Proinflammatory Bacterial Peptidoglycan as a Cofactor for the Development of Central Nervous System Autoimmune Disease

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Upon stimulation by microbial products through TLR, dendritic cells (DC) acquire the capacity to prime naive T cells and to initiate a proinflammatory immune response. Recently, we have shown that APC within the CNS of multiple sclerosis (MS) patients contain peptidoglycan (PGN), a major cell wall component of Gram-positive bacteria, which signals through TLR and NOD. In this study, we report that *Staphylococcus aureus* PGN as a single component can support the induction of experimental autoimmune encephalomyelitis (EAE) in mice, an animal model for MS. Mice immunized with an encephalitogenic myelin oligodendrocyte glycoprotein peptide in IFA did not develop EAE. In contrast, addition of PGN to the emulsion was sufficient for priming of autoreactive Th1 cells and development of EAE. In vitro studies demonstrate that PGN stimulates DC-mediated processes, reflected by increased Ag uptake, DC maturation, Th1 cell expansion, activation, and proinflammatory cytokine production. These data indicate that PGN-mediated interactions result in proinflammatory stimulation of Ag-specific effector functions, which are important in the development of EAE. These PGN-mediated processes may occur both within the peripheral lymph nodes as well as in the CNS and likely involve recognition by TLR on DC. Thus, PGN may provide a physiological trigger of DC maturation, and in this way disrupt the normal tolerance to self Ag. As such, PGN signaling pathways may serve as novel targets for the treatment of MS. *The Journal of Immunology,* **2005, 174: 808–816.**

Multiple sclerosis (MS)[2] is considered to be a chronic autoimmune disease of the CNS of unknown etiology critically driven by $CD4^+$ Th1 cells. However, the presence of autoreactive T cells is clearly not the single determining factor for the development of MS, because myelin-reactive T cells are also found in healthy individuals (1, 2). This implies that other factors play an important role in the initiation and perpetuation of pathological autoantigen-specific immune processes.

Bacteria and viruses are prominent cofactors that have been implicated in autoimmune disease initiation and persistence (3). As such, it has been shown that MS relapses are frequently associated with antecedent infections (4, 5), which in some cases are bacterial (6). In experimental autoimmune encephalomyelitis (EAE), an animal model for MS, a strong adjuvant containing killed whole *Mycobacterium tuberculosis* (CFA) is required for disease induction. When IFA emulsion is being used, animals will not develop EAE (7, 8). Microbial components, such as CpG-DNA and LPS, are physiological stimuli of EAE development via innate receptors on APC (9–11). These data indicate that noninfectious microbial components contribute to disease development in EAE and likely in MS.

Peptidoglycan (PGN) is an important bacterial cell wall component implicated in chronic inflammation (12). We have previously demonstrated that PGN isolated from sterile human spleen stimulates T cell proliferation and cytokine production (13). Furthermore, we have shown that phagocytic cells may distribute PGN to sites of chronic inflammation. At these sites, PGN-containing dendritic cells (DC) and macrophages (Mϕ) are present in high numbers and express costimulatory molecules and cytokines, as revealed by extensive in situ analysis of MS brain tissue (14) and rheumatoid arthritis synovial tissue (15, 16). In conclusion, these data support the concept that biologically active PGN can be transported by APC to lymphoid tissues and sites of chronic inflammation. Therefore, we hypothesized that PGN acts as a supporting factor of autoimmune processes in MS.

As a mechanism of action, PGN can activate the innate immune system through binding of extracellular and intracellular receptors (17–19). Extracellular PGN has been claimed to bind TLR2 in association with CD14, expressed on the cell surface of APC. Some internalized TLR ligands can also engage TLR in phagosomes (20). In addition, two new intracellular receptors for PGN fragments, NOD1/2 (CARD4/15) have been described recently (21, 22). Although high numbers of immunocompetent PGN-containing APC were found in MS brain tissue by in situ analysis, a functional role for PGN in the pathogenesis of MS remains to be elucidated. Therefore, we assessed the role of PGN in the development of mouse EAE. To achieve this, mice were immunized with autoantigen emulsified in IFA supplemented with PGN. We used PGN derived from *Staphylococcus aureus*, which does not contain the motif required for NOD1 ligation (22), but contains the motif necessary for NOD2 ligation (23, 24). Mice were assessed for EAE development, and draining lymph nodes were analyzed

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[2] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; DC, dendritic cell; Mϕ, macrophage; MHC-II, MHC class II; PGN, peptidoglycan; sPGN, soluble PGN; iPGN, insoluble PGN; NAMLAA, *N*-acetylmuramyl-L-alanine amidase; PGRP-L, PGN recognition protein-L; MOG, myelin oligodendrocyte glycoprotein; TCM, cell culture medium; DAP, diaminopimelic acid.

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for the presence of PGN-containing cells. Because DC play a prominent role in linking the innate and adaptive immune response, we assessed the functional role of PGN in Ag uptake, DC maturation, and T cell polarization. Taken together, our data show that proinflammatory PGN stimulates the development of autoimmune-mediated processes in EAE.

Materials and Methods

Mice

Female C57BL/6 mice (8–12 wk old) and BALB/c mice (6–10 wk old) were purchased from Harlan (Horst, The Netherlands). $OVA_{323–339}$-specific, MHC class II (MHC-II)-restricted, TCR transgenic (OT-II) mice, backcrossed on the C57BL/6 background, were bred within the facility. All animal experiments were performed with approval of the Erasmus University MC committee (Rotterdam, The Netherlands) for animal ethics. Mice were housed under specified pathogen-free conditions and received water and food ad libitum. Paralyzed mice with EAE scores >2.5 were afforded easier access to food and water.

Peptides, Abs, and PGN

Myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) was a kind gift from Dr. U. Günthert (Basel University, Basel, Switzerland). Soluble PGN (sPGN) was prepared from *S. aureus* by gel-permeation chromatography (25), and insoluble PGN (iPGN) was prepared with standard methods. The LPS content of both PGN fractions was rigorously checked and was <13 pg of LPS per milligram of PGN as measured by *Limulus* amebocyte lysate assay, which are background values. Absence of LPS was further confirmed by in vitro culture with mouse bone marrow-derived DC. Purification and identification of the biologically active components in the sPGN fractions are currently being performed, and might reveal other TLR2 agonists as recently described by Travassos et al. (26).

