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TLR7 Engagement on Dendritic Cells Enhances Autoreactive Th17 Responses via Activation of ERK

Qing Xiao,*† Xue Li,*† Deming Sun,‡ Huanfa Yi,‡ Xiaoxiao Lu,* and Hong Nian*

In this study, we showed that TLR7 activation significantly promoted interphotoreceptor retinoid-binding protein (IRBP)-specific Th17 responses by upregulating RORγt, IL-17, GM-CSF, and IL-23R expression in experimental autoimmune uveitis mice. In vivo administration of CL097 activated dendritic cells (DCs) and endowed them with an increased ability to activate IRBP-specific Th17 cells. CL097-treated DCs (CL097-DCs) formed a cytokine milieu that favored the generation and maintenance of Th17 cells by stimulating IL-1β, IL-6, and IL-23 expression. Furthermore, IRBP-specific T cells from immunized mice injected with CL097-DCs produced more IL-17 and transferred more severe experimental autoimmune uveitis than did those from mice injected with DCs. The enhanced immunostimulatory activities of CL097-DCs depended on JNK, ERK, and p38 activation. Blockade of ERK, but not p38 or JNK, completely abolished the Th17 responses induced by CL097-DCs. Collectively, our findings suggest that CL097 treatment significantly promotes autoreactive IL-17+ T cell responses through enhancing DC activation, which is mediated, at least in part, via the activation of ERK signaling. The Journal of Immunology, 2016, 197: 3820–3830.

TLRs bridge the cross-talk between innate and adaptive immunity, primarily via the activation of dendritic cells (DCs). Upon activation, DCs drive Th1 or Th17 cell differentiation by providing antigenic, costimulatory, and cytokine signals (10). TLRs were reported to differentially regulate pathogenic Th responses. Shi et al. (11) demonstrated that activation of TLR4 with LPS preferentially stimulates IL-17 production, whereas ligation of TLR9 and TLR3 using CpG or polyinosinic-polycytidylic acid [poly(I:C)] primarily stimulates pathogenic Th1 cells in ocular autoimmunity. Among the TLR family, TLR7 is activated by ssRNAs and is strongly expressed by DCs. TLR7 was implicated in a variety of autoimmune disorders (12–14), and engagement of TLR7 endows human DCs with the ability to regulate the effector function of Th17 cells (15, 16). Recently, targeting cell surface TLR7 in DCs was shown to be a promising strategy for treating autoimmune diseases (17). However, little is known about the mechanism by which TLR7 engagement regulates interphotoreceptor retinoid-binding protein (IRBP)-specific Th17 cells in EAU. More recently, a high copy number of TLR7 was shown to be associated with increased risk for uveitis in humans (18), suggesting the importance of determining the underlying contribution of TLR7 to cellular signals and interactions in the development of EAU.

MAPK signaling is among the critical pathways activated by innate stimuli in DCs. MAPKs, comprising ERK, JNK and p38, represent fundamental mechanisms for cellular responses to various extracellular signals (19, 20). Excessive activation of MAPKs is involved in autoimmune diseases, and inhibitors of these pathways were evaluated as new therapeutic approaches in autoimmune diseases (21, 22). Recent work identified the function of intrinsic ERK phosphorylation in T cells, including an involvement in Th17 development (23, 24). However, whether ERK regulates the cross-talk between innate and adaptive immune responses in EAU remains largely unknown.

CL097 is a potent TLR7 agonist in mice (25–28). In the current study, we addressed the role of TLR7 engagement in EAU development and associated mechanisms using a well-established EAU model. We found that TLR7 ligation enhanced EAU development by increasing the generation of Th17 effector cells. Moreover, TLR7 ligation promoted Th17 cell development by enhancing RORγt expression. Importantly, we demonstrated that
TLR7 engagement led to enhanced DC-mediated Th17 responses, primarily via modulation of ERK activity in DCs.

