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Cutting Edge: TLR3 Stimulation Suppresses Experimental Autoimmune Encephalomyelitis by Inducing Endogenous IFN-β

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Experimental autoimmune encephalomyelitis is a well-characterized model of cell-mediated autoimmunity. TLRs expressed on APCs recognize microbial components and induce innate immune responses, leading to the elimination of invading infectious agents. Certain TLR agonists have been reported to have adjuvant properties in CNS autoimmune inflammatory demyelination. We report in this study that TLR3 stimulation by polyinosinic-polycytidylic acid, a double-stranded RNA analog, suppresses demyelination in a murine experimental autoimmune encephalomyelitis model. Disease suppression is associated with the induction of endogenous IFN-β and the peripheral induction of the CC chemokine CCL2. These data indicate that a preferential activation of the MyD88-independent, type I IFN-inducing TLR pathway has immunoregulatory potential in this organ-specific autoimmune disease. The Journal of Immunology, 2006, 177: 7505–7509.

Experimental autoimmune encephalomyelitis (EAE) is an animal model for multiple sclerosis (MS), a common inflammatory demyelinating disease of the CNS. Both diseases may be triggered by myelin-reactive CD4+ T cells and are ameliorated by immunomodulatory treatments (1). TLRs recognize bacterial and viral products and activate APCs to produce inflammatory cytokines through MyD88-dependent pathways. In contrast, TLR-mediated induction of type I IFNs can be activated in a MyD88-independent fashion via IFN regulatory factor (IRF)-3 and other IFN-responsive factors (2). Different from other known TLRs, TLR3 only signals through a MyD88-independent pathway that uses the adaptor molecules Toll/IL-1R domain-containing adaptor protein (Mal)/MyD88-adaptor-like protein (Mal) and Toll/IL-1R domain-containing adaptor protein inducing IFN-β (TRIF). TLR3 stimulation leads to the transcription of late phase NF-κB and the translocation of IRF3, thereby inducing the production of type I IFN and IFN-responsive genes.

Recent reports have demonstrated the immune adjuvant properties of certain TLR agonists, including TLR2, TLR4, and TLR9, in EAE (3–5). Therefore, negative regulation of TLR pathways could be used to limit autoimmune responses (6). However, the direct immunoregulatory properties of TLR signaling pathways in autoimmune demyelination remain to be explored. Such studies are relevant to human disease, as IFN-β, a type I IFN induced in APCs by TLR stimuli, is used for the treatment of MS. Its mechanisms of action include induction of IL-10, inhibition of the expansion of encephalitogenic T cells, reduced production of inflammatory cytokines, and reduction of blood-brain barrier permeability (7, 8).

We report in this study for the first time that polyinosinic-polycytidylic acid (poly I:C), a mimic of double-stranded viral RNA that stimulates TLR3, suppresses a murine model of relapsing EAE. Disease suppression is associated with increased levels of IFN-β and the CC chemokine CCL2. These findings indicate that activation of specific TLR pathways has therapeutic potential in organ-specific autoimmunity.

Materials and Methods

Mice and EAE induction

Six- to 8-wk-old female SJL/J mice were purchased from The Jackson Laboratory and EAE was induced as described (9). Mice received a s.c. inoculation of the proteolipid protein (PLP) peptide PLP139–151 (100 μg/mouse) in CFA (containing 1 mg/ml Mycobacterium tuberculosis H37 Ra) at two sites on the back. Mice were scored daily for clinical signs of EAE according to a published clinical scale (10). Experimental procedures were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University (Philadelphia, PA).

Treatment

Poly I:C (potassium salt; Sigma-Aldrich) (100 μg in 200 μl of PBS) or PBS were administered i.p. at the indicated times (three injections every second day). In selected experiments, mice also received a neutralizing rat anti-mouse IFN-β Ab (Yamasu Shiyu) or a rat anti-CCL2 Ab (BD Biosciences).

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Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; BMDC, bone marrow dendritic cell; IRF, IFN regulatory factor; LFB, Luxol fast blue; Mal, MyD88-adaptor-like protein; MS, multiple sclerosis; p.i., postimmunization; PLP, proteolipid protein; poly I:C, polyinosinic-polycytidylic acid; TRIF, Toll/IL-1R domain-containing adaptor protein inducing IFN-β.

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Histology

Mice were sacrificed at different times postimmunization (p.i.) and perfused transcardially with a solution of 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Histological analysis of spinal cords for demyelination (Luxol fast blue (LFB)) and inflammatory infiltration (H&E staining) were performed as described (10). Numbers and severity of lesions were quantitated as described (10).