PGN of all bacterial species is composed of two alternating sugar residues, *N*-acetyl glucosamine and *N*-acetyl muramic acid, forming glycan strands. These strands are connected via stem peptides that differ from one bacterial species to another, and that have the sequence L-alanine-D-isoglutamine-L-lysine-D-alanine-D-alanine in *S. aureus*. In addition, PGN from *S. aureus* contains pentaglycine bridges. The complete PGN network is a highly complex ordered network, the detailed structure of which is a subject of debate to this day (27). The two main hydrolyzing enzymes capable of PGN degradation are lysozyme and *N*-acetylmuramyl-L-alanine amidase (NAMLAA), which is identical with PGN recognition protein-L (PGRP-L) (28, 29). Lysozyme hydrolyzes the bond between the two sugar moieties, whereas NAMLAA/PGRP-L hydrolyzes the lactate bond between *N*-acetyl muramic acid and the amino group of the first L-alanine.

Induction and clinical evaluation of EAE

C57BL/6 mice were immunized s.c., at four sites (axillar and inguinal), with 200 μg of $MOG_{35–55}$ in 0.1 ml of PBS emulsified in an equal volume of CFA containing 200 μg of *M. tuberculosis* (H37/Ra; Difco Laboratories). Additionally, mice were injected i.p. with 200 ng of *Bordetella pertussis* toxin in 0.2 ml of PBS on days 0 and 2 after immunization (Sigma-Aldrich). Mice were weighed and scored for clinical signs of EAE daily according to the following scoring system: 0, no disease; 0.5, partial tail paralysis; 1, complete tail paralysis; 1.5, limb weakness without tail paralysis; 2, limb weakness and tail paralysis; 2.5, partial limb and tail paralysis; 3, complete hind or front limb paralysis; 3.5, paraplegia; 4, quadriplegia; 5, death due to EAE.

Histology

The immunohistochemical techniques used in this study have previously been described in detail (30, 31). In brief, frozen sections of 6 μm were thaw-mounted on gelatin-coated glass slides and stored overnight in a humidified atmosphere. Sections were air-dried for 1 h and fixed at room temperature in fresh acetone containing 0.02% (v/v) H_2O_2. Then the slides were air-dried for 10 min, washed with PBS, and incubated with predetermined optimal dilutions of reagents overnight at 4°C in a humidified atmosphere. Secondary and tertiary reagents were applied and incubated for 1 h at room temperature. Between each incubation, slides were washed twice with PBS/0.05% Tween 20. HRP converted 3-amino-9-ethyl-carbazole into a bright red precipitate upon incubation for 10 min. Incubation of slides with naphthol-AS-MX phosphate (Sigma-Aldrich) and fast blue BB base (Sigma-Aldrich) for 30 min at 37°C resulted in a blue precipitate indicating alkaline phosphatase activity. As a negative control, the primary Ab was omitted. Nuclei were counterstained by hematoxylin.

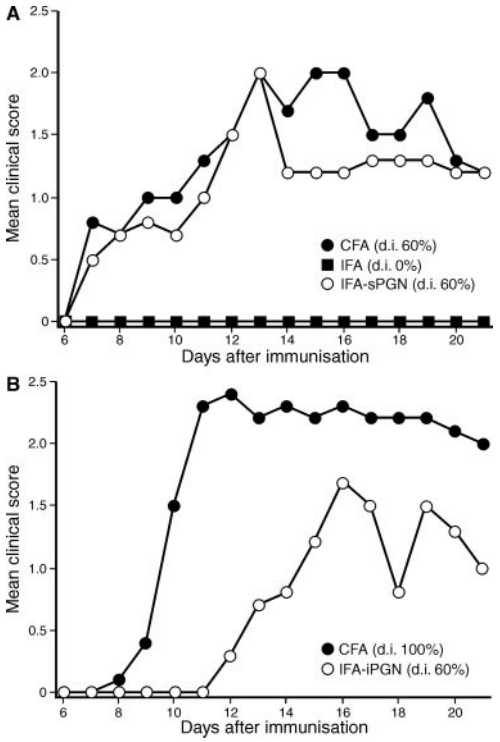

FIGURE 1. Both sPGN and iPGN are potent adjuvants for the induction of EAE. *A* and *B*, In two separate experiments, C57BL/6 mice were immunized with $MOG_{35–55}$ emulsified in CFA (●). In *A*, mice were immunized with $MOG_{35–55}$ in IFA (■), or IFA-25 μg sPGN (○). In *B*, mice were immunized with IFA-400 μg iPGN (○). Mice were weighed and scored for clinical signs of EAE daily. Five animals were used for each group. Graphs show mean clinical scores of animals that developed EAE. d.i., Disease incidence.

Four to 7 wk after immunization, mice were euthanized using CO_2, and brain tissues were snap frozen in liquid nitrogen and stored at −80°C. To determine the cellular infiltration in the CNS, immunohistochemical stainings were performed on frozen sections with anti-mouse B220 (RA-3-6B2; BD Pharmingen), anti-human cross-reactive to mouse CD3 (DakoCytomation), and anti-mouse MHC-II (M5/114). For CD3, donkey anti-rabbit Ig-biotin (Amersham Biosciences) was used as a conjugate. For B220 and MHC-II, rabbit anti-rat IgG-biotin (DakoCytomation) was used as a conjugate. As a tertiary step, slides were incubated with ABComplex-HRP (DakoCytomation).

To assess the presence of PGN within the draining lymph nodes, we isolated axillary and inguinal lymph nodes at 4 and 96 h after immunization. Lymph nodes were snap frozen in liquid nitrogen and stored at −80°C. A double-staining procedure was used to determine which cell types contain PGN. PGN-containing cells were detected with mAb 15704-biotin (Gentaur Molecular Products) raised against *S. aureus* PGN, followed by streptavidin-FITC (BD Biosciences). DC were detected by incubation with anti-mouse CD11c-PE (HL3; BD Biosciences).

