The American Journal of Pathology, Vol. 169, No. 5, November 2006

DOI: 10.2353/ajpath.2006.060143

Immunopathology and Infectious Disease

Phagocytes Containing a Disease-Promoting Toll-Like Receptor/Nod Ligand Are Present in the Brain during Demyelinating Disease in Primates

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Recent studies claim a central role for Toll-like receptor (TLR) ligands in stimulating autoimmune disease by activation of antigen-presenting cells in the target organ, but it is unclear if and how TLR ligands reach target organs. Most evidence comes from rodent models, and it is uncertain whether this principle holds in primates. Here we identify which cells contain peptidoglycan (PGN) in multiple sclerosis brain and in two nonhuman primate experimental autoimmune encephalomyelitis (EAE) models with different disease courses: acute (rhesus monkey) versus chronic disease (marmoset). Because persistence of TLR ligands in the central nervous system might be consequential for disease progression, we also determined the expression of two major PGN-degrading enzymes, ie, lysozyme and *N*-acetylmuramyl-L-alanine amidase. Distinct phagocyte subsets, including granulocytes, macrophages, and dendritic cells, contained PGN in the brain and coexpressed the inflammatory cytokine interleukin-12. The number of phagocytes carrying PGN increased in acute and chronic EAE compared with control animals, with the highest number of PGN-containing cells in acute EAE brain. Lytic enzymes were scarcely expressed in monkey and multiple sclerosis brain, favoring PGN persistence. PGN stimulated interleukin-12p70 release by leukocytes from all three primate species. The presence of PGN in the inflamed brain may have major implications because TLR2/Nod ligation potentially promotes inflammation and disease progression. ***(Am J Pathol 2006, 169:1671–1685; DOI: 10.2353/ajpath.2006.060143)***

The normal repertoire of human and nonhuman primates contains significant numbers of potentially encephalitogenic T cells.[1–3] Under homeostatic conditions, these cells are kept dormant by control mechanisms involving regulatory Tr1 cells. Whether autoreactive Th1 cells or regulatory Tr1 cells are induced depends on the maturation state of the antigen-presenting cells (APCs), eg, dendritic cells (DCs), in peripheral lymphoid organs.[4–6] The development of autoimmune encephalomyelitis depends on additional activation signals to central nervous system (CNS)-infiltrating autoreactive T cells from resident APCs. Such additional signals can be provided by CpG-Toll-like receptor (TLR)-9 interaction.[7,8] Different from mice, neither the myeloid DCs in the peripheral compartment nor the resident brain APCs (microglia) of humans[9] or rhesus monkeys (J.J. Bajramovic, unpublished data) express TLR9. Hence, data obtained in mice on the disruption of tolerance by stimulation with CpG oligonucleotides[7,10,11] cannot be easily extrapolated to primates (including humans). Therefore, we here investigate alternative TLR ligands in the primate CNS.

Peptidoglycan (PGN) is a major cell wall component of gram-positive bacteria, and we have shown that PGN has strong proinflammatory effects in animal models of multiple sclerosis (MS).[12] PGN stimulates the innate immune system by binding to the Toll-like receptor (TLR)2/6, although this concept has recently been challenged.[13] Recently identified intracellular PGN receptors are the Nod1 (Card4) and Nod2 (Card15) signaling receptors.[14–16]

Supported by the Dutch Multiple Sclerosis Research Foundation (project 03-513 MS and 04-540 MS).

Accepted for publication July 18, 2006.

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PGN can induce signal transduction via TLR2 and Nod1/2 receptors within cells and via TLR2/6 in association with CD14 on the cell surface. Activation of these receptors results in the induction of cell maturation and the production of several chemokines and proinflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1, and IL-6.[17–19]

PGN is a complex structure formed by three-dimensional cross-linked layers of glycan backbones with peptide bridges.[20] The glycan strands of PGN in all bacterial species are composed of two alternating sugar residues, *N*-acetyl glucosamine (GlcNAc) and *N*-acetyl muramic acid (MurNAc). Stem peptides cross-link these glycan chains and differ in composition between bacteria strains. PGN is digested by enzymes with different substrate specificities of which lysozyme and *N*-acetylmuramyl-L-alanine amidase (NAMLAA) are the best characterized. Lysozyme is present in granules of phagocytes[21–23] and partially degrades PGN by hydrolyzing the bond between GlcNAc and MurNAc, resulting in solubilized PGN. We have previously identified and functionally characterized NAMLAA,[24–27] which was recently shown to be identical to the PGN recognition protein long form (PGRP-L),[28–30] NAMLAA is expressed by neutrophilic granulocytes but not by monocytes and resting macrophages under normal conditions. PGN is cleaved by NAMLAA/PGRP-L between the MurNAc residue and the first L-alanine of the stem peptide.

Because of its complex structure, PGN is highly resistant to degradation under physiological conditions. Recently, it was shown that PGN-induced responses were comparable in macrophages from NAMLAA/PGRP-L-deficient and wild-type mice.[31] Most likely, several PGN-degrading enzymes are necessary for complete PGN degradation. *In vitro* studies clearly show that the combined degradation of PGN by lysozyme and NAMLAA reduces the proinflammatory potential of PGN.[26] Because macrophages and DCs contain lysozyme but lack NAMLAA, incomplete PGN degradation may result in the persistence of intracellular bioactive PGN.

We have proposed that PGN may act as a costimulating factor for the development of autoimmune disease in the absence of infection or bacterial replication. APCs redistribute PGN from the mucosa[32–34] to secondary lymphoid organs and to sites of chronic inflammation and may stimulate autoimmune processes locally. We have previously described PGN-containing APCs in sites of chronic inflammation in multiple sclerosis MS brain tissue,[35] rheumatoid arthritis synovial tissue,[36,37] and bowel wall tissue of patients with Crohn's disease.[38] In rodent animal models, PGN has been shown to facilitate development of autoimmune disease. Injection of PGN derived from different bacterial strains can induce chronic arthritis and colitis in susceptible rodents.[39,40] Moreover, we have demonstrated that PGN induces DC maturation and contributes to the development of mouse experimental autoimmune encephalomyelitis (EAE), an animal model for MS.[12]

In this study, we examined EAE in rhesus monkeys (*Macaca mulatta*)[41] and marmoset monkeys (*Callithrix jacchus*)[42] to further investigate the relevance of PGN in autoimmune encephalomyelitis. These nonhuman primates are outbred and are genetically and immunologically closely related to humans.[43] On immunization with myelin components marmoset monkeys develop EAE with a similar pathology and disease course compared with that of MS patients. EAE-affected rhesus monkeys develop large lesions in the brain, which contains abundant numbers of neutrophils.[44] This severe inflammatory necrosis is associated with an acute disease course. In contrast, marmosets develop a relapsing-remitting or primary-progressive disease course with perivascular lesions and demyelinated areas containing mainly macrophages, T cells, and B cells in the CNS. The fundamentally distinct clinical and pathological presentation of EAE might represent different forms of human encephalomyelitis. Rhesus monkey EAE more closely resembles acute disseminated encephalomyelitis, whereas marmoset EAE resembles chronic MS.[41,42,45]

To address whether the TLR/Nod ligand PGN is carried into primate brain, in this study we assessed whether the presence, location, and numbers of PGN-containing cells in brain tissue from rhesus monkeys with acute EAE differs from marmoset monkeys with a chronic disease course. In both models, we determined whether the number of PGN-containing cells in the brain was related to the development of EAE. In addition, we identified the cell types containing PGN in both human (primate) and nonhuman primate brain. Because NAMLAA and lysozyme are enzymes involved in PGN clearance, we assessed whether these PGN-degrading enzymes were differentially expressed in MS and nondemented control brain tissues and in EAE-affected versus non-EAE-affected monkey brain tissue. To obtain functional insight into the role of PGN in rhesus monkey and marmoset EAE, we assessed *in vitro* whether leukocytes from rhesus monkey and marmoset monkeys are responsive to stimulation with PGN from *Staphylococcus aureus*, known to precipitate EAE in mice.

Materials and Methods

Nonhuman Primate Tissues

Brain tissue was obtained from marmosets and rhesus monkeys raised at the Biomedical Primate Research Centre (Rijswijk, The Netherlands), as described previously.[44,46] Brain tissues used for this study had been neuropathologically well characterized. All brain tissues were obtained from previous studies; hence, no animals were sacrificed solely for the purpose of the current study. All experimental procedures with live animals had been reviewed and approved by the institutional animal care and use committee. EAE was induced in marmoset monkeys by immunization with 20 mg of whole human myelin or 0.1 mg of recombinant human myelin oligodendrocyte glycoprotein (rhMOG) emulsified in complete Freund's adjuvant (CFA). As adjuvant controls, two randomly selected monkeys were immunized with 1 mg of ovalbumin (Sigma Chemical Co., St. Louis, MO) in CFA, a protocol used for induction of arthritis. EAE was induced in rhesus monkeys by immunization with 0.32 mg of rh-

MOG_{1-125}, or 0.1 mg of MOG_{34-56} in CFA.[44] As adjuvant controls, rhesus monkeys (n = 2 monkeys) were immunized with 3 to 5 mg of bovine type II collagen in CFA.[47] Animals immunized with myelin antigen were examined daily for clinical symptoms of EAE. We used the following scoring system for disease: 0 = no clinical EAE signs; 0.5 = apathy, loss of appetite, and altered walking pattern without ataxia; 1 = lethargy and/or anorexia; 2 = ataxia, sensory loss/blindness; 2.5 = hemi- or paraparesis; 3 = hemi- or paraplegia; 4 = quadriplegia; and 5 = death attributable to EAE. All rhesus monkeys immunized with $rhMOG_{1-125}$ (n = 7) developed a hyperacute disease course, with a 24- to 48-hour time period from the onset to full-blown disease.[44] Rhesus monkeys that were immunized with MOG_{34-56} in CFA developed a heterogeneous disease course.[41] The four monkeys that were included in this study were asymptomatic at day 28 after immunization. These monkeys were challenged with MOG_{34-56} in incomplete Freund's adjuvant (IFA) (see Table 1, animals 8 and 9) or with an irrelevant peptide MOG_{4-26} in IFA (Table 1, animals 6 and 7). After one or two homologous challenges both monkeys developed acute clinical signs of EAE within 7 days after challenge and were sacrificed. The two rhesus monkeys challenged with irrelevant peptide remained asymptomatic. From the 37 marmoset monkeys that were immunized with myelin antigens, 28 developed EAE. In the other nine marmoset monkeys, EAE development had been successfully blocked by experimental treatment with new immunotherapeutic reagents. Details regarding immunogens, day of sacrifice, and development of EAE for both marmoset and rhesus monkeys are shown in Tables 1 and 2, respectively.

