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Lipopolysaccharide Injection Induces Relapses of Experimental Autoimmune Encephalomyelitis in Nontransgenic Mice via Bystander Activation of Autoreactive CD4<sup>+</sup> Cells<sup>1</sup>

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Infections sometimes associate with exacerbations of autoimmune diseases through pathways that are poorly understood. Ag-specific mechanisms such as cross-reactivity between a microbial Ag and a self-Ag have received no direct support. In this study, we show that injection of LPS induces experimental autoimmune encephalomyelitis in TCR-transgenic mice and relapse of encephalomyelitis in normal mice. This form of treatment induces proliferation and cytokine production in a fraction of effector/memory Th lymphocytes in vitro via physical contact of Th cells with CD4<sup>+</sup> LPS-responsive cells. TCR-mediated signals are not necessary; rather what is required is ligation of costimulatory receptors on Th cells by costimulatory molecules on the CD4<sup>+</sup> cells. This form of bystander activation provides an Ag-independent link between infection and autoimmunity that might fit the clinical and epidemiological data on the connection between infection and autoimmunity better than the Ag-specific models. *The Journal of Immunology, 2005, 175: 959–966.*

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3* Abbreviations used in this paper: MS, multiple sclerosis; CsA, cyclosporin A; EAE, experimental autoimmune encephalomyelitis; ICOS, inducible costimulator; MBP, myelin basic protein; PT, pertussis toxin; SC, spleen cell; SEB, staphylococcal enterotoxin B; CM, complete medium.

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Materials and Methods

Antigens

Peptides MBP<sub>Act-11</sub> (AcASQKRPSQRSK) and MBP<sub>85–99</sub> (ENPVVHFFKNIVTPR) were synthesized as described previously (17). Lysed *Salmonella typhimurium* strain C5 Nalr were from U. E. Schaible (Max-Planck-Institut für Infektionsbiologie, Berlin, Germany). *S. typhimurium* LPS (ATCC source strain 7823, purified by gel filtration) and OVA were obtained from Sigma-Aldrich. Some experiments were also repeated with ultrapure LPS from *Salmonella abortus equi* (ALX-581-009; Alexis), which is free from potentially TLR2-stimulating contaminations.
Results

Immunization with S. typhimurium or LPS induces EAE in Tα- mice

We had shown earlier that a TCR that recognizes MBP<sub>Ac1–11</sub>/I-A<sup>α</sup> also recognizes a large number of microbial peptides. One of these peptides was derived from S. typhimurium and immunization with this peptide induced EAE in mice whose T cells exclusively express receptor (T<sup>α</sup>- cells) (17). Immunization of T<sup>α</sup>- mice with lysed S. typhimurium/CFA followed by PT induced EAE with the same incidence, similar kinetics, and severity as immunization with the MBP<sub>Ac1–11</sub> peptide (Fig. 1 and Table I). However, presentation and recognition of the cross-reactive peptide was not necessary. Immunization with LPS from S. typhimurium also induced EAE in the T<sup>α</sup>- mice (Fig. 1 and Table I). Different from another transgenic model (31), the T<sup>α</sup>- mice did not develop EAE when immunized with PBS/CFA followed by PT (Fig. 1 and Table I). LPS-induced EAE was T cell dependent because LPS immunization did not induce EAE in littermates of the T<sup>α</sup>- mice that do not express the MBP<sub>Ac1–11</sub>-specific transgenic TCR (T<sup>α</sup>- mice) (Table I).

No difference in the extent of inflammation and demyelination was detectable on H&E- and Luxol Fast Blue-stained sections between MBP<sub>Ac1–11</sub>- or LPS-induced EAE. Furthermore, immunohistochemistry for T cells, B cells, and macrophages revealed no discernable differences in the composition of the inflammatory infiltrates (Fig. 2).

