Platelets Contribute to the Pathogenesis of Experimental Autoimmune Encephalomyelitis


Rationale: Multiple sclerosis (MS) and its mouse model, experimental autoimmune encephalomyelitis (EAE), are autoimmune disorders of the central nervous system (CNS). The function of platelets in inflammatory and autoimmune pathologies is thus far poorly defined.

Objective: We addressed the role of platelets in mediating CNS inflammation in EAE.

Methods and Results: We found that platelets were present in human MS lesions as well as in the CNS of mice subjected to EAE but not in the CNS from control nondiseased mice. Platelet depletion at the effector-inflammatory phase of EAE in mice resulted in significantly ameliorated disease development and progression. EAE suppression on platelet depletion was associated with reduced recruitment of leukocytes to the inflamed CNS, as assessed by intravital microscopy, and with a blunted inflammatory response. The platelet-specific receptor glycoprotein Ibα (GPIbα) promotes both platelet adhesion and inflammatory actions of platelets and targeting of GPIbα attenuated EAE in mice. Moreover, targeting another platelet adhesion receptor, glycoprotein IIb/IIIa (GPIIb/IIIa), also reduced EAE severity in mice.

Conclusions: Platelets contribute to the pathogenesis of EAE by promoting CNS inflammation. Targeting platelets may therefore represent an important new therapeutic approach for MS treatment. (Circ Res. 2012;110:1202-1210.)

Key Words: platelets ■ experimental autoimmune encephalomyelitis ■ vascular inflammation ■ autoimmune disease

Multiple sclerosis (MS) is an inflammatory degenerative disease of the central nervous system (CNS).1–5 MS and its mouse counterpart, experimental autoimmune encephalomyelitis (EAE), are initiated by infiltration of the neuronal tissue by T-cells autoreactive to antigens of the myelin sheath.6–9 The subsequent breakdown of the blood-brain barrier (BBB) facilitates the recruitment of further inflammatory effector cells such as mononuclear cells and macrophages10 and the activation of resident inflammatory microglial cells that contribute to the development of CNS lesions.11 Inhibiting the inflammatory response is a promising therapeutic approach in EAE and MS.1,9 For instance, leukocyte integrins or their endothelial counterreceptors that contribute to inflammatory cell recruitment represent therapeutic targets in EAE and MS.9,12,13

Editorial, see p 1157

Besides their well-established role in hemostasis and thrombosis, platelets contribute to inflammatory processes. On inflammatory stimulation, platelets rapidly adhere to the endothelium or to the subendothelial extracellular matrix at
sites of vascular endothelial injury.14–17 Several platelet adhesion receptors, such as the glycoprotein (GP) VI, the integrin GPIIb/IIIa (also designated as αIIbβ3-integrin or CD41/CD61-integrin; CD indicates cluster of differentiation), or the GP Ib/IX/V complex, mediate platelet adhesion or aggregation.18 Platelet-derived chemokines and cytokines can trigger inflammation in a paracrine fashion, whereas the direct adhesive interactions of platelets with endothelial cells and inflammatory cells promote leukocyte recruitment to the inflamed tissue. Distinct platelet adhesion receptors have been implicated in these processes, including P-selectin, junctional adhesion molecule-C, or GPIb. The latter receptor is a component of the GP Ib/IX/V complex that acts as the von Willebrand Factor (vWF) receptor mediating platelet adhesion to the inflamed endothelial surface and the subendothelial matrix, whereas the interaction of GP Ib with the leukocyte integrin Mac-1 promotes leukocyte/platelet interactions and thereby inflammatory cell recruitment. Platelets are thus recognized as an important link between hemostasis and inflammation.14,15,19–27

A recent microarray approach analyzing differentially expressed genes in plaques from patients with MS revealed an upregulation of the message of the platelet-specific GPIIb in patients with chronic demyelinating disease.28 Furthermore, a recent report demonstrated increased activation of platelets in the peripheral blood from patients with MS.29 These observations prompted us to investigate whether platelets could play a role in the pathogenesis of EAE. The present work demonstrates for the first time that platelets constitute a substantial component of the inflammatory response in the course of EAE by promoting the inflammatory response in the CNS. Targeting platelets or their interactions with inflammatory cells may thus represent a novel therapeutic approach to ameliorate inflammatory lesions in MS.

Methods


Results

Platelets Are Present in the CNS of Mice After Induction of EAE and in Human MS Lesions

To address a potential contribution of platelets to the inflammatory lesions of EAE, we first assessed for the presence of platelets in the CNS after EAE induction. EAE was induced in mice using myelin oligodendrocyte glycoprotein (MOG). At different time points after EAE induction, we analyzed the inflamed spinal cords for the presence of platelets as assayed by the detection of the platelet-specific CD41 (GPIIb) and GP Ib (CD42b). Before euthanizing the mice and extracting the spinal cords, mice were perfused to efficiently remove circulating blood including circulating platelets, thus allowing for the assessment of only tissue-associated platelets or platelets permanently adherent on the endothelium of the inflamed CNS vessels. First, as compared with noninflamed spinal cords, we found elevated levels of CD41 mRNA and GP Ib mRNA in the inflamed spinal cords of mice on day 21 after EAE induction, representing the effector phase of the disease (Figure 1A and 1B). These results are in line with a previous report that demonstrated an upregulation of CD41 mRNA in chronic human MS lesions.28 Second, using immunohistochemistry with an antibody for the platelet-specific
GPIb, we could visualize platelets in inflamed spinal cords, in parts localizing in inflamed areas containing activated microglial cells, which were stained by the marker Iba-1 (Figure 1C and 1D). Furthermore, the presence of platelets in inflamed spinal cords was assessed by Western blot analysis for platelet specific GPIIb. Platelets were detectable at the effector phase of the disease (ie, after clinical disease onset), whereas no platelets were found in noninflamed spinal cord tissues (Online Figure I). In addition to inflamed spinal cords, platelets were detectable in inflamed brain sections of mice subjected to EAE (Online Figure I).

Moreover, we assessed for the presence of platelets in lesions from human MS patients. Four cases of patients with MS were analyzed. All cases displayed active demyelination plaques including perivascular chronic inflammatory infiltrates composed of lymphocytes and plasma cells, macrophage infiltration, myelin breakdown with relative preservation of axons, and gliosis. Positive platelet staining with anti-human CD42b was observed in vessels and capillaries, in areas of hemorrhage, and in the extracellular space, which could represent potentially extravasated platelets. Normal adjacent brain areas as well as sections from normal human brain tissue displayed minimal to no positive platelet staining. Sections stained using IgG control antibody were also negative (Figure 2). Furthermore, a chronic active plaque from a 54-year-old female patient with clinically definite MS also displayed abundant platelets as assessed by GPIb staining (Online Figure I).