Brain Tissue Samples of MS Patients and Nondemented Control Patients

Human autopsy brain tissue was provided by The Netherlands Brain Bank (Coordinator Dr. R. Ravid, Amsterdam, The Netherlands). Information regarding MS patients and nondemented control patients is shown in Table 4.

Staging of MS Brain Lesions

As described previously,[48] brain lesions were characterized according to an internationally accepted staging system based on inflammation and demyelination criteria.[49,50] Preactive lesions are characterized by HLA-II-expressing clusters of activated microglia cells and few perivascular inflammatory cells. Preactive lesions do not contain phagocytic cells possessing myelin breakdown products or areas of demyelination. Active lesions are characterized by demyelinated areas. Phagocytic cells in these areas contain myelin breakdown products, which can be visualized by oil red O, a histochemical staining for neutral lipids. In these lesions, strong expression of HLA-II is present on perivascular and parenchymal macrophage.

Monoclonal Antibody 2E9 against PGN

2E9 is a murine monoclonal antibody (mAb) (IgG3) raised against a pure fraction of PGN polysaccharides isolated from normal human feces.[51] The specificity of this mAb has been extensively investigated and confirmed using various assays as described previously.[35,51]

Immunohistochemistry

Immunohistochemical staining was performed as described,[48] with slight modifications. Sections from marmoset and rhesus monkey tissues were fixed for 10 minutes at room temperature in 4% paraformaldehyde in phosphate-buffered saline (PBS) with 0.02% (v/v) H_2O_2 in PBS to eliminate endogenous peroxidase activity. Human brain tissue sections were fixed for 10 minutes at room temperature in fresh acetone containing 0.02% (v/v) H_2O_2. Remaining endogenous peroxidase activity was revealed by staining with 4-chloro-1-naphtol-phosphate (Sigma), which results in a dark blue precipitate.

PGN-containing cells were demonstrated by staining with mAb 2E9-biotin. The specificity of the murine mAb 2E9, raised against normal human feces PGN polysaccharides, has been well documented previously.[35,51] mAb 2E9 recognizes PGN-containing cell wall fragments, such as lysozyme-solubilized cell walls of different gram-positive bacteria.[35,51] NAMLAA-containing cells were detected by staining with biotinylated monoclonal mouse anti-human NAMLAA-biotin IgG1 (AAA4) as described above.[27] mAb AAA4, raised against human serum amidase, specifically recognizes NAMLAA.[27,30] Lysozyme-containing cells were detected by staining with polyclonal rabbit anti-human lysozyme antibody (Ab-1; Neomarkers, Fremont, CA), followed by biotinylated donkey anti-rabbit antibody (Amersham Biosciences, Buckinghamshire, UK). Incubations with secondary and tertiary reagents were done for 1 hour at room temperature. Specific staining for PGN, NAMLAA, and lysozyme was revealed by peroxidase-linked avidin (DAKO, Glostrup, Denmark) and 3-amino-9-ethylcarbazole (Sigma) as chromogen, resulting in a bright red staining.

Nuclei were counterstained by hematoxylin. As negative controls, the primary antibody was omitted, and isotype-matched control antibodies of irrelevant specificity were used. The controls did not display binding activity in tissue of monkeys and MS patients. Reactive human tonsil sections were included in each staining procedure as positive control tissue. Sections were evaluated by two independent observers.

Immunofluorescent Double Labeling

A double-staining procedure was used to determine which cell types contain PGN and NAMLAA. In brief, all tissues were fixed with acetone, as described above. To reduce autofluorescence, slides were incubated with 0.1% sodium borohydride (Sigma) in MQ and 0.3 mol/L glycine (Sigma) in PBS. PGN-containing cells were detected with mAb 2E9-fluorescein isothiocyanate (FITC)

and NAMLAA-containing cells with mAb AAA4-FITC. mAbs CD11b (Leu-15, macrophages, microglia, DCs, granulocytes), IL-12p40/p70 (BD Biosciences, San Jose, CA), and CD83 (DC) (HB15a; Immunotech, Marseille, France) were conjugated to Zenon AF594 (Molecular Probes Europe, Leiden, The Netherlands) according to the manufacturer's instructions. Neutrophils were detected by staining with mouse anti-neutrophil elastase mAb (NP57, DAKO) followed by anti-mouse Ig-TRITC (DAKO). Because the available antibodies against human macrophage did not cross-react with rhesus monkey and marmoset macrophage, we were unable to determine the exact percentage of macrophage within the population of PGN-containing cells. However, the combination of CD11b, CD83, and neutrophil elastase as markers still allowed for a confident estimate of macrophages/microglia, DCs, and granulocytes. Histochemical staining for lipids was performed with 0.3% Sudan black B (BDH Laboratory Supplies, Poole, UK) in 70% ethanol, thereby reducing autofluorescence.

Quantitation of PGN-Containing Cells

The number of PGN-containing cells in the parenchyma and infiltrates of brain tissue derived from marmoset and rhesus monkeys were quantitated by two independent observers. The maximum number of PGN-containing cells in the infiltrates was determined per tissue section. The number indicates the mean number of two tissue sections, quantified by two independent observers. The surface area of the section was determined using a VIDAS-RT image analysis system (Kontron Elektronik GmbH/Carl Zeiss, Weesp, The Netherlands). Area measurements were performed using a 1.6-fold magnification objective. The frequency of PGN-containing cells in the brain parenchyma was calculated by dividing the number of PGN-containing cells in the tissue section by the surface area of the section.

Peptidoglycan

Soluble PGN (sPGN) from *Staphylococcus aureus* was prepared by gel-permeation chromatography.[52] The content of LPS in sPGN was assessed to be <15 pg/mg (acceptable background values), using a TLR4/MD2-transfected HEK293 cell line, with IL-8 production as a read-out. sPGN induced dose-dependent production of IL-8 by TLR2-transfected HEK293 cells (PGN μg/ml; IL-8 pg/ml, 20 μg/ml; 421 pg/ml, 10 μg/ml; 363 pg/ml, 5 μg/ml; 184 pg/ml, 2.5 μg/ml; 84 pg/ml, 1.25 μg/ml; 20 pg/ml, 0.6 μg/ml; 5 pg/ml, 0;0). There was no LTA contamination as revealed by high-pressure liquid chromatography analysis. At present, purification and identification of biologically active components in sPGN are being performed and may reveal other TLR2 agonists, as described by Travassos and colleagues.[13]

Stimulation of Peripheral Blood Mononuclear Cells (PBMCs) and IL-12p70 Enzyme-Linked Immunosorbent Assay

Blood samples from rhesus monkeys and marmoset monkeys were collected as described previously, using lithium-heparin-precoated tubes.[53] PBMCs were isolated by Ficoll (LSM Lymphocyte Separation Medium; ICN Biomedicals, Aurora, OH) and cells were cultured in 25 mmol/L HEPES-buffered RPMI 1640 (Life Technologies, Glasgow, UK) supplemented with 10% fetal calf serum (ICN Biomedicals), penicillin, and streptomycin (Invitrogen, Paisley, UK), Glutamax (Invitrogen), and β-mercaptoethanol (Invitrogen). sPGN was sonicated for 20 minutes and immediately added to the culture. Cells were seeded (4×10^5/well in 0.5 ml) with or without sPGN (range 1 to 5 to 10 μg/ml) in 48-well plates (BD-Falcon, Franklin Lakes, NJ) for 21 hours. Supernatants were harvested and diluted 1:1 in PBS/1% bovine serum albumin (Sigma) and stored at -20°C for further analysis. According to the manufacturer's instructions, concentration of IL-12p70 was determined by using monkey-specific IL-12 enzyme-linked immunosorbent assay kits (U-Cytech, Utrecht, The Netherlands).

Statistical Evaluation

Statistical evaluation was performed using SPSS 11 software (SPSS, Chicago, IL). The Mann-Whitney *U*-test was used to analyze differences. A value of $P < 0.05$ was considered statistically significant.

Results

Presence of TLR/Nod Ligand in CNS Correlates with Infiltration

Brain tissues of rhesus monkeys and marmoset monkeys were stained for PGN and NAMLAA- and lysozyme-containing cells. Animals were divided into different groups, based on immunization procedure and development of clinical EAE (Table 1, rhesus monkeys; and Table 2, marmoset monkeys). In agreement with previous findings,[41,44] large infiltrates were found in rhesus monkey EAE brain tissue compared with smaller infiltrates in marmoset EAE brain tissue (Figure 1, A–C). As expected, a positive correlation was found ($r = 0.67$ rhesus monkey and $r = 0.43$ marmoset) between the size of infiltrates and the maximum number of PGN-containing cells in infiltrates. The following two sections describe the assessment of PGN, NAMLAA, and lysozyme in rhesus monkey and marmoset brain tissue.

Elevated Numbers of Cells Containing PGN and NAMLAA in the Infiltrates and Parenchyma of Rhesus Monkeys with EAE

Infiltrates

As expected, infiltrates were absent in brain tissue of rhesus monkeys ($n = 2$) immunized with collagen in CFA

Table 1. Rhesus Monkey-Derived Brain Tissues

Animal	Immunogen	Clinical EAE	Day of sacrifice	EAE score
1, 2	Collagen II	−	100	0
3, 4	phMOG4-26,1× boost phMOG34-56	−	138	0
5	phMOG34-56, 2× boost phMOG34-56	+	112	5
6[a]	phMOG34-56, 1× boost phMOG34-56	+	35	5
7	rhMOG1-125	+	24	4
8	rhMOG1-125	+	17	4
9[a]	rhMOG1-125	+	18	4
10	rhMOG1-125	+	39	3
11	rhMOG1-125	+	32	4
12	rhMOG1-125	+	35	2.5

rhMOG1−125, recombinant peptide representing the complete extracellular N-terminal domain of human MOG; phMOG34−56, synthetic peptide representing an extracellular N-terminal domain of human MOG.
[a]Brain tissues that were used for double staining.

as an adjuvant reference group without CNS involvement (Table 3). In one of two rhesus monkeys immunized with myelin antigens, but without EAE, one infiltrate (medium/large size) was present in the brain with only a few PGN-containing cells (Table 3). In contrast, animals with clinical EAE had many PGN- and NAMLAA-containing infiltrates, with high numbers of positive cells in the infiltrates (Figure 1C; Figure 2, A and B; and Table 3). There was no clear correlation between the severity of EAE (Table 2) and the number of PGN- and NAMLAA-containing cells (Table 3). The maximum number of PGN-containing cells in these rhesus monkey brain infiltrates was much higher compared with the maximum number found in infiltrates of marmoset EAE brain tissue, which can be explained by the differences in size of the infiltrates (Figure 1D, Table 3).

