Regulation of Experimental Autoimmune Uveoretinitis by Anti-Delta-Like Ligand 4 Monoclonal Antibody

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Purpose. To investigate the involvement of δ-like ligand (Dll)4 in the development of experimental autoimmune uveoretinitis (EAU) in B10.RII mice.

Methods. B10.RII mice were immunized with interphotoreceptor retinoid binding protein (IRBP) peptide 161–180 in complete Freund’s adjuvant together with intraperitoneal injection of Bordetella pertussis toxin. mRNA expressions of Notch receptors and their ligands in the eye were evaluated. To investigate the involvement of Dll in EAU, anti-Dll1, anti-Dll4, or control antibody (Ab) was intraperitoneally injected during both the induction and the effector phases or only the effector phase. Alternatively, mice were intraperitoneally injected with γ-secretase inhibitor (GSI) or the control vehicle during the induction phase. Fourteen days after immunization, the eyes and spleens were harvested. The eyes were used for histologic and/or cytokine mRNA expression analysis, whereas the spleens were used for flow cytometric analysis, and antigen-recall proliferation and cytokine assays.

Results. Expression of Notch1, 2, 4, and Dll4 in the eye were upregulated by EAU induction. Anti-Dll4 Ab treatment during both the induction and effector phases, but not only the effector phase, significantly reduced the severity of EAU. IFN-γ, IL-12p35, IL-17A, and TGF-β mRNA expression in the eye were significantly attenuated by treatment with anti-Dll4 Ab. Splenocytes from anti-Dll4 Ab-treated mice showed significantly less proliferation and IL-17 production on antigen stimulation. Also, the severity of EAU was significantly reduced by γ-secretase inhibitor treatment during the induction phase.

Conclusions. Dll4-mediated Notch signaling during the sensitization is critical for the development of EAU. This can be a novel prophylactic target for autoimmune uveitis. (Invest Ophthalmol Vis Sci. 2011;52:8224–8230) DOI:10.1167/iovs.11-77756

Uveitis, a form of intraocular inflammation, causes retinal damage that can result in blindness in severe cases.¹ Depending on the etiology, uveitis can be classed as endogenous or exogenous.² Because endogenous uveitis is accompanied by systemic autoimmune conditions, the underlying mechanisms are thought to be autoimmune in nature.³ This is further supported by the fact that experimental autoimmune uveoretinitis (EAU), a model of human uveitis, can be induced by immunization with retinal autoantigens in adjuvants.⁴ EAU can be also induced by the adoptive transfer of antigen (Ag)-specific T-cells,⁶ indicating that EAU is a T-cell-mediated disease. Of the various T-cell subsets, IFN-γ-producing Th1 cells,⁷,⁸ and IL-17-producing Th17 cells⁹,¹⁰ appear to be the key players in EAU development.

The involvement of T-cells in EAU has been investigated by modulating the level of T-cell activation. One way of achieving this is by treating animals with antibodies (Abs) specific for costimulatory molecules. Shao et al. demonstrated that stimulation of 4-1BB by treatment with agonistic anti-4-1BB Abs significantly suppressed EAU when the treatment was given during the sensitization period.¹¹ Usui et al. reported that inducible costimulator and/or B7-related protein-I interactions are crucial for EAU development.¹² Thus, it appears that costimulatory molecules participate in the induction of EAU.

Notch, a family of four heterodimeric transmembrane receptors, regulates cell fate decisions during the development of many organs.¹³ Notch signaling is initiated by the interaction between the receptor and a ligand from the Jagged or Delta family.¹⁴ Among its various properties, Notch is also involved in the induction of peripheral T-cell responses. Although the effects of Notch signaling on T-cell activation, proliferation, and polarization remain unclear, T-cell polarization and the resulting cytokine profiles may depend on the ligand that interacts with the Notch receptor.¹⁵–¹⁹ For example, Jagged1 promotes IL-4 expression and stimulates T helper cell 2 (Th2) responses,¹⁵ whereas δ-like ligand (Dll)1 and Dll4 induce differentiation along a Th1 pathway.¹⁷,¹⁸ In accordance with the latter notion, we previously demonstrated that treatment with an anti-Dll4 Ab exacerbates the severity of Th2-mediated experimental allergic conjunctivitis by downregulating Th1 cytokines and upregulating Th2 cytokines.²⁰ The aim of the present study was to investigate the role of Dll4 in the development of Th1 and/or Th17-mediated EAU.

