared to healthy controls. Alternatively, differences between acute \textit{in vitro} and chronic \textit{in vivo} treatment might be important determinants of the latter finding. Of note, increased systemic inflammation in elderly might also explain the observed positive correlation between age and CD169 expression on monocytes in healthy controls. The lack of this correlation in MS.
patients is likely due to the high inflammatory burden in these individuals, leading to a plateau in CD169 expression.

We show that CD169+ phagocytes are increased in the circulation and CNS of EAE-affected animals and that temporal depletion of CD169+ cells markedly reduces severity of clinical symptoms in the initial stages of EAE disease. These findings indicate that targeting CD169 is an effective strategy to suppress neuroinflammation. Our findings are in line with a previous study showing that total CD169 knockout mice display a reduced EAE severity during the initial disease stages of EAE 15. However, the temporal depletion approach we used excludes that potential developmental abnormalities due the embryonic CD169 deficiency impact EAE disease severity. Future research is needed to further define the underlying molecular and cellular mechanisms that govern the reduced EAE disease severity upon CD169+ cell depletion.

Previous studies reported that several members of the siglec family are expressed on NK cells, e.g. Siglec-3 33 and Siglec-7 34. In this study, we show that CD169 (Siglec-1) is also expressed on CD56+CD7+ NK cells and that the percentage of these cells, as well as CD169 abundance on these cells, is increased in MS patients. In general, ligation of Siglecs attenuates the activation of NK cells 33, 34. More research is warranted to define whether ligation of CD169 has a similar inhibitory impact on NK cell function. Interestingly, an increasing amount of studies indicate that NK cells are implicated in MS pathology 35. Therefore, we cannot exclude that depletion of CD169-expressing CD56+CD7+ NK cells partially accounts for the reduced disease severity in our experiments.

In summary, findings in this report suggest that specific targeting of CD169+ cells might hold therapeutic value for early targeting of neuroinflammation. Considering its expression by early activated microglia, our findings further argue for CD169 as being a marker with diagnostic value.
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Competing interests
The other authors declare that they have no competing interests.

Authors’ contributions
JB and EB performed the experiments, analyzed the data and wrote the manuscript. EL, DE, and JvH provided experimental materials. EL, PD, DE, JvH, JH, and NH revised the manuscript. JvH, JH, and NH participated in the design and coordination of the project.
References


Figure legends

Figure 1: CD169–expressing phagocytes are increased in the circulation and CNS of EAE animals. (a,b) Spinal cord tissue of EAE animals (20 dpi) stained for CD169 and Iba-1 (a) or CD169 and CD11b/c (b) (both 20x magnification). (c) Spinal cord tissue of EAE animals (30 dpi) stained for CD169 and Iba1 (40x magnification). (d,e) Quantification of the amount of CD169+ cells per image (d) and the percentage of Iba1+ cells expressing CD169 (e) in spinal cord tissue of control and EAE mice (acute (20dpi), and chronic phase (30 dpi)). For quantification, 20 images per animal with 9 animals per time point were used (20x magnification). (f,g) PBMC isolated from control (n=7) and EAE mice (acute phase (20 dpi), n=6; chronic phase (30 dpi), n=9) were stained for CD14 and CD169. Flow cytometry was used to assess the % of CD14+ cells expressing CD169 (f) and the abundance of CD169 on these cells (g). Data are presented as mean ± SEM.

Figure 2: Transient depletion of CD169-expressing cells reduces EAE severity. (a) CD169-DTR mice and WT mice were sacrificed 2 or 8 days after intraperitoneal DTR administration. Splenic sections were stained with CD169 and dapi (20x magnification). One representative image is shown. (b) Spinal cord tissue of PBS and DT-treated CD169-DTR EAE animals (30 dpi) stained for CD169 and CD11b/c (20x magnification). EAE animals were treated with PBS or DT every other 5 days, starting 5 dpi. Images shows CD169 immunoreactivity on phagocytes 5 days post the last DT treatment (c) MOG-immunized CD169-DTR animals were treated every other 5 days (arrows) starting 5 dpi with PBS (n=4; black) or diphertheria toxin (DT, n=8). Neurological score and weight was assessed daily. Data represent the mean ± SEM. (d,e). The cumulative disease index (d) and disease onset (e) of immunized CD169-DTR mice treated with PBS or DT. Data represent the mean ± SEM.
Figure 3: The number of circulating CD169+ phagocytes is increased in MS patients. (a,b) PBMCs isolated from MS patients (n=57) and healthy controls (n=19) were stained for CD14 and CD169. Flow cytometry was used to assess the percentage of CD14+ cells expressing CD169 (a) and the abundance of CD169 on these cells (b). MS patients were subdivided based on their treatment regime; no treatment (n=19), natalizumab (n=10), beta-interferon (n=22), and Alemtuzumab (n=6). Data are presented as mean ± SEM.

Figure 4: CD169 is highly expressed in MS brain tissue. (a-g) CNS tissue of non-neurological controls (white matter) and (chronic) active MS lesions were stained for CD169. (a,b) Overview images of an active (a) and chronic active (b) MS lesion (2.5x magnification). (c) NAWM of non-demented control stained for CD169 (40x magnification). (d-h) Representative images of perilesional area of active MS lesion (d), lesion center of active MS lesion (e), perivascular cuff of active MS lesion (f), active rim of chronic active MS lesion (g), and inactive center of chronic active MS lesion (h, all 40x magnification).

Figure 5: CD169 is highly expressed on myelin-containing phagocytes in MS lesions. (a) Fluorescent staining of active MS lesion (green, Iba1; red; CD169; 20x magnification). (b) Fluorescent staining of active MS lesion (green, PLP; red; CD169; 20x magnification). (c) Quantification of the percentage of Iba1+ cells expressing CD169 in active MS lesions. For quantification, 10 images per tissue or MS lesions were used (20x magnification). Data are presented as mean ± SEM.

Supplementary figure 1: Inflammatory stimuli increase the expression of CD169 on macrophages. RAW264.7 cells were stimulated with LPS (100 ng/ml, a), IFNα (500 U/ml, a), IFNβ (500 U/ml, a), or IFNγ (500 U/ml, a) for 24 or 48 hours. Flow cytometry was used to define the expression of CD169. Data are presented as mean ± SEM.
Supplementary figure 2: The number of circulating CD56+CD7− monocyte/DC-like cells and CD56+CD7+ NK cells expressing CD169 is increased in MS patients. (a,d) PBMCs isolated from MS patients (n=59) and healthy controls (n=19) were stained for CD56, CD7, and CD169. Flow cytometry was used to assess the percentage of CD56+CD7− monocyte/DC-like cells and CD56+CD7+ NK cells expressing CD169 (a,c), and the abundance of CD169 on these cells (b,c). PBMC of MS patients were subdivided based on their treatment regime; no treatment (n=19), natalizumab (n=10), beta-interferon (n=22), and Alemtuzumab (n=6). Data are presented as mean ± SEM.
Figure 1
Figure 2
Figure 3
Figure S1
Figure S2