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Encephalitogenicity of Complete Freund’s Adjuvant Relative to CpG Is Linked to Induction of Th17 Cells

Justine T. Tigno-Aranjuez,* Ritika Jaini,† Vincent K. Tuohy,*‡ Paul V. Lehmann,*† and Magdalena Tary-Lehmann1,*‡

For decades, CFA has been the classic adjuvant for the induction of experimental autoimmune encephalomyelitis (EAE). Its encephalitogenic activity has been originally linked to the induction of Th1 responses. CpG, which is also a potent Th1 inducer, has been suggested by some studies to be comparably encephalitogenic. In this study, using the SJL proteolipid protein (PLP) 139–151 peptide EAE model, we show that active immunizations using CFA but not CpG 1826/IFA as an adjuvant induced disease. Passive induction of EAE resulted in severe disease when cells were transferred from PLP in CFA-primed mice but resulted in only a mild, transient disease when cells originated from PLP in CpG 1826/IFA-primed mice. In accordance with these findings, immunizations using CFA but not CpG 1826/IFA as an adjuvant elicited a delayed-type hypersensitivity response. ELISPOT analysis revealed that CFA promoted the differentiation of much higher levels of IFN-γ-secreting cells and also induced Ag-specific proliferation to the same extent. The severity of EAE in PLP in CFA-immunized mice was reduced when IL-17 was neutralized in vivo, demonstrating the crucial role of this cytokine in disease induction. The data show that immunizations using the autoantigen in CpG 1826/IFA result in very low frequencies of Ag-specific IL-17 cells, suggesting a lower risk of Th17-mediated pathology when using this adjuvant. The Journal of Immunology, 2009, 183: 5654–5661.

Negative selection of autoreactive T cells in the thymus is frequently incomplete (1). Such T cells can migrate into the periphery and persist as naive T cells. It is believed that one of the potential mechanisms by which autoimmunity occurs is through activation of such T cells during cross-reactive infections or by immunizations (through the processes of molecular mimicry or bystander activation) (2). Indeed, many studies have shown an association between the occurrence of certain infectious diseases and the development of autoimmunity (reviewed in Ref. 3). The discovery of such a link has fueled interest in elucidating the pathogenic molecules involved in autoimmune disease exacerbation and the effects of such molecules on the immune system. Interestingly, in experimental autoimmune encephalomyelitis (EAE),† an animal model for multiple sclerosis, the classical method of inducing disease involves the administration of an autoantigen in CFA that is heat-killed mycobacteria in oil. In some cases, this is even accompanied by administration of pertussis toxin from Bordetella pertussis (4). It has been proposed by other groups that certain bacterial components, such as bacterial DNA or LPS, are capable of replacing the requirement for CFA in mediating EAE (5–7).

However, despite the fact that potentially autoreactive T cells always exist in considerable numbers in healthy individuals and that Ag exposures that result in the activation of such T cells are likely to be common, the occurrence of autoimmune disease is not a frequent event. This suggests that not only do naive autoreactive T cells need to be activated into effector cells, but to mediate disease they must differentiate into the appropriate effector class. For decades, Th1 cells were thought to be the pathogenic T cell population responsible for the destruction observed in EAE (8). Th1 cells are described as secreting IFN-γ and IL-2, and mediating delayed-type hypersensitivity (DTH) reactions (9–11). Positive DTH reactions have been associated with the development of good cellular immunity against foreign Ags. In the context of autoimmunity, however, DTH is considered harmful (12, 13). The hypothesis that Th1 cells were detrimental in the setting of autoimmunity continued despite results obtained from studies using IFN-γ-deficient mice and IFN-γ neutralization experiments, which still showed disease induction (14, 15). Recent work over the past few years, however, has implicated Th17 cells both in mediating autoimmune-related pathology and in eliciting DTH (16–18). Th17 cells are described as secreting IL-17, IL-6, TNF-α, IL-1β, IL-22, IL-21, and inflammatory chemokines, which function in the recruitment of neutrophils and macrophages to sites of infection (19). The importance of IL-17 in autoimmunity was initially demonstrated by Komiyama et al. (20). Using mice that were deficient in IL-17, this group was able to show that EAE occurred with delayed onset and severity compared with wild-type (WT) mice (20).