LPS induction induces EAE relapses in SJL mice

Only the T<sup>α</sup>- mice that harbor a quasi-monoclonal T cell repertoire developed EAE upon LPS immunization. Naive nontransgenic SJL, PL/J, or B10.PL mice were resistant (Table I). To determine whether previous activation and expansion of MBP-specific Th cells made genetically unaltered SJL mice susceptible to LPS-induced EAE, we immunized SJL mice with MBP<sub>85–99</sub>. This induced monophasic EAE in 65% of the SJL mice ~2 wk after the immunization. Almost all of the mice had recovered 40 days after the immunization (Table II). On day 45, we injected the mice with LPS or PBS followed by PT. Only 2 of the 20 PBS-injected mice suffered a relapse of EAE (Table II) and the clinical}

\[\text{FIGURE 1. Immunization with LPS induces EAE in T}^\alpha\text{ mice. T}^\alpha\text{ mice were immunized with MBP}<_{\text{Ac1–11}}, \text{LPS, S. typhimurium lysate, or PBS, followed by PT. Data represent mean EAE scores (±SEM). Data shown are pooled from three independent experiments, each containing at least five mice per group. Animals were sacrificed when their score reached 4 or higher, and their score was kept at 5 for the remainder of the experiment.}\]
symptoms were mild (score 2). In contrast, immunization with LPS induced a relapse in 13 of 28 mice (p < 0.02, Table II). Similar results were obtained when we injected the mice with ultrapure LPS (data not shown), demonstrating that the LPS effect was not due to contaminating TLR2 ligands. The mean clinical score (2.5) was higher than during the first episode of EAE and some of the mice had severe symptoms (score 4). Ten of 17 mice that had developed EAE upon MBP immunization on day 0 developed an EAE relapse following the LPS immunization on day 45. Similarly, 5 of 11 mice of the mice that had not developed EAE upon MBP immunization on day 0 developed EAE following the LPS immunization (p = 0.7). Therefore, a previous EAE episode is not necessary to make SJL mice susceptible to LPS-induced EAE, rather the T cell activation induced by MBP immunization is both necessary and sufficient. In contrast to the MBP-immunized mice, none of the SJL mice that were immunized with OVA on day 0 and with LPS on day 45 developed EAE (Table II). Together, these results indicate that MBP-specific effector/memory T cells, but not naive T cells or effector/memory T cells that recognize irrelevant, non-CNS Ags such as OVA, mediate bystander-induced EAE.

MBP<sub>85–99</sub>-immunized SJL mice had perivascular and subpial inflammatory infiltrates of T cells, macrophages, B cells, and granulocytes (Fig. 3, a–e). Mice injected with PBS at day 40 and without clinical relapse showed subpial scars at day 75, but no evidence of recent inflammation (Fig. 3, f–k), whereas mice immunized with LPS and with clinical signs of relapsing disease had extensive, highly cellular subpial lesions with polymorphonuclear granulocytes and macrophages expressing the early macrophage marker S100A9 as evidence of recent invasion of inflammatory cells into the lesion (Fig. 3, l–p).

**LPS induces bystander activation of Th cells in vitro**

LPS is not known to have direct effects on murine Th cells but potently induces the expression of cytokines and costimulatory molecules in cells of the innate immune system. To investigate the immunological mechanisms leading to LPS-induced EAE, we examined whether LPS-induced activation of the innate immune cells caused Th cell activation in vitro. Therefore, we cultured SC from T<sup>+</sup>α<sup>-</sup> mice in CM alone or with MBP<sub>Ac1–11</sub> or LPS. LPS induced proliferation of 1.0–1.5% of the CD4<sup>+</sup> T cells (Fig. 4a). This was significantly above background proliferation (CM alone: 0.2–0.4%, p < 0.015 in five independent experiments). LPS-induced T cell proliferation depended on the presence of CD4<sup>+</sup> cells and was not detectable when CD4<sup>+</sup> cells were depleted from the cultures (our unpublished data). LPS also induced up-regulation of both CD69 and CD25 in T cells; T cells from MBP-immunized mice had a significantly higher percentage of CD69<sup>+</sup> and CD25<sup>+</sup> cells than T cells from normal mice (Table II). These results indicate that MBP-specific effector/memory T cells, but not naive T cells or effector/memory T cells that recognize irrelevant, non-CNS Ags such as OVA, mediate bystander-induced EAE.