Uptake of OVA by DC

DC were prepared as previously described (32). Briefly, bone marrow was flushed with RPMI 1640 (Invitrogen Life Technologies) from femurs and tibiae of C57BL/6 mice. Cells were washed, enumerated, and plated in bacteriological 100-mm diameter petri dishes. Cell culture medium (TCM) was RPMI 1640 supplemented with gentamicin (60 μg/ml; Invitrogen Life Technologies), 2-ME (5×10^{-5} mol/L; Sigma-Aldrich) and 5% (v/v) FCS (Biocell Laboratories). At day 0 of the culture, the cells were seeded at a concentration of 2×10^6/dish in medium containing recombinant mouse GM-CSF (200 IU/ml; kindly provided by K. Thielemans (University of Brussels, Brussels, Belgium)). At day 3, TCM containing 200 IU/ml recombinant mouse GM-CSF was added. At days 6 and 8, half of the medium was collected and centrifuged, and the pellet was resuspended in TCM containing 200 IU/ml recombinant mouse GM-CSF. At day 9, 100 μl TCM containing 1×10^5 DC were plated in round-bottom 96-well plates for 30 min at 4 or 37°C. DC were pulsed with different concentrations (0,

Table I. *Clinical parameters of EAE induced by sPGN or iPGN*

Adjuvant	PGN (μg/animal)	Disease Incidence (%) $n = 5$	Onset Day[a]	Maximum Score[a]	Cumulative Score[a] (day 20)
CFA	n.a.[b]	60	8.7 ± 2.9	2.0	20.2 ± 8.8
IFA	0	0	0	0	0
IFA-sPGN	25	60	7.0	2.0	15.8 ± 8.8
	250	0	0	0	0
CFA[c]	n.a.	100	9.5 ± 1.6	2.7 ± 0.5	24.0 ± 4.6
IFA-iPGN	25	0	0	0	0
	200	0	0	0	0
	250	40	12.0	2.0	11.0 ± 4.2
	400	60	13.7 ± 1.5	1.7 ± 0.6	9.8 ± 6.7
	600	0	0	0	0

[a] Represented values are from animals that developed EAE.
[b] n.a., Not applicable.
[c] Mean values of two separate experiments.

0.01, 0.1, 1 mg/ml) of OVA-FITC (Molecular Probes). The endotoxin level of FITC-OVA determined by a *Limulus* amebocyte lysate assay (BioWhittaker) was <0.001 μg, which was previously reported not to affect DCs (33). DC were stimulated with 10 μg/ml sPGN or iPGN. Cells were stained for 30 min at 4°C with anti-CD11c-allophycocyanin (HL3; BD Biosciences) dissolved in PBS containing 0.5% BSA and 0.01% sodium azide. Uptake of OVA-FITC was determined on CD11c-allophycocyanin-gated cells by flow cytometry. Dead cells and debris were excluded using propidium iodide.

DC maturation assay

At day 9 of the culture, DC were pulsed overnight with either 100 ng/ml LPS, or 10 μg/ml sPGN or iPGN. To exclude LPS contamination of the PGN preparation, polymyxin B was added to the culture. Cells were centrifuged, and supernatants were collected for cytokine analysis (IL-6, IL-10, IL-12p70, and TNF-α). The maturation state of DC was determined by staining for 30 min with mAbs anti-CD11c-allophycocyanin, anti-MHC-II-FITC (M5/114.5.2), in combination with anti-CD40-PE (3/23), anti-CD80-PE (16-10A1), and anti-CD86-PE (GL-1) (all obtained from BD Biosciences) diluted in PBS containing 0.5% BSA and 0.01% sodium azide. Maturation of DC was determined on CD11c-allophycocyanin-gated cells by flow cytometry. Dead cells and debris were excluded using propidium iodide.

OVA-specific T cell proliferation assay

DC were obtained from bone marrow culture as described above. At day 9 of culture, DC were plated in 24-well plates containing 2 ml of TCM, and pulsed with 10 mg/ml OVA-Worthington (Biochemical) for 24 h. At the dose we used in our experiments, the endotoxin level of OVA measured by a *Limulus* amebocyte lysate assay (BioWhittaker) was <0.001 μg. Simultaneously, DC were stimulated with 100 ng/ml LPS (*Escherichia coli*, strain O26:B6; Sigma-Aldrich), 10 μg/ml sPGN, or iPGN. OT-II cells were purified as described previously (32). Briefly, lymph nodes and spleens were collected from OT-II mice. After RBC lysis, cells were labeled with CFSE. Cells were enumerated, and dead cells, stained for trypan blue, were excluded. After 24 h, DC were washed and 1×10^4 DC were cocultured with 1×10^5 CFSE-labeled OVA TCR transgenic $CD4^+$ OT-II T cells in round-bottom 96-well plates containing 200 μl of TCM. Cells were centrifuged, and supernatants were harvested and stored at −20°C for cytokine analysis. Cells were resuspended and incubated with anti-Vα2-biotin and Vβ5.1/5.2-PE TCR mAbs (eBioscience) for 30 min at 4°C. Biotinylated anti-Vα2 was detected using streptavidin-allophycocyanin (BD Biosciences). Percentages of proliferating OVA-specific $CD4^+$ T cells were determined by gating on cells that were double positive for Vα2 and Vβ5.1/5.2. Dead cells and debris were excluded using propidium iodide.

MOG_{35-55}-specific T cell proliferation assay

For evaluation of MOG_{35-55}-specific lymph node cell proliferation, mice were immunized with MOG_{35-55} in adjuvant. Mice were sacrificed, and draining inguinal, brachial, and axillar lymph nodes were isolated at 33 days after immunization. Lymph node cells were cultured for 4 days in 96-well plates in 200 μl of RPMI 1640 supplemented with 10% heat-inactivated FCS (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker). Cells (4×10^5/well) were cultured at 37°C

