



Notch ligand Delta-like4 inhibits the development of murine experimental allergic conjunctivitis

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ABSTRACT

Background: To investigate the involvement of Notch ligands in the development of experimental allergic conjunctivitis (EC) in mice.

Methods: To induce EC, wild-type (WT) or IFN- γ -deficient (GKO) BALB/c mice were immunized with ragweed (RW) in alum followed by RW challenge in eye drops. Twenty-four hours after RW challenge, the conjunctivas, spleens and blood were harvested to evaluate conjunctival eosinophil infiltration, RW-specific cytokine production and serum Ig levels, respectively. Abs against Notch ligands (anti-Jagged1, anti-Jagged2, anti-Delta-like (Dll)1 and anti-Dll4) were injected intraperitoneally into EC-developing mice during the induction or effector phase. As a control, normal hamster IgG (nhlgG) was injected.

Results: Treatment with anti-Dll4 Ab but not the other Abs during the induction phase significantly augmented the severity of EC, as measured by the conjunctival eosinophil infiltration. Anti-Dll4 Ab treatment also significantly upregulated RW-recall IL-4 production and suppressed serum IgE and IgG1 levels. However, anti-Dll4 Ab treatment during the induction phase did not significantly affect the severity of EC in GKO mice. None of the Abs significantly affected the severity of EC, splenocyte cytokine production, or serum Ig levels when administered during the effector phase.

Conclusions: These observations suggest that Dll4, a Notch ligand, plays a role in suppressing the development of EC, possibly by providing a negative signal for Th2 development during the induction phase. In addition, IFN- γ may participate in the augmentation of EC by anti-Dll4 Ab treatment.

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1. Introduction

A typical pathological feature of severe forms of allergic conjunctivitis (AC) such as vernal keratoconjunctivitis (VKC) is the massive infiltration of inflammatory cells (predominantly eosinophils) into the conjunctiva [1]. In addition, the adjunct tissue cornea is damaged by the conjunctival-infiltrating eosinophils, presumably because of their release of mediators such as major basic protein [2]. The resulting corneal tissue damage, which can include corneal ulceration, can lead to vision loss [3]. Consequently, ophthalmologists are greatly interested in the immunological mechanisms behind VKC.

The mechanism by which VKC develops has been investigated by using animal models of AC [4]. By using short ragweed pollen (RW) as the triggering Ag, our group has established a murine model of AC in which we have designated experimental allergic conjunctivitis (EC) [5]. We first showed that EC is predominantly mediated by CD4⁺ T cells, especially Th2 cells [6], rather than IgE [5]. We then asked how T cells participate in the development of EC. For this, we performed a series of experiments examining the role of a variety of T cell-related costimulatory molecules in the development of EC. We found that these costimulatory molecules play a positive [7] and a negative [8] role in the development of EC depending on how the molecule promotes the T-cell deviation toward Th2 [7] and Th1 [8], respectively.

Notch receptors and ligands are expressed in developing and mature lymphocytes and lymphoid tissues and have been shown to play a crucial role in early B- and T-lymphocyte development [9,10]. More recently, it was demonstrated that Notch ligands, which are composed of Jagged and Delta-like (Dll) groups, influence mature T-cell function in that the Dll group directs T-cell polarization toward

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Th1 while the Jagged group induces Th2 responses [11,12]. Thus, on the basis of our observations described above, it is possible that Notch ligands are actively involved in the development of allergic diseases such as AC. However, to date, there are few reports examining the involvement of Notch ligands in the development of allergic diseases [13]. Therefore, we asked here whether and how Notch ligands participate in the development of EC, a model of allergic diseases.