**FIGURE 2.** Similar histopathology of EAE induced by MBP<sub>Ac1–11</sub> or LPS. T<sup>+</sup>α<sup>-</sup> mice were immunized with MBP<sub>Ac1–11</sub> (upper panels) or LPS (lower panels) followed by PT. Mice that had reached a clinical stage 3 were sacrificed and prepared for histological analysis. Prominent perivascular and subpial inflammation is present in both groups (H&E staining, a and e). Immunohistochemistry for Mac-3 (b and f), CD3 (c and g), or B220 (d and h) shows no significant difference in cellular composition of the inflammatory infiltrate between the two groups of mice. Data shown are representative for at least three mice per treatment group. Scale bar, 50 μm.
TNF-α in response to LPS (Fig. 4c). SJL mice that had been immunized with MBP/CFA s.c. followed by PT i.v. responded similarly to SJL mice that had been immunized with MBP/CFA s.c. followed by PBS i.v. Thus, previous in vivo exposure to PT was not necessary for susceptibility to LPS-induced bystander activation (Fig. 4d).

Taken together, effector/memory Th cells of different Ag specificities are susceptible to LPS-induced bystander activation. However, only autoreactive effector/memory Th cells cause disease upon LPS-induced bystander activation (Table II).

**LPS does not act directly on CD4⁺ T cells**

Unsorted SC produced IFN-γ and TNF-α in response to LPS but not in CM alone (Fig. 5a). We purified CD4⁺ cells from MBP85–99-immunized SJL mice by MACS (purity >98%). When the purified CD4⁺ cells were cultured along with CD4⁻ cells and LPS, the fraction of cytokine-producing CD4⁺ cells was the same as in total SC (Fig. 5a). In contrast, purified CD4⁺ cells alone did not respond to LPS (Fig. 5a). Thus, LPS has no direct effect on Th cells. Instead, LPS-induced bystander activation of Th cells depends on the activation of CD4⁺ cells by LPS.

**LPS-induced bystander activation depends on cell-cell contact and is not mediated by soluble factors**

LPS-induced bystander activation of Th cells could be mediated either by soluble factors produced by the LPS-responsive CD4⁺ cells or by cell-cell contact between the LPS-responsive cells and the Th cells. To distinguish between these possibilities, we placed MACS-purified CD4⁺ cells from MBP85–99-immunized SJL mice in the upper chamber of a Transwell system. The lower chamber contained CM alone, CM and LPS, or LPS and CD4⁺ cells. When CD4⁺ cells were cultured in the upper chamber and APC in the lower chamber, the CD4⁺ cells no longer produced IFN-γ or TNF-α in response to LPS (Fig. 5a). The sorted CD4⁺ cells in the upper chamber were viable as demonstrated by the fact that they produced both IFN-γ and TNF-α upon stimulation with anti-CD3/anti-CD28 (our unpublished data). Supernatants from LPS-stimulated SC did not induce IFN-γ- or TNF-α production in the CD4⁺ cells (our unpublished data). Furthermore, addition of the selective p38 MAPK inhibitor SB203580, which inhibits IL-12- and IL-18-induced IFN-γ production of murine Th1 cells (32), had no effect on LPS-induced cytokine production in the CD4⁺ effector/memory cells (our unpublished data). Taken together, LPS-induced bystander activation of CD4⁺ cells depends on physical contact of Th cells with LPS-responsive APC.

**CsA does not inhibit bystander activation**

Since LPS-induced bystander activation of CD4⁺ cells is contact dependent, we asked whether TCR-mediated signaling was necessary. CsA inhibits TCR expression of IFN-γ (32). We cultured SC from naive or MBP85–99-immunized SJL mice with SEB, MBP85–99, or LPS in the presence or absence of CsA and

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**Table II. Induction of EAE relapse in nontransgenic SJL mice**

<table>
<thead>
<tr>
<th>Immunization Day 0</th>
<th>EAE Incidence</th>
<th>EAE Prevalence Day 40</th>
<th>Immunization Day 40</th>
<th>EAE incidence upon second immunization*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP85–99</td>
<td>15/25 (60%)</td>
<td>5/25ᵇ</td>
<td>PBS</td>
<td>2/20ᵃ</td>
</tr>
<tr>
<td>MBP85–99</td>
<td>24/35 (69%)</td>
<td>7/35ᵇ</td>
<td>LPS</td>
<td>15/28ᵃ</td>
</tr>
<tr>
<td>OVA</td>
<td>0/8</td>
<td>0/8</td>
<td>LPS</td>
<td>0/8</td>
</tr>
</tbody>
</table>

*Includes only those mice that newly developed symptoms upon the II° immunization.
ᵇ The clinical score was 1 for all mice that still displayed clinical symptoms of EAE on day 40 and remained at this level throughout the observation time.
ᵃ, p < 0.02.