For many years, CFA has been considered a strong type 1 polarizing adjuvant. CpG oligodeoxynucleotides (ODNs) are sequences of unmethylated bacterial DNA that have previously been shown to function as type 1 adjuvants for CD4+ T cells, resulting

Neg...
in similar effects as CFA (21, 22). CpG ODN and CFA immunizations with hen egg lysozyme were both shown to induce IFN-γ- and IFN-γ-dependent Ab isotypes such as IgG2a (21). Despite these similarities, it is interesting to note that in the setting of autoimmunity, the encephalitogenic potential of CpG ODNs is still under question (5–7, 23–26). Given these findings, it was our aim to do a comparative analysis on the two adjuvants, CFA and CpG 1826/I/FA, and examine their effects on the induction of EAE, cytokine secretion, proliferation, regulatory T cell (Treg) induction, and elicitation of DTH.

In this study, we show that the immunization of mice with autoantigen in CFA but not CpG 1826/I/FA is capable of inducing EAE when used for the active induction of disease. Passive transfer of in vitro restimulated cells from CFA-immunized mice resulted in severe disease compared with the mild, transient disease when cells were transferred from CpG 1826/I/FA-immunized mice. We show that both adjuvants are capable of inducing the type 1 cytokines IFN-γ and IL-2 upon Ag recall and that both adjuvants induce similar levels of Ag-specific proliferation. However, only CFA immunizations resulted in IL-17 production and DTH. Furthermore, in vivo neutralization of IL-17 in mice immunized with proteolipid protein (PLP) peptide 139–151 in CFA reduced EAE disease severity. No differences in Treg frequencies were observed between the two groups. In summary, unlike CFA, CpG 1826/I/FA is an adjuvant that does not induce active disease, DTH, or high levels of the autoimmune-associated cytokine IL-17. These results provide valuable unappreciated differences between two seemingly similar adjuvants and suggest that CpG ODNs are safe to use in therapeutic settings where a Th17 response may be detrimental.

Materials and Methods

Mice, immunizations, peptides, and oligonucleotides

SJL/J female mice (6–8 wk old) were purchased from The Jackson Laboratory. Mice were maintained in the Cleveland Clinic Lerner Research Institute Biological Resources Unit (Cleveland, OH) and housed under pathogen-free conditions. Mice were immunized with 100 μg of Ag s.c. in the flank. The PLP peptide 139–151 (HSLKGLGHLPDPKF) was synthesized by Princeton Biomolecules. IFA was obtained by mixing one part maniide monoooleate (Sigma-Aldrich) and nine parts paraffin oil (EM Science). CFA was obtained by mixing 5 mg/ml heat-killed Mycobacterium tuberculosis H37Ra (Difco) into IFA. The ODN was used was CpG ODN 1826 (CpG 1826) with the sequence 5′-tcattgatctgccgtg-3′ (small letters indicate phosphorothioate linkage; bold letters indicate CpG dinucleotides). CpG 1826 was synthesized by Midland Certified Reagents and emulsified along with peptide in IFA to deliver 35 μg/mouse (Cpg1826/I/FA). All animal procedures were performed as written in a protocol reviewed and approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

Induction of EAE

For active EAE induction, SJL/J mice were immunized with 100 μg of PLP 139–151 in CFA s.c. in the flank. Mice were scored daily using the following scoring system: 1, flaccid tail; 2, paraparesis; 3, hind limb paralysis; 4, quadriplegia; and 5, moribund state or death. To ensure that mice who developed paralysis received adequate nutrition, Napa Nectar was supplied to the mice. Mice were examined daily. Data are represented as ear swelling (millimeters after challenge) and measured using a digital micrometer (Mitutoyo). After measurement, ear biopsies were then taken for histologic examination. Data are represented as ear swelling (millimeters after challenge − millimeters before challenge).

Thymidine proliferation on assay

Fourteen days after immunization, splenocytes were harvested from mice, depleted of RBC by ammonium chloride treatment, and 1 × 10⁶ cells were cultured with the indicated concentrations of PLP 139–151. On day 3, 50 μl of a 0.02mCi/ml solution of 3H (MP Biomedicals) was added to the wells and incubated for an additional 16 h at 37°C before harvesting and reading on a beta counter reader (PerkinElmer). Data are represented as stimulation indices in which proliferative responses to the Ag are divided by proliferative responses in the medium.

DTH assay

At day 14 postimmunization, mice were challenged with 10 μg of soluble Ag injected intradermally in the ear. The left ear was injected with the Ag of interest while the right ear was injected with an irrelevant Ag. Swelling was assessed 24 h postchallenge and measured using a digital micrometer (Mitutoyo). After measurement, ear biopsies were then taken for histologic examination. Data are represented as ear swelling (millimeters after challenge − millimeters before challenge).