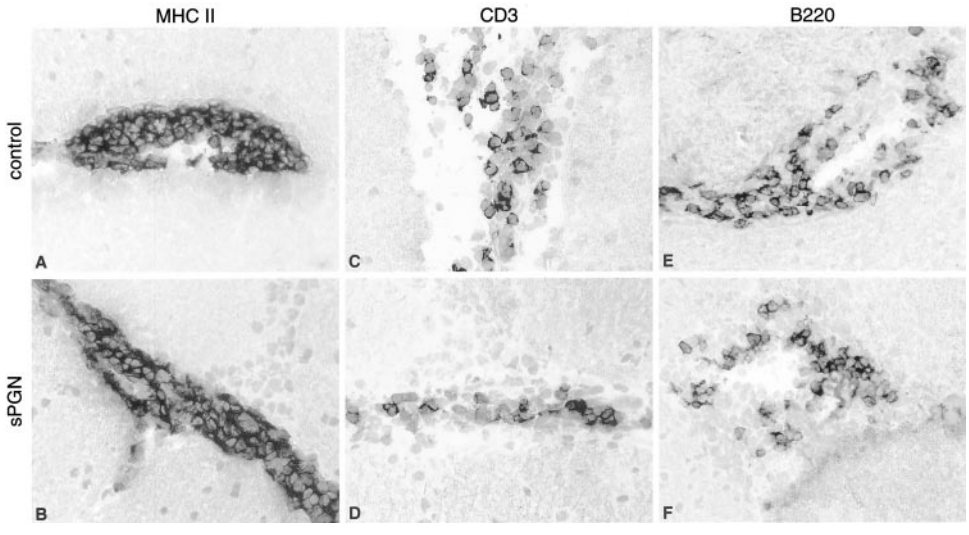

FIGURE 2. Immunization with PGN-containing adjuvant induces classical EAE histopathology. Brain tissues were isolated between 4 and 7 wk after immunization. Frozen brain sections were stained with hematoxylin in combination with MHC-II (*A* and *B*), CD3 (*C* and *D*), and B220 (*E* and *F*).

Table II. *Histological analysis of infiltrates in EAE brain tissue*

Adjuvant	PGN (μg/animal)	Number[a]	Size[b]
CFA	n.a.[c]	>10	+
	n.a.	>10	+
	n.a.	4–10	+
	n.a.	4–10	+
	n.a.	>10	+
IFA-sPGN	25	>10	+
	25	4–10	+
	25	>10	+
IFA-iPGN	250	4–10	−/+
	250	4–10	+
	400	1–3	−/+
	400	1–3	−/+
	400	4–10	−/+

[a] Number, Number of inflammatory foci.
[b] Infiltrate size, −, no infiltrating cells; −/+, 1–5 cells; +, 6–20 cells.
[c] n.a., Not applicable.

and 5% CO_2 in the presence or absence of 10 μg/ml $MOG_{35–55}$. After 72 h of culture, 1 μCi of [^{3}H]thymidine (Amersham Biosciences) was added for 16 h. Incorporation of [^{3}H]thymidine was measured in triplicate using a filtermat harvester and a beta plate counter (PerkinElmer). Supernatants from $MOG_{35–55}$ proliferation assays were harvested 96 h after culture, centrifuged, and stored for further cytokine analysis at −20°C.

Cytokine measurement by ELISA

According to the manufacturer's instructions, concentrations of IL-4, IL-10, IL12-p70, IFN-γ, and TNF-α were determined by using OptEIA ELISA kits (BD Biosciences).

Statistical evaluations

Statistical evaluation was performed using SPSS 11 software. The Mann-Whitney *U* test was used to analyze differences in cytokine production and T cell proliferation. A value of $p < 0.05$ was considered statistically significant.

Results

Both sPGN and iPGN are potent adjuvants for the induction of EAE. PGN is a known proinflammatory stimulator of the innate immune response, but very little is known about the possible role of PGN in the development of MS and EAE. To determine whether PGN by itself is a sufficient proinflammatory stimulus for the development of EAE, we added highly purified sPGN or iPGN from *S. aureus* to an emulsion of IFA and $MOG_{35–55}$. In three independent experiments, standard induction of EAE by immunization of mice with $MOG_{35–55}$ in CFA resulted in an EAE incidence of 60–100%, with a normal EAE course (Fig. 1 and Table I). Mice immunized with $MOG_{35–55}$ in IFA, as a negative control, did not develop EAE, as expected. In contrast, addition of sPGN or iPGN to IFA/$MOG_{35–55}$ induced EAE with a disease incidence from 40 to 60%. Different dosages of sPGN and iPGN were used for EAE induction. High dosages of 250 μg of sPGN and 600 μg of iPGN did not induce EAE (Table I). Importantly, however, lower PGN dosages induced EAE with an incidence of 40–60%. The lowest effective dose for EAE induction with sPGN (25 μg/animal) was 10 times lower than with iPGN, in keeping with the higher biological activity described for sPGN previously. These data demonstrate that PGN is a potent adjuvant and can substitute whole *M. tuberculosis* in the induction for EAE.

PGN-adjuvant induces classical EAE histopathology

Brain tissue was examined by histology to assess whether the signs of paralysis of mice immunized with PGN-containing adjuvant are associated with a characteristic EAE histopathology. In brain tissue of animals that developed EAE, infiltrates were found containing $CD3^+$ T cells, $B220^+$ B cells, and MHC-II^+ APC (Fig. 2). A comparable number of infiltrates and infiltrating cells were detected in EAE mice injected with CFA and IFA-sPGN, whereas the infiltrate size and number of infiltrating cells in IFA-iPGN-injected EAE mice was slightly lower (Table II). These data confirm that paralysis induced with PGN-containing adjuvant is associated with a characteristic EAE histopathology.

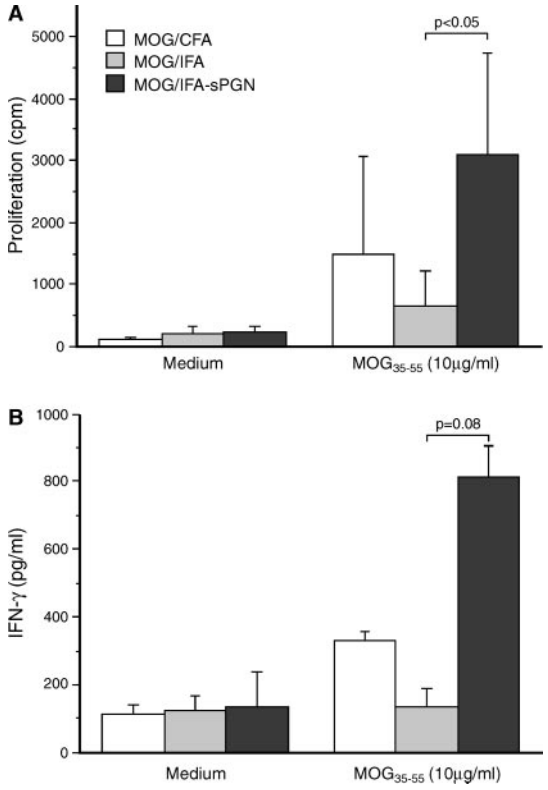

FIGURE 3. Immunization with PGN-containing adjuvant stimulates the development of $MOG_{35–55}$-specific Th1 cells. Draining lymph nodes from five individual mice per group were harvested at day 33 after immunization. *A*, Cells were stimulated in vitro with 10 μg/ml $MOG_{35–55}$, and proliferation was measured after 96 h of culture by [^{3}H]thymidine incorporation. *B*, Supernatants were harvested after 96 h of culture and measured by ELISA for IFN-γ content. Mean values per group ± SD are shown.