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**FIGURE 3.** LPS-induced EAE relapses in SJL mice. Histological analysis reveals perivascular and subpial infiltrates (H&E staining, a) comprised of Mac-3-positive macrophages (b), CD3-positive T cells (c), few B220-positive B cells (arrows, d), and some S100A-positive polymorphonuclear granulocytes and early invading macrophages (e) in SJL mice immunized with MBP85–99 suffering from the first bout of disease (day 20 after immunization). In animals immunized with PBS/CFA on day 40 after the first immunization, subpial scars (f) with foamy macrophages (g), perivascular T cell cuffing (arrows, h), few B cells (arrows, i) and almost no S100-positive granulocytes and macrophages (arrow, k) are recognized (day 75 after immunization). Mice immunized with LPS/CFA on day 40 after the first immunization show extensive, highly cellular subpial inflammation (l, H&E; m, anti-Mac-3, macrophages; n, anti-CD3, T cells; o, anti-B220, B cells) with abundant S100A9-positive cells (p), indicating recent recruitment of inflammatory cells into the lesion (day 75 after immunization). Representative spinal cord cross-sections were chosen for photography. All animals depicted had equal clinical EAE scores (level 2) at their first bouts, permitting a direct comparison of lesional pathology. Scale bar, 50 μm.
determined the cytokine production of CD4\(^+\) cells. CsA inhibited the IFN-\(\gamma\) production in response to either SEB or MBP\(_{85-99}\) almost completely (Fig. 5b). In contrast, CsA did not inhibit the IFN-\(\gamma\) or TNF-\(\alpha\) production of naive Th cells in response to LPS (data not shown) and only marginally inhibited the LPS-induced IFN-\(\gamma\) production of effector/memory Th cells (Fig. 5b). Thus, LPS-induced bystander activation of Th cells proceeds via signaling pathways distinct from the TCR-mediated triggering of calcineurin. The APC used in these experiments could have still been loaded with Ag. To test this possibility, we performed experiments in which APC (CD4\(^+\)CD8\(^-\)) from either naive or immunized animals were cultured with T cells from either naive or immunized
animals. When Th cells from unimmunized animals (Thn) were cultured along with APC from either unimmunized (APCn) or immunized (APCi) animals, there was no detectable IFN-γ production in response to either medium or MβP85.90 and little IFN-γ production in response to LPS (Fig. 5c, left panels). Only Th cells from immunized animals (Thi) produced IFN-γ in response to MβP85.90 and that response was stronger when both the Th and the APC came from immunized mice (Fig. 5c, right panels). Supporting the data shown in Fig. 4c, Th cells from immunized animals produced more IFN-γ in response to LPS than Th cells from unimmunized animals (Thn) did. Interestingly, the response was slightly stronger when both the Th and the APC were from previously immunized mice. Yet, the pertinent point is that a significant percentage of Th cells from immunized animals produced IFN-γ when cultured with APC from unimmunized animals and LPS.

**Costimulatory molecules of the B-7 family are necessary for LPS-induced bystander activation**

We next asked whether the costimulatory molecules were essential for LPS-induced bystander activation. SC from MβP85.90-immunized SJL mice were cultured in CM alone or with LPS in the presence or absence of different mAbs or combinations thereof. Blockade of the ICAM-1:LFA-1 interaction with anti-CD54 reduced the LPS-induced IFN-γ-production by ~25% (Fig. 6b). Neither anti-CD80 nor anti-CD86, anti-ICOSL, or CTLA4-Ig alone influenced the LPS-induced cytokine production strongly (Fig. 6), but the combination of these reagents reduced the number of cytokine-producing CD4+ cells by ~50% (anti-B7 family, Fig. 6). Addition of anti-CD54 to that combination only marginally reduced the IFN-γ-production further. Taken together, LPS-induced bystander activation of Th cells is at least partly mediated by the contact between costimulatory ligands of the B7 family on APC, some of which are up-regulated in response to LPS, and their receptors on CD4+ Th cells.