2. Materials and methods

2.1. Mice

Wild-type (WT) BALB/c mice were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). IFN- γ -deficient BALB/c (GKO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were kept in specific pathogen-free conditions at the animal facility of Kochi Medical School and age- and gender-matched mice were used when they were 6 to 12 weeks old. All research adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Reagents

RW was purchased from Polysciences, Inc. (Warrington, PA). RW extract was obtained from LSL Co. LTD. (Tokyo, Japan). Aluminum hydroxide (alum) was purchased from Sigma (St. Louis, MO). The anti-mouse Jagged1, anti-mouse Jagged2, anti-mouse Dll1 and anti-mouse Dll4 mAbs (all hamster IgG) were prepared as previously described [14–16]. These Abs were purified from ascites by using protein G columns. The Ab preparations contained less than 100 pg/ml endotoxin. Normal hamster IgG (nhIgG) was obtained from MP Biomedicals Inc. (Aurora, OH).

2.3. EC induction by active immunization and treatment with Abs

RW adsorbed on alum was injected into the left hind-footpad and the tail base. Fifty μ l of the emulsion (50 μ g of RW and 675 μ g of alum) was injected into each site. The WT and GKO mice were injected intraperitoneally with 200 μ g of purified anti-Jagged1 Ab, anti-Jagged2 Ab, anti-Dll1 Ab, anti-Dll4 Ab, or nhIgG ($n = 10$ per group in WT mice and $n = 5$ per group in GKO mice) on days 0, 2, 4, 6 and 8 after immunization (induction phase treatment). In a separate experiment, the actively immunized WT mice ($n = 10$ per group) were injected intraperitoneally with 200 μ g of Ab only once 2 h before the RW challenge (effector phase treatment). Ten days after active immunization, the eyes of the immunized mice were challenged with RW in PBS (2 mg in 10 μ l per eye). Twenty-four hours later, the eyes and blood were harvested for histological analysis and measurement of serum Ig levels, respectively. The spleens were also harvested for cytokine assays and splenocyte transfer.

2.4. EC induction by adoptive transfer of splenocytes from actively immunized and Ab-treated mice

Splenocytes harvested from the WT mice described above were cultured with 5 μ g/ml RW extract at a concentration of 10^7 cells per ml in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, ICN Biomedical Japan Co., Tokyo, Japan), 5×10^{-5} M 2-mercaptoethanol (2-ME), 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. After incubation for 72 h at 37 °C in a humidified atmosphere with 5% CO₂, 2×10^7 splenocytes were injected intraperitoneally into naïve BALB/c mice ($n = 8$ mice per group). Four days after the transfer, the eyes of the recipient mice

were challenged with RW in PBS (2 mg in 10 μ l per eye). Twenty-four hours later, the eyes were harvested for histological analysis.

2.5. EC induction by adoptive transfer of RW-primed splenocytes treated in vitro with anti-Dll4 Ab

WT mice were actively immunized with RW in alum and 10 days later, spleens were harvested for splenocyte culture as above. The splenocytes were cultured with 5 μ g/ml RW extract in the presence of either anti-Dll4 Ab or nhIgG (10 μ g/ml). Three days later, 2×10^7 splenocytes were injected intraperitoneally into naïve BALB/c mice ($n = 8$ mice per group). Four days after the transfer, the eyes of the recipient mice were challenged with RW in PBS. Twenty-four hours later, the eyes were harvested for histological analysis.

2.6. Histological analysis

The eyes including the conjunctivas were harvested and fixed in 10% buffered formalin. Vertical 2 μ m-thick sections were cut and stained with Giemsa. Infiltrating eosinophils in the lamina propria mucosae of the tarsal and bulbar conjunctivas throughout each section were counted by two blinded observers. The sections counted were those of the central portion of the eye, which included the pupil and optic nerve head. The data are presented as means $\pm$ SEM of all the mice examined.

2.7. Measurement of Ig levels in serum

Twenty-four hours after RW challenge of actively immunized mice, blood was collected and serum was prepared. Total IgE and IgG1 levels in the serum were assessed by ELISA, as detailed before [5] and the data are presented as Ig concentrations.