Materials and Methods

Mice and Reagents

B10.RII mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were housed under specific pathogen-free conditions at the Kochi Medical School animal facility. All animals were treated according to the institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Interphotoreceptor retinoid binding protein (IRBP) peptide 161–180 (SGIPYIISYLHPGN-TILHVD) was produced by Scrum Inc. (Tokyo, Japan) by consignment synthesis. HPLC purity of the peptide was > 95%. Complete Freund’s adjuvant (CFA) containing 2.5 mg/mL Mycobacterium tuberculosis was obtained from Difco (Baltimore, MD) and pertussis toxin (PTX) was obtained from Sigma-Aldrich (St. Louis, MO). The anti-mouse Dll1 (HMD1–5) and anti-mouse Dll4 (HMD4–2) monoclonal Abs (mAbs)
Induction of EAU
To induce EAU, mice (8–12 weeks of age) were immunized subcutaneously with an emulsion (200 µL) containing 100 µg IRBP peptide 161–180 in CFA. Pertussis toxin (1.0 µg) was concurrently injected intraperitoneally as an additional adjuvant.

Treatment with Abs
EAU was induced as described above. Mice were intraperitoneally injected with 200 µg of anti-Dll1, anti-Dll4, or nhIgG (n = 10 per group) every other day from the day of immunization until Day 12 (both the induction and effector phases). Alternatively, to examine the role of Dll4 in the effector phase of EAU, 200 µg of anti-Dll4 (n = 5) or nhIgG (n = 6) was injected intraperitoneally every other day from Day 8 to Day 12 (only the effector phase).

Treatment with GSI
EAU was induced as described above. GSI was administered daily via intraperitoneal injection (5 mg/kg per day, dissolved in dimethyl sulfoxide [DMSO]) beginning 3 days before EAU induction and ending 2 days after EAU induction (n = 4). Control mice received the same volume of DMSO (n = 8).

Histopathological Evaluation
The eyes were enucleated on Day 14 after IRBP immunization and fixed for 48 hours in 4% buffered paraformaldehyde until processing. Fixed and dehydrated tissues were embedded in paraffin, and 2 µm vertical sections were stained with hematoxylin and eosin. The intensity of EAU was scored from zero to four in a blinded manner according to the histopathological grading for murine EAU as described previously.

Quantitative Assessment of Notch Receptors, Their Ligands, Retinoid Related Orphan Nuclear Receptor C, and Cytokine mRNA Levels
To evaluate the kinetic changes of expression levels of Notch receptors and their ligands, the eyeballs from EAU-developing mice that were not treated with any Abs or GSI were enucleated 7, 14, 21, and 28 days after EAU induction. As a control, the eyeballs were collected from naïve mice. To evaluate the effect of anti-Dll4 Ab on EAU, the eyeballs were collected from nhlgG- or anti-Dll4 Ab-treated EAU-induced mice at the time of kill. The corneas and lenses were removed (n = 3 per group). Total RNA was prepared (mirVana mRNA Isolation Kit; Ambion, Carlsbad, CA) according to the manufacturer’s instructions. RNA was treated with genomic DNA wipeout buffer (Qiagen, Hilden, Germany) for 2 minutes at 42 °C and 0.5 µg was used to synthesize cDNA using a real-time transcription kit (Quantitect; Qiagen). The levels of Notch receptors (Notch1, 2, 3, and 4), their ligands (Jagged1, Jagged2, Dll1, and Dll4), retinoid-related orphan nuclear receptor C (Rorc), IFN-γ, IL-4, IL-6, IL-12p35, IL-17A, IL-17F, and transforming growth factor (TGF)-β expression in the ocular tissues were measured quantitatively according to the manufacturer’s protocol (Applied Biosystems, Carlsbad, CA), and assayed with a real-time PCR system (ABI)
StepOnePlus; Applied Biosystems). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used for normalization. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in triplicate, including the no-template controls. Relative expression was calculated using the comparative cycle threshold method.