Platelets Contribute to EAE Pathogenesis

Because platelets were present in the inflamed CNS after EAE induction, we then examined their impact on EAE by using pharmacological depletion of platelets. Efficient platelet depletion was achieved with an intraperitoneal injection of rabbit anti-mouse platelet serum (depletion of >97% at 24 hours after injection) (Online Figure II), as previously described. Platelet depletion lasted for at least 72 hours (data not shown). The platelet-depleting serum did not alter the number of circulating total leukocytes (Online Figure II), as previously described. Neither control nor platelet-depleting serum had any immune-stimulatory effects, as assessed by tumor necrosis factor-α and C3 levels in the serum of mice (data not shown).

We performed platelet depletion either in the immunization phase or in the inflammatory effector phase of EAE, when clinical disease is evident. In the former case, mice received an intraperitoneal injection of platelet-depleting serum or control serum at 2 different time points, on days 2 and 6, whereas in the latter case, mice received platelet-depleting serum or control serum on days 12 and 16 after immunization (day 12 represents the average begin of clinical disease in EAE, that is, the effector phase). Whereas platelet depletion during the...
immunization phase of disease did not affect EAE development (Figure 3A), depletion of platelets resulted in a significant and persistent reduction in EAE disease severity 2 days after application of the depleting agent (Figure 3B). Consistent with reduced EAE disease severity on platelet depletion in the effector phase, we found ameliorated loss of axonal integrity, as assessed by axonal staining using the neurofilament-200 (NF-200) as a marker, in mice that received the platelet-depleting serum as compared with mice receiving control serum (Online Figure III). Moreover, reduced demyelination was also found on platelet depletion as analyzed by Luxol fast blue staining of the spinal cords (Online Figure III). Taken together, our findings suggest a role of platelets in the effector phase of EAE, whereas platelets do not interfere with the immunization phase of EAE.

Platelets Contribute to the Inflammatory Response During EAE Pathogenesis

The data that platelet depletion during the effector phase of EAE reduced EAE severity indicated that platelets contribute to inflammation in the course of EAE. Indeed, inflammation in EAE was blunted on platelet depletion on days 12 and 16 after immunization, as evidenced by studying different components of the inflammatory response of EAE. First, activation of resident microglial cells is a marker of the inflammatory response in the course of EAE, and activated microglia contribute to CNS inflammation and myelin sheath lesions.11 Interestingly, we observed a significant reduction of microglia in the inflamed spinal cord on platelet depletion in mice as compared with control mice (Figure 4A and 4B). Second, we performed microarray analysis of the inflamed spinal cords from EAE mice that were treated with control or platelet-depleting serum. Several inflammatory and immune factors and pathways were significantly downregulated in the inflamed spinal cords on platelet depletion. Selected proinflammatory factors such as cytokines, chemokines, or adhesion molecules that were significantly downregulated in the spinal cords on platelet depletion are summarized in Online Table I. We confirmed the downregulation of selected proinflammatory factors (the chemokines CCL-2, CCL-5, the chemokine receptor CXCR-4, the cytokine IL-1β, the macrophage marker CD68, and the adhesion molecule ICAM-1) in the spinal cord on platelet depletion by real-time PCR analysis (Figure 4C).

Third, platelets contributed to inflammatory cell recruitment in the course of EAE. We observed decreased accumulation of inflammatory cells to the inflamed CNS on platelet depletion. In particular, mice treated with the platelet-depleting or the control serum on days 12 and 16 after immunization were euthanized on day 21. Immediately before euthanasia, mice were perfused with saline to remove circulating cells, the inflamed spinal cords were extracted, leukocytes were isolated, and their total number was counted. Flow cytometry analysis of the leukocyte populations isolated from inflamed spinal cords was performed for CD45 and CD11b, and we assessed the numbers of CD45highCD11b+ cells representing recruited monocytes/macrophages.34–36 On platelet depletion, a significant decrease in the numbers of CD45highCD11b+ cells in the inflamed spinal cord after EAE induction was observed (Figure 5A). In addition, we studied the recruitment of different leukocyte subpopulations into the inflamed spinal cord of mice that received platelet-depleting or control serum by performing quantitative PCR analysis for the mRNA of CD4, CD8, and CD11b. On platelet depletion, the mRNA levels of CD4, CD8, and CD11b were reduced as compared with mice receiving control serum, implying reduced recruitment of the respective cells to the inflamed spinal cord (Online Figure IV). Moreover, the level of granulocytes was significantly reduced after platelet depletion, as assessed by ELISA for myeloperoxidase (Online Figure IV). That platelet depletion expectedly resulted in reduced platelet accumulation in the inflamed CNS was demonstrated by the marked decrease in platelet-specific GPIb mRNA in the inflamed CNS of mice receiving the platelet-depleting serum as compared with the control serum (data not shown).

To substantiate the impact of platelets on leukocyte recruitment to the inflamed CNS in vivo, we induced EAE in mice and assessed the effect of platelet depletion on leukocyte recruitment to the CNS by spinning disc intravital microscopy.15,37 Fourteen days after EAE immunization mice were treated without or with the platelet-depleting reagent and 24 hours thereafter, leukocyte recruitment to the inflamed CNS was assessed by intravital microscopy. A 70% and 50% reduction in the number of rolling leukocytes and of firmly
adherent leukocytes, respectively, was observed in the vessels of the inflamed CNS of thrombocytopenic mice, as compared with control mice (Figure 5B through 5D and Online Videos I and II). In the absence of EAE disease, there was virtually no leukocyte adhesion to the vessel wall independent of whether or not platelets were depleted (Online Table II).

Together, these findings suggest that platelets substantially contribute to EAE disease severity in mice by promoting the inflammatory response in the course of EAE, through promoting leukocyte recruitment to the inflamed spinal cord, and the upregulation of multiple inflammatory cytokines, chemokines, and adhesion molecules.

**Targeting Platelet GPIbα Ameliorates EAE in Mice**

Our findings thus far demonstrated that platelets are present in the inflamed CNS tissue after EAE induction and contribute to disease severity by promoting the inflammatory response, including inflammatory cell recruitment to the inflamed CNS. We therefore addressed next whether targeting specific platelet receptors could represent a therapeutic approach in EAE. Platelet GPIbα is a component of the GPIb/IX/V complex that interacts with vWF-mediating platelet interactions with the vessel wall and thereby platelet recruitment, and contributes to inflammatory actions of platelets in part due to its propensity to bind to leukocyte integrin Mac-1 and promote leukocyte/platelet adhesion and leukocyte recruitment. We therefore engaged reagents blocking GPIbα in EAE. First, we used a blocking Fab to GPIbα that is capable of blocking platelet–vessel wall interactions and thus platelet accumulation. To evaluate the therapeutic potential of GPIbα blockade in EAE, we treated mice with the blocking Fab to platelet GPIbα at the beginning of the effector phase of the disease, on days 12, 14, and 16, and found a prolonged amelioration of EAE (Figure 6A). In the human disease setting, treatment is given after diagnosis of the disease. To mimic this situation, we also treated mice with the Fab to GPIbα after disease onset, resulting in a transient but significant clinical recovery from EAE, as compared with Fab control treatment (Figure 6B).