In vivo IL-17 neutralization

For in vivo IL-17 neutralization assays, 6- to 8-wk old female SJL/J mice were immunized with 100 μg of PLP 139–151 in CFA s.c. in the flank. One hundred micrograms of an anti-mouse IL-17 neutralizing Ab (MAB421 clone 50104.11; R&D Systems) was administered i.p. at days 6, 10, and 14. Control groups were injected with 100 μg of an IgG2a isotype control Ab (MAB006 clone 54447.11; R&D Systems) at the same time points. Mice were scored daily as previously mentioned.
for immunohistochemical detection of demyelination. Among the 1826/IFA or CFA adjuvant. Eight weeks after the first signs of

Mice were immunized with 100 μg of PLP 139–151 or PBS administered in either CFA or in 10, 35, or 100 μg of CpG 1826/IFA. Mice were monitored daily for disease scores (see Materials and Methods for scoring details). Results are shown for one of two experiments using 10 mice per group. All data points that terminated earlier than the duration of the 30-day scoring period due to overlap on the x-axis had a clinical score of 0.

Treg staining

Fourteen days after immunization, splenocytes were harvested from mice and subsequently stained using FITC-CD4, PE-CD25, and PECy5-Foxp3 as suggested by the manufacturer using a mouse Treg staining kit (eBioscience).

Graphical software and statistical analysis

Prism software (GraphPad) was used both for generating graphs and for performing statistical analysis. Where two groups were compared, a Student t test was used to verify significance. When three or more groups were analyzed, a one-way ANOVA (with Tukey’s post hoc test) was used to verify significance (where the cutoff for significance was set to p < 0.05). Each experiment was performed a minimum of two times. The numbers of mice (n) used in each group is noted below the graph or in the graph legend.

Results

Active immunizations using PLP 139–151 in CpG 1826/IFA do not induce clinical signs of EAE

Mice were immunized with 100 μg PLP 139–151 in either CpG 1826/IFA or CFA adjuvant. To rule out the possibility that the clinical results were a factor of the dose of CpG administered, three different doses of CpG 1826 were used: 10, 35, or 100 μg of CpG 1826 per immunization. Mice were monitored and scored daily for signs of disease for 30 days. As shown in Fig. 1, only mice immunized with PLP 139–151 in CFA developed clinical signs of EAE. Clinical signs of EAE were absent in all CpG 1826/IFA-immunized groups regardless of the dose of CpG 1826 used for immunization.

Active immunizations using PLP 139–151 in CpG 1826/IFA do not induce histological signs of EAE

Mice were immunized with 100 μg of PLP 139–151 in either CpG 1826/IFA or CFA adjuvant. Eight weeks after the first signs of disease (in the PLP 139–151 in CFA group), mice were sacrificed for immunohistochemical detection of demyelination. Among the three different CpG dose groups, the mice receiving 35 μg CpG 1826/IFA were used for immunohistochemical staining. When the spinal cords were stained with Luxol fast blue, demyelination (indicated by loss of Luxol fast blue stain) was detected in the CFA- but not in the CpG 1826/IFA-immunized group (Fig. 2, C vs D, lower magnification; G vs H, higher magnification). Serial sections were also stained using a second myelin-specific marker called Black-Gold. A similar loss of myelin was observed with CFA but not CpG 1826/IFA-immunized mice confirming the demyelination observed using Luxol Fast Blue (Fig. 2, K vs L, lower magnification; O vs P, higher magnification).

No demyelination was observed for mock-immunized mice (Fig. 2, first two columns on the left).

Passive transfer of cells from PLP 139–151 in CpG 1826/IFA-immunized mice induce mild and transient EAE compared with the severe disease that results from the transfer of cells from PLP 139–151 in CFA-immunized mice