Flow Cytometric Analysis

Spleens were collected from naïve and EAU-developing mice and red blood cell (RBC)-depleted splenocytes were prepared. These splenocytes were incubated for 30 minutes on ice with FITC- or biotin-labeled Abs at optimal concentrations. After the incubation, the cells were washed with cold 2% fetal calf serum (FCS)-PBS. In the case of biotin-labeled Abs, the cells were then incubated with streptavidin-PE or streptavidin-FITC for 10 minutes and then washed again. These cells were then analyzed (FACScan flow cytometer; Becton Dickinson, Mountain View, CA) and acquisition and analysis were performed using commercially-available software (CellQuest Pro software; BD Biosciences).

Cellular Proliferation Assay

RBC-depleted splenocytes (3 × 10^5 cells per well) from anti-Dll4 or nhlgG-treated mice were cultured with IRBP peptide 161 to 180 (1, 5, or 25 μg/mL) in 96-well flat-bottom plates in 0.2 mL RPMI 1640 medium supplemented with 5% FCS and 50 μM 2-mercaptoethanol (2-ME). After an 80-hour incubation at 37 °C in a humidified atmosphere containing 5% CO2, the cultures were pulsed for 16 hours with 0.5 μCi per well [3H]thymidine (Japan Atomic Energy Research Institute, Tokai, Japan). The cultures were then harvested and the incorporated radioactivity was measured using standard techniques. The data were expressed as cpm.

Measurement of Cytokines in the Culture Supernatants

RBC-depleted splenocytes (10^7 cells per mL) from anti-Dll4 Ab or nhlgG-treated mice were cultured for 48 hours with IRBP peptide 161 to 180 (25 μg/mL) in 96-well flat-bottom plates, in which each well contained 0.2 mL RPMI 1640 medium supplemented with 10% FCS and 2-mercaptoethanol. The levels of IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IL-17A, and IFN-γ were measured (Bioplex system; Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Data were expressed as the mean ± SEM (pg/mL).

Statistical Analysis

The severity of EAU grading was analyzed using the Kruskal-Wallis test with the Bonferroni and/or Dunn test or Wilcoxon rank sum test.
TABLE 1. Cell Compartments in the Spleen

<table>
<thead>
<tr>
<th>Cell Compartment</th>
<th>nhlgG</th>
<th>Dll4</th>
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<tbody>
<tr>
<td>CD3+</td>
<td>48.12 ± 2.75</td>
<td>43.11 ± 1.84</td>
</tr>
<tr>
<td>CD4+</td>
<td>29.96 ± 1.74</td>
<td>28.03 ± 1.60</td>
</tr>
<tr>
<td>CD4+CD25+</td>
<td>5.11 ± 4.51</td>
<td>5.25 ± 3.24</td>
</tr>
<tr>
<td>B220+</td>
<td>45.94 ± 2.31</td>
<td>50.93 ± 3.11</td>
</tr>
<tr>
<td>F4/80+</td>
<td>5.32 ± 1.88</td>
<td>5.78 ± 4.26</td>
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At the time the animals were killed, spleens were collected from EAU-developing mice treated with either nhlgG or anti-Dll4 Ab and RBC-depleted splenocytes were prepared to evaluate cell compartments by flow cytometry. Data are presented as % of each cell compartment (CD3+ T-cells, CD4+ T-cells, B220+ B-cells, F4/80+ macrophages and CD4+CD25+ regulatory T-cells) out of whole splenocytes. n = 6 per group.

qRT-PCR and cellular proliferation and/or cytokine production by splenocytes were analyzed using an unpaired t-test or Mann-Whitney U test, depending on whether the data were parametrically distributed. A P value of < 0.05 was regarded as significant.