T cell proliferation and cytokine measurements

Splenocytes were obtained from EAE or healthy mice sacrificed and perfused with 0.1 M PBS (pH 7.4) and were cultured at a density of 2.5 x 10^6/ml in RPMI 1640 medium containing 10% FCS in the presence or absence of 60 μg/ml PLP139–151 or 2.5 μg/ml Con A. After 48 h of culture, T cell proliferation was measured by [3H]thymidine incorporation as described (10). Cytokine concentrations were measured in supernatants by quantitative ELISA (R&D Systems and BD Biosciences) or by cytometric bead array (BD Biosciences) according to the manufacturers’ recommendations. The range of detection for each cytokine was 20–500 pg/ml.

Isolation of CNS cells and flow cytometry

Mononuclear cells from the CNS of EAE mice were isolated after extensive perfusion by Percoll gradient centrifugation (10). Flow cytometric analysis of single cell suspensions for surface and intracytoplasmic staining was performed as described (10). Data were acquired on a FACS Aria flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Generation of bone marrow-derived dendritic cells (BMDCs)

BMDCs were generated by culturing bone marrow cells with GM-CSF for 9 days and subsequent induction of maturation with 1 μg/ml LPS or the indicated concentrations of poly I:C overnight as described (11).

Real-time PCR

Quantification of IFN-β was performed in pooled spleen cell samples from different treatment groups (12). The internal control 18S rRNA was used as housekeeping gene. Change in expression was reported as 2ΔΔCT, where CT is threshold cycle and ΔΔCT = ΔCT sample – ΔCT controls.

Statistical analysis

A nonparametric Mann-Whitney U test was used for comparisons of clinical scores, numbers of pathological lesions, proliferative responses, and cytokine levels between different groups.

Results and Discussion

We report in this study that poly I:C, a TLR3 agonist and a potent inducer of IFN-β (13), suppresses relapsing EAE, an animal model for MS and organ-specific autoimmunity. To test the hypothesis of EAE suppression by a potent inducer of IFN-β, we immunized female SJL/J mice with PLP139–151 and administered poly I:C (100 μg/mouse/day) or PBS on day 5, 7 and 9 p.i. (arrows). Data represent average daily scores ± SE (*, p < 0.05) and are representative of three independent experiments. B, Quantitative pathological analysis of spinal cord lesions. Data represent mean number of foci of inflammation (H&E) or demyelination (LFB) ± SEM (n = 6 per group). *, p < 0.05; ***, p < 0.01. C, Representative microphotographs for inflammation (H&E staining) and demyelination (LFB staining). Original magnifications were ×20.

Because poly I:C is a strong inducer of IFN-β (14), an effective immunoregulator in EAE (7) and MS, we wanted to determine the treatment effects on IFN-β production. Levels of IFN-β produced by spleen cells cultured in the above conditions were below detection. Therefore, we measured IFN-β mRNA levels in pooled spleen cells by real-time PCR. Levels of IFN-β mRNA expression were increased in poly I:C-treated mice as compared with controls (Fig. 2D).

To assess whether IFN-β is involved in suppression of EAE by poly I:C, we performed in vivo neutralization experiments. EAE protection by poly I:C was reversed by an anti-IFN-β Ab (Fig. 3A), indicating a role for IFN-β in mediating this effect of poly I:C. In spleen cells obtained from sacrificed mice and cultured ex vivo, anti-IFN-β also reversed the production of CCL2 observed in poly I:C-treated mice (Fig. 3B). Next, we assessed the role of CCL2, a macrophage chemoattractant for which both promoting and inhibitory roles have been reported in EAE (15).
Neutralization of CCL2 completely reversed the inhibitory effect of poly I:C (Fig. 3 C).

Together, these data indicate that the suppressive effect of poly I:C is at least partly mediated by IFN-β and CCL2. Our findings of increased levels of endogenous IFN-β are consistent with the known IFN-β-inducing activity of poly I:C and with the hypothesis that IFN-β would reduce inflammatory demyelination (7, 8). By contrast, CCL2 has been reported to have both disease-promoting and protective effects (16, 17) in EAE. Mice deficient for CCL2 are resistant to EAE (18). However, transgenic overexpression of CCL2 in the CNS protects against EAE (17). In addition, CCL2 promotes the differentiation of Th2 cells and is involved in the induction of immune tolerance by the oral administration of myelin Ags (16). Our data support a role for this chemokine in the induction of tolerance and immunosuppression in EAE. Induction of CCL2 in the peripheral compartment of the immune system may reduce the concentration gradient to the CNS, thereby limiting the influx of inflammatory cells from the periphery. This model is supported by reports of increased serum levels of CCL2 in MS patients after IFN-β treatment (19).