Materials and Methods

Animals and reagents

B10RIII mice (5 wk old) were purchased from the Jackson Laboratory (Bar Harbor, ME), and female C57BL/6 (B6) mice (10 wk) were purchased from Vital River Laboratory Animal Technology (Beijing, China). All procedures were carried out in accordance with the regulations stipulated by the Animal Use and Protection Committee at Tianjin Medical University and conformed to The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Related Research. A truncated form of murine IRBP peptide, 20–40, was synthesized and purified by Sangon (Shanghai, China). Mycobacterium tuberculosis strain H37RA was obtained from Difco (Detroit, MI). Pertussis toxin (PTX) was purchased from Sigma (St. Louis, MO). Recombinant murine IL-12 and IL-23 were purchased from R&D Systems (Minneapolis, MN). FITC- or PE-conjugated Abs against mouse CD11c, CD25, MHC class II (MHCII), CD69, and FoxP3 were purchased from BioLegend (San Diego, CA). FITC-conjugated CD40 and CD80 were obtained from BD Biosciences (San Jose, CA). The p38 inhibitor SB203580 and JNK inhibitor SP600125 were purchased from Sigma. The ERK inhibitor U0126, anti-phospho-p38 Ab (3D7), anti-phospho-SAPK/JNK Ab (G9), and anti-phospho-ERK1/2 Ab (E10) were purchased from Cell Signaling Technology (Danvers, MA). The mouse TLR-2 agonist PGN, TLR-4 agonist LPS, TLR-3 agonist poly(I:C), TLR7 engagement led to enhanced DC-mediated Th17 responses,De novo synthesis of the uveitogenic peptide consisting of aa 1–20 of human IRBP (IRBP 1–20) and aa 161–180 of human IRBP (IRBP 161–180) was used for B6 and B10RIII mice, respectively. The peptides were emulsified in CFA, IFA, or IFA containing the TLR7 ligand CL097. Doses of CL097 used for in vivo immunization were 50, 100, or 150 μg per mouse. Concurrently, 200 ng of PTX (Sigma) was injected i.p.

For adoptive transfer, recipient mice were injected i.p. with 2 × 10⁶ activated T cells, prepared as described previously (29, 30).

Scoring of EAU

Mice were examined three times a week for clinical signs of EAU by indirect fundoscopy. The pupils were dilated with 0.5% tropicamide and 1.25% phenylephrine hydrochloride ophthalmic solutions, and fundoscopic grading of disease was performed using the scoring system reported by Thura et al. (31). For histopathologic evaluation, whole eyes were collected at the end of the experiment, fixed in 4% paraformaldehyde in PBS, and embedded in paraffin. Five-micrometer sections were cut through the papillary optic nerve plane and stained with standard H&E. The presence or absence of disease was evaluated by blind observers who examined six sections cut at different levels for each eye. The severity of EAU was graded on a scale of 0 to 4, based on cellular infiltration and structural changes (32).

Generation of bone marrow–derived DCs

Bone marrow–derived DCs (BMDCs) were generated as described previously (22). In brief, bone marrow cells were flushed from the femurs and tibiae of B6 mice. The cells (1 × 10⁶) were cultured in 24-well plates in medium supplemented with 10 ng/ml of GM-CSF and 10 ng/ml of rIL-4 (both from R&D Systems). Nonadherent cells were removed carefully, and fresh medium was added every 2 d. On day 7, nonadherent cells were collected for phenotyping. In some experiments, DCs were preincubated with SB203580, U0126, or SP600125 for 1 h and then with CL097. Because these inhibitors were dissolved in DMSO, DCs incubated with DMSO alone were used as a control.

DC isolation

Single-cell suspensions prepared from spleens of mice immunized with IFA/Ag or IFA/Ag+CL097 were incubated for 10 min at 4°C with PE-conjugated anti-mouse CD11c Ab and then for 15 min at 4°C with FITC- or PE-MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD11c⁺ cells were enriched using MACS cell selection, following the manufacturer’s instructions (Miltenyi Biotec). The purity of the isolated cell fraction was determined by flow cytometric analysis using FITC-conjugated anti-CD11c Ab (BioLegend).