PGN-containing adjuvant promotes $MOG_{35–55}$-specific Th1 cell polarization

It has been well established that EAE induction by immunization with an encephalitogenic Ag in CFA results in the generation of autoantigen-specific $CD4^+$ IFN-γ-producing T cells (34). To address whether the PGN component of bacteria is able to generate $MOG_{35–55}$-specific Th1 cells, we determined $MOG_{35–55}$-specific T cell proliferation and IFN-γ, IL-4, and IL-10 production at day 33 after immunization. Lymph node cells from mice immunized with $MOG_{35–55}$ in CFA proliferated dose dependently and produced IFN-γ upon in vitro restimulation with $MOG_{35–55}$ (Fig. 3). Mice immunized with $MOG_{35–55}$ in IFA also showed Ag-specific proliferation, but only low amounts of IFN-γ were produced. Addition of sPGN to the immunization mixture resulted in increased Ag-specific proliferation and IFN-γ production compared with immunization with IFA/$MOG_{35–55}$. IL-4 and IL-10 were undetectable in any of the groups. These data show that PGN creates an inflammatory environment. In combination with autoantigen presentation, PGN can effectively induce autoantigen-specific Th1 cells.

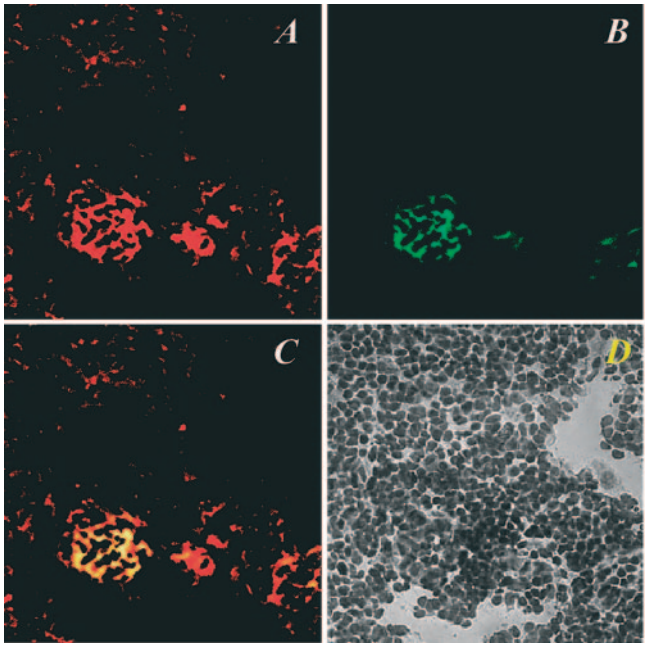

FIGURE 4. PGN is redistributed from the site of immunization to DC in draining lymph nodes. Draining lymph nodes were isolated at 4 h after s.c. immunization with $MOG_{35–55}$ in IFA-sPGN. Frozen sections were stained by immunofluorescence for DC (CD11c in red, *A*) and for PGN (green, *B*). Overlay in *C* demonstrates clusters of DC containing PG. *D* is a light-microscopic image of the same area.

PGN is redistributed from the site of immunization to the draining lymph nodes

To determine whether PGN is transported from the site of immunization to secondary lymphoid organs during EAE development, we injected PGN in combination with IFA and $MOG_{35–55}$ and isolated draining lymph nodes at different time points after immunization. Two mice per group were injected with either 25 μg of sPGN or 400 μg of iPGN in IFA/$MOG_{35–55}$ emulsions, dosages of PGN optimal for EAE induction. Two naive mice were used as negative controls. Axillary, brachial, and inguinal lymph nodes were isolated and frozen at 4 and 96 h after immunization. Per mouse, four lymph nodes were analyzed for the presence of PGN-containing cells. In naive mice, individual PGN-containing cells were occasionally found in three of eight lymph nodes (38%), in agreement with previous observations (35, 36). In contrast, we detected several clusters of PGN-containing cells in >90% of the draining lymph nodes from mice immunized with sPGN and iPGN. The number of PGN-containing clusters did not vary between the time points (data not shown). These data indicate that APC can take up and transport PGN from the site of immunization to the draining lymph nodes within hours. Double-labeling experiments identified cells in the PGN-containing clusters as DC expressing CD11c (Fig. 4). PGN-containing clusters may thus contribute to the development of a proinflammatory environment to help generate $MOG_{35–55}$-specific Th1 cells.

Stimulation of DC by PGN increases protein uptake and induces DC maturation

At the site of immunization, PGN can be recognized by different cell types, i.e., DC, Mϕ, and granulocytes. Because PGN was mainly observed in DC in the lymph nodes, we assessed whether PGN stimulates protein uptake, cell maturation, T cell stimulation, and polarization by this APC subset crucial for priming of T cells. For these experiments, immature mouse bone marrow-derived DC were used. To examine the effects of PGN on protein uptake, DC were incubated with OVA conjugated to FITC in the presence or absence of 10 μg/ml PGN at 4 or 37°C. Different concentrations of OVA-FITC (0, 0.01, 0.1, and 1 mg/ml) were applied, and uptake was determined by flow cytometry. Incubation at 4°C showed a dose-dependent passive uptake of OVA-FITC (data not shown). Addition of PGN did not alter the uptake of OVA-FITC at 4°C (Fig. 5*A*). When cells were cultured at physiological temperature (37°C), both sPGN and iPGN stimulated the uptake of OVA-FITC (Fig. 5*B*).