Parenchyma

A significantly higher number ($P < 0.05$) of PGN-containing cells was found in the parenchyma of EAE brain tissue compared with non-EAE brain tissue (Figure 1E). In contrast to NAMLAA expression in the infiltrates, only a few NAMLAA-containing cells were present in the parenchyma of rhesus monkey brain tissues. Taken together, brain tissue of rhesus monkeys with EAE contains large infiltrates with abundant numbers of PGN-containing cells and high numbers of NAMLAA-expressing cells. The number of NAMLAA-expressing cells is consistent with the abundant number of neutrophils present in these lesions. In contrast, only occasional lysozyme-expressing cells were present near blood vessels.

Elevated Numbers of Cells Containing PGN in the Infiltrates and Parenchyma of Marmoset Monkeys with EAE

Infiltrates

As in rhesus monkeys with EAE, the number of PGN-containing cells in infiltrates of marmoset monkeys with EAE was significantly higher ($P < 0.05$) than in animals without EAE (Figure 1, B and D). One of two control marmosets immunized with ovalbumin in CFA had two small infiltrates in the brain, which did not contain PGN. In seven of nine marmosets immunized with myelin antigens but without EAE, brain infiltrates (of medium size, number 2.9 ± 2 per section) were present with a few PGN-containing cells (1 ± 0.8 per infiltrate). In 25 of 27 marmosets with EAE, brain infiltrates (3.3 ± 2.6 per section) were present with a modest number of PGN-containing cells (10.5 ± 12.5 per infiltrate). In all marmoset groups a similar frequency of PGN-containing cells (13.7 ± 17.5 per 100 mm^2) was present in the brain parenchyma (Figure 1F).

Parenchyma

Few cells within perivascular infiltrates expressed NAMLAA in EAE and non-EAE marmoset tissues (Figure 2C). No NAMLAA-containing cells were detected in the parenchyma of the brain. In brain tissue of marmoset monkeys we could not detect lysozyme-containing cells, whereas positive cells were found in human tonsil tissue. These data indicate that the antibody we used against human lysozyme is most likely not cross-reactive with marmoset lysozyme. In summary, these data show that PGN-containing cells are present in a significantly higher number in brain infiltrates of EAE marmoset monkeys compared with non-EAE marmoset monkeys, whereas NAMLAA is only scarcely expressed.

Various Phagocytic Subsets Contain PGN and NAMLAA in Monkey Brain Tissue

PGN is present within macrophages/microglia, DCs, and granulocytes in MS brain tissue.[35] The cell types containing PGN and NAMLAA in marmoset and rhesus monkey EAE brain tissue were identified by double staining for CD11b (macrophages, microglia, DCs, granulocytes), CD83 (DC), and neutrophil elastase (neutrophils). EAE-affected brain tissues from marmoset ($n = 3$) and rhesus monkeys ($n = 2$) were selected based on representative numbers of PGN- and NAMLAA-containing cells.

PGN was mostly present in $CD11b^+$ cells in both rhesus monkey (84 to 87%) and marmoset (88 to 100% of PGN-containing cells) EAE brain tissue (Figure 3, A and B, respectively). Many neutrophils (62 to 65%) and also

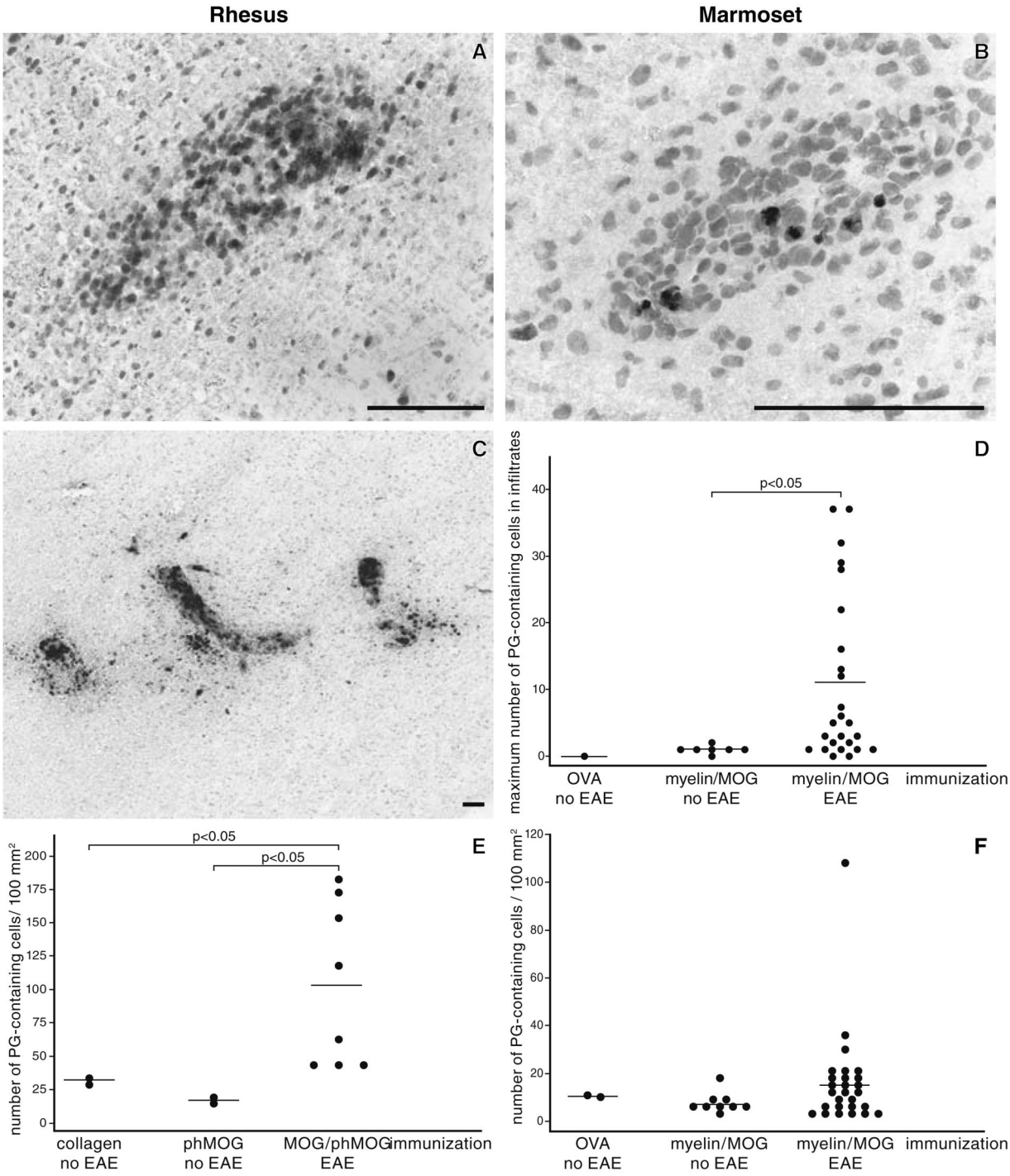

Figure 1. PGN-containing cells are present in rhesus monkey and marmoset brain. We assessed whether EAE development is associated with elevated numbers of PGN-containing cells in the brain and whether the number of PGN-containing cells differs in brain tissue from rhesus monkeys with acute EAE compared with marmoset monkeys with chronic EAE. Left column, rhesus brain tissue; right column, marmoset brain tissue. Significantly higher numbers of cells in infiltrates of marmoset EAE brain tissue (**D**) contain PGN when compared with control brain tissue. **C:** Many PGN-containing infiltrates are present in rhesus EAE brain tissue. Significantly elevated numbers of PGN-containing cells are present in the parenchyma of rhesus (**E**) but not in marmoset (**F**) EAE brain tissue, compared with control brain tissue. Many cells in infiltrates of rhesus EAE brain tissue (**A**) (animal 6) and a modest cell number in infiltrates of marmoset EAE brain tissue (animal 10) contain PGN (**B**, **D**). Difference in frequency of PGN-containing cells likely reflects the distinct CNS pathologies in marmoset versus rhesus, with high abundance of neutrophils in rhesus monkeys with EAE. Note in **E** the maximum number of PGN-containing cells in infiltrates of marmoset monkeys is demonstrated, whereas for rhesus monkey brain tissue the maximum number of PGN-containing cells in infiltrates is depicted in Table 3. Scale bars = 100 μm.

DCs (16%) in rhesus monkey brain contained PGN. PGN was not detected within DCs in marmoset brain tissue. The number of PGN- and NAMLAA-containing granulocytes in marmoset tissues could not be determined because the available antibodies against human granulocytes did not cross-react with marmoset granulocytes.

In rhesus monkey EAE brain tissue, NAMLAA was mostly expressed by neutrophils (43 to 80% of NAMLAA-