Real-time quantitative RT-PCR

Total RNA from cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The first-strand cDNA was synthesized with a reverse transcription kit (Fermentas, Burlington, ON, Canada). All gene transcripts were analyzed by quantitative RT-PCR (Q-PCR) with SYBR Green Master Mix using an ABI 7900 HT Sequence Detection System (both from Applied Biosystems, Foster City, CA). Gene-specific primers for Q-PCR are listed in Table I. GAPDH was used as endogenous control in all experiments. For each sample, the relative abundance of target mRNA was calculated from the obtained ΔCt values for target and the endogenous reference gene GAPDH using the following formula: relative mRNA expression = 2^(ΔCt(target) - ΔCt(ref))

Intracellular staining and ELISA

For intracellular staining, cells were exposed to 50 ng/ml of PMA, 1 μg/ml of ionomycin, and 1 μg/ml of brefeldin A (Sigma) for 4 h and then were washed, fixed, permeabilized overnight with buffer (Cytofix/Cytoperm; eBioscience, San Diego, CA), intracellularly stained with Abs, and analyzed on a flow cytometer (FACS Calibur; BD Biosciences). The cytokines in the culture supernatants were detected with commercially available ELISA kits (R&D Systems).

Western blot analysis

Total protein was extracted from 2.5 × 10⁶ cells. The protein concentrations were assessed using BCA kits (Biorega, Tianjin, China), according to the manufacturer’s instruction. The total proteins (50 μg) were added to Laemmli sample buffer, and the samples were heated to 100°C for 10 min, loaded into sample wells, resolved on SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. The membranes were blocked with Abs specific for phospho-p38, phospho-JNK, phospho-Erk (all from Cell Signaling Technology) or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) and an HRP-linked secondary Ab. Signals were detected with ECL. Prime Western blotting Detection Reagent (GE Healthcare), according to the manufacturer’s instructions. The bands were scanned with a Multispectral Imaging System (UVP, Upland, CA) and analyzed by Quantity One software (Bio-Rad, Hercules, CA).

DC administration

Bone marrow–derived DCs from B6 mice were incubated with CL097 (2.5 μg/ml) for 24 h and then CL097-treated DCs (CL097-DCs) or untreated DCs (5 × 10⁶ cells/0.1 ml per mouse) were injected i.p. into B6 mice on the day of immunization.

Statistical analysis

All experiments were performed at least three times. The figures show results from a representative experiment. Data are presented as mean ± SD and were analyzed with GraphPad Prism 5.0. The two-tailed Student t test was used to determine statistical significance; p values < 0.05 were considered statistically significant.

Results

TLR7 engagement on DCs, but not on T cells, enhances IRBP-specific Th17 responses in vitro

We first compared the effect of different TLR agonist–stimulated DCs on IRBP-specific Th17 responses. BMDCs stimulated or not with different TLR agonists, including PGN (TLR2), poly(I:C) (TLR3), LPS (TLR4), CL097 (TLR7), and CpG (TLR9), were cocultured with purified IRBP-specific T cells isolated from immunized B6 mice under Th17-polarizing conditions in the presence of immunizing Ag. Five days later, T cells were analyzed for intracellular IL-17 and IFN-γ expression by flow cytometry (FACS). As shown in Fig. 1A and 1B, the percentages of Ag-specific IL-17⁺ T cells were increased significantly in the groups treated with LPS, PGN, or CL097 (16.8, 15.9, and 14.6%, respectively) and were increased slightly in the poly(I:C)-treated group (10.9%) compared with the control group (7.5%); no significant difference was noted in the CpG-treated group (7.6%). In contrast, the percentage of IFN-γ⁺ T cells did not change profoundly among these groups. Cytokine...
assay by ELISA showed that the production of IL-17 was increased significantly by treatment with LPS, PGN, poly(I:C), or CL097 but not CpG (Fig. 1C). These data indicate that stimulation of TLR2, TLR4, and TLR7 was more effective than other TLRs at enhancing Th17 responses, which is in line with the report by Shi et al. (11) showing that LPS and PGN, but not CpG, preferentially stimulate IL-17 production. A dose-titration assay showed that CL097 increased IL-17 and IFN-\(\gamma\) production in a dose-dependent manner with significant effect at doses \(\geq 2.5\) \(\mu\)g/ml (Fig. 1D).

TLR7 is also expressed at low levels in CD4+ T cells (32). Therefore, we examined whether CL097 directly activated autoreactive T cells. To address this, T cells isolated from immunized mice were preincubated or not with CL097, PGN, or LPS for 12 h, washed, and cocultured with APCs in the presence of Ag under Th17-polarizing conditions for 48 h. The production of IL-17 was measured by ELISA. (E) Autoreactive T cells were preincubated with CL097, PGN, or LPS for 12 h, washed, and cocultured with APCs in the presence of Ag under Th17-polarizing conditions for 48 h. The production of IL-17 was measured by ELISA. (F) Autoreactive T cells derived from EAU mice were stimulated with CL097 (1 \(\mu\)g/ml) for 12 h, and CD69 expression was assessed by flow cytometry. Data are representative of at least three independent experiments. *\(p<0.05\), **\(p<0.01\).