Second, we engaged the antibody anti-M2 that was raised against the binding site of Mac-1 for GPIbα and specifically blocks this interaction and thereby platelet-dependent inflammatory cell recruitment. Anti-M2 antibody blocked the adhesion of primary bone marrow–derived macrophages to immobilized surface-adherent platelets in vitro (Online Figure V), without affecting further Mac-1–dependent adhesive interactions, for example, to fibrinogen or ICAM-1.

![Image](http://circres.ahajournals.org/DownloadedFrom/hftp/ncres.ahajournals.org/)

**Figure 4. Platelet depletion reduces the inflammatory response during EAE.** To analyze the influence of platelet depletion on the inflammatory response in EAE, the disease was induced in wild-type mice; on days 12 and 16 after induction mice were treated with control serum (control) or with platelet-depleting serum. A and B, On day 21, staining for microglia cells using the antibody to the marker Iba-1 was performed. The area of microgliosis was reduced in spinal cords on platelet depletion. Representative images showing microgliosis areas are depicted. B, The area of microgliosis was analyzed by morphometry. Six spinal cords per group and 3 nonconsecutive sections per spinal cord were analyzed. Data are mean±SEM. *P<0.05. C, Spinal cords from control treated or platelet-depleted mice were isolated on day 21 after induction of EAE. Real-time PCR analysis for the mRNA levels of the chemokines CCL-2, CCL-5, and CCL-19, the chemokine receptor CXCR-4, the cytokine IL-1β, the macrophage marker CD68, and the adhesion molecule ICAM-1 was performed. Data are mean±SEM (n=5 mice per group) and are shown as percent of control. The mRNA expression of the respective molecule in spinal cords from control-treated mice represents the 100% control. *P<0.05 as compared with control serum-treated animals.
Online Figure V), as previously described. Treatment with antibody anti-M2 on days 12, 14, and 16 resulted in a significant and prolonged reduction of clinical EAE symptoms as compared with control antibody (Figure 6C). When the blocking Fab to GPIb\n/H9251 was used in combination with the antibody anti-M2, no additive inhibitory effect was observed, indicating that both reagents interfere with the same pathway of platelet-mediated inflammation in EAE (Online Figure VI).

To assess whether the function of platelets in EAE can be solely attributed to GPIb or the GPIb-Mac-1 interaction molecularly, we engaged an inhibitor of the major platelet adhesive receptor GPIIb\nIIIa. Blocking Fab to GPIIb\nIIIa provided a significant reduction in EAE disease severity (Figure 7). Together, these data demonstrate that targeting platelet GPIb or other platelet receptors, such as GPIIb\nIIIa, could represent a specific therapeutic strategy for EAE treatment.

Discussion
The inflammatory response is a major component of MS pathogenesis and thus an important therapeutic target. We demonstrated unequivocally that platelets contribute substantially to the inflammatory response and pathogenesis of EAE and provide clear evidence that targeting platelets is a novel therapeutic strategy in EAE and thereby potentially in human MS.

Previous reports have shown the message of the platelet-specific receptor CD41 (\n/H9251 IIb-integrin, GPIIb) in chronic lesions of patients with multiple sclerosis, the presence of platelets in murine neuroinflammation, as well as the relevance of platelet-derived interleukin-1\n for cerebrovascular inflammation. A recent study has demonstrated increased levels of platelet activation in the peripheral blood of MS patients. Consistently, we were able to demonstrate that platelets are present in the inflamed vessels and the inflamed parenchyma of the CNS during EAE in mice; in addition, platelets were detected by immunohistology in human chronic active MS lesions.

EAE is a chronic inflammatory disease of the nervous system associated with destruction of myelin sheaths and axonal damage. The myelin sheath damage is mediated by the inflammatory response, including recruited inflammatory cells, a process facilitated by the disruption of the BBB. Recruited monocytes/macrophages participate in the phagocytosis of myelin and secrete proinflammatory cytokines. Our experiments using platelet depletion in the course of EAE demonstrated unequivocally that platelets contribute substantially to the inflammatory cell recruitment and thereby to the exacerbation of EAE disease severity. Platelet depletion
suppressed EAE when applied in the effector phase of the disease, whereas platelet depletion during the immunization phase did not influence EAE disease induction. These data point to a role of platelets in the inflammatory phase of EAE and in EAE disease exacerbation rather than in EAE disease induction. Platelets may aggravate inflammation in EAE because they are capable of enhancing monocyte adhesion to endothelial cells. Alternatively, platelets could locally secrete proinflammatory mediators, activating recruited inflammatory cells or resident microglia. As pointed out by our mRNA microarray analysis of inflamed spinal cords, the inflammatory response was blunted in the absence of platelets. Different proinflammatory factors including chemokines, cytokines, and endothelial adhesion molecules were reduced in the spinal cords of mice that received the platelet-depleting serum. It is likely that these different pathways are interconnected with each other, which merits further investigation.

We also demonstrated that the platelet adhesion receptor GPIbα may represent a therapeutic target in EAE and MS. Platelet GPIbα mediates both platelet–vessel wall interactions and platelet accumulation as well as platelet-dependent leukocyte recruitment. Blocking Fab to GPIbα as well as an antibody that specifically interferes with the interaction of GPIbα with leukocyte Mac-1 suppressed EAE in mice. This is in accordance with previous findings that blocking antibodies to Mac-1 ameliorated clinical severity of EAE in mice without affecting the number of sick animals, whereas EAE development was reduced in Mac-1−/− mice. However, the inhibitory effect on EAE mediated by blocking platelet GPIbα is not restricted to the GPIbα/Mac-1 interaction. Presumably, alternative GPIb-mediated pathways, such as platelet adhesion to vWF, may participate in the function of platelets in EAE, which should be addressed in future studies. Moreover, blockade of another platelet adhesion receptor, GPIIIbIIa, also reduced EAE disease severity. Thus, the contribution of platelets to EAE is probably multifaceted. We think that strategies to interfere with platelet accumulation and platelet-leukocyte interactions, for example, by targeting GPIbα, GPIIIbIIa, as shown here, or other platelet adhesion receptor systems, such as the interaction between P-selectin and its ligand on leukocytes, P-selectin glycoprotein ligand-1, might represent therapeutic approaches in EAE and MS.

Besides the engaged model for primary progressive EAE disease (in the present study), which reflects a disease subtype of human MS, there is also a relapsing-remitting mouse model, representing a more common MS subtype.
remitting EAE in future investigations to further substantiate the therapeutic potential of platelet-targeting therapies in MS.

Finally, a recent study demonstrated the crosstalk between coagulation and brain inflammation. In this study, a proteomic approach identified tissue factor and protein C inhibitor within chronic active lesions, and thrombin inhibition was used to reduce EAE severity. Our present data underline this connection between coagulation, platelets, and neutrophil activation and suggest that targeting platelets and their receptors may represent a novel attractive therapeutic approach to ameliorate the inflammatory response in MS.