To assess the effects of PGN on DC maturation, we stimulated DC with sPGN or iPGN. As a positive control, DC were stimulated with LPS. Maturation of DC was determined by the expression of MHC-II, CD40, CD80, and CD86, and by the induction of several cytokines. Both sPGN and iPGN stimulated expression of MHC-II, CD40, CD80, and CD80. Stimulation of DC with sPGN efficiently induced expression of MHC-II, CD40, CD80 and CD86 to a similar or higher level as compared with LPS stimulation. These molecules were also induced by iPGN, to a similar or lower level compared with LPS (Fig. 6*A*). Both sPGN and iPGN induced production of several cytokines, but with a different pattern of production. Whereas sPGN induced production of all proinflammatory cytokines measured, iPGN induced TNF-α, but not IL-6 and IL-12 (Fig. 6*B*). IL-10 was not produced under these conditions (data not shown). As predicted, addition of the LPS antagonist polymyxin B effectively inhibited LPS-induced maturation marker expression and cytokine production (Fig. 6*B*), whereas no inhibition was seen when cells were stimulated with PGN, demonstrating that the effects of PGN were not the result of LPS contamination. Taken together, these data demonstrate that both forms of PGN can stimulate protein uptake and induce DC maturation in vitro.

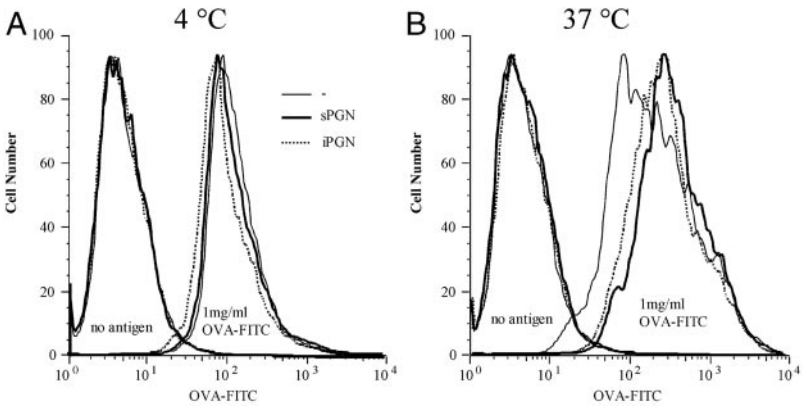

FIGURE 5. Stimulation of DC by PGN increases protein uptake. Immature DC were cultured in the absence or presence of OVA-FITC. DC were either not stimulated (thin lines) or stimulated with sPGN (thick lines) or iPGN (dotted lines). Uptake of OVA-FITC was determined at 4°C (*A*) or 37°C (*B*) after 30 min of incubation. Histograms represent the number of CD11c-gated cells.

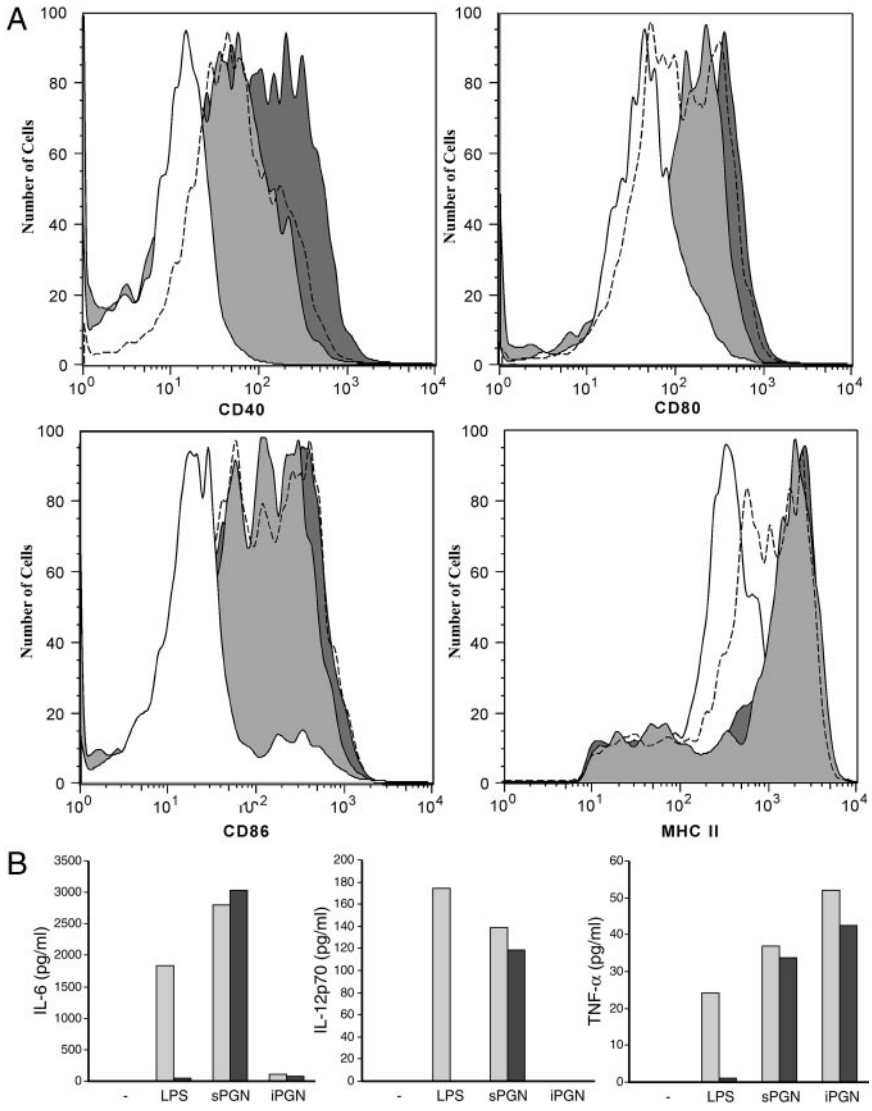

FIGURE 6. PGN induces maturation of bone marrow-derived DC. Immature DC were either not stimulated (white histogram, closed line) or stimulated with LPS (white histograms, dotted line), sPGN, or iPGN (light-gray and dark-gray histograms, respectively). *A*, Maturation of DC was determined by measuring expression of MHC-II, CD40, CD80, and CD86 by flow cytometry. Histograms represent the number of CD11c-gated cells. *B*, Supernatants were harvested, and production of IL-6, IL-12p70, and TNF-α was determined by ELISA. Black bars represent cells that were incubated with polymyxin B to exclude LPS contamination.