**FIGURE 1.** CL097-DCs promoted IRBP-specific IL-17+ cell responses. (A–C) DCs were stimulated with CL097 (2.5 \(\mu\)g/ml), poly(I:C) (50 \(\mu\)g/ml), PGN (10 \(\mu\)g/ml), CpG (10 \(\mu\)g/ml), or LPS (500 ng/ml) for 24 h, washed, and cocultured with IRBP-specific T cells isolated from immunized mice under Th17-polarizing conditions in the presence of Ag. (A) IL-17+ or IFN-\(\gamma\)+ T cells were determined by flow cytometry. (B) Percentages of IL-17+ T cells among T cells. (C) ELISA of IL-17 secreted by T cells 48 h after stimulation with Ag. (D) DCs stimulated with different dosages of CL097 or LPS (500 ng/ml) were cocultured with T cells isolated from EAU mice under Th17- or Th1-polarizing conditions, and IL-17 and IFN-\(\gamma\) production was measured by ELISA. (E) Autoreactive T cells were preincubated with CL097, PGN, or LPS for 12 h, washed, and cocultured with APCs in the presence of Ag under Th17-polarizing conditions for 48 h. The production of IL-17 was measured by ELISA. (F) Autoreactive T cells derived from EAU mice were stimulated with CL097 (1 \(\mu\)g/ml) for 12 h, and CD69 expression was assessed by flow cytometry. Data are representative of at least three independent experiment. *\(p<0.05\), **\(p<0.01\).
APCs under Th17- or Th1-polarizing conditions. Supernatants were harvested for analysis of cytokine secretion by ELISA, and T cells were collected for analysis of intracellular IL-17 and IFN-γ expression by FACS. As shown in Fig. 3A and 3B, mice treated with IRBP1–20/IFA+CL097 had significantly higher percentages of IL-17+ and IFN-γ+ T cells compared with the IRBP/IFA group (12.6% versus 0.3% and 7.6% versus 4.2%, respectively). In parallel, the production of IL-17 and IFN-γ by IRBP-specific T cells was increased significantly in the CL097 group compared with the IFA group (Fig. 3C). These results indicate that TLR7 activation promotes Th1 and Th17 responses in vivo.

To determine whether CL097 enhanced Th1 and Th17 autoimmune responses in EAU by affecting DC functions, we compared the Th1- and Th17-stimulatory activity of DCs isolated from mice treated with IRBP1–20/IFA or IRBP1–20/IFA+CL097 had significantly higher percentages of IL-17+ and IFN-γ+ T cells compared with the IRBP/IFA group (12.6% versus 0.3% and 7.6% versus 4.2%, respectively). In parallel, the production of IL-17 and IFN-γ by IRBP-specific T cells was increased significantly in the CL097 group compared with the IFA group (Fig. 3C). These results indicate that TLR7 activation promotes Th1 and Th17 responses in vivo.

To determine whether CL097 enhanced Th1 and Th17 autoimmune responses in EAU by affecting DC functions, we compared the Th1- and Th17-stimulatory activity of DCs isolated from mice treated with IRBP1–20/IFA or IRBP1–20/IFA+CL097. Splenic DCs isolated from the spleens of IRBP1–20/IFA and IRBP1–20/IFA+CL097 mice were incubated for 48 h with responder T cells from immunized B6 mice, and cytokine levels in the supernatants were measured by ELISA. As shown in Fig. 3D, DCs from IRBP1–20/IFA+CL097-treated mice induced higher levels of IL-17 and IFN-γ compared with DCs from IRBP1–20/IFA-treated mice. Further analysis revealed that IRBP1–20/IFA+CL097-treated mice exhibited a significantly increased percentage of CD40+CD11c+ DCs compared with IRBP1–20/IFA-treated mice (Fig. 3E). Because the CD25+ DC subset is known to have a strong stimulatory effect on Th17 cells (34), we also examined whether CL097 administration would affect the differentiation of CD25+ DCs. To this end, freshly isolated splenic DCs from IRBP1–20/IFA- or IRBP1–20/IFA+CL097-treated mice were tested for coexpression of CD25 and CD11c by flow cytometry. As seen in Fig. 3E, the percentage of CD25+ DCs was greatly increased (1%) in IRBP1–20/IFA+CL097-treated mice compared with IRBP1–20/IFA-treated mice (0.1%).