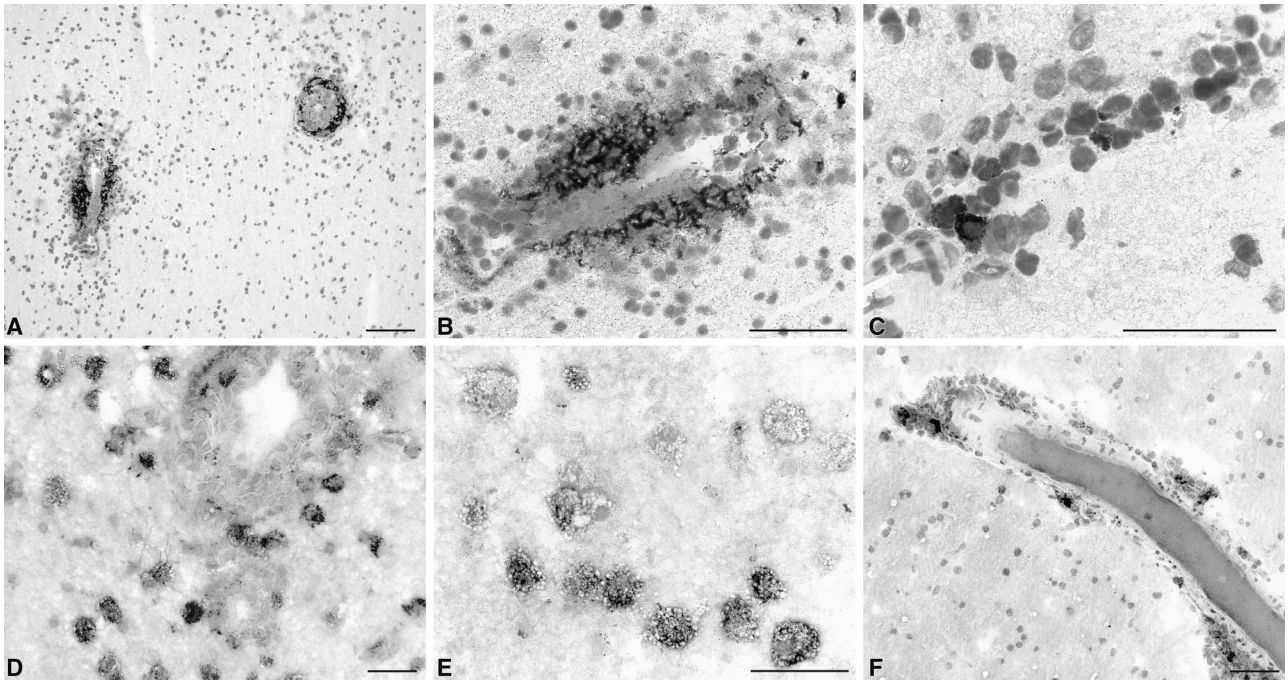

Figure 2. Restricted NAMLAA expression in MS and monkey EAE-affected brain. **A** and **B:** In rhesus EAE brain tissue (animal 6), many perivascular infiltrates are present with a moderate to high number of NAMLAA-containing cells. **C:** In marmoset EAE brain tissue (animal 10) some NAMLAA-containing cells are localized near blood vessels. **D:** Foamy macrophages in active MS brain lesions (sample 97-160) express NAMLAA. **E:** Expression of NAMLAA is restricted to a certain subpopulation of foamy macrophages. **F:** Occasionally, perivascular cells also express NAMLAA in MS brain tissue (sample 00-082). Scale bars = 100 μm.

containing cells), to a lesser extent by $CD11b^+$ cells (25 to 48%) and by some DCs (7 to 8%). NAMLAA in marmoset EAE brain tissue was occasionally expressed by $CD11b^+$ cells (0.5 to 1%), but not by DCs. Comparing the number of $CD11b^+$ PGN-containing cells with $CD11b^+$ NAMLAA-containing cells suggests that PGN and NAMLAA are generally present in different cells, ie, PGN is mostly present in cells that express CD11b, whereas NAMLAA is mostly expressed in $CD11b^-$ cells. Furthermore, PGN may persist intracellularly in brain infiltrates because of the restricted expression or lack of lysozyme and/or NAMLAA, occurring both in rhesus and marmoset monkeys with EAE.

Restricted Expression of NAMLAA and Lysozyme in MS Brain

Previously, the number of PGN-containing cells was determined in MS and non-MS control brain tissue.[35] To determine whether PGN persistence in MS brain is associated with the expression of PGN-degrading enzymes, we assessed whether NAMLAA- and lysozyme-containing cells are also localized at sites of inflammation in MS brain and in control brain tissue. Subsequently, we determined whether expression of NAMLAA and lysozyme in MS brain parallels the expression in marmoset and

Table 2. Marmoset Monkey-Derived Brain Tissues

Animal	Immunogen	Clinical EAE	Day of sacrifice	Mean EAE score ± SD (range)
1	OVA	−	49	0
2	OVA	−	49	0
3	None	−	n.a.	0
4	None	−	n.a.	0
5 to 8	MOG	−	53 to 155	0
9 to 20	MOG	+	44 to 112	2.3 ± 1 (0 to 3)
9[a]	MOG	+	49	1.8
10[a]	MOG	+	49	1.9
12	MOG	+	67	2.5
19	MOG	+	67	2.5
20	MOG	+	44	0
21 to 25	Myelin	−	86	0
26 to 41	Myelin	+	41 to 93	2.4 ± 1.2 (0 to 3.5)
36[a]	Myelin	+	77	0

[a]Brain tissues that were used for double staining; n.a., not applicable.

Table 3. High Numbers of PGN-Containing Cells in Rhesus Monkey EAE Brain Tissue

				PGN-infiltrates		NAMLAA-infiltrates	
Animal	Immunogen	EAE	Infiltrate size	/total infiltrates	PGN cells	/total infiltrates	NAMLAA cells
1	Collagen II	−	−	0/0	0	0/0	0
2	Collagen II	−	−	0/0	0	0/0	0
3	[a]phMOG34-56	−	−	0/0	0	0/0	0
4	[a]phMOG34-56	−	++/+++	1/1	2	0/0	0
7	rhMOG1-125	+	+	1/1	1	0/0	0
5	[b]phMOG34-56	+	++	6/13	27	1/13	5
8	rhMOG1-125	+	++/+++	2/3	50	0/3	0
9	rhMOG1-125	+	++	15/22	54	12/22	13
10	rhMOG1-125	+	+++	9/9	151	9/9	25
11	rhMOG1-125	+	+++	11/11	264	5/11	50
6	[b]phMOG34-56	+	++	32/32	173	25/32	50
12	rhMOG1-125	+	+++	10/10	>300	10/10	100

rhMOG1-125, recombinant peptide representing the complete extracellular N-terminal domain of human MOG; phMOG34-56, synthetic peptide representing an extracellular N-terminal domain of human MOG. Infiltrate size: +, small; ++, medium (>30 cells); +++, large (>100 cells).
[a]Booster with phMOG4-26.
[b]Booster with phMOG34-56.

rhesus brain tissue. In MS brain (n = 30 lesions from n = 9 patients) with different lesion stages (Table 4), a few to a moderate number of NAMLAA-containing cells were found. These cells had a foamy macrophage appearance and were localized within active lesions (Figure 2D). Within the same active lesions, many NAMLAA-negative foamy cells were also present, indicating that myelin-phagocytosing cells do not all or continuously produce NAMLAA (Figure 2E). Occasionally, some NAMLAA-containing cells were present near blood vessels (Figure 2F). Preactive perivascular infiltrates and parenchymal areas of the brain did not contain NAMLAA. NAMLAA-containing cells were absent in nondemented control brain tissues (n = 2 patients). Lysozyme-containing cells were

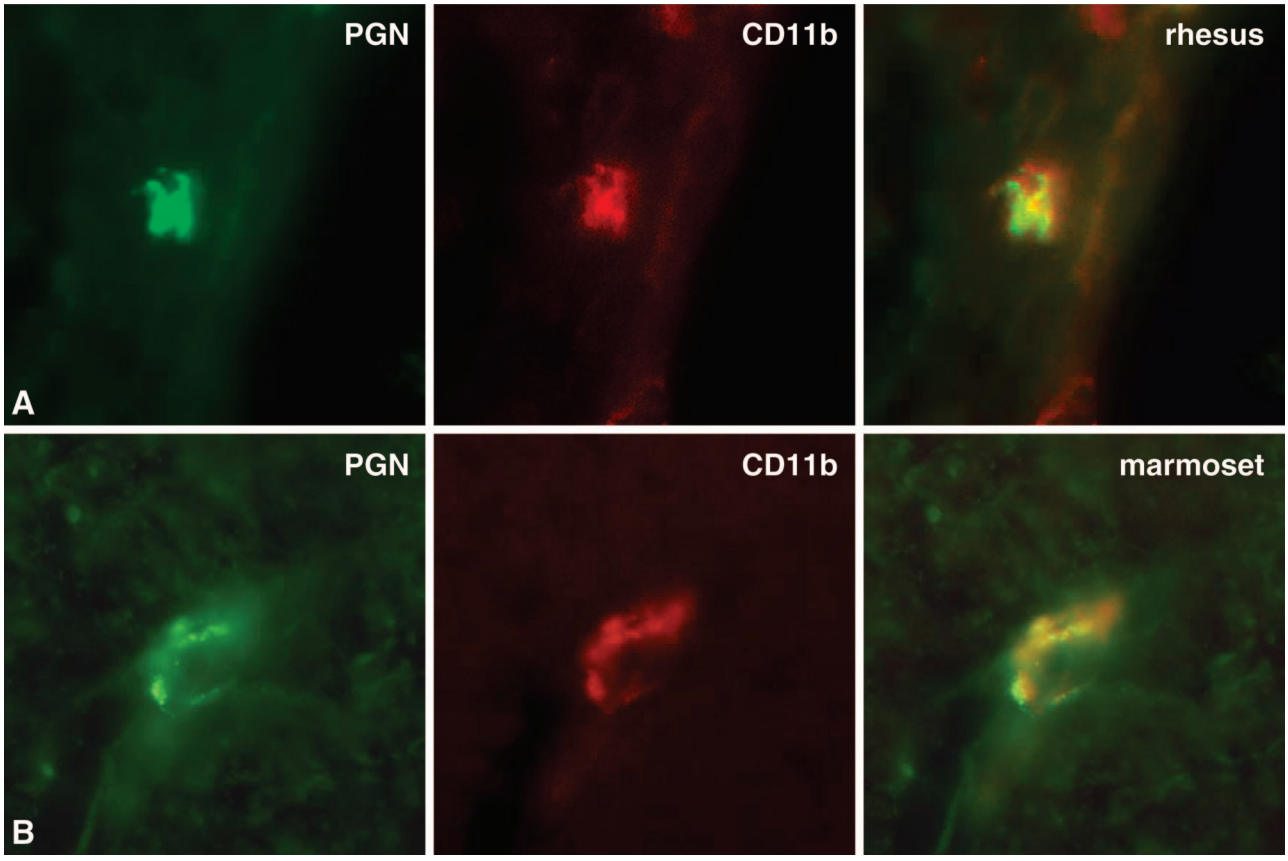

Figure 3. PGN-containing cells in rhesus monkey and marmoset brain express CD11b. Double staining of brain tissue of rhesus monkeys (**A**, top) shows that 84 to 87% of PGN-containing cells (green) express CD11b (red). In brain tissue of marmoset monkeys (**B**, bottom), 88 to 100% of the PGN-containing cells expressed CD11b.