Ten days after immunization, splenocytes and lymph node cells were obtained from mice that had either received PLP 139–151 in 35 μg of CpG 1826/IFA or PLP 139–151 in CFA. These cells were cultured for 3 days with 20 μg/ml PLP 139–151. After re-stimulation, cells were washed, counted for viability, and adaptively transferred i.p. into naive recipients. Recipient mice were monitored daily for signs of disease. Mice who had received cells from PLP 139–151 in CFA developed severe disease (which was statistically higher than that of the PLP 139–151 in CpG 1826/IFA-immunized recipients from day 8 onwards) (Fig. 3). At the peak of disease in the PLP 139–151 in CFA-immunized group, 11 of 14 mice had to be sacrificed to relieve pain and distress (Fig. 3, shown as crosses). However, mice that had received cells from PLP 139–151 in CpG 1826/IFA developed a mild transient disease that was reduced to a mean clinical score of <1 at the end of the scoring period (Fig. 3).
Mice were immunized with 100 μg of PLP-139–151 in CFA or 35 μg of CpG 1826/IFA adjuvant results in comparable Ag-specific IL-17 production. Immunization of PLP 139–151 in either CFA or CpG/IFA adjuvant immunizations, PLP peptide administered using CpG 1826/IFA actually showed higher frequencies of IL-2-secreting cells upon Ag recall compared with CFA (p = 0.0031). When IL-17 responses were measured, CFA immunizations resulted in much greater PLP-specific IL-17 responses compared with PLP in CpG 1826/IFA immunizations. These differences in Ag-specific IL-17 secretion were highly statistically significant (p < 0.001). The average Ag-specific response for each cytokine is also shown in Table I. Therefore, despite comparable IFN-γ secretion induced by both immunizations, PLP 139–151 immunizations in CFA induce much greater Ag-specific IL-17 production.

**Immunization of PLP 139–151 in either CFA or CpG/IFA adjuvant result in comparable Ag-specific IFN-γ release, but only CFA immunizations induce Ag-specific IL-17 production**

Mice were immunized with 100 μg of PLP 139–151 in either 35 μg of CpG 1826/IFA or CFA adjuvant. The dose of CpG used was determined from a previous report in which a comparably strong induction of IFN-γ was observed using either 30 or 100 μg of CpG 1826/IFA for immunization (21). Fourteen days later, splenocytes were harvested to perform an ELISPOT assay. Fig. 5 shows Ag-specific cytokine recall responses against PLP 139–151. Both CFA and CpG 1826/IFA immunizations resulted in IFN-γ and IL-2 production showing no significant differences between the frequencies of IFN-γ-secreting cells at this time point. However, despite the production of appreciable amounts of IL-2 by both adjuvant immunizations, PLP peptide administered using CpG 1826/IFA actually showed higher frequencies of IL-2-secreting cells upon Ag recall compared with CFA (p = 0.0031). When IL-17 responses were measured, CFA immunizations resulted in much greater PLP-specific IL-17 responses compared with PLP in CpG 1826/IFA immunizations. These differences in Ag-specific IL-17 secretion were highly statistically significant (p < 0.001). The average Ag-specific response for each cytokine is also shown in Table I. Therefore, despite comparable IFN-γ secretion induced by both immunizations, PLP 139–151 immunizations in CFA induce much greater Ag-specific IL-17 production.

**In vivo neutralization of IL-17 reduces disease severity in mice immunized with PLP 139–151 in CFA**

To demonstrate the dominant role of IL-17 in disease induction in vivo, we performed in vivo IL-17 neutralization experiments on PLP 139–151 in CFA-immunized mice. PLP 139–151 in CFA-immunized mice received three doses of anti-mouse IL-17 neutralizing Ab (on days 6, 10, and 14), whereas a control group received a matched amount of isotype control. Fig. 6 shows a statistically significant reduction in the clinical scores when IL-17 is neutralized vs the isotype control treated group from day 13 to day 21.

**FIGURE 3.** Adoptive transfer of splenocyte cultures from PLP 139–151 in CFA but not CpG 1826/IFA results in severe clinical signs of EAE. SJL/J mice were immunized s.c. with 100 μg of PLP-139–151 or PBS administered in either CFA or 35 μg of CpG 1826/IFA. Splenocytes and lymph node cells from immunized mice were stimulated with PLP-139–151 for 3 days before adoptive transfer into naive recipients. Recipient mice were monitored daily for disease scores. Combined results are shown for two experiments using seven recipient mice per group. Crosses represent mice that had to be euthanized due to prolonged inability to reach food and water. *, P < 0.05 for student’s t tests performed for each time point.