To further determine the mechanisms by which CL097 enhanced the autoimmune Th cell responses, we compared gene expression of the lineage-related transcription factors and cytokines in autoreactive T cells from immunized mice treated or not with CL097. As shown in Fig. 3G, T cells from the IRBP1–20/IFA+CL097-treated group had significantly higher mRNA expression of IL-17, RORγt, T-bet, GM-CSF, IFN-γ, and IL-23R than did those from the IRBP1–20/IFA group, whereas the expression of IL-21 did not change significantly between these two groups.

**FIGURE 2.** Treatment with CL097 accelerated EAU. (A and B) B10RIII mice were immunized with 75 μg of uveitogenic peptide IRBP161–180 emulsified in IFA, CFA, or IFA containing CL097 (50, 100, or 150 μg per mouse). All mice received an additional injection of PTX (200 ng per mouse) on day 0. (A) Clinical scores of EAU. Data are mean ± SD for six mice per group. (B) In vivo–primed T cells were stimulated with IRBP161–180 in the presence of syngeneic APCs under Th17- or Th1-polarizing conditions, and IL-17 and IFN-γ levels in the culture supernatant were assessed by ELISA. Data are representative or the average of analysis of six mice for each group (two mice per group per experiment, three independent experiments). *p < 0.05, **p < 0.01. (C) Histo-pathology of eyes from the groups immunized with IFA/Ag, IFA/Ag+CL097 (100 μg per mouse), or CFA (H&E; scale bars, 100 or 200 μm, respectively). Normal retina with intact photoreceptors (*) and choroid (C) were observed in the eye of IFA/Ag-immunized mouse (disease grade 0). In contrast, retina with disorganized photoreceptors and thickening of the choroid were observed in the eye of IFA/Ag+CL097-immunized mouse (disease grade 2–3).
CL097 promotes the maturation of DCs and enhances the expression of Th17-polarizing cytokines in DCs via MAPKs

Given that DC maturation plays an important role in the polarization of Th cells (35), we examined whether CL097 affected DC maturation and function. We first assessed the expression of MHCII and costimulatory molecules in unstimulated or CL097-DCs by flow cytometry. The data showed that the expression of MHCII, CD80, CD86, and CD40 was increased in CL097-DCs (Fig. 4A).

Because DC-produced cytokines, such as IL-23, IL-1β, and IL-6, strongly affect Th cell development (36, 37), we next explored whether TLR7 activation affected the expression of these cytokines in DCs. DCs were cultured for 4–24 h in the absence or presence of CL097, and cells were subjected to Q-PCR. As indicated in Fig. 4B, CL097 treatment significantly enhanced IL-1β, IL-23, IL-6, and IL-12 gene expression in DCs (Table I). Kinetic analysis revealed that the expression of IL-23, IL-12, and IL-1β genes in CL097-DCs reached peak levels at 4 h after stimulation, whereas the maximal expression of IL-6 was observed at 8 h.

Previous work suggested that MAPKs are potent inducers of TLR-mediated IL-6, IL-1β, and IL-23 production by DCs (36, 38). To determine the potential role of these MAPKs in CL097-induced proinflammatory cytokine production by DCs, the phosphorylation levels of MAPKs in CL097-DCs were detected by Western blot. As shown in Fig. 4C, CL097 induced a dramatic phosphorylation of ERK at 15 min after treatment, a modest phosphorylation of JNK at 15 min, 30 min, and 1 h, and a weak phosphorylation of p38 at 15 min and 1 h. To further assess the role of MAPK activation in the observed cytokine gene expression changes induced by CL097 in
DCs, we used well-known pharmacological inhibitors of these MAPKs. DCs were treated with SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), or U0126 (ERK inhibitor) for 1 h and then stimulated with CL097 for 4 h. As shown in Fig. 4D, p38-specific inhibitor significantly attenuated CL097-induced IL-6 and IL-1β mRNA expression, whereas it had a slight inhibitory effect on IL-23, IL-6, IL-1β, and IL-12 mRNA expression.