PGN-OVA-pulsed DC stimulate T cell proliferation and Th1 cell polarization

After Ag uptake and migration of DC to the draining lymph nodes, mature DC will prime naive T cells to proliferate and differentiate along a certain pathway, depending on the nature of the stimulus. To address whether sPGN and iPGN are able to stimulate Ag-specific T cell proliferation and Th1 cell polarization, we used an in vitro model system. Bone marrow-derived DC were cultured with OVA in combination with LPS, sPGN, or iPGN. After 24 h, the DC were incubated with CFSE-labeled naive $OVA_{323-339}$ TCR transgenic $CD4^+$ T cells. OVA-pulsed DC that were stimulated by LPS, sPGN, or iPGN, induced an increase in the OVA-specific T cell proliferation compared with unstimulated cells (Fig. 7, *B–E*). Furthermore, both forms of PGN induced significantly increased levels of IFN-γ compared with both nonstimulated and LPS-stimulated DC (Fig. 7*F*). Only low levels of IL-4 and IL-10 were detected in all OVA-pulsed DC-T cell cultures (data not shown). In summary, these data show that PGN stimulates Ag-specific T cell proliferation and Th1 cell development.

Discussion

Bacterial and viral components stimulate innate immune responses that potentially contribute to the development and persistence of autoimmune disease (3, 5). Although it is very difficult to demonstrate mechanistic links between microbial agents and MS, it has been shown epidemiologically that antecedent infections are associated with an increased risk of relapse development (4, 5). Components of bacterial agents may function as physiological adjuvants and stimulate autoantigen-specific adaptive immune responses. In this study, we show that PGN, a large bacterial cell wall component, can act as an environmental factor promoting CNS autoimmune disease development via innate immunity.

PGN from a vast array of different bacterial species can be derived from different anatomical sites, including all mucosa permanently exposed to the outside world. During bacterial infection, PGN can be released, either from bacterial cells upon replication or upon uptake and processing by APC. In the absence of infections, the major bacterial load is located at mucosal sites. At these sites, i.e., the gut, DC may sample bacteria through the intestinal epithelium and subsequently migrate to secondary lymphoid organs (37, 38). Even under normal circumstances, some PGN can be detected in the blood circulation, within the liver, and within lymphoid tissues, reflecting physiologic processes dealing with exposure to bacterial components (13, 39–45).

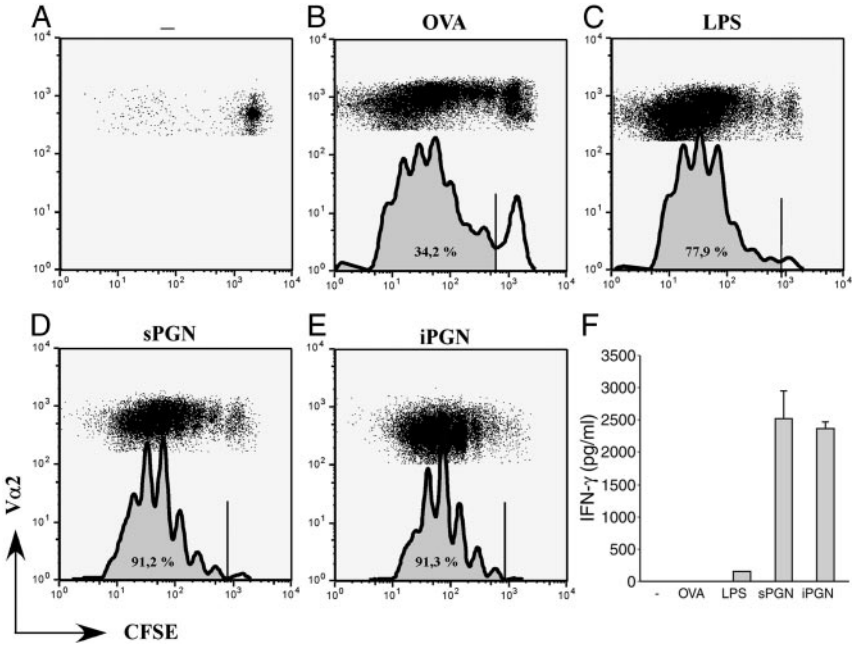

FIGURE 7. PGN stimulates Ag-specific T cell proliferation and Th1 cell polarization. Immature DC were not stimulated (*A*) or stimulated with OVA (*B*) combined with LPS (*C*), sPGN (*D*), or iPGN (*E*) for 24 h. $OVA_{323–339}$ TCR transgenic $CD4^+$ T cells were incubated with all DC populations. *A–E*, After 6 days of culture, $OVA_{323–339}$-specific T cell proliferation was assessed by flow cytometry using CFSE. *F*, Supernatants were harvested, and production of IFN-γ was determined by ELISA. Percentages indicate the number of divided $OVA_{323–339}$ $CD4^+$ T cells. Histograms represent the number of cell divisions.

We therefore hypothesized that bacterial PGN can contribute to disease development and progression in MS and EAE in the absence of infection or bacterial replication. To functionally test this hypothesis, we determined whether EAE could be induced by immunization of mice with an encephalitogenic peptide ($MOG_{35–55}$) emulsified in IFA supplemented with *S. aureus* PGN, asking whether PGN by itself is a sufficient proinflammatory cofactor for induction of EAE.

For our studies, we used two different types of *S. aureus* PGN. We observed functional differences between sPGN and iPGN in vivo as well as in vitro. Although EAE could be induced with both types of PGN, the effective dose for EAE induction was 10-fold lower for sPGN compared with iPGN. In vitro stimulation of DC with iPGN only induced TNF-α production, whereas sPGN could induce IL-6 and IL-12 in addition to TNF-α. These data implicate that partially degraded PGN is more effective in exerting proinflammatory effects than iPGN.