**FIGURE 4.** Immunizations using PLP 139–151 administered in CFA but not CpG 1826/IFA result in DTH. SJL/J mice were immunized s.c. with 100 μg of PLP-139–151 or OVA protein administered in either CFA or 35 μg of CpG 1826/IFA. Fourteen days later, 10 μg of soluble Ag was injected intradermally into the ears of immunized mice (left ear was challenged with PLP 139–151, right ear was challenged with OVA). A, Ear swelling was measured immediately before and 24 h after the injection of peptide. B–E, H&E staining showing cell infiltration in CFA immunized mice upon challenge with relevant Ag. Low magnification images were acquired under ×5 original magnification, whereas higher magnification images (insets) were acquired under ×40 original magnification. Scale bars represent 50 μm. Combined results are shown for two experiments using 10 mice per group.

**CFA but not CpG immunizations with an encephalitogenic PLP peptide result in appreciable DTH**

Because the inflammatory reaction in EAE against neuroantigens is reminiscent of a DTH response, we performed ear swelling assays to assess DTH. Similarly as described previously, mice were immunized with 100 μg of PLP 139–151 in 35 μg of CpG1826/ IFA or PLP 139–151 in CFA. Fourteen days later, mice were challenged intradermally with 10 μg of soluble relevant Ag on one ear and with a similar amount of soluble irrelevant Ag on the other ear. Ear thickness was measured using a digital micrometer immediately before challenge and 24 h after. Fig. 4 shows that appreciable swelling is observed when CFA but not CpG 1826/IFA was used for immunization. The level of ear swelling using PLP 139–151 in CFA was higher when compared with the swelling measured in mice that were challenged with the irrelevant Ag or the swelling measured when CpG 1826/IFA was used as an adjuvant (p < 0.05) (Fig. 4A). These results are also shown histologically in Fig. 4, B–E. Mice immunized with PLP 139–151 in CFA show evidence of massive cellular infiltrate upon challenge with relevant Ag, whereas no such infiltrate is observed in mice immunized with PLP 139–151 in CpG 1826/IFA (Fig. 4, B vs D). Minimal infiltrate in the ear is observed when both groups of mice are challenged with irrelevant Ag (Fig. 4, C and E).

**Immunization of PLP 139–151 in either CFA or CpG/IFA adjuvant result in comparable Ag-specific IFN-γ release, but only CFA immunizations induce Ag-specific IL-17 production**

Mice were immunized with 100 μg of PLP 139–151 in either 35 μg of CpG 1826/IFA or CFA adjuvant. The dose of CpG used was determined from a previous report in which a comparably strong induction of IFN-γ was observed using either 30 or 100 μg of CpG 1826/IFA for immunization (21). Fourteen days later, splenocytes were harvested to perform an ELISPOT assay. Fig. 5 shows Ag-specific cytokine recall responses against PLP 139–151. Both CFA and CpG 1826/IFA immunizations resulted in IFN-γ and IL-2 production showing no significant differences between the frequencies of IFN-γ-secreting cells at this time point. However, despite the production of appreciable amounts of IL-2 by both adjuvant immunizations, PLP peptide administered using CpG 1826/IFA actually showed higher frequencies of IL-2-secreting cells upon Ag recall compared with CFA (p = 0.0031). When IL-17 responses were measured, CFA immunizations resulted in much greater PLP-specific IL-17 responses compared with PLP in CpG 1826/IFA immunizations. These differences in Ag-specific IL-17 secretion were highly statistically significant (p < 0.001). The average Ag-specific response for each cytokine is also shown in Table I. Therefore, despite comparable IFN-γ secretion induced by both immunizations, PLP 139–151 immunizations in CFA induce much greater Ag-specific IL-17 production.
ENCEPHALITGENICITY OF CFA vs CpG IS LINKED TO Th17 INDUCTION

We used a thymidine incorporation assay to measure PLP-specific proliferation in mice immunized with either PLP 139–151 in CFA or PLP 139–151 in 35 μg of CpG/IFA. Fig. 7 shows comparable dose-dependent, Ag-specific proliferative responses for both groups. Therefore, despite different encephalitogenic potential, both CFA and CpG/IFA immunizations induce similar levels of proliferation against PLP 139–151.

Both CFA and CpG/IFA immunizations result in comparable frequencies of peripheral CD4+CD25+Foxp3+ Treg cells

To rule out the possibility that diminished Th17 frequencies with CpG/IFA immunizations may be a result of the induction of Tregs as suggested by others (29), we performed CD4+CD25+Foxp3+ staining of splenocytes from mice that were mock immunized or immunized with either PLP 139–151 in CFA or PLP 139–151 in 35 μg of CpG/IFA. Fig. 8 shows no statistical differences in the frequencies of CD4+CD25+Foxp3+ cells among any of the groups. This would suggest that CpG/IFA immunizations work independently of Tregs in conferring resistance against EAE.