Table 4. Clinical and Neuropathological Data of MS Patients and Controls

Case	Sex	Age (years)	pm time (hour:minute)	Disease duration (years)	Progressive MS	Number of lesions (*n*)/lesion stage
00/024	F	52	8:25	12	Yes	(1) Preactive (1) Active demyelinating
00/82	M	34	<42:00	Unknown	Unknown	(4) Preactive
00/120	F	69	13:20	23	Yes	(1) Preactive
01/018	F	48	08:10	9	Yes	(2) Preactive (1) Active demyelinating (1) Chronic inactive
97/123	M	46	03:45	23	Yes	(1) Preactive (3) Active demyelinating
97/160*	F	40	07:00	11	Yes	(2) Preactive (2) Active demyelinating
98/176	M	83	07:05	52	Yes	(1) Preactive
99/051	F	45	10:55	14	Yes	(1) Preactive (1) Active demyelinating (1) Chronic active
99/066	M	69	16:45	43	Yes	(3) Preactive
96/013	F	68	10:30	n.a.	n.a.	Nondemented control No lesions
96/078	F	87	08:00	n.a.	n.a.	Nondemented control No lesions

*From this patient two different tissue samples were stained. pm, post-mortem; M, male; F, female; n.a., not applicable.

absent in all human brain tissues with the exception of occasional intravascular cells. In the positive control tissue, human tonsil, lysozyme-containing cells were present, confirming that the absence of lysozyme in human brain tissue cannot be explained by failure of the staining. In summary, NAMLAA was expressed by a subpopulation of cells with a foamy macrophage appearance in active MS lesions and lysozyme was not expressed in MS and control brain.

Proinflammatory S. aureus*-Derived sPGN Stimulates IL-12p70 Production by Primate PBMCs*

Previously, we have demonstrated that sPGN, purified from the pathogenic bacterium *S. aureus*, exerts proinflammatory effects on mouse bone marrow-derived DCs.[12] Moreover, sPGN efficiently induced EAE in mice, when admixed with MOG_{35-55} encephalitogenic peptide in IFA.[12] To further substantiate the pathogenic relevance of PGN, we determined whether highly purified *S. aureus* sPGN, as used in our previously described mouse studies, can induce the prototypical Th1-promoting cytokine IL-12 by nonhuman primate PBMCs. IL-12 is an important proinflammatory cytokine in the development of murine and nonhuman primate EAE.[54–56] PBMCs from marmoset monkeys ($n = 2$), rhesus monkeys ($n = 2$), and one human donor, as an internal control, were stimulated with different concentrations of *S. aureus* sPGN. As expected, *S. aureus* sPGN induced IL-12p70 production by human PBMCs in a dose-dependent way (Figure 4). Both rhesus monkeys ($n = 2$) and the marmoset ($n = 1$) PBMCs also produced elevated levels of IL-12p70 on sPGN stimulation, in a dose-dependent manner (Figure 4). In conclusion, *in vitro* sPGN from *S. aureus* induces a prototypical proinflammatory cytokine thought to be crucially involved in demyelinating disease. To determine whether this also occurs during inflammation in the CNS, we determined whether PGN-containing APCs in the brain also produce IL-12p40/p70. Indeed, 95 to 100% of PGN-containing

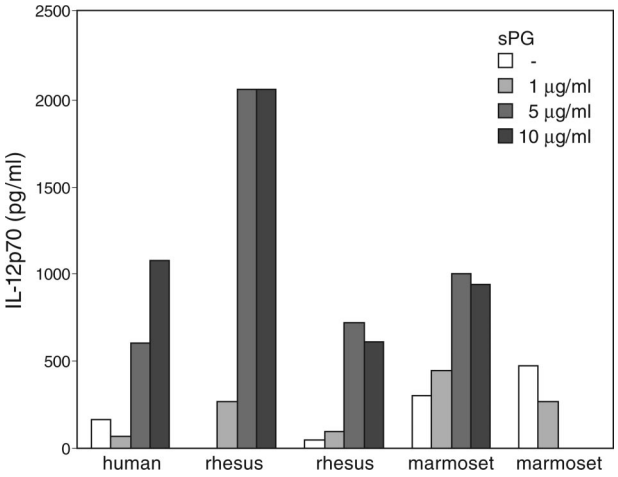

Figure 4. *S. aureus*-derived sPGN induces IL-12p70 production by marmoset and rhesus monkey APCs. To demonstrate the functional potential of highly purified PGN to induce proinflammatory cytokines, PBMCs from human ($n = 1$), marmoset ($n = 2$), or rhesus monkeys ($n = 2$) were stimulated with different concentrations of sPGN for 21 hours. Supernatants were harvested, and IL-12p70 was determined by enzyme-linked immunosorbent assay.

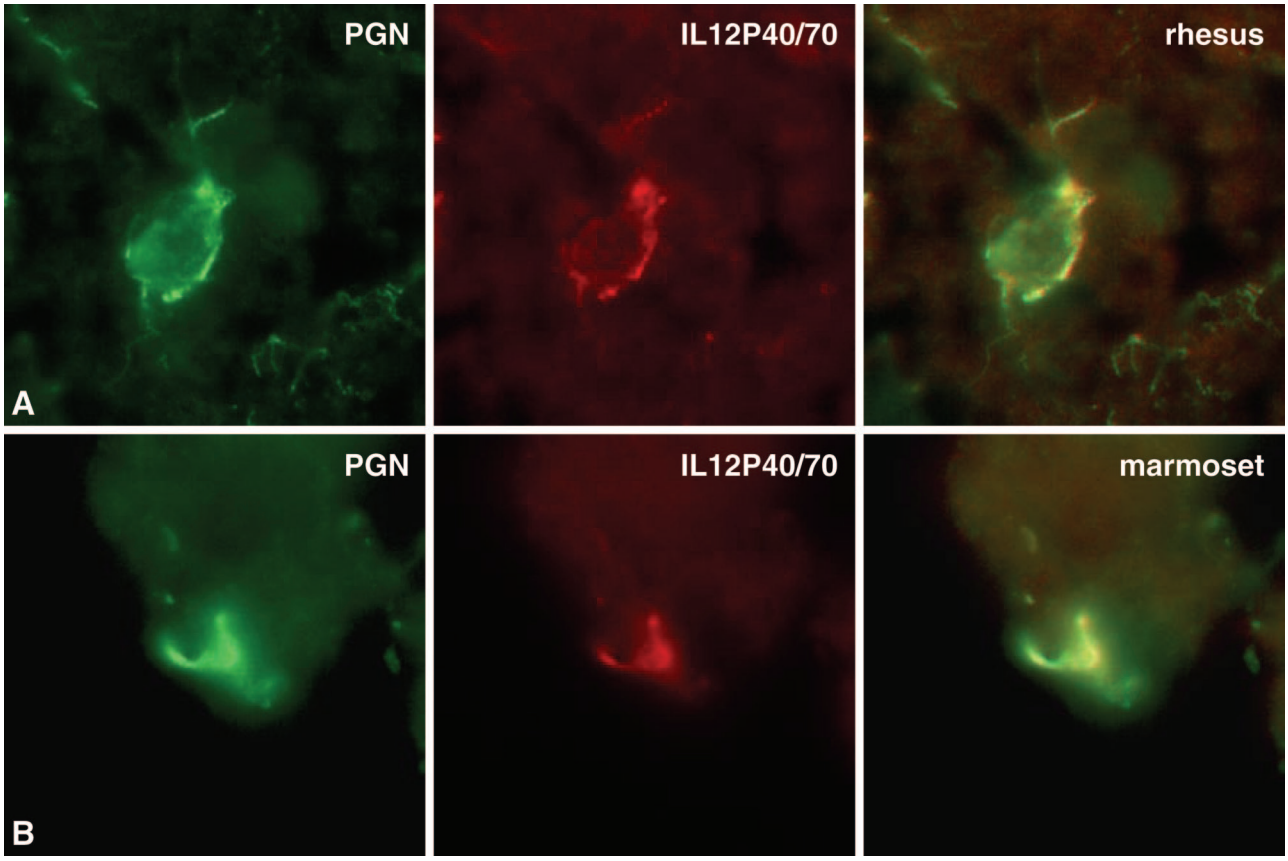

Figure 5. IL12p40/p70 is expressed by PGN-containing cells in the brain of rhesus and marmoset monkeys. Double staining for PGN and IL-12p40/p70 of both rhesus and marmoset brain tissue (**A** and **B**, top and bottom panels, respectively) shows that 95 to 100% of PGN-containing cells (green) express IL-12p40/p70 (red).

cells in both rhesus and marmoset brain produce IL-12p40/p70 (Figure 5, A and B, respectively).

Discussion

This study demonstrates that phagocytes containing the TLR/Nod ligand PGN are present in the brain of primates with demyelinating disease. Recent studies in mouse EAE models have shown that innate immune responses within the CNS are important in the expression of autoimmune disease. However, the species differences between TLR expression on CNS APCs in primates hampers direct translation of this principle to MS. Therefore, we used two distinct nonhuman primate EAE models to bridge the immunological gap between the EAE mouse and the MS patient. Bacterial components can stimulate APCs to produce a plethora of proinflammatory cytokines and chemokines and stimulate the development of antigen-specific Th1 cells. Bacterial PGN, which stimulates cells via extracellular and intracellular receptors (TLR and Nod), can possibly activate APCs of the CNS. Proinflammatory PGN can be redistributed from the periphery into sites of chronic inflammation, such as rheumatoid joints[37] and MS brain.[35] In relation to MS, we compared EAE-affected rhesus monkeys, which develop an acute disease course with marmoset EAE monkeys, which develop chronic EAE.[41]

We show here that in both monkey models for MS, EAE development is associated with a significantly elevated number of PGN-containing cells in the brain. EAE-affected rhesus monkeys develop large brain lesions with many PGN-containing cells, whereas EAE-affected marmoset monkeys develop infiltrates of medium size with a modest number of PGN-containing cells. We do not claim that PGN-containing APCs specifically traffic to the CNS as a consequence of PGN properties. Instead we hypothesize that PGN is always present in a certain percentage of circulating APCs in the blood and that infiltrating APCs therefore carry PGN to any site of inflammation in a Trojan horse-like way. During EAE, these cells will therefore accumulate in the CNS and subsequently contribute to or even enhance autoimmune processes. We therefore hypothesize a causal relation between the number of PGN-containing cells and inflammation. The current data do not illustrate in which stage(s) of the disease PGN-containing cells exacerbate inflammation. The fact that PGN-containing cells are present in perivascular cuffs in both MS and EAE in nonhuman primates implies that these cells can contribute to early disease processes. However, PGN-containing cells are also found within the brain parenchyma, which is likely to be associated with more advanced pathology. The persistence of PGN in MS and monkey EAE brain tissue might be because of the restricted expression of both NAMLAA and lysozyme. The

fact that a TLR/Nod ligand is transported into the brain during autoimmune encephalomyelitis and MS may have major consequences for disease development and perpetuation, as discussed below.