**Discussion**

In this report, we show that LPS injection induces EAE relapses in genetically unaltered mice and bystander activation of Th cells. Previous reports had either demonstrated adjuvant effects of TLR ligands, such as LPS in vitro (33–37), or the induction of autoimmune disease through systemic injection of PT or CpG in transgenic mice (31, 38). Addition of LPS to the in vitro culture enhances the encephalitogenic potential of MBP-specific T cells upon adoptive transfer into syngeneic recipients (33, 35). In otherwise EAE-resistant B10.S mice, EAE can be induced passively if CpG or LPS plus IFN-γ are added to the in vitro culture before adoptive transfer (34). Moreover, if lymph node cells from mice or rats that had been tolerated toward myelin Ags are cultured in the presence of CpG in vitro, they become encephalitogenic upon adoptive transfer into syngeneic recipients (36, 37). Taken together, myelin-specific cells that are unable to adoptively transfer EAE if cultured with myelin Ags alone can be rendered encephalitogenic through in vitro culture with myelin Ags in the presence of CpG or LPS. Reports on the active induction of EAE by injection of Ag- nonspecific stimuli in vivo have thus far been restricted to transgenic mouse models. EAE can be induced by injection of PT alone in some, but not all, strains of mice transgenic for a MBP-specific TCR (31), and CpG injection induces EAE in a fraction of B10.S mice that express a proteolipid protein-specific TCR (38). Our finding that LPS injection induces EAE in Tαα mice (Fig. 1) confirms and extends these reports. The important novel finding presented in this report is the active induction of EAE relapses in normal mice upon LPS injection without the need for adoptive transfer of in vitro-cultured myelin-specific cells.

Immunization with PBS/CFA followed by PT i.v. did not induce EAE in Tαα mice or EAE relapses in SJL mice. Thus, LPS is necessary in both models and EAE is not due to the injection of PT, which has several known EAE-promoting effects, such as increasing Th1 responses and enhancing the T cells’ access to the CNS (39–41).

**A novel mechanism for Ag-independent T cell activation**

Bystander activation has been used to describe different phenomena. We use this term to indicate T cell activation that occurs independently of Ag recognition by the TCR. In accordance with earlier studies (42–46), we found that memory/effector T cells were more susceptible to TCR-independent bystander activation than naive Th cells. However, LPS-induced bystander activation of CD4+ cells described here differs from the cytokine-driven bystander activation depicted in the earlier studies (32, 42–49) in several important aspects. Most important, soluble factors such as cytokines do not mediate LPS-induced bystander activation. This is also different from the in vitro adjuvanticity of the TLR ligands LPS or CpG, which were observed in adoptive transfer models of EAE. Those effects could be mimicked by addition of IL-12 to the culture (34, 36, 37). In contrast to a recent study that reported the detection of TLR4 mRNA in MACS-purified CD4+CD25+ T cells (50), we did not find any direct LPS effects on highly purified CD4+ T cells.

Instead, physical contact between LPS-responsive CD4+ cells and CD4+ Th cells is necessary for LPS-induced bystander activation of Th cells. The interaction between costimulatory molecules of the B7 family on LPS-activated CD4+ cells and their receptors on Th cells is an important but not the only mechanism for LPS-induced bystander activation of Th cells. Blockade of the
costimulatory ICAM-1:LFA-1 interaction (51) also reduced the LPS-induced cytokine production significantly (see Fig. 6). Furthermore, enhanced costimulation via members of the TNFR family (24) may well play a role in LPS-induced bystander activation of Th cells. Taken together, our data show that under certain circumstances costimulatory signals provided by activated APC can induce T cell activation in the absence of TCR triggering. Similar observations have been made with “superagonistic” Abs against CD28 in rats (52, 53) and with pairs of Abs against human CD2 (54). The question remains, which regulatory processes usually prevent T cell activation by costimulatory signals alone and under which circumstances in vivo costimulatory signals alone suffice to cause T cell activation. It is conceivable that bystander activation in vivo may have beneficial effects. For example, bystander activation might contribute to the maintenance of T cell memory similar to what has been described for B lymphocytes (55).