Discussion

CpG ODNs and CFA are two adjuvants described to possess similar type 1 polarizing properties. In work by Chu et al. it was demonstrated that the immunization of hen egg lysozyme in CpG/IFA resulted in the production of both Ag-specific IFN-γ and IFN-γ-dependent Ab isotypes upon Ag recall (21). These type 1 responses were at a level comparable to or exceeding that of CFA. This Th1-polarizing activity of CpG has also been demonstrated using other foreign Ags and even autoantigens as immunogens (5–7, 30). In a report by Segal et al. immunization of SJL/J mice with MBP 87–106 in either CFA or CpG/IFA resulted in Ag-specific IFN-γ release and proliferation (6). Because of these similarities and the fact that CFA is commonly used to induce EAE, it was assumed that both CFA and CpG were equally encephalitogenic.

In the work by Segal et al., CpG/IFA not only resulted in similar IFN-γ and proliferative responses as compared with CFA but also induced EAE of equal severity upon the transfer of in vitro autoantigen-restimulated cells (that is, upon passive induction of EAE) (6). In contrast, the results of the present study show that adoptive transfer experiments using cells from autoantigen in CpG/IFA-primed mice resulted in only a mild, transient disease. No mention of active disease was made in the paper by Segal et al (6). In the present study, we did not observe any clinical or histological signs of disease during active induction of EAE. There are several possible explanations for the discrepancies observed between these two studies. First, the increased encephalitogenicity of CpG-primed cells in passive EAE may be due to changes in T cell differentiation during the in vitro culture period. For example, reactivation was performed in serum-containing medium that includes TGF-β and possibly other cytokines that could affect T cell differentiation (31). Second, during adoptive transfers, substantially higher numbers of neuroantigen-specific T cells are injected into the recipient compared with what would be induced in an active immunization. The transfer of very high numbers of Ag-specific cells may have increased the previously insignificant numbers of Th17 (or Th1) cells to nonphysiologically high levels, resulting in clinical disease. Other differences include the use of a different neuroantigen by Segal (myelin basic protein peptide 87–106) and that these experiments were performed in different mouse colonies, the microbial environments of which may have a profound effect on T cell responses (6). Any of these factors individually, or in combination, may explain the different outcomes of these two studies.

Dissection of the signals and receptors required for the elicitation of a pathogenic autoimmune response has been a very active area of research. A lot of focus has been directed on determining which TLRs (and TLR agonists) are involved in EAE, primarily because of the very striking finding that MyD88−/− mice are resistant to EAE (thereby implicating all TLRs except TLR3) (32, 33). Because the effects of CFA have been attributed predominantly to signaling through TLRs 2, 4, and 9, and the effects of CpG have been attributed to signaling through TLR9, mice deficient in these various TLRs have been used in the context of EAE. The results of these studies with regard to the role of TLR9 are conflicting. On the one hand, a study by Prinz et al. demonstrated that the immunization of TLR9 knockout mice with myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 in CFA resulted in delayed disease onset, less severe disease, and decreased disease incidence compared with WT animals, suggesting that in the WT setting, signaling through this receptor contributes to disease (32). Conversely, in a study by Marta et al., immunization of TLR9 KO
mice using MOG protein in CFA led to exacerbation of disease compared with WT mice, suggesting a regulatory role for this receptor (33). In both of these studies, however, CFA was used for inducing a pathogenic response. The role of TLR9 in such CFA-triggered responses is not fully understood. CFA reportedly contains CpG DNA, but to what extent this CpG contributes to the encephalitogenic role of CFA is presently unknown. Mycobacteria are primarily composed of glycoproteins and glycolipids and DNA only constitutes a minor component of this organism, with CpG DNA comprising an even smaller fraction (34, 35). Nonetheless, what our study clearly shows is that the activation of TLR9 by CpG alone is not sufficient to engage Th17 cells (Fig. 5) and EAE (Figs. 1–3). Therefore, our data do not permit us to make conclusions as to the role of TLR9 in EAE (for example, TLR9 activation in combination with other signals) but does suggest that TLR9 activation alone is not critically involved in Th17 generation. TLR9 may have other roles in the disease (as suggested by the study by Prinz et al.; Ref. 32), but this is beyond the scope of the present work.