RESULTS

Kinetic Changes of Notch Receptors and Their Ligands in the Eyes during the Development of EAU

To investigate the kinetic changes of Notch receptors and their ligands expressed in the eye, miRNA expressions of Notch receptors and their ligands were evaluated by qRT-PCR. Expression of Notch1, 2, 4, and Dll4 were significantly upregulated 28 days after EAU induction (Fig. 1).

Expression of Dll1 and Dll4 in Naïve Splenocytes

To determine which types of cells express Dll1 and Dll4, splenocytes were collected from naive mice and were subjected for flow cytometric analysis. Percentages of Dll1+/CD11c− and Dll1+/F4/80− cell compartment were 2.13 ± 0.53% and 2.09 ± 0.66%, respectively (Fig. 2). Percentages of Dll4+/CD11c− and Dll4+/F4/80− cell compartment were 1.27 ± 0.29% and 1.55 ± 0.44%, respectively (Fig. 2).

Administration of Anti-Dll4 Ab during Both the Induction and Effector Phases Suppresses the Severity of EAU

To investigate whether Dll1 and Dll4 participate in the development of EAU, mice immunized with IRBP peptide were treated with anti-Dll1 Ab, anti-Dll4 Ab, or nhlgG during both the induction and effector phases. Inflammatory cell infiltration and destruction of the retinal structure were both less marked in anti-Dll4 Ab-treated mice (Fig. 3A). Treatment with anti-Dll4 Ab, but not anti-Dll1 Ab, significantly suppressed the histologic score of EAU (Fig. 3B). Therefore, differences in immunologic parameters between the nhlgG-treated group and the anti-Dll4 Ab-treated group were investigated.

Cytokine mRNA Expression in the Eye

Because IFN-γ and IL-17 play pivotal roles in the development of EAU, qRT-PCR analysis was performed to evaluate the cytokine expression levels in the eye. The expression of IFN-γ, IL-12p35, IL-17A, and TGF-β were significantly attenuated in the anti-Dll4 Ab-treated group compared with the nhlgG-treated group, whereas the levels of IL-4, IL-6, IL-17F,
and Rorc were not significantly different between the two groups (Fig. 4).

**Administration of Anti-Dll4 Ab during Both the Induction and Effector Phases Does Not Affect the Cell Populations within the Spleen**

To investigate how the intraperitoneal injection of anti-Dll4 Ab affected systemic immune responses, the effects of anti-Dll4 Ab treatment on specific splenocyte populations within the spleen were investigated. The percentage of CD3⁺ T-cells, CD4⁺ T-cells, B220⁺ B-cells, F4/80⁺ macrophages, and CD4⁺CD25⁺ regulatory T-cells within the total splenocyte populations did not significantly differ between the nhlgG-treated and the anti-Dll4 Ab-treated groups (Table 1, n = 6 per group).

**Administration of Anti-Dll4 Ab during Both the Induction and Effector Phases Suppresses Ag-Specific Proliferation and IL-17 Production of Splenocytes**

Ag (IRBP peptide)-specific proliferation and cytokine production of splenocytes was also examined. Ag-specific proliferation of splenocytes from mice injected with anti-Dll4 Ab was significantly lower than that from the nhlgG-treated mice at the concentrations of 5 and 25 μg/mL of IRBP peptide (Fig. 5A). With regard to cytokines, Ag-specific IL-17A production was significantly suppressed in splenocytes from anti-Dll4 Ab-treated mice (Fig. 5E), whereas production of IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, and IFN-γ was not significantly affected (Figs. 5B–5D, 5F–5I).