**FIGURE 4.** CL097 enhanced the activation of DCs, and inhibition of MAPKs suppressed CL097-induced proinflammatory cytokine gene expression. (A) DCs were incubated alone or with CL097 (2.5 μg/ml) for 12 h. The expression of MHCII, CD86, CD80, and CD40 was analyzed by flow cytometry. (B) Kinetics of proinflammatory cytokine IL-6, IL-12, IL-23, and IL-1β mRNA expression of CL097-DCs. DCs were stimulated with CL097 for the indicated times (4, 8, 12, and 24 h) followed by Q-PCR to detect the expression levels of IL-6, IL-12, IL-23, and IL-1β mRNA. (C) DCs were incubated with medium only or with CL097 (2.5 μg/ml) for 5, 15, or 30 min or for 1, 3, or 6 h, and expression of p-ERK, p-p38, and p-JNK was assessed by Western blot. (D) DCs were incubated with DMSO only or p38 inhibitor SB203580, JNK inhibitor SP600125, or ERK inhibitor U0126 for 1 h before the addition of CL097 (2.5 μg/ml). IL-23, IL-6, IL-1β, and IL-12 mRNA expression was determined by Q-PCR 4 h later. Data are representative of at least three independent experiments. *p < 0.05.

**Table I.** Sequences of primers used in this study for real-time RT-PCR

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<tr>
<th>Gene Name</th>
<th>Forward Primer Sequence (5'-3')</th>
<th>Reverse Primer Sequence (5'-3')</th>
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<td>GAPDH</td>
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<td>GCCACCATGACATAAGCTTCA</td>
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<tr>
<td>IL-23p19</td>
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</tr>
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<td>IL-26p35</td>
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<td>GCAAAAAGCTGAGACACCA</td>
</tr>
<tr>
<td>IL-1β</td>
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<td>GCCAGGCGCCAGGTCAAAGGG</td>
</tr>
<tr>
<td>IL-6</td>
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<td>TTGGAGCTGATATCTCTGCAGTGA</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>ACTGGGTTGGTTCTTGCGAGT</td>
<td>GCAGTTGCGCTCCATGTAGTA</td>
</tr>
<tr>
<td>GM-CSF</td>
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<tr>
<td>IL-23R</td>
<td>CAGAGGAAGATCCCTCATGCAGGAATG</td>
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on IL-23 expression. The specific inhibitor for JNK significantly reduced IL-6 mRNA expression, but not IL-1β and IL-23 mRNA expression, in DCs induced by CL097. Inhibition of ERK signaling significantly inhibited the expression of IL-1β and IL-23, but not IL-6, induced by CL097.

**ERK signaling is involved in IL-17 production by uveitogenic T cells mediated by CL097-DCs**

To further test whether MAPKs are involved in the ability of CL097-DCs to drive Th17 responses, DCs were treated or not with specific p38, ERK, and JNK inhibitors (SB203580, U0126, or SP600125, respectively) for 1 h before CL097 stimulation, washed, and cocultured with IRBP-specific T cells in the presence of Ag. The ability of CL097-DCs to induce enhanced intracellular IL-17 expression (Fig. 5A, 5B), increased secretion of IL-17 (Fig. 5D), and elevated mRNA expression of Th17-associated genes (Fig. 5C) was almost completely blocked when ERK was inhibited. In contrast, inhibition of p38 or JNK only partially reduced IL-17 production and Th17-related gene expression induced by CL097 in the same setting.