Implications of TLR Stimulation in Tolerance Regulation by APCs

Under normal conditions, autoreactive T cells are under tight control to prevent the development of autoimmune disease. Even high numbers of activated autoreactive T cells inside the target organ are not sufficient to induce autoimmune disease.[57] However, autoimmune disease can develop once the autoantigen is presented by resident APCs of the CNS to autoreactive T cells in the presence of costimulation by TLR or CD40 ligands.[10,57–59] Both in the periphery and at the inflammatory site, immature APCs have a major role in the maintenance of T-cell tolerance to self-antigens. For example, introduction of the $PLP_{139-151}$ T-cell receptor transgene into B10.S mice did not result in spontaneous EAE development. However, when APCs from B10.S $PLP_{139-151}$ T-cell receptor transgenic mice were activated by systemic TLR ligand administration, 33% of these mice developed EAE.[60] Moreover, TLR-induced activation of CNS APCs promoted EAE development in C57BL/6 mice.[7] It has recently been demonstrated that naïve myelin-specific T cells become activated in inflamed CNS by local APCs[61] and can cause disease progression by epitope spreading.[62,63] By activation of local APCs, PGN can possibly function as a trigger for T cells directed against endogenous myelin epitopes and thereby contribute to the perpetuation of demyelinating disease.

With respect to the nonhuman primate EAE models, we show here a dose-dependent induction of IL-12p70 by sPGN of peripheral blood leukocytes from both marmoset and rhesus monkeys. Moreover, we demonstrate that PGN-containing cells in brain tissue of rhesus and marmoset monkeys with EAE also produce IL-12p40/p70. The p40 moiety of IL-12 is shared by the related family member IL-23 and plays an important role in the development of EAE.[64] It was recently demonstrated that treatment of EAE-affected marmoset monkeys with a mAb against anti-IL-12p40 resulted in inhibition of lesion development.[65] Its importance is furthermore underscored by the fact that mice with an astrocyte-targeted expression of IL-12p70 developed EAE after immunization with CFA and toxin, without specific autoantigen in the emulsion.[55] Thus, PGN may contribute to inflammation and disease progression by promoting the production of Th1-stimulating proinflammatory cytokines in the CNS and in secondary lymphoid organs. Interestingly, in EAE-susceptible mouse strains, APCs have a higher activation state compared with APCs from EAE-resistant stains.[60] In accordance, we are currently testing the hypothesis that the more acute EAE in rhesus monkeys is attributable to a higher sensitivity of rhesus monkey APCs to TLR/Nod-induced activation than that of marmoset APCs. This suggests that TLR/Nod genes may form a genetic susceptibility trait for acute demyelinating disease.

Activation of Resident and Infiltrated APCs by TLR/Nod Ligation in the CNS

In MS,[35] and rhesus monkey EAE brain tissue, PGN is present within macrophages, DCs, and neutrophils, suggesting that CNS-infiltrating phagocytes are responsible for the transport of PGN into the CNS. In the chronic marmoset EAE model, neutrophils are rarely found within the CNS, and PGN is detected mostly within macrophages. PGN can induce proinflammatory signals through ligation of TLR2,[14,15,66] and/or Nod receptors.[16,67] It has been described that various TLRs are expressed on resident APCs in the MS brain.[9] PGN-induced responses in murine astrocytes are TLR2-dependent,[68] and murine microglia produce numerous proinflammatory cytokines and chemokines after PGN stimulation.[69,70] These data demonstrate that different resident cell types in the brain produce proinflammatory mediators on stimulation with PGN. In addition, blood-derived PGN-containing, brain-infiltrating cells may locally produce proinflammatory mediators via TLR/Nod signaling pathways. We are currently assessing coexpression of TLR and Nod by PGN-containing cells using antibodies that became available recently.

Persistence of PGN in the CNS Is Related to Restricted Expression of Degrading Enzymes

Previously, it was shown that radioactively labeled PGN monomers accumulated over time in brain and intestine of mice,[71] and injected arthritogenic PGN was retained for several weeks in spleen, liver, and synovial tissue of rats.[72] These data demonstrate that PGN can be transferred into and retained at inflammatory sites. To understand the persistence of PGN in MS and monkey EAE brain tissue, we determined the expression of NAMLAA and lysozyme. These enzymes together are capable of abolishing the proinflammatory capacity of PGN.[26] Lysozyme expression in macrophages can be strongly down-modulated during brain disease as was previously described for HIV-associated dementia.[73]

In the CNS of MS patients and EAE-affected monkeys, one might expect many lysozyme-expressing cells, because infiltrates contain many phagocytosing cells and lysozyme is known to be present within granules of phagocytes.[21–23] However, in this study, we could not detect lysozyme-containing cells in infiltrates or in the parenchyma of MS brain tissue. Occasional intravascular cells containing lysozyme were found in nondemented controls, in MS and in rhesus monkey EAE brain tissue, which is in agreement with previous findings in MS brain tissue.[74,75] Also NAMLAA is expressed in a restricted manner in MS- and EAE-affected marmoset brain, whereas in EAE-affected rhesus monkeys many NAMLAA-containing cells are present in the brain. Disease development in marmoset monkeys and MS patients is more chronic compared with rhesus monkeys, which develop an acute encephalomyelitis accompanied by large necrotic brain lesions with many neutrophils and macrophages.[41] These differences in pathology of MS,

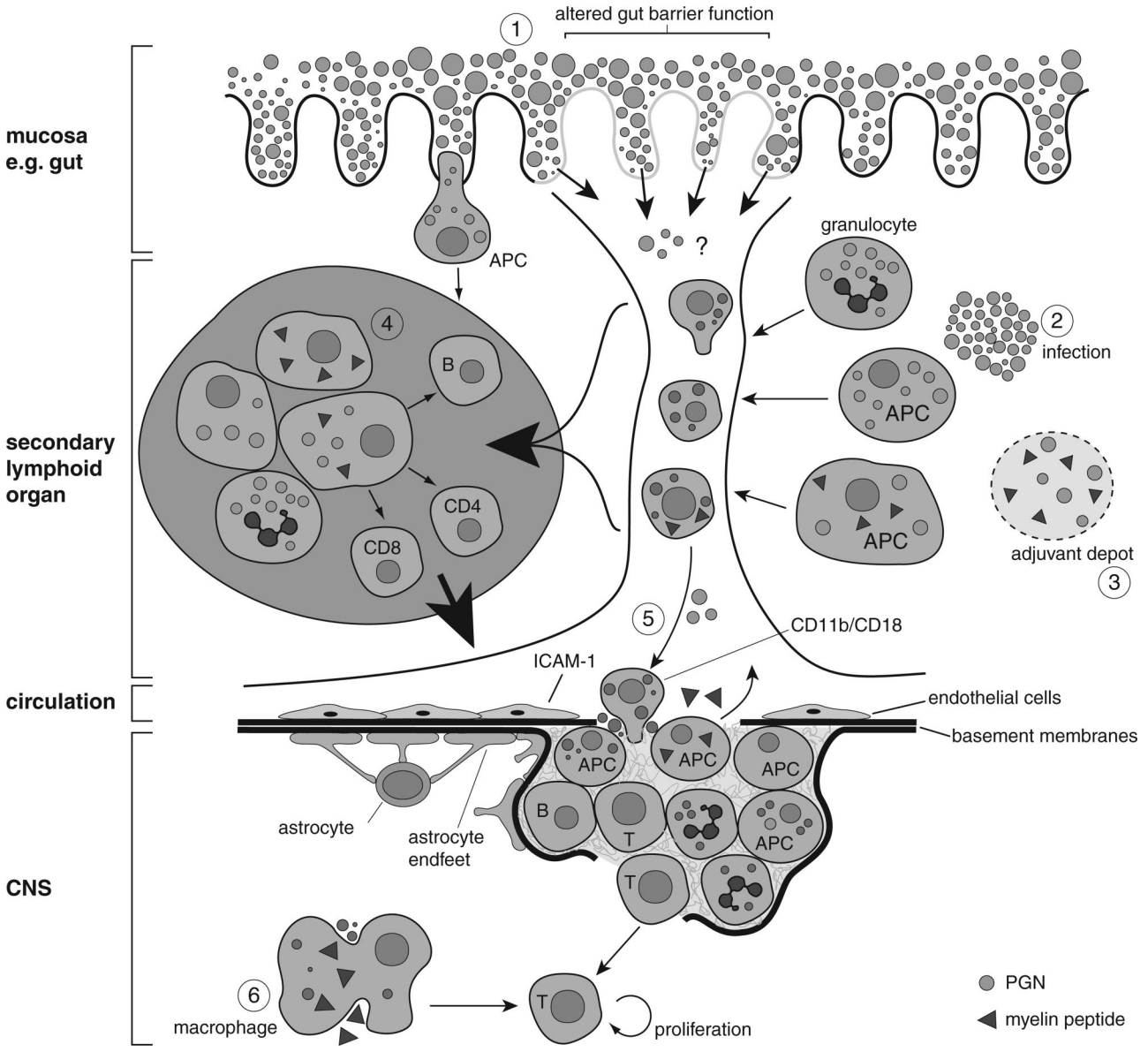

Figure 6. Proinflammatory PGN as a co-factor in MS and EAE. This figure models the concepts and findings of this study in relation to key references supporting the numbered items. The four relevant anatomical compartments are shown from top to bottom. Sources of PGN are the normal mucosal flora, notably from the gut (**1**) and infection (**2**), mimicked by adjuvant administration in EAE induction (**3**). The uptake of gut compounds by DC extending protrusions throughout the gut epithelium has been demonstrated recently by several groups.[33,34,76] Studies by us and others have demonstrated the presence of PGN in secondary lymphoid organs (**4**),[77–79] including within DCs,[12] as well as the proinflammatory action in human spleen.[80] Migration of APC-containing PGN through the circulation (**5**) has also been documented.[81,82] Our current and previous[35] studies have confirmed the presence of PGN in the CNS of primates with MS and EAE (**6**). Finally, it has been demonstrated *in vitro* that TLR/Nod ligands stimulate microglia[83–85] *in vitro* to produce inflammatory cytokines.

marmoset EAE, and rhesus monkey EAE most likely account for the different numbers of NAMLAA- and PGN-containing cells. In conclusion, these findings show that intracellular PGN persists in MS and monkey brain tissue. Persistence of PGN correlates with the absence of NAMLAA- and lysozyme-expressing cells in MS- and EAE-affected monkeys. Local conditions in the CNS may be responsible for the restricted expression of these enzymes, and the subsequent absence of one of the two enzymes may be responsible for the persistence of biologically active PGN fragments.