The mechanisms leading to the preferential generation of autoantigen-specific Th1 cells upon CpG 1826/IFA administration and to that of Th1/Th17 cells upon CFA administration were not examined in this particular study. However, numerous other studies have been published examining the innate immune signals induced by these two adjuvants. For example, a number of reports have been published on the role of CpG in inducing IL-12, a cytokine promoting the differentiation of naive T cells into the Th1 lineage (36–39). On the other hand, CFA possesses various other components such as muramyl dipeptide and lipoproteins/lipopeptides that have been shown to signal through NOD2 (nucleotide-binding oligomerization domain 2) and TLR2, respectively, and could possibly synergize for IL-23 to promote Th17 survival and expansion (40–42). A recent study also implicated another CFA component, trehalose dimycolate (also known as cord factor), as

![Graph 1](image1.png)

**FIGURE 6.** In vivo neutralization of IL-17 significantly reduces the severity of clinical disease in mice immunized with PLP 139–151 in CFA. SJL/J mice were immunized s.c. with 100 μg of PLP 139–151 in CFA and injected i.p. with 100 μg of anti-IL-17 neutralizing Ab or matched isotype control Ab at days 6, 10, and 14. Results are shown for one of two experiments using 10 mice per group. *, P < 0.05 for student’s t tests performed for each time point.

![Graph 2](image2.png)

**FIGURE 7.** Immunizations using PLP 139–151 administered in either CFA or CpG 1826/IFA induce similar levels of proliferation. SJL/J mice were immunized with 100 μg of PLP 139–151 or PBS in either CFA or 35 μg of CpG 1826/IFA s.c. Splenocytes from immunized mice were harvested 14 days later and stimulated with PLP 139–151 for 4 days. Tritiated thymidine was added during the last 16 h of culture. Combined results are shown for two experiments using eight mice per group. Results are represented as stimulation index (cpm Ag/cpm medium).

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<th>IL-2 Recall Response</th>
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<td>Mean response</td>
<td>106.1 ± 54</td>
<td>140.3 ± 45</td>
<td>78.5 ± 34</td>
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a SJL/J mice were immunized with 100 μg of PLP 139–151 in CFA or CpG 1826/IFA s.c. Splenocytes from immunized mice were harvested 14 days later and used in 24-h IFN-γ, IL-2, and IL-17 ELISPOT assays. Results are expressed as cytokine-secreting cells per 1 × 10⁶ plated splenocytes. Combined results are shown for two experiments using three or six mice per group.
An immunization with PLP 139–151 administered in either CFA or CpG 1826/IFA induces similar frequencies of CD4⁺CD25⁺Foxp3⁺ Treg cells. SJL/J mice were immunized with 100 μg of PLP 139–151 or PBS in either CFA or 35 μg of CpG 1826/IFA s.c. Splenocytes from immunized mice were harvested 14 days later and stained with FITC-CD4, PE-CD25, and PECy5-Foxp3. Dot plots and frequencies shown are gated on CD4⁺ events. Representative histograms are shown on the left and combined results from four independent experiments were used to calculate percentages on the right (n = 8/group).

In summary, we have shown that the differences in encephalitogenicity between CFA and CpG 1826/IFA immunizations can be linked to the induction of Th17 cells. Immunizations using PLP 139–151 in CFA resulted in induction of both Th17 and Th1 cells, whereas immunizations using PLP 139–151 in CpG 1826/IFA resulted in the induction of predominantly Th1 cells. Induction of Th17 using CFA correlated with DTH reactions, which were absent in CpG 1826/IFA immunized mice. When examining the ability of these two adjuvants to induce disease, we observed that upon active immunization, CFA but not CpG 1826/IFA resulted in EAE as evidenced by clinical scores and histology. The severity of disease could be significantly reduced by in vivo IL-17 neutralization. When adoptive transfer experiments were performed, cells from CFA-immunized mice resulted in severe disease, whereas cells from CpG 1826/IFA-immunized mice resulted only in a mild, transient disease. The protective effect of the immunization of autoantigen in CpG was not reflected by increased Treg frequencies. These findings suggest that CpG has a lower potential for inducing autoimmune disease as a result of selection for low frequencies of Ag-specific Th17 cells. These are encouraging results for the use of CpG ODNs without eliciting a pathogenic IL-17 response.

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Disclosures
The authors have no financial conflict of interest.

References