**Administration of Anti-Dll4 Ab during the Effector Phase Does Not Affect the Severity of EAU**

To investigate whether Dll4 participates in the development of EAU during the effector phase, mice immunized with the IRBP peptide were treated with anti-Dll4 Ab or nhlgG every other day from Day 8 to Day 12. Inflammatory cell infiltration and destruction of the retinal structure were observed at a similar level between the two groups of mice (Fig. 6A). Histologic grading of EAU confirmed that the severity of EAU was not significantly different between the two groups (Fig. 6B). Expression levels of cytokine mRNA in the eye and Ag-specific cytokine production by splenocytes did not differ between the two groups (data not shown).

**GSI Treatment during the Induction Phase Suppresses the Severity of EAU**

The effect of Notch inhibition on EAU induction was investigated. GSI was administered to EAU-induced mice during the induction phase (from Day −3 to Day 2) and inflammatory cell infiltration and destruction of retinal structures were examined. The results show that both were less marked in GSI-treated mice compared with controls (Fig. 7A). In addition, the EAU score was significantly reduced after treatment with GSI (Fig. 7B).

**DISCUSSION**

The results of the present study show that treatment with anti-Dll4 Ab during both the induction and effector phases or treatment with GSI during the induction phase significantly suppressed the severity of EAU. In contrast, treatment with anti-Dll4 Ab during the effector phase did not affect the severity of EAU. Taken together, it appears that the Dll4-mediated Notch signaling is critical for EAU development during the induction phase.

The mechanism underlying suppression of EAU by treatment with anti-Dll4 Ab during both the induction and effector phases may be mediated by attenuation of Ag-specific T-cell proliferation and IL-17 production, because IL-17-producing T-cells, B220⁺ B-cells, F4/80⁺ macrophages, and CD4⁺CD25⁺ regulatory T-cells within the total splenocyte populations did not significantly differ between the two groups (data not shown).
Th17 cells play a crucial role in EAU development. Recently identified innate lymphoid cells (ILCs) represent a novel family of hematopoietic effectors participating in innate immune responses. Interestingly, it was revealed that ILCs produce IL-5, IL-13, IL-17, and IL-22 which are cytokines produced by a certain subset of T-cells. Thus, it remains to be determined whether decreased production of IL-17 is due to suppressed function of ILCs.

With regard to the relationship between IL-17 production and EAU, it was recently demonstrated that by using TCR-δ knockout mouse, γδ T-cells play a crucial role in the generation and activation of IL-17-producing autoreactive T-cells which mediate EAU. More recently, the same group further elucidated that γδ T-cells regulate the generation of β2 TCR+ IL-17-producing uveitogenic T-cells. It was also reported that NK1.1 T cells play a role in the development of EAU. Depletion of NK1.1 T cells by injection of anti-CD161c Ab attenuated the severity of EAU. Additionally, NKT-cells are identified to regulate the development of EAU through inhibition of IL-17 production. Thus, it appears that γδ T-cells, NK cells, and NKT-cells play a major role in the development of IL-17-mediated EAU. We found that the proportion of these cell compartments in splenocytes was not significantly affected by anti-Dll4 Ab treatment (data not shown). However, it is possible that these cells may be affected functionally by treatment with anti-Dll4 Ab. Further studies are necessary to evaluate whether anti-Dll4 Ab affect the function of γδ T-cells, NK cells, and NKT-cells.