PGN as a Co-Factor in MS and EAE

PGN-containing cells may stimulate autoimmune-mediated processes both in the CNS and in the secondary lymphoid organs. Figure 6 models this notion, including the acquisition of PGN by APCs at different mucosa and their migration to both secondary lymphoid organs and the CNS. Although PGN or PGN fragments may also gain access to these tissue compartments in soluble form, this seems less plausible for intact PGN because this is such a large particulate structure. Although there is much ev-

idence from independent studies for all features of the model in Figure 6, a causal functional relationship between PGN and disease activity is hard to establish. To this end, we recently demonstrated that PGN stimulates autoimmune-mediated processes in mouse EAE development. Adding purified PGN from *S. aureus* to IFA and MOG35–55 was a sufficient proinflammatory stimulus to break to T-cell tolerance.[12] Taken together, given the persistence of PGN in APCs in the CNS during autoimmunity, and the stimulatory capacity of PGN in EAE development, prevention of PGN trafficking and activity may diminish the effects of autoimmune disease in humans.

Acknowledgments

We thank Prof. Dr. U. Zähringer (Research Center Borstel, Borstel, Germany) and Dr. L. van der Fits for valuable comments; T. van Os for microphotography and preparation of figures; and Dr. R. Ravid (coordinator) for providing the human brain tissue from the Netherlands Brain Bank in Amsterdam.

References

1. Pette M, Fujita K, Kitze B, Whitaker JN, Albert E, Kappos L, Wekerle H: Myelin basic protein-specific T lymphocyte lines from MS patients and healthy individuals. Neurology 1990, 40:1770–1776
2. MeinL E, Hoch RM, Dornmair K, de Waal Malefyt R, Bontrop RE, Jonker M, Lassmann H, Hohlfeld R, Wekerle H, 't Hart BA: Encephalitogenic potential of myelin basic protein-specific T cells isolated from normal rhesus macaques. Am J Pathol 1997, 150:445–453
3. Villoslada P, Abel K, Heald N, Goertsches R, Hauser SL, Genain CP: Frequency, heterogeneity and encephalitogenicity of T cells specific for myelin oligodendrocyte glycoprotein in naive outbred primates. Eur J Immunol 2001, 31:2942–2950
4. Mahnke K, Knop J, Enk AH: Induction of tolerogenic DCs: 'you are what you eat.' Trends Immunol 2003, 24:646–651
5. Lutz MB, Schuler G: Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol 2002, 23:445–449
6. Steinman RM, Hawiger D, Nussenzweig MC: Tolerogenic dendritic cells. Annu Rev Immunol 2003, 21:685–711
7. Darabi K, Karulin AY, Boehm BO, Hofstetter HH, Fabry Z, LaManna JC, Chavez JC, Tary-Lehmann M, Lehmann PV: The third signal in T cell-mediated autoimmune disease? J Immunol 2004, 173:92–99
8. Prinz M, Garbe F, Schmidt H, Mildner A, Gutcher I, Wolter K, Piesche M, Schroers R, Weiss E, Kirschning CJ, Rochford CD, Bruck W, Becher B: Innate immunity mediated by TLR9 modulates pathogenicity in an animal model of multiple sclerosis. J Clin Invest 2006, 116:456–464
9. Bsibsi M, Ravid R, Gveric D, van Noort JM: Broad expression of Toll-like receptors in the human central nervous system. J Neuropathol Exp Neurol 2002, 61:1013–1021
10. Ichikawa HT, Williams LP, Segal BM: Activation of APCs through CD40 or Toll-like receptor 9 overcomes tolerance and precipitates autoimmune disease. J Immunol 2002, 169:2781–2787
11. Ho PP, Fontoura P, Ruiz PJ, Steinman L, Garren H: An immunomodulatory GpG oligonucleotide for the treatment of autoimmunity via the innate and adaptive immune systems. J Immunol 2003, 171:4920–4926
12. Visser L, Jan de Heer H, Boven LA, van Riel D, van Meurs M, Melief MJ, Zähringer U, van Strijp J, Lambrecht BN, Nieuwenhuis EE, Laman JD: Proinflammatory bacterial peptidoglycan as a cofactor for the development of central nervous system autoimmune disease. J Immunol 2005, 174:808–816
13. Travassos LH, Girardin SE, Philpott DJ, Blanot D, Nahori MA, Werts C, Boneca IG: Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Rep 2004, 5:1000–1006
14. Girardin SE, Sansonetti PJ, Philpott DJ: Intracellular vs extracellular recognition of pathogens—common concepts in mammals and flies. Trends Microbiol 2002, 10:193–199
15. Underhill DM, Ozinsky A, Hajjar AM, Stevens A, Wilson CB, Bassetti M, Aderem A: The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature 1999, 401:811–815
16. Girardin SE, Travassos LH, Herve M, Blanot D, Boneca IG, Philpott DJ, Sansonetti PJ, Mengin-Lecreulx D: Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. J Biol Chem 2003, 278:41702–41708
17. Michelsen KS, Aicher A, Mohaupt M, Hartung T, Dimmeler S, Kirschning CJ, Schumann RR: The role of toll-like receptors (TLRs) in bacteria-induced maturation of murine dendritic cells (DCS). Peptidoglycan and lipoteichoic acid are inducers of DC maturation and require TLR2. J Biol Chem 2001, 276:25680–25686
18. Lawrence C, Nauciel C: Production of interleukin-12 by murine macrophages in response to bacterial peptidoglycan. Infect Immun 1998, 66:4947–4949
19. Wang ZM, Liu C, Dziarski R: Chemokines are the main proinflammatory mediators in human monocytes activated by Staphylococcus aureus, peptidoglycan, and endotoxin. J Biol Chem 2000, 275:20260–20267
20. Dmitriev BA, Toukach FV, Schaper KJ, Holst O, Rietschel ET, Ehlers S: Tertiary structure of bacterial murein: the scaffold model. J Bacteriol 2003, 185:3458–3468
21. Cramer DW, Ravnikar VA, Craighill M, Ng WG, Goldstein DP, Reilly R: Mullerian aplasia associated with maternal deficiency of galactose-1-phosphate uridyl transferase. Fertil Steril 1987, 47:930–934
22. Mutasa HC: Combination of diaminobenzidine staining and immunogold labeling: a novel technical approach to identify lysozyme in human neutrophil cells. Eur J Cell Biol 1989, 49:319–325
23. Miyauchi J, Sasadaira H, Watanabe K, Watanabe Y: Ultrastructural immunocytochemical localization of lysozyme in human monocytes and macrophages. Cell Tissue Res 1985, 242:269–277
24. Hoijer MA, de Groot R, van Lieshout L, Jacobs BC, Melief MJ, Hazenberg MP: Differences in N-acetylmuramyl-L-alanine amidase and lysozyme in serum and cerebrospinal fluid of patients with bacterial meningitis. J Infect Dis 1998, 177:102–106
25. Hoijer MA, Melief MJ, Calafat J, Roos D, van den Beemd RW, van Dongen JJ, Hazenberg MP: Expression and intracellular localization of the human N-acetylmuramyl-L-alanine amidase, a bacterial cell wall-degrading enzyme. Blood 1997, 90:1246–1254
26. Hoijer MA, Melief MJ, Debets R, Hazenberg MP: Inflammatory properties of peptidoglycan are decreased after degradation by human N-acetylmuramyl-L-alanine amidase. Eur Cytokine Netw 1997, 8:375–381
27. Hoijer MA, Melief MJ, Keck W, Hazenberg MP: Purification and characterization of N-acetylmuramyl-L-alanine amidase from human plasma using monoclonal antibodies. Biochim Biophys Acta 1996, 1289:57–64
28. Wang ZM, Li X, Cocklin RR, Wang M, Fukase K, Inamura S, Kusumoto S, Gupta D, Dziarski R: Human peptidoglycan recognition protein-L is an N-acetylmuramoyl-L-alanine amidase. J Biol Chem 2003, 278:49044–49052
29. Gelius E, Persson C, Karlsson J, Steiner H: A mammalian peptidoglycan recognition protein with N-acetylmuramoyl-L-alanine amidase activity. Biochem Biophys Res Commun 2003, 306:988–994
30. Zhang Y, van der Fits L, Voerman JS, Melief MJ, Laman JD, Wang M, Wang H, Li X, Walls CD, Gupta D, Dziarski R: Identification of serum N-acetylmuramoyl-l-alanine amidase as liver peptidoglycan recognition protein 2. Biochim Biophys Acta 2005, 1752:34–46
31. Xu M, Wang Z, Locksley RM: Innate immune responses in peptidoglycan recognition protein L-deficient mice. Mol Cell Biol 2004, 24:7949–7957
32. Kraehenbuhl JP, Hopkins SA, Kerneis S, Pringault E: Antigen sampling by epithelial tissues: implication for vaccine design. Behring Inst Mitt 1997, (98):24–32
33. Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP, Ricciardi-Castagnoli P: Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol 2001, 2:361–367