Acknowledgments

We thank the cooperative Human Tissue Network (CHTN) of the University of Pennsylvania for providing us with normal human brain tissue. The authors would like to acknowledge D. Winkler, J. Hammer, and G. Sanchez-Howard for help with mouse work, Dr. F. Boisset for help with immunohistochemistry, and Dr. R. Hodes and Dr. D.S. Singer for critically reading the manuscript.

Sources of Funding

This research was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute (T.C.), and National Institute on Aging (K.G.B.), the National Institutes of Health grants HL57506, HL085816, and HL073852 (D.I.S.), the National Multiple Sclerosis Society grant RG3411B4/1 (J.W.R.), Health grants HL57506, HL085816, and HL073852 (D.I.S.), the National Institutes of Health, National Cancer Institute (T.C.), and the Volkswagen Foundation (Lichtenberg program). The junctional adhesion molecule 3 (JAM-3) on human platelets is a counterreceptor for the leukocyte integrin Mac-1. The junctional adhesion molecule 3 (JAM-3) on human platelets is a counterreceptor for the leukocyte integrin Mac-1.

Disclosures

D.I.S. and Y.W. are coinventors of small-molecule inhibitors targeting the Mac-1/GPIbα interaction.

References

6. Bettelli E, Pagany M, Weiner HL, Lintoning C, F.L.I., is supported by the IZKF program of the University of Tübingen (1868-0-0), the Tuebingen National Multiple Sclerosis Society grant RG3411B4/1 (J.W.R.), and the German Research Foundation and the Novartis Foundation for Therapeutic Research (T.C.).
7. Results of the University of Vienna.
9. Team AE. Platelets in EAE.
11. Langer et al. Platelets in EAE.


Platelets Contribute to the Pathogenesis of Experimental Autoimmune Encephalomyelitis


*Circ Res.* 2012;110:1202-1210; originally published online March 27, 2012; doi: 10.1161/CIRCRESAHA.111.256370

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/110/9/1202

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2012/03/27/CIRCRESAHA.111.256370.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org//subscriptions/
Supplemental Material

Platelets contribute to the pathogenesis of experimental autoimmune encephalomyelitis

Harald F. Langer1,2,*, Eun Young Choi1,3,4,5,*, Hong Zhou6, Rebecca Schleicher2, Kyoung-Jin Chung1,3,4, Zhongshu Tang7, Kerstin Göbel8, Khalil Bdeir9, Antonios Chatzigeorgiou3,4, Connie Wong6, Sumeena Bhatia1, Michael J. Kruhlak1, John W. Rose10, James B. Burns10, Kenneth E. Hill10, Hongchang Qu9, Yongqing Zhang11, Elin Lehrmann11, Kevin G. Becker11, Yunmei Wang12, Daniel I. Simon12, Bernhard Nieswandt13, John D. Lambris9, Xuri Li7, Sven G. Meuth8, Paul Kubes6 and Triantafyllos Chavakis1,3,4

1Experimental Immunology Branch, Center for Cancer Research, National Cancer Institute (NCI), National Institutes of Health (NIH), Bethesda, MD, USA; 2Medizinische Klinik III, Kardiologie und Kreislaufforschung, Eberhard Karls-Universität Tübingen, Tübingen, Germany; 3Division of Vascular Inflammation, Diabetes and Kidney, Department of Internal Medicine III and 4Institute of Physiology, University of Dresden, Germany; 5Department of Medicine, Graduate School, University of Ulsan, Seoul, Republic of Korea; 6Immunology Research Group, Department of Physiology and Biophysics, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada; 7National Eye Institute, NIH, Rockville, MD, USA; 8Neurological Clinic - Inflammatory Disorders of the Nervous system and Neurooncology / Institute of Physiology, University of Münster, Germany; 9Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA, USA; 10Neurovirology Research Laboratory, VASLCHCS, Salt Lake City, UT, USA; 11Gene Expression and Genomics Unit, National Institute on Aging, NIH, Baltimore, MD, USA; 12University Hospitals Harrington-McLaughlin Heart & Vascular Institute and Case Cardiovascular Center, Case Western Reserve University School of Medicine, Cleveland, OH, USA; 13Chair of Vascular
Medicine and Rudolf Virchow Center, DFG Research Center for Experimental Biomedicine, University of Würzburg, Germany

*, contributed equally to the work
Reagents

Recombinant mouse granulocyte-macrophage colony stimulating factor (GM-CSF) was obtained from Endogen (Rockford, IL). The antibody to GPIbα (pOp/B) and rat anti-mouse Fab to GPIbα were generated in our laboratory and previously described.\(^1\) Rat IgG Fab fragment was from Rockland Inc. (Gilbertsville, PA). Alexa488-conjugated anti-rabbit IgG and nuclear dye Hoechst 33342 were from Molecular Probes (Eugene, OR). For induction of EAE in mice we used Myelin oligodendrocyte glycoprotein (MOG\(_{35-55}\)) from American Peptide Inc (Sunnyvale, CA). Incomplete Freund’s adjuvant (IFA) was from SIGMA, \textit{M. Tuberculosis} H37 RA was from DIFCO Laboratories (Detroit, MI) and Pertussis Toxin was from Calbiochem (La Jolla, CA). Platelet depleting serum or control serum from rabbit was used previously\(^2\) and purchased from Accurate Chemical & Scientific Corporation (Westbury, NY). A chicken anti-neurofilament-200 antibody was from Abcam and DAPI was from Invitrogen.

Induction of EAE in mice

EAE experiments were approved by the National Cancer Institute Animal Care and Use Committee. EAE was induced in 8- to 12-week-old female C57BL/6 mice by subcutaneous (s.c.) immunization with 400 \(\mu\)g MOG\(_{35-55}\) in emulsion with incomplete Freund’s adjuvant (IFA) and 5 mg/ml inactivated \textit{M. tuberculosis} as described before.\(^3\)\(^{--}\)\(^5\) Furthermore, the animals received an intraperitoneal (i.p.) injection of 400 ng Pertussis Toxin at day 0 and day 2 after injection of MOG. Mice were assessed and scored daily by an observer blinded to the protocol: 0, no clinical disease; 1, tail
weakness; 2, hind limb weakness; 3, incomplete (one-sided) hindlimb paralysis; 4, complete hind limb paralysis; 5, hind limb paralysis and forelimb paralysis; 6, moribund or dead. Only mice with a score of at least 1 for more than 2 consecutive days were judged to have onset of EAE and were considered. Mice were treated with i.p. injections of Fab to GPIbα or control Fab (75 µg per mouse) on days 12, 14 and 16 or on days 15, 17 and 19 after injection of MOG, or with anti-platelet serum or control serum intraperitoneally (each 0.5ml/kg body weight; from Accurate Chemical and Scientific Corp.) on day 12 and day 16, or with i.v. injections of anti-M2 antibody or control rabbit antibody (each 47 µg/mouse) on days 12, 14 and 16, or with an anti-GPIIbIIIa FAb or control Fab intravenously on day 12 and subsequently intraperitoneally on days 14 and 16.