To better characterize the mechanisms of EAE suppression, we studied the ability of poly I:C to induce IFN-β, CCL2, and TNF-α in vitro. Spleen cells obtained from nonimmunized SJL/J mice and stimulated with poly I:C in vitro produced low amounts of CCL2 and TNF-α (not shown). Levels of IFN-β were below detection by ELISA in these conditions. Thus, we performed these experiments in BMDCs, which produce higher amounts of IFN-β as well as other cytokines. Bone marrow cells were cultured in the presence of GM-CSF for 9 days and then supplemented with poly I:C (or LPS as a positive control) overnight to induce DC maturation as previously reported (11). Poly I:C induced the maturation of BMDCs as indicated by the increased expression of CD40 and CD86 (Fig. 4 A). There was a potent, dose-dependent induction of IFN-β and CCL2 (Fig. 4, B and C). Thus, poly I:C induced IFN-β and CCL2 both in vivo (Fig. 2) and in vitro. TNF-α was also induced, indicating that, in addition to IFN-β inducing pathways, NF-κB-mediated signaling was also activated (Fig. 4 C).

Next, we determined the relationship between IFN-β and CCL2 induction by poly I:C. In other experimental paradigms, poly I:C has been reported to induce both IFN-β and CCL2 (20). To determine whether the induction of IFN-β and CCL2 by poly I:C was reciprocally dependent, we used in vitro neutralization experiments. Blocking CCL2 production with a neutralizing Ab had no effect on production of IFN-β. By contrast, neutralization of IFN-β blocked the production of CCL2 (Fig. 4 D). These data indicate that induction of CCL2 by poly I:C was IFN-β-dependent. Interestingly, production of TNF-α was not blocked by anti-IFN-β and anti-CCL2, alone or in combination, suggesting that distinct pathways are involved in the induction of TNF-α by poly I:C (Fig. 4 D).

Previous reports have shown that TLR signaling mediates certain proinflammatory effects of adjuvants (3, 4, 21) and pertussis toxin (22), which are often used to promote autoactivity in EAE. In this study we demonstrate immunoregulatory properties of TLR pathways in EAE. Our finding of increased IFN-β and CCL2 (Fig. 2) with a tendency toward increased levels of IL-1, IL-6, IL-10, IL-12, and IL-18 suggests a predominant effect of poly I:C treatment on APCs, which produce such cytokines. By contrast, there were modest or no effects on T cell proliferation and T cell-derived cytokines such as IFN-γ, IL-4, and IL-5. This result is consistent with a recent report in which poly I:C did not induce T cell proliferation when used as an adjuvant component in EAE (3). In contrast to the mycobacterial components in that study, the TLR4 agonist LPS and the TLR2 agonist zymosan, poly I:C did not confer full adjuvant, EAE-inducing properties to IFA (3).
Mal and TRIF and leads to the activation of IRF3 and late phase NF-κB. In addition to induction of IFN-β, our consistent findings of increased levels of TNF-α suggest that both the IRF3 and NF-κB pathways are induced by poly I:C injection in vivo. Because TNF-α has both proinflammatory and anti-inflammatory effects in EAE (23), we can speculate that the anti-inflammatory and neuroprotective effects of TNF-α at least partly contribute to disease protection.

The data presented here provide evidence for the activation of a MyD88-independent immunoregulatory pathway by poly I:C. Interestingly, when we administered LPS in a similar treatment protocol, there was delayed clinical onset of EAE without significant effects on disease severity. Both IFN-β and TNF-α were induced in spleen cells (our unpublished results). LPS activates TLR4 and uses both MyD88-dependent and -independent pathways (6). The MyD88-dependent pathway signals via IL-1R-associated kinase (IRAK)-4, IRAK-1, and TNFR-associated factor (TRAF)-6, leading to the activation of early phase NF-κB. A balance between the relative activation of the MyD88-independent pathway (“immunoregulatory”) and the MyD88-dependent pathway (“proinflammatory”) may thus determine the predominant effect of TLR stimulation on EAE. Our data provide evidence for the activation of a MyD88-independent immunoregulatory pathway by poly I:C. Consistent with these findings, we found increased severity of chronic EAE in C57BL/6 mice deficient for TLR3 (D. Fitzgerald, T. Touil, G. X. Zhang, A. Rostami, and B. Gran, manuscript in preparation).

We conclude that selective stimulation of TLR signaling pathways holds promise for the treatment of CNS inflammatory demyelination.

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Disclosures
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References