High dosages of both sPGN and iPGN were not able to induce EAE. The reasons for this are not clear but may be related to dose-dependent induction of Th1 vs Th2 responses by administration of TLR ligands in vivo (33). Furthermore, it has been shown that apoptosis of Mϕ is induced by PGN, as described by Fukui et al. (46). As recently shown, TLR engagement triggers DC apoptosis through up-regulation of the proapoptotic Bcl-2 family member Bim. In this way, both innate and acquired immune system signals cooperate to determine DC lifespan, and hence also the longevity of T-DC interaction (47, 48).

L-Lysine or diaminopimelic acid (DAP) at position 3 of the stem peptide are important structural components of PGN. DAP-type PGN is present in all Gram-negative bacteria, whereas most Gram-positive bacteria contain lysine-type PGN (49). The intracellular PGN-sensing molecules NOD1/2 discriminate between lysine-type and DAP-type PGN (50). NOD2 is a general sensor for both Gram-positive and Gram-negative bacteria, through the recognition of muramyl dipeptide, the minimal motif found in all PGNs (23, 24). In contrast to NOD2, NOD1 specifically recognizes DAP-type PGN (21, 22). Bacteria containing L-lysine can induce chronic arthritis, whereas bacteria with other peptides at this position are nonarthritogenic (51). Several other studies have also identified the presence of L-lysine as typical for proinflammatory PGN (52–54). Considering the arthritogenic effects of L-lysine at position 3 of the stem peptide, one might expect similar findings in EAE development. Indeed, we have now shown that EAE can be induced with lysine-type *S. aureus*-derived PGN as adjuvant.

Already at 4 h after immunization, PGN-containing clusters could be detected in the draining lymph nodes. These clusters persisted at least for 7 days after immunization. Others have also shown the persistence of PG-containing components until at least 63 days after injection of PGN-polysaccharides by muramic acid detection, a specific component of PGN, through gas chromatography and mass spectrometry (40). PGN degradation is mainly dependent on the enzymes lysozyme and NAMLAA/PGRP-L (55). Recently, it was found that the PGRP-L is an amidase (28, 29) and is identical with NAMLAA, which we have characterized in detail previously (55, 56). In contrast to granulocytes, Mϕ and DC lack NAMLAA/PGRP-L, which may result in incomplete or delayed PGN degradation and persistence of intracellular PGN. This may explain why DC in MS brain tissue and in EAE lymph node tissues contain PGN. The fact that PGN is observed both in lymph nodes and in the CNS is important, because PGN may act as a physiological proinflammatory adjuvant by stimulating autoantigen-specific immune responses. Indeed, in the current study, we could demonstrate that PGN-containing adjuvant enhanced $MOG_{35–55}$-specific lymph node cell proliferation and IFN-γ production at 4 wk after immunization.

DC recognize specific motifs on pathogens by expressing a diverse repertoire of pathogen-associated recognition receptors. Upon bacteria-induced stimulation, DC mature, migrate to the circulation, and prime the adaptive arm of the immune response by

initiating the development of effector T cells in secondary lymphoid organs (57). Because we detected PGN within DC in MS brain, rheumatoid arthritis synovium, and EAE lymphoid tissue, we addressed the functional role for PGN in EAE development by in vitro simulation of the different processes involving DC that occur during immunization for EAE development.

Early events after immunization include uptake of adjuvant and Ag by DC, inducing their maturation. These processes can be accelerated by whole *M. tuberculosis*-containing adjuvant as has been demonstrated in vivo (7). Also bacterial components like CpG-DNA are able to increase Ag uptake, provided that the Ag is covalently linked to CpG-DNA (58–60). In concordance with these studies, we now demonstrate that PGN increases Ag uptake by DC.

Upon stimulation with TLR ligands or cytokines, DC will mature and migrate to secondary lymphoid tissues to initiate T cell activation. It has been demonstrated that *S. aureus* PGN may serve as a maturation signal for murine DC, by inducing the expression of CD86 and MHC-II, and the production of MIP-2 and proinflammatory cytokines (61, 62). Also, human monocytes secrete several chemokines (IL-8, MIP-1α) and proinflammatory cytokines (TNF-α, IL-1, IL-6, and IL-12) after PGN stimulation, as shown by cDNA array analysis (63). In agreement with these studies, we now show that both forms of *S. aureus*-derived PGN induce DC maturation, as demonstrated by enhanced expression of MHC-II and costimulatory molecules, and production of proinflammatory cytokines. These proinflammatory cytokines, induced upon TLR stimulation, can exert direct effects on T cell polarization (i.e., IL-12) (64, 65) and T cell regulation (i.e., IL-6) (66) and have been implicated in the pathogenesis of MS as well as EAE (67–69).

Classical EAE induction is dependent on the presence of autoantigen and proinflammatory stimuli from adjuvant components (whole *M. tuberculosis*). A combination of both factors will result in the development of autoantigen-specific $CD4^+$ Th1 cells within the secondary lymphoid organs. Not much is known about the effect of PGN on T cell proliferation and differentiation. A recent report proposed that Th cell polarization is dependent on differential TLR ligation (70). Our in vitro studies demonstrate that OVA-pulsed DC did induce T cell proliferation but did not induce Th1 cell development. However, when OVA-pulsed DC were coincubated with PGN, DC were able to induce OVA-specific Th1 cell development, which confirms previous reports (71). In addition, PGN-stimulated OVA-DC increased the expansion of OVA-specific T cells, compared with OVA-pulsed DC alone. These data provide evidence that PGN stimulates DC-mediated processes and serves as a potent proinflammatory trigger for the development of autoantigen-specific Th1 cells.

In this study, we show that the TLR-NOD2 ligand *S. aureus* PGN acts as a proinflammatory factor that is involved in the development of autoimmune disease of the CNS. The contribution of PGN on disease development is likely dependent on DC-mediated processes, because PGN is located within DC in MS brain tissue and mouse EAE lymph node tissue. We have demonstrated that PGN is able to potentiate different DC-mediated processes such as Ag uptake, DC maturation, and subsequent induction and stimulation of autoantigen-specific Th1 cell development. Targeting PGN receptors or their respective signaling pathways may well become a novel therapeutic strategy for the treatment of autoimmune disease in humans.

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