To study the inflammatory response in the spinal cord tissue on day 21 after induction of EAE, spinal cords were removed after systemic perfusion of mice with PBS to get rid of circulating cells. Spinal cord tissues were grinded and flushed through a nylon mesh (pore size 70 µm). Subsequently, a Percoll gradient centrifugation was performed, cells were counted using a hemacytometer and a single cell suspension of leukocytes in the spinal cord tissue was prepared. Cells were incubated with anti-CD11b and anti-CD45 for 30 min and analyzed by flow cytometry. CD45<sup>high</sup>CD11b<sup>+</sup> cells represent recruited monocytes / macrophages as previously described.<sup>7-9</sup>

For immunofluorescence analysis or immunohistochemistry of spinal cord tissues, extracted spinal cords were fixed with PFA and consecutively incubated in PBS/15% sucrose and PBS/30% sucrose. After blocking with BSA/5% goat serum
and permeabilization using 0.3% Triton X-100, tissue sections were incubated with rabbit anti-mouse Iba-1 (1:250, Wako Chemicals, Richmond) and rat anti-mouse CD42 (1:50, Emfret Analytics, Eibelstadt, Germany) for 2h at 37°C. After washing with PBS, samples were incubated with Alexa488-conjugated anti-rabbit IgG for immunofluorescence analysis of areas of microgliosis for 1.5 h at room temperature. After washing, nuclei were counterstained with Hoechst Dye (1:2000). For immunohistochemistry, after incubation with primary antibodies, samples were incubated with secondary peroxidase or alkaline phosphatase conjugated antibodies and corresponding substrates using staining kits according to the instructions of the manufacturer (Vector laboratories, Burlingame, CA).

For studying axonal integrity, the sections were fixed with 4% PFA for 15 min and washed 3x10 min with PBS. The sections were blocked with PBS supplemented with 5% normal goat serum and 0.5% Triton X-100, followed by incubation with chicken anti-neurofilament-200 antibody (Abcam, 1:10,000) at 4 °C overnight. After 3x10 min wash with PBS, Alexa 488-goat anti-chicken antibody (Invitrogen, 1:500) was applied to the sections and kept for 2 h at 4 °C. After another 3x10 min wash with PBS, the sections were mounted with antifade reagent with DAPI (Invitrogen). The antibodies were diluted in PBS supplemented with 5% normal goat serum and 0.1% Triton X-100. Mosaic images of the whole spinal cord section were captured using an AxioVision system (Zeiss) and the MosaiX function. In addition, spinal cords from EAE mice were removed, placed in paraformaldehyde (Sigma, MO) and staining for myelin (Luxol fast blue) was performed on 8 μm frozen coronal sections (Histoserve, Gaithersburg, MD).
In some experiments, spinal cords were collected on day 21 after EAE induction, protein lysates were prepared and MPO concentration was determined by ELISA according to the manufacturer’s protocol (R and D systems). Moreover, lysates from spinal cords at different time points post EAE induction were analyzed by western blot for platelet specific antigens. To this end, spinal cords were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% Na-deoxycholate, 0.1% SDS, 25 mM NaF, 1 mM sodium orthovanadate, and protease inhibitors) and 20-50 µg of protein was loaded and separated on 4-12% SDS gels (NUPAGE, Invitrogen), followed by 2 h transfer at 4°C. Membranes were blocked with 5% skim milk in TBS-0.05% Tween-20, washed twice with TBS-0.05% Tween-20. Membranes were incubated with primary antibodies at 4°C overnight. The following conditions were used: anti-mouse GPIIb (1:50, BD Biosciences) in 5% skim milk or anti-actin (Sigma). Primary antibodies were washed extensively with TBS-0.05% Tween-20 and membranes were incubated with secondary HRP conjugated antibodies 1:1000 dilution in 5% skim milk for 45 min RT. After washing with TBS-0.05% Tween-20, membranes were developed with ECL (Pierce).

Intravital microscopy after EAE induction

Animals were anesthetized by i.p. injection of a mixture of 10 mg/kg xylazine and 200 mg/kg ketamine hydrochloride. A cranial window was made using a high-speed drill (Fine Science Tools) and the dura mater was removed to expose the underlying cerebral microcirculation. To observe leukocyte rolling and adhesion, tail vein was cannulated and Rhodamine 6G (0.5 mg/kg body weight) was injected i.v.. Leukocyte
endothelial interactions were visualized using BX51W1 spin disk confocal microscope (Olympus, Canada). Three different postcapillary venules with a diameter between 30 and 70 \( \mu m \) were chosen for observation. Throughout the experiment, rectal temperature was continuously monitored and kept at 36\(^\circ\)C to 37\(^\circ\)C with a heating pad (Fine Science Tools). All experiments were recorded for later playback analysis. Rolling leukocytes were defined as those cells moving at a velocity less than that of erythrocytes. Cells were considered adherent if they remained stationary for at least 30 s. For platelet depletion, mice were pretreated intraperitoneally with rabbit anti-mouse thrombocyte serum (0.5 ml/kg body weight, Accurate Chemical and Scientific Corp.). Four hours before intravital microscopy, this procedure depleted over 97% of platelets from the circulation (data not shown).

**Isolation of primary mouse cells**

Bone marrow–derived macrophage isolation and murine platelet isolation were performed as described before.\(^{11-13}\)

**Adhesion assays**

To evaluate the adhesion of primary bone marrow-derived macrophages to platelets, isolated platelets (1x10\(^7\)/well) were allowed to adhere to 96-well plates overnight followed by blocking with BSA (3%). Macrophages (2x10\(^4\) cells/well) were added to the wells and incubated for 40 min. After washing twice with PBS, adherent cells were counted by direct phase contrast microscopy. Adhesion of macrophages to platelets was studied in the absence or presence of anti-M2 antibody specifically
raised against the binding site of Mac-1 to GPIb.\textsuperscript{6} Similarly, adhesion of mouse macrophages was tested to immobilized fibrinogen (10\(\mu\)g/ml, Molecular Probes) or ICAM-1 (10\(\mu\)g/ml, R&D Systems) without or with anti-M2 (10\(\mu\)g/ml) or IgG control (10\(\mu\)g/ml, rabbit IgG, Dako).