**CL097-stimulated DCs enhance IRBP-specific Th17 responses and the ability of IRBP-specific Th17 cells to induce EAU**

Next, we evaluated whether CL097-DCs influenced Th17 development in vivo. DCs treated or not with CL097 were adoptively transferred i.p. to B6 mice on the day of immunization. At 13 d postimmunization, uveitogenic T cells were isolated and restimulated with IRBP1–20 and APCs under nonpolarizing conditions in the presence of Ag. As shown in Fig. 6A and 6B, the percentages of IL-17+ T cells and the secretion of IL-17 and IFN-γ increased significantly in mice injected with CL097-DCs in comparison with mice given injection of DCs. Further Q-PCR

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**FIGURE 5.** Suppression of ERK inhibited CL097-DC–mediated IL-17 production by IRBP-specific T cells. (A) DCs were incubated with vehicle only or p38 inhibitor SB203580, JNK inhibitor SP600125, or ERK inhibitor U0126 for 1 h before the addition of CL097 (2.5 μg/ml). After 24 h, DCs were cocultured with uveitogenic T cells isolated from IRBP-immunized B6 mice in the presence of Ag. IL-17+ or IFN-γ+ cells were analyzed by flow cytometry. (B) Percentages of IL-17+ T cells among uveitogenic T cells. (C) Q-PCR analysis of IL-17 and RORγt expression. (D) ELISA of IL-17 secreted by T cells 48 h after stimulation with Ag. Data are representative of three independent experiment. *p < 0.05.
analysis revealed that the expression of IL-17, ROR-γt, T-bet, and IFN-γ was also upregulated significantly in freshly isolated uveitogenic T cells from mice given injections of CL097-DCs (Fig. 6C).

To determine whether CL097-DCs also affect the pathogenic activity of activated Th17 cells, T cells were isolated from spleen and draining nodes of immunized B6 mice injected with DCs or CL097-DCs. After 48 h of in vitro stimulation with Ag and APCs under Th17-polarizing conditions, the activated T cells were adoptively transferred to naive B6 mice, and EAU scores were monitored by fundoscopy. As shown in Fig. 6D, T cells from CL097-DC–treated mice induced more severe EAU in recipient mice than did those from DC-treated mice.

CL097 treatment reduces the percentages of regulatory T cells in vivo

An enhanced immune response could be due to reduced percentages of regulatory T cells (Tregs). To examine this possibility in our system, we examined the proportions of CD4+Foxp3+ Tregs in the T cells from the spleen and lymph nodes of IFA/Ag or IFA/Ag+CL097 immunized mice. As shown in Fig. 7A and 7B, the percentage of CD4+Foxp3+ T cells was decreased significantly in the T cells from CL097-treated mice compared with untreated mice (4.1 ± 0.16% versus 5.7 ± 0.32%, respectively).

We also examined the effect of CL097-treated DCs on Treg development in vivo. CL097-DCs or untreated DCs were adoptively transferred i.p. to B6 mice on the day of immunization. T cells were isolated 13 d later, and Foxp3 expression was determined by FACs. As indicated in Fig. 7C and 7D, mice injected with CL097-DCs displayed significantly reduced proportions of Tregs compared with mice injected with DCs (2.36 ± 0.33% versus 3.83 ± 0.15%, respectively).

Discussion

TLRs are key players in autoimmune disorders. In this study, we explored the ability of different TLR ligand–activated DCs to modulate uveitogenic Th17 development and found that TLR7 ligation significantly promoted autoreactive Th17 responses by enhancing IL-17 and ROR-γt expression in EAU. Our results showing that TLR7 activation enhanced the clinical signs of EAU by promoting Ag-specific CD4+ T cell responses are in agreement with most recent findings in other experimental models of autoimmune diseases, such as autoimmune diabetes and multiple sclerosis (25, 39, 40).

Our finding that engaging TLR7 on DCs led to DCs with an increased ability to promote Th17 responses is consistent with several previous reports (15, 41, 42). However, the TLR7 agonist SA-2 is capable of suppressing the IL-17 response by eliciting IL-10 and IL-10–inducing cytokine production by DCs (16). Hence, it is likely that the effect of TLR7 activation on Th17 responses might be influenced by multiple factors, such as the type of TLR7 ligands, the dosages and timing of administration, or a combination of these. In addition to their ability to regulate adaptive immunity indirectly through stimulating innate responses, TLRs were shown to directly modulate the function of T cells. TLR2 and TLR4 signaling in CD4+ T cells was reported to directly promote Th17 responses (43, 44). Our results are in accordance with these reports, showing that TLR4 and TLR2 ligation in T cells significantly enhanced IL-17 production in autoreactive T cells. A recent publication by Dominguez-Villar et al. (45) demonstrated that engagement of TLR7 expressed on human CD4+ T cells reduced IL-17 production in HIV infection. However, in the EAU model, we found that TLR7 engagement on autoreactive T cells did not significantly affect T cell activation and IL-17 production. The discrepancy in these findings may be due to different experimental setting or
We found that CL097 significantly promoted the expression and polarization of pathogenic Th17 development, respectively (11, 49). DCs, are critically involved in the initiation, expansion, and stabilization of pathogenic Th17 development. Indeed, CL097-DCs significantly enhanced autoreactive Th17 responses in EAU mice. It was reported that 10% of splenic CD11c+ DCs display increased percentages of CD25+ DCs compared with untreated ones, and a significant percentage of BMDCs expressed higher CD25 when the culture medium was supplemented with CL097 (2.5 μg/ml) (data not shown), suggesting that CL097 treatment may enhance differentiation of CD25+ DCs and, hence, lead to increased Th17 responses. However, more research is needed to unravel the relationship between the increased generation of CD25+ DCs and the enhanced autoreactive Th17 responses in CL097-treated mice.