As described above, both Dll1 and Dll4 induce differentiation along a Th1 pathway. However, the results of the present study showed that Ag-specific IFN-γ production by splenocytes was not significantly suppressed by anti-Dll4 Ab treatment. Recently, it was shown that Dll4 regulated initial stages of Th2 differentiation and Th2 cytokine production in established allergic responses. We also previously demonstrated that anti-Dll4 Ab treatment increased Th2 cytokine production in experimental allergic conjunctivitis. In this study, however, anti-Dll4 Ab treatment did not affect Th2 cytokine production by splenocytes. Taken together, these results suggest that treatment with anti-Dll4 Ab was less likely to affect systemic Th1/Th2 balance, which then affects the severity of EAU. In contrast to splenocytes, IFN-γ production in the eye at Day 14 of EAU was significantly suppressed by anti-Dll4 Ab treatment. The discrepancy of IFN-γ levels between the spleen and the eye remains to be elucidated. Because IL-17A and IL-17F are detectable in the eye during the early stages of EAU and IFN-γ is detected later, IL-17-producing Th17 cells can be the initiator and IFN-γ-producing Th1 cells are the secondary infiltrating cells in EAU induced by CFA immunization. Therefore, it can be considered that systemic attenuation of Ag-specific Th17 cells by anti-Dll4 Ab treatment led to the reduction of secondary Th1 influx into the eye. IL-17-producing Th17 cells play a pivotal role in EAU development. In terms of the relationship between Dll1 and IL-17, it was reported that Dll4 enhanced the differentiation of Th17 cells because Dll4 upregulated Rorc expression in T-cells and both the Rorc and Il17 gene promoters are direct transcriptional targets for Notch. Indeed, the results of the present study show that treatment with anti-Dll4 Ab significantly downregulated IL-17A mRNA expression in the eye and Ag-specific IL-17A production by splenocytes in EAU-induced mice. It was also demonstrated that Dll4 influences the generation of IL-17-producing T-cells in the presence of additional skewing cytokines, IL-6, and TGF-β. Thus, reduced expression of TGF-β mRNA in the eye may also be involved in downregulation of IL-17 mRNA expression in the eye. More recently, it was shown that anti-Dll4 mAb treatment decreased IL-17 mRNA expression in mice with Thelier’s murine encephalomyelitis virus-induced demyelinating disease. Taken together, our data here further support the notion that Dll4-mediated Notch signaling is critical for Th17 differentiation and the development of Th17-mediated autoimmune disease.

In addition to T-cells, both Notch receptors and ligands are expressed also in dendritic cells (DC) and bacterial products not only upregulate the expression of Notch ligands in DC but also can activate Notch signaling in macrophages. Thus, Notch signaling in innate immune cells may also participate in the induction and perpetuation of EAU. We found that Dll1 and Dll4 are expressed, although not abundantly, in CD11c+ and F4/80+ splenocytes from naïve B10.RIII mice. It was recently demonstrated that Notch signaling initiated by Jagged1, but not Dll1 or Dll4, induced the maturation of DC. Thus, it is unlikely that anti-Dll4 Ab treatment affected DC maturation and cytokine production in this study. It remains to be elucidated how Dll4 molecules in F4/80+ cells participate in the development of EAU. Factors that cause enhanced or decreased expression of Dll4 molecules have not been investigated extensively, although it was recently reported that IL-33 induced a significant decrease in Dll4 mRNA expression in the mucosa of BALB/c mice. Further studies are necessary to determine the factors to affect expression of Dll molecules.

As previously reported, inhibition of Notch signaling by GSI suppressed Th1 immune responses and the development of experimental autoimmune encephalomyelitis (EAE). Later, Jurynczyk et al. demonstrated that selective inhibition of Notch3, but not Notch1, abrogated both the Th1 and Th17 responses of proteolipid protein-reactive effector T-cells in experimental autoimmune encephalomyelitis. In the present study, EAU-induced mice were treated with GSI to examine the involvement of Notch in EAU development. In agreement with the data obtained by anti-Dll4 Ab treatment during both the induction and effector phases, GSI treatment significantly suppressed the severity of EAU. Importantly, GSI treatment was given only during the sensitization period (from Day 3 to Day 2 of EAU induction). Together with the data showing that treatment with anti-Dll4 Ab during the effector phase alone did not affect the severity of EAU, it appears that the signal through Notch is important for Ag sensitization in EAU development. Furthermore, these data suggest that the mechanism underlying the suppression of EAU by anti-Dll4 Ab treatment is, at least in part, due to inhibition of Notch signaling.

In conclusion, Dll4-mediated Notch signaling during the induction phase is critical for the development of EAU. Because anti-Dll4 Ab treatment suppressed the induction of Ag-specific Th17 cells and the development of EAU, Dll4 has a potential as a prophylactic target for T-cell mediated sight-threatening intraocular inflammatory diseases such as uveitis.

References