**Reverse transcriptase-PCR and real-time RT-PCR.**

Total RNA was isolated from spinal cord tissue as described before.\textsuperscript{12} Briefly, total RNA was prepared using a Trizol based RNA preparation protocol. Reverse transcription of the RNA was performed using ImProm-II reverse transcription system (Promega) and subsequently the RT-generated cDNA samples were subjected to real-time PCR amplification using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) and a QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA). Primers were designed using the Primer Express oligo design software (Applied BioSystems, Foster City, CA), and synthesized by Integrated DNA Technologies (Coralville, IA). The primers used were as follows: actin, 5'-cgtggggccgccttaggcacca-3' and 5'-ttggccttagggttcaggggg-3'; CD41, 5'-tggcttcatccccacaaca-3' and 5'-tttctgaaggactggca-3'; TNF-\(\alpha\), 5'-gggtaggagagcttagtg-3' and 5'-ttggccttagggttcaggggg-3'; IL-1\(\beta\), 5'-cattgtggctgttggaagc-3' and 5'-caactgcactacaggctcc-3'; CXCR4, 5'-tcatctttggggtcagc-3' and 5'-actccatgagcagaggctcc-3'; CCL-2, 5'-gctgaccccaagaaggaatg-3' and 5'-gtgcttgaggtggttggga-3'; CCL-5, 5'-ctcaccatatggcgggac-3' and 5'-ttctctctggtggctagc-3'; CCL-19, 5'-
ttcagcctgctgctctctg-3’ and 5’- accctgagcccttcatt-3’; ICAM-1, 5’- ttcacactgaatgccagctc-3’ and 5’-gtctgctgagacccctcttg-3’. The following PCR cycle was used: 95°C for 15 min; 50 cycles, each cycle containing 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Dissociation curve analysis was performed on all PCR products to ensure that specific PCR products were generated. PCR reactions for each sample were performed in duplicate. Data were analyzed using the comparative C_T method,^{12,14} and the levels of specific target mRNA were expressed as relative fold changes (fold change = 2 ^ \Delta \Delta C_T, where \Delta \Delta C_T = (C_T, target - C_T, actin)_{Time x} - (C_T, target - C_T, actin)_{Time 0}.

**RNA isolation and microarray analysis**

The spinal cords of mice subjected to EAE that received the control or platelet depleting serum were collected after transcardiac perfusion. Total RNA was isolated using Trizol reagent (Invitrogen) according to the standard protocol and used to generate biotin-labeled complementary RNA using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX). For hybridization, the labeled nucleic acid made from total RNA was subjected to Mouse Ref-8 beadchips containing > 24,000 Ref sequences per array (Illumina, Inc., San Diego, CA). Microarray data were analyzed using DIANE 6.0. ANOVA tests were done to eliminate the genes with larger variances within each comparing group. Genes were determined to be differentially expressed after calculating the Z ratio, which indicates the fold-difference between experimental groups, and false discovery rate (fdr), which controls for the expected proportion of false rejected hypotheses. Individual genes
with p value <=0.05, absolute value of Z ratio >=1.5 or <=-1.5 and fdr <= 0.3 were considered significantly changed. The same cutoff criteria are applied for Gene Ontology (GO) selection (Analysis of microarray data using Z score transformation\textsuperscript{15}).

**Detection of platelets in human MS tissue**

The MS plaque depicted in Online Figure I is from a patient previously reported in an earlier study.\textsuperscript{16} MS brain tissues were obtained from the Rocky Mountain MS Center (RMMSC) (Englewood, CO, courtesy of Dr. Ron Murray). The research with this autopsy tissue was approved as exempt by the IRB in accordance with DHHS Federal regulation 45CFR46. The plaque presented in this study is a chronic active type 1 lesion (active inflammation and demyelination).\textsuperscript{17} The tissue was frozen and stored at -70°C. The tissue was partially thawed and dissected into segments with visible plaques and serial sections were sliced with a thickness of 20 microns using a cryostat. The sections were mounted (2 per glass slide) and stored at -20°C. Slides were rehydrated with dH2O overnight at 4°C and equilibrated in phosphate-buffered saline (PBS). Confocal immunofluorescent microscopy was performed as previously described \textsuperscript{16,18}. Briefly, tissues were permeabilized with 0.2% Triton X-100 in PBS and blocked with Image-It FX signal enhancer solution (Molecular Probes, Eugene, OR). To detect platelets, purified monoclonal mouse antibody against human CD42b (BD Biosciences) (1:800 dilution) was utilized. The primary antibody was incubated overnight in a humidified chamber at 4°C. Thereafter, the secondary donkey anti-mouse IgG specific for both heavy and light chains
conjugated to Cy 5 (Jackson) (1:800 dilution) was added for 1 h at room temperature (RT). Propidium iodide (PI) (5.0 μM) was added to detect cell nuclei. Negative controls to assess background staining were performed with 10 μg/ml normal mouse serum (Pierce). Coverslips were mounted using ProLong Gold anti-fade mounting media (Molecular Probes Inc.). Resolution of lipofuscin and lipids was accomplished by a combination of mid-range laser excitation, broad pinhole and neutral density filter from the Argon laser. This allowed for the optical isolation of the lipofuscin/lipid particles by using their bi-fringment refractive properties. We then utilized the back light scattering and interference contrast to acquire an image. Lipids were auto-fluorescent and visualized with 457/517 nm Argon and the 615 nm HeNe laser, but not observed with the 605 nm laser. This methodology was verified with Oil-red-O staining of serial sections. Images were acquired with Personal Confocal Microscopy PCM-2000 (Nikon, Melville, NY) using a Nikon E800 upright microscope with Pan Fluor oil emersion lenses. The Personal Confocal Image program (PCI, Compix, Cranberry Township, PA) was used to acquire digital images.\textsuperscript{16,18}

Moreover, four cases from a different center (University of Pennsylvania, Division of Neuropathology) \textbf{with histological evidence of active demyelinating plaques (inspected by a neuropathologist of the University of Pennsylvania)} from patients with multiple sclerosis were studied. All four cases studied were from patients clinically diagnosed with multiple sclerosis with oligoclonal IgG bands at cerebrospinal fluid examination, periventricular plaques by MRI and with a dominant lesion by MRI that was biopsied to rule
out the remote possibility of tumor. Histopathological examination showed that the lesions that were biopsied represented active demyelinating plaques. All cases displayed acute demyelination with perivascular chronic inflammatory infiltrates composed of lymphocytes and plasma cells, macrophage infiltration, myelin breakdown with relative preservation of axons and gliosis. Five µm sections cut from paraffin-embedded brain tissues were deparaffinized, incubated in 10 mM sodium citrate buffer, pH 6.0 at sub-boiling temperatures for 10 min to unmask antigen, endogenous peroxidase was blocked with 0.3% H₂O₂ and the sections were incubated with mouse anti-CD42b (clone 42CO1) monoclonal antibodies or IgG1 isotype control antibodies (1 µg/ml, Thermo Scientific) for 16 hrs at 4°C. Biotinylated anti-mouse (Vector Labs, 1:200) IgG was added followed by HRP-conjugated streptavidin. Peroxidase was detected using the avidin-biotin complex ABC Kit (Vector Labs) counterstained with hematoxylin. As a control, 4 cases of normal human brain tissue were studied; these samples were provided by the cooperative Human Tissue Network (CHTN) of the University of Pennsylvania.