2.8. Measurement of cytokines in the culture supernatants

RBC-depleted splenocytes (10^7 cells/ml) were cultured for 48 h with 25 μ g/ml RW extract in 96-well flat-bottom plates in a final volume of 0.2 ml RPMI 1640 medium supplemented with 10% FCS and 2-ME. In a separate experiment, anti-Dll4 Ab or nhIgG (10 μ g/ml) was added to the culture. The levels of IL-2, IL-4, IL-5, IL-10, IL-12 and IFN- γ in the culture supernatants were measured by using Luminex100 (Luminex Corporation, Austin, TX) and the Bio-Plex Cytokine Assay Panel (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturers' instructions.

2.9. Statistical analysis

Differences between Ab-treated and control IgG-treated mice in terms of their cytokine production, serum Ig levels, and infiltrating eosinophil numbers were tested for significance by ANOVA and Scheffe's F-test or Student's *t* test. *P* values less than 0.05 were considered significant.

3. Results

3.1. Treatment with anti-Dll4 Ab during the induction phase exacerbates the severity of EC

To investigate whether Jagged and Dll play a role in the development of EC during the induction phase, actively immunized BALB/c mice were injected with anti-Jagged1, anti-Jagged2, anti-Dll1 or anti-Dll4 Ab 5 times every other day from the day of immunization. Ten days after the immunization, the mice were challenged with RW in eye drops and 24 h later, the conjunctivas were harvested