Figure 7. CL097 treatment reduced the percentages of Tregs in vivo. (A and B) T cells isolated from IFA/Ag-, IFA/Ag+CL097-, or CFA/Ag-immunized mice were analyzed for the percentages of CD4+Foxp3+ cells by flow cytometry. (C and D) T cells isolated from CFA/Ag+DC- or CFA/Ag+CL097-DC-immunized mice were analyzed for the percentages of CD4+Foxp3+ cells by flow cytometry. Data are representative or the average of analysis of six to nine mice (two or three mice per group per experiment, three independent experiments). *p < 0.05, **p < 0.01.

MAPK signaling pathways are central for DC activation. MAPKs were shown, in various systems, to influence cytokine secretion by DCs, including mediating the expression of Th17-polarizing cytokines IL-1β, IL-23, and IL-6 (36, 50). Our results showed that CL097 induced the activation of ERK, JNK, and p38 MAPKs in DCs. Using specific pharmacological MAPK inhibitors, we found that p38 participated in CL097-induced IL-6 and IL-1β, but not IL-23, mRNA expression; JNK was involved in IL-6, but not IL-1β and IL-23, mRNA expression; and ERK was responsible for upregulated expression of IL-1β and IL-23, but not IL-6, induced by CL097. Taken together, these results indicate that JNK, p38, and ERK MAPKs are activated by CL097 treatment but may play different roles in CL097-mediated cytokine production by DCs. Inhibition of ERK induced a trend toward reduced IL-6 expression, but it was without statistical significance in our system. This observation was unexpected, because previous reports showed that ERK activation induced by PGN or ok-432 significantly upregulated IL-6 expression in DCs or human monocytes (36, 51). In response to CL097, MAPKs are phosphorylated and activate the transcription factor AP-1 (52), which can enhance the activation of IL-23, IL-1β, and IL-6 promoters, resulting in increased expression of IL-23, IL-1β, and IL-6 in DCs.

It is interesting to note that blockade of ERK, but not p38 or JNK, markedly suppressed CL097-DC–induced autoreactive Th17 responses, suggesting that CL097 promotes IL-17 expression in developing uveitogenic T cells primarily via the ERK pathway. This is consistent with a previous report showing that ERK is...
CD44^CD25^ Tregs were demonstrated to play an important role in controlling the occurrence of autoimmune diseases (55–57). Activated Tregs prevent or attenuate EAU (58–60). Dysfunction of Tregs was also reported in active uveitis patients (60–62). We observed a significant decrease in Tregs in the CL097-treated group compared with the control group, implying an inhibiting effect of TLR7 activation on Treg generation; this is in agreement with recent reports showing that TLR7 activation negatively regulates Tregs (39, 63). The reduction in the percentages of Tregs may account, in part, for the more severe disease in CL097-treated mice.

In conclusion, we demonstrated that TLR7 ligation positively regulated DC activation by enhancing the production of Th17-polarizing cytokines, such as IL-23, IL-1β, and IL-6, resulting in the enhancement of Th17 development and, thereby, amplifying associated autoimmune. Our data support previous studies on the critical regulatory function of TLR7 on autoimmune responses and indicate a new molecular mechanism for DC-mediated Th17 responses to CL097 treatment, most likely through positively regulating the activity of ERK.

Disclosures

The authors have no financial conflicts of interest.

References