Data presentation and statistics

Comparisons between group means were performed using Student $t$-test or Mann-Whitney-U analysis as appropriate. $P<0.05$ was considered statistically significant.
**Online Table I: Genes involved in immune and inflammatory responses that are significantly downregulated upon platelet depletion in the course of EAE.**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Accession</th>
<th>Definition</th>
<th>Z ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL5</td>
<td>NM_013653.2</td>
<td>chemokine (C-C motif) ligand 5 (Ccl5)</td>
<td>-8.30</td>
</tr>
<tr>
<td>CLEC7A</td>
<td>NM_020008.1</td>
<td>C-type lectin domain family 7, member a (Clec7a)</td>
<td>-6.86</td>
</tr>
<tr>
<td>CD68</td>
<td>NM_009857.1</td>
<td>CD68 antigen (Cd68)</td>
<td>-6.49</td>
</tr>
<tr>
<td>IL1B</td>
<td>NM_008361</td>
<td>interleukin 1 beta (Il1b)</td>
<td>-6.16</td>
</tr>
<tr>
<td>CXCL13</td>
<td>NM_018866.1</td>
<td>chemokine (C-X-C motif) ligand 13 (Cxc13)</td>
<td>-6.00</td>
</tr>
<tr>
<td>CCL7</td>
<td>NM_013654.2</td>
<td>chemokine (C-C motif) ligand 7 (Ccl7)</td>
<td>-5.74</td>
</tr>
<tr>
<td>TLR2</td>
<td>NM_011905.2</td>
<td>toll-like receptor 2 (Tlr2)</td>
<td>-5.58</td>
</tr>
<tr>
<td>CXCL10</td>
<td>NM_021274.1</td>
<td>chemokine (C-X-C motif) ligand 10 (Cxc10)</td>
<td>-5.26</td>
</tr>
<tr>
<td>CCL19</td>
<td>NM_011888.2</td>
<td>chemokine (C-C motif) ligand 19 (Ccl19)</td>
<td>-5.14</td>
</tr>
<tr>
<td>CD40</td>
<td>NM_170702.2</td>
<td>CD40 antigen (Cd40), transcript variant 5</td>
<td>-4.89</td>
</tr>
<tr>
<td>CXCR4</td>
<td>NM_009911.2</td>
<td>chemokine (C-X-C motif) receptor 4 (Cxcr4)</td>
<td>-4.70</td>
</tr>
<tr>
<td>LY9</td>
<td>NM_008534.2</td>
<td>lymphocyte antigen 9 (Ly9)</td>
<td>-4.64</td>
</tr>
<tr>
<td>ICAM1</td>
<td>NM_010493.2</td>
<td>intercellular adhesion molecule 1 (Icam1)</td>
<td>-4.60</td>
</tr>
<tr>
<td>CEBPB</td>
<td>NM_009883.3</td>
<td>CCAAT/enhancer binding protein (C/EBP), beta (CebpB)</td>
<td>-4.56</td>
</tr>
<tr>
<td>TLR13</td>
<td>NM_205820.1</td>
<td>toll-like receptor 13 (Tlr13)</td>
<td>-4.40</td>
</tr>
<tr>
<td>TLR7</td>
<td>NM_133211.3</td>
<td>toll-like receptor 7 (Tlr7)</td>
<td>-4.15</td>
</tr>
<tr>
<td>LY86</td>
<td>NM_010745.1</td>
<td>lymphocyte antigen 86 (Ly86)</td>
<td>-4.14</td>
</tr>
<tr>
<td>CD14</td>
<td>NM_009841.3</td>
<td>CD14 antigen (Cd14)</td>
<td>-4.11</td>
</tr>
<tr>
<td>CCL3</td>
<td>NM_011337.2</td>
<td>chemokine (C-C motif) ligand 3 (Ccl3)</td>
<td>-4.08</td>
</tr>
<tr>
<td>CCL2</td>
<td>NM_011333.3</td>
<td>chemokine (C-C motif) ligand 2 (Ccl2)</td>
<td>-3.78</td>
</tr>
<tr>
<td>TNF</td>
<td>NM_013693.1</td>
<td>tumor necrosis factor (Tnf)</td>
<td>-3.75</td>
</tr>
<tr>
<td>CCL4</td>
<td>NM_013652.2</td>
<td>chemokine (C-C motif) ligand 4 (Ccl4)</td>
<td>-3.66</td>
</tr>
<tr>
<td>CCR5</td>
<td>NM_009917.2</td>
<td>chemokine (C-C motif) receptor 5 (Ccr5)</td>
<td>-3.66</td>
</tr>
<tr>
<td>CSF1R</td>
<td>NM_001037859.2</td>
<td>colony stimulating factor 1 receptor (Csf1r)</td>
<td>-3.63</td>
</tr>
<tr>
<td>CCL1</td>
<td>NM_011328.2</td>
<td>chemokine (C-C motif) ligand 1 (Ccl1)</td>
<td>-3.58</td>
</tr>
<tr>
<td>NCF1</td>
<td>NM_010876.2</td>
<td>neutrophil cytosolic factor 1 (Ncf1)</td>
<td>-3.56</td>
</tr>
<tr>
<td>LY96</td>
<td>NM_016923.1</td>
<td>lymphocyte antigen 96 (Ly96)</td>
<td>-3.43</td>
</tr>
<tr>
<td>C3</td>
<td>NM_009778.1</td>
<td>complement component 3 (C3)</td>
<td>-3.11</td>
</tr>
<tr>
<td>IFNG</td>
<td>NM_008337.3</td>
<td>interferon gamma (Ifng)</td>
<td>-2.84</td>
</tr>
<tr>
<td>TLR6</td>
<td>NM_011604.2</td>
<td>toll-like receptor 6 (Tlr6)</td>
<td>-2.77</td>
</tr>
<tr>
<td>TLR1</td>
<td>NM_030682.1</td>
<td>toll-like receptor 1 (Tlr1)</td>
<td>-2.75</td>
</tr>
<tr>
<td>CD97</td>
<td>NM_011925.1</td>
<td>CD97 antigen (Cd97)</td>
<td>-2.35</td>
</tr>
<tr>
<td>IL1A</td>
<td>NM_010554</td>
<td>interleukin 1 alpha (Il1a)</td>
<td>-2.34</td>
</tr>
<tr>
<td>ITGB2</td>
<td>NM_008404.4</td>
<td>integrin beta 2 (Itgb2)</td>
<td>-2.34</td>
</tr>
<tr>
<td>IRAK2</td>
<td>NM_172161.2</td>
<td>interleukin-1 receptor-associated kinase 2 (Irak2)</td>
<td>-2.27</td>
</tr>
<tr>
<td>MYD88</td>
<td>NM_010851.2</td>
<td>myeloid differentiation primary response gene 88 (Myd88)</td>
<td>-2.25</td>
</tr>
<tr>
<td>TLR4</td>
<td>NM_021297.2</td>
<td>toll-like receptor 4 (Tlr4)</td>
<td>-2.18</td>
</tr>
<tr>
<td>CD97</td>
<td>NM_011925.1</td>
<td>CD97 antigen (Cd97)</td>
<td>-2.01</td>
</tr>
<tr>
<td>TNFRSF1A</td>
<td>NM_011609.3</td>
<td>tumor necrosis factor receptor superfamily, member 1a (Tnfrsf1a)</td>
<td>-2.01</td>
</tr>
</tbody>
</table>
Online Table II: Number of adherent leukocytes in mice without EAE after platelet depletion as assessed by intravital microscopy of the spinal cord