**Induction of autoimmunity by Ag-independent T cell activation**

Bystander activation of autoreactive Th cells occurs independently of TCR Ag recognition and fits the clinical and epidemiological data on the connection between infection and autoimmunity. A variety of infections increase the risk for exacerbations in MS patients (8–11), but despite extensive efforts no specific pathogen has been identified as culprit (2, 3, 7, 19). Bystander activation of autoreactive Th cells occurs independently of TCR Ag recognition and fits the clinical and epidemiological data on the connection between infection and autoimmunity better than supposed Ag-specific mechanisms. The following scenario could link infection and autoimmunity via bystander activation: autoreactive T cells are part of the normal repertoire (56–58). The frequency of these autoreactive Th cells is one of the genetically determined factors that contribute to susceptibility to autoimmune diseases (59). Survival and expansion of autoreactive Th cells can be supported either by recognition of self-Ag and overt autoimmune attacks or, clinically silent, by the recognition of cross-reactive microbial peptides (18, 21). Once the number of autoreactive T cells has reached a certain threshold, as in the monoclonal T α- mice or following MBP immunization in the nontransgenic mice, or in patients who have already suffered previous episodes of MS, TCR-independent stimuli such as LPS-induced bystander activation can trigger sufficient numbers of autoreactive T cells to cause autoimmune damage. This scenario is different both from the Ag-dependent adjuvant effects of LPS (60–63) and from bystander damage which can occur in virally infected mice with a monoclonal T cell repertoire (64–66). Both the Ag-specific activation of the monoclonal T cells and the simultaneous viral infection at the site of tissue damage are necessary conditions for the induction of bystander damage which can result in diabetes (64), keratitis (65), or encephalitis (66). In sharp contrast, TCR-mediated signals are not required for the LPS-induced bystander activation of Th cells described here.

Only 50% of the LPS-immunized SJL mice had EAE relapses. Similarly, <10% of the infectious episodes in the clinical studies were associated with MS exacerbations (8–11). One explanation is that immunoregulatory mechanisms prevent clinically overt autoimmunity. Alternatively, the number of autoreactive Th cells, which are bystander activated, may be too small to cause damage, and finally some infectious agents may lack the potential to induce bystander activation. That only about one-quarter of MS exacerbations are associated with clinically apparent infections (8–11) may be due to the fact that some infections are clinically inapparent. Alternatively, additional mechanisms, which are not triggered by infections, could cause exacerbations. The latter possibility is supported by pathological evidence suggesting that there are four distinguishable subgroups of MS (67), each of which may have different immunopathological mechanisms and different susceptibility for infection-induced immunopathology. Even so, aggressive therapy and prophylaxis of infections to prevent bystander activation of autoreactive T cells could be a useful approach in comprehensive treatment of MS patients.

Exacerbations of MS are associated with many different infections, including Gram-positive bacteria and viruses that do not possess LPS (8–11). We found that bacterial lipopolysaccharides, which are expressed by Gram-positive bacteria, can induce EAE relapses in SJL mice with similar incidence and severity as LPS (V.S. and T.K., unpublished data), and it remains to be established whether other pathogen-associated molecular patterns such as dsRNA are able to induce bystander activation of autoreactive Th cells.

In summary, LPS induces the proliferation and cytokine production of Th cells independently of TCR signaling. This bystander activation of Th cells is not mediated by soluble factors and requires the physical contact between LPS-responsive CD4+ cells and Th cells. LPS immunization induces exacerbations of EAE in genetically unaltered normal mice. Bystander activation of autoreactive Th cells is an Ag-independent mechanism that fits the clinical and epidemiological data on the connection between infection and autoimmunity better than Ag-specific models.

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**Disclosures**

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**References**