34. Macpherson AJ, Uhr T: Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science 2004, 303:1662–1665
35. Schrijver IA, van Meurs M, Melief MJ, Ang CW, Buljevac D, Ravid R, Hazenberg MP, Laman JD: Bacterial peptidoglycan and immune reactivity in the central nervous system in multiple sclerosis. Brain 2001, 124:1544–1554
36. Melief MJ, Hoijer MA, Van Paassen HC, Hazenberg MP: Presence of bacterial flora-derived antigen in synovial tissue macrophages and dendritic cells. Br J Rheumatol 1995, 34:1112–1116
37. Schrijver IA, Melief MJ, Tak PP, Hazenberg MP, Laman JD: Antigen-presenting cells containing bacterial peptidoglycan in synovial tissues of rheumatoid arthritis patients coexpress costimulatory molecules and cytokines. Arthritis Rheum 2000, 43:2160–2168
38. Klasen IS, Melief MJ, van Halteren AG, Schouten WR, van Blankenstein M, Hoke G, de Visser H, Hooijkaas H, Hazenberg MP: The presence of peptidoglycan-polysaccharide complexes in the bowel wall and the cellular responses to these complexes in Crohn's disease. Clin Immunol Immunopathol 1994, 71:303–308
39. Sartor RB, Rath HC, Lichtman SN, van Tol EA: Animal models of intestinal and joint inflammation. Baillieres Clin Rheumatol 1996, 10:55–76
40. Onta T, Sashida M, Fujii N, Sugawara S, Rikiishi H, Kumagai K: Induction of acute arthritis in mice by peptidoglycan derived from gram-positive bacteria and its possible role in cytokine production. Microbiol Immunol 1993, 37:573–582
41. Brok HP, Bauer J, Jonker M, Blezer E, Amor S, Bontrop RE, Laman JD, 't Hart BA: Non-human primate models of multiple sclerosis. Immunol Rev 2001, 183:173–185
42. 't Hart BA, van Meurs M, Brok HP, Massacesi L, Bauer J, Boon L, Bontrop RE, Laman JD: A new primate model for multiple sclerosis in the common marmoset. Immunol Today 2000, 21:290–297
43. Bontrop RE, Otting N, Slierendregt BL, Lanchbury JS: Evolution of major histocompatibility complex polymorphisms and T-cell receptor diversity in primates. Immunol Rev 1995, 143:33–62
44. Kerlero de Rosbo N, Brok HP, Bauer J, Kaye JF, 't Hart BA, Ben-Nun A: Rhesus monkeys are highly susceptible to experimental autoimmune encephalomyelitis induced by myelin oligodendrocyte glycoprotein: characterisation of immunodominant T- and B-cell epitopes. J Neuroimmunol 2000, 110:83–96
45. 't Hart BA, Laman JD, Bauer J, Blezer E, van Kooyk Y, Hintzen RQ: Modelling of multiple sclerosis: lessons learned in a non-human primate. Lancet Neurol 2004, 3:588–597
46. Brok HP, Uccelli A, Kerlero De Rosbo N, Bontrop RE, Roccatagliata L, de Groot NG, Capello E, Laman JD, Nicolay K, Mancardi GL, Ben-Nun A, 't Hart BA: Myelin/oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis in common marmosets: the encephalitogenic T cell epitope pMOG24-36 is presented by a monomorphic MHC class II molecule. J Immunol 2000, 165:1093–1101
47. Bakker NP, van Erck MG, Otting N, Lardy NM, Noort RC, 't Hart BA, Jonker M, Bontrop RE: Resistance to collagen-induced arthritis in a nonhuman primate species maps to the major histocompatibility complex class I region. J Exp Med 1992, 175:933–937
48. Visser L, de Vos AF, Hamann J, Melief MJ, van Meurs M, van Lier RA, Laman JD, Hintzen RQ: Expression of the EGF-TM7 receptor CD97 and its ligand CD55 (DAF) in multiple sclerosis. J Neuroimmunol 2002, 132:156–163
49. 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
50. van Waesberghe JH, Kamphorst W, De Groot CJ, van Walderveen MA, Castelijns JA, Ravid R, Lycklama a Nijeholt GJ, van der Valk P, Polman CH, Thompson AJ, Barkhof F: Axonal loss in multiple sclerosis lesions: magnetic resonance imaging insights into substrates of disability. Ann Neurol 1999, 46:747–754
51. Kool J, De Visser H, Gerrits-Boeye MY, Klasen IS, Melief MJ, Van Helden-Meeuwsen CG, Van Lieshout LM, Ruseler-Van Embden JG, Van den Berg WB, Bahr GM: Detection of intestinal flora-derived bacterial antigen complexes in splenic macrophages of rats. J Histochem Cytochem 1994, 42:1435–1441
52. Rosenthal RS, Dziarski R: Isolation of peptidoglycan and soluble peptidoglycan fragments. Methods Enzymol 1994, 235:253–285
53. Brok HP, Hornby RJ, Griffiths GD, Scott LA, Hart BA: An extensive monoclonal antibody panel for the phenotyping of leukocyte subsets in the common marmoset and the cotton-top tamarin. Cytometry 2001, 45:294–303
54. Laman JD, van Meurs M, Schellekens MM, de Boer M, Melchers B, Massacesi L, Lassmann H, Claassen E, 't Hart BA: Expression of accessory molecules and cytokines in acute EAE in marmoset monkeys (Callithrix jacchus). J Neuroimmunol 1998, 86:30–45
55. Lassmann S, Kincaid C, Asensio VC, Campbell IL: Induction of type 1 immune pathology in the brain following immunization without central nervous system autoantigen in transgenic mice with astrocyte-targeted expression of IL-12. J Immunol 2001, 167:5485–5493
56. Brok HP, van Meurs M, Blezer E, Schantz A, Peritt D, Treacy G, Laman JD, Bauer J, 't Hart BA: Prevention of experimental autoimmune encephalomyelitis in common marmosets using an anti-IL-12p40 monoclonal antibody. J Immunol 2002, 169:6554–6563
57. Lang KS, Recher M, Junt T, Navarini AA, Harris NL, Freigang S, Odermatt B, Conrad C, Ittner LM, Bauer S, Luther SA, Uematsu S, Akira S, Hengartner H, Zinkernagel RM: Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. Nat Med 2005, 11:138–145
58. Conant SB, Swanborg RH: Autoreactive T cells persist in rats protected against experimental autoimmune encephalomyelitis and can be activated through stimulation of innate immunity. J Immunol 2004, 172:5322–5328
59. Garza KM, Chan SM, Suri R, Nguyen LT, Odermatt B, Schoenberger SP, Ohashi PS: Role of antigen-presenting cells in mediating tolerance and autoimmunity. J Exp Med 2000, 191:2021–2027
60. Waldner H, Collins M, Kuchroo VK: Activation of antigen-presenting cells by microbial products breaks self tolerance and induces autoimmune disease. J Clin Invest 2004, 113:990–997
61. McMahon EJ, Bailey SL, Castenada CV, Waldner H, Miller SD: Epitope spreading initiates in the CNS in two mouse models of multiple sclerosis. Nat Med 2005, 11:335–339
62. McRae BL, Vanderlugt CL, Dal Canto MC, Miller SD: Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. J Exp Med 1995, 182:75–85
63. Vanderlugt CL, Neville KL, Nikcevich KM, Eagar TN, Bluestone JA, Miller SD: Pathologic role and temporal appearance of newly emerging autoepitopes in relapsing experimental autoimmune encephalomyelitis. J Immunol 2000, 164:670–678
64. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ: IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med 2005, 201:233–240
65. 't Hart BA, Brok HP, Remarque E, Benson J, Treacy G, Amor S, Hintzen RQ, Laman JD, Bauer J, Blezer EL: Suppression of ongoing disease in a nonhuman primate model of multiple sclerosis by a human-anti-human IL-12p40 antibody. J Immunol 2005, 175:4761–4768
66. Uronen-Hansson H, Allen J, Osman M, Squires G, Klein N, Callard RE: Toll-like receptor 2 (TLR2) and TLR4 are present inside human dendritic cells, associated with microtubules and the Golgi apparatus but are not detectable on the cell surface: integrity of microtubules is required for interleukin-12 production in response to internalized bacteria. Immunology 2004, 111:173–178
67. Viala J, Sansonetti P, Philpott DJ: Nods and 'intracellular' innate immunity. C R Biol 2004, 327:551–555
68. Esen N, Tanga FY, DeLeo JA, Kielian T: Toll-like receptor 2 (TLR2) mediates astrocyte activation in response to the Gram-positive bacterium Staphylococcus aureus. J Neurochem 2004, 88:746–758
69. Kielian T, McMahon M, Bearden ED, Baldwin AC, Drew PD, Esen N: S. aureus-dependent microglial activation is selectively attenuated by the cyclopentenone prostaglandin 15-deoxy-delta12,14-prostaglandin J2 (15d-PGJ2). J Neurochem 2004, 90:1163–1172
70. Olson JK, Miller SD: Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. J Immunol 2004, 173:3916–3924
71. Ladesic B, Perovic S, Hrsak I: Pharmacokinetics of an immunomodulator peptidoglycan monomer in mice after intravenous administration. Int J Immunopharmacol 1993, 15:145–150
72. Zhang X, Rimpilainen M, Šimelyte E, Toivanen P: Enzyme degradation and proinflammatory activity in arthritogenic and nonarthritogenic Eubacterium aerofaciens cell walls. Infect Immun 2001, 69:7277–7284
73. Sun B, Rempel HC, Pulliam L: Loss of macrophage-secreted lysozyme in HIV-1-associated dementia detected by SELDI-TOF mass spectrometry. AIDS 2004, 18:1009–1012

74. Ulvestad E, Williams K, Mork S, Antel J, Nyland H: Phenotypic differences between human monocytes/macrophages and microglial cells studied in situ and in vitro. J Neuropathol Exp Neurol 1994, 53:492–501
75. Adams CW, Poston RN, Buk SJ: Pathology, histochemistry and immunocytochemistry of lesions in acute multiple sclerosis. J Neurol Sci 1989, 92:291–306
76. Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, Vyas JM, Boes M, Ploegh HL, Fox JG, Littman DR, Reinecker HC: CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science 2005, 307:254–258
77. Schrijver IA, Melief MJ, Eulderink F, Hazenberg MP, Laman JD: Bacterial peptidoglycan polysaccharides in sterile human spleen induce proinflammatory cytokine production by human blood cells. J Infect Dis 1999, 179:1459–1468
78. Gilbart J, Fox A: Elimination of group A streptococcal cell walls from mammalian tissues. Infect Immun 1987, 55:1526–1528
79. Stimpson SA, Esser RE, Cromartie WJ, Schwab JH: Comparison of in vivo degradation of 125I-labeled peptidoglycan-polysaccharide fragments from group A and group D streptococci. Infect Immun 1986, 52:390–396
80. Schrijver IA, Melief MJ, Markusse HM, Van Aelst I, Opdenakker G, Hazenberg MP, Laman JD: Peptidoglycan from sterile human spleen induces T-cell proliferation and inflammatory mediators in rheumatoid arthritis patients and healthy subjects. Rheumatology 2001, 40:438–446
81. Lehtonen L, Eerola E, Oksman P, Toivanen P: Muramic acid in peripheral blood leukocytes of healthy human subjects. J Infect Dis 1995, 171:1060–1064
82. Sartor RB, Bond TM, Compton KY, Cleland DR: Intestinal absorption of bacterial cell wall polymers in rats. Adv Exp Med Biol 1987, 216A:835–839
83. Jack CS, Arbour N, Manusow J, Montgrain V, Blain M, McCrea E, Shapiro A, Antel JP: TLR signaling tailors innate immune responses in human microglia and astrocytes. J Immunol 2005, 175:4320–4330
84. Kielian T, Mayes P, Kielian M: Characterization of microglial responses to Staphylococcus aureus: effects on cytokine, costimulatory molecule, and Toll-like receptor expression. J Neuroimmunol 2002, 130:86–99
85. Kielian T, Esen N, Bearden ED: Toll-like receptor 2 (TLR2) is pivotal for recognition of S. aureus peptidoglycan but not intact bacteria by microglia. Glia 2005, 49:567–576