<table>
<thead>
<tr>
<th></th>
<th>Ctrl Serum</th>
<th>Pttl depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Mean</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Ctrl Serum</th>
<th>Pttl depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.6666667</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Mean</td>
<td>0.1666667</td>
<td>0</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>0.1666667</td>
<td>0</td>
</tr>
</tbody>
</table>

cell rolling  cell adhesion
Legends to Online Figures

**Online Figure I.** (A, B, C) Brain sections from mice without EAE (A) or 19 days post induction of EAE (B, C). Tissues were analyzed for the platelet specific marker CD42 (GPIb, red) and the microglial marker Iba-1 (green) by immunofluorescence staining. The arrows indicate platelets. (C) shows a higher magnification of the insert from (B). (D) Confocal microscopy of a chronic active MS plaque from the cerebrum demonstrates the presence of platelets identified by staining with CD42b specific primary antibody and a Cy-5 (red) labeled secondary antibody. Nuclei, stained with propidium iodide are blue. Auto-fluorescent accumulations of lipofuscin/myelin in the chronic active plaque (yellow) were also present. Scale Bar: 20 µm. (E) EAE was induced in C57Bl/6 and at day 0 (no EAE), day 9, day 16 and day 30 spinal cords were lysed and analyzed by western blot for platelet GPIIb. Isolated platelets from peripheral mouse blood served as positive control. Densitometric analysis was performed to calculate the ratio of GPIIb/actin on d0, d9, d16, d25 and d30. The ratio of GPIIb/actin was set as 1. One representative western blot is shown, similar results were obtained in 3 independent.

**Online Figure II.** Injection of antiplatelet serum during EAE results in significant platelet depletion as compared to injection of control serum, but has no effect on peripheral leukocyte count.
Online Figure III. (A, B, C) To analyze the influence of platelet depletion on spinal cord demyelination, EAE was induced in WT mice and on days 12 and 16 post induction mice were treated with control serum (control) or with platelet-depleting serum. On day 21, axonal staining in spinal cords was performed with the anti-neurofilament-200 (NF-H) antibody and reduced demyelination was observed in the spinal cords of mice upon platelet depletion. Data are mean +/- SEM (n=5). * indicates p<0.05 as compared to mice treated with control serum. (D) EAE was induced in C57Bl/6 and mice were treated with platelet depleting or control serum at day 12 and day 16. On day 21 mice spinal cords were stained with Luxol fast blue to delineate areas of demyelination. Area of demyelination was evaluated in 6 spinal cords using computer assisted analysis software as described in materials and methods. Data are mean +/- SEM and are shown as % of control. The control serum treated group represents the 100% control. * indicates p<0.01.

Online Figure IV. (A) To analyze the influence of platelet depletion on the cellular composition of inflammatory cells in EAE spinal cords, the disease was induced in WT mice and on days 12 and 16 post induction mice were treated with control serum (control) or with platelet-depleting serum. On day 21 after induction of EAE mice were perfused with PBS to get rid of circulating cells and spinal cords were isolated. Real-time PCR analysis for the mRNA levels of CD4, CD8 and CD11b was carried out. Data are mean +/- SEM (n=5 mice per group) and are shown as percent of control. The mRNA expression of the respective molecule in spinal
cords from control treated mice represents the 100 % control. * indicates p<0.05 as compared to control serum treated animals.  (B) Similarly, myeloperoxidase (MPO) concentration was determined by ELISA on day 21 post EAE induction in spinal cords from mice treated with control or platelet depletion serum. Data are mean +/- SEM (n=4). * indicates p<0.05 as compared to MPO concentration of mice treated with control serum.

**Online Figure V.**  (A) Adhesion of mouse bone marrow derived macrophages to immobilized BSA or surface-adherent platelets was studied in the absence or presence of control IgG or of anti-M2 antibody specifically blocking the Mac-1/GPIbα interaction (each antibody at 10µg/ml). Data are mean +/- SEM and are shown as number of adherent macrophages per field (n=3-4). * indicates p<0.05 as compared to control IgG.  (B, C) Adhesion of mouse macrophages to (B) immobilized fibrinogen (10µg/ml) or (C) ICAM-1 (10µg/ml) was studied in the absence or presence of control IgG or of anti-M2 antibody (each at 10µg/ml). Data are mean +/- SEM (n=3); no significant differences were observed between the two groups.

**Online Figure VI.**  EAE was induced in WT mice. Mice were treated i.p. with Fab to GPIbα (GPIb Fab) or control Fab (each 75 µg/mouse, Control Fab) on days 12, 14 and 16. Some mice were additionaly treated i.v. with anti-M2 antibody or control antibody (each 47 µg/mouse) on days 12, 14 and 16. Clinical disease scores are shown. Data are mean +/- SEM (n= 10 mice per group). * indicates p<0.05 as compared to control Fab group on the same day.
Online Video I.
To test for the impact of platelets on leukocyte recruitment to the inflamed CNS, EAE was induced in WT mice, after 2 weeks mice were treated with platelet-depleting serum and 24 h thereafter intravital microscopy was carried out. The video sequence is taken from one experiment representative for the experiments described in figure 5B-D).

Online Video II.
Similarly to supplemental Video 2, EAE was induced in WT mice, after 2 weeks mice were treated with control serum and 24 h thereafter intravital microscopy was carried out.

Reference List


Langer et al., suppl Figure I

<table>
<thead>
<tr>
<th>孤立的血小板</th>
<th>d0</th>
<th>d9</th>
<th>d16</th>
<th>d25</th>
<th>d30</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPIIb:actin</td>
<td>1</td>
<td>0.87</td>
<td>140.5</td>
<td>256.5</td>
<td>165.9</td>
</tr>
</tbody>
</table>

EAE脊髓
**Control Plt depletion**

**NF-H staining density**

**Demyelination area (% of control)**

---

**A**

Control

Deplete

**B**

Control

Deplete

---

**C**

NF-H staining density

**D**

Demyelination area (% of control)
A

Langer et al., suppl Figure IV

![Graph A](image)

- mRNA expression (% of control)
- CD4, CD8, CD11b
- Control, Plt depletion

B

![Graph B](image)

- MPO (ng/ml)
- Control, platelet depletion

* Indicates statistical significance.