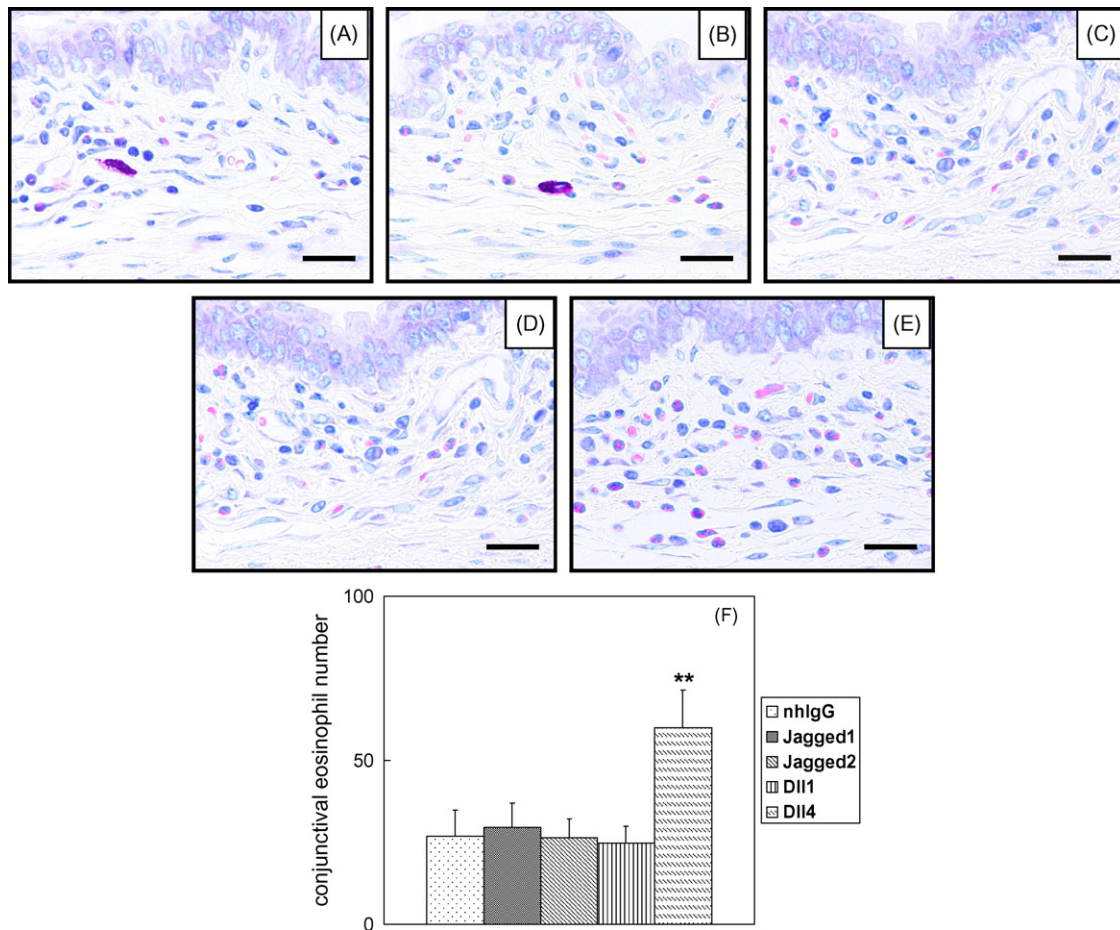


Fig. 1. Augmentation of EC by anti-Dll4 Ab treatment during the induction phase. Balb/c WT mice were actively immunized with RW in alum and then challenged 10 days later with RW in eye drops to induce EC. The mice were intraperitoneally injected with 200 μ g of nhlgG (A), anti-Jagged1 Ab (B), anti-Jagged2 Ab (C), anti-Dll1 Ab (D) or anti-Dll4 Ab (E) 5 times every other day starting on the day of immunization. Twenty-four hours after RW challenge, the conjunctivas were harvested for histological analysis. (A–E) Microphotographs of the conjunctiva. Giemsa staining. Bar = 20 μ m. (F) Conjunctival eosinophil numbers of 10 mice in each group are shown as means $\pm$ SEM. ** P < 0.01 as compared to nhlgG group.

to evaluate conjunctival eosinophil numbers. Treatment with anti-Dll4 Ab (Fig. 1E), but not anti-Jagged1 (Fig. 1B), anti-Jagged2 (Fig. 1C), or anti-Dll1 (Fig. 1D) Ab, apparently increased conjunctival eosinophil numbers as compared to the control group that had been injected with nhlgG (Fig. 1A). Cell counting confirmed that there was a significant difference in conjunctival eosinophil number between the anti-Dll4 Ab-treated group and the other groups (Fig. 1F). Since EC is induced by Th2 cells, but not Th1 cells [6], we investigated whether the anti-Dll4 Ab-induced exacerbation of EC is mediated by its ability to modulate the Th1/Th2 balance. Compared to the control group, anti-Dll4 Ab treatment significantly upregulated the RW-recall production of IL-4 by splenocytes and slightly decreased IFN- γ production, although the latter effect did not achieve statistical significance (Fig. 2A). Relative to the effect of nhlgG, the anti-Dll1 Ab also significantly suppressed IL-10 (Fig. 2A).

To further investigate how anti-Notch ligand Ab treatment affected systemic immune responses, splenocytes from EC-developing mice treated in the induction phase with the Abs were transferred into naïve BALB/c mice and the recipient mice were challenged with RW to induce EC. The transfer of splenocytes from the anti-Dll1 or anti-Dll4 Ab-treated mice induced significantly more severe EC than the splenocytes from nhlgG-treated mice (Fig. 2B). In addition, all the anti-Notch ligand Ab-treated mice had significantly lower total serum IgE levels than the nhlgG-treated

mice (Fig. 2C). Moreover, compared to the control nhlgG-treated mice, the anti-Jagged2 Ab-treated mice produced significantly higher total serum IgG1 while significantly lower levels were observed in the anti-Jagged1-, anti-Dll1- and anti-Dll4-treated mice (Fig. 2C).

3.2. Treatment with anti-Notch ligand Abs during the effector phase does not affect the severity of EC

Next, to investigate whether Jagged and Dll play a role in the development of EC during the effector phase, the Abs were injected intraperitoneally once 2 h prior to RW challenge. Although conjunctival eosinophil numbers tended to be increased by anti-Notch ligand Ab treatment, these changes did not achieve statistical significance (Fig. 3A). With regard to splenocyte cytokine production, IFN- γ production was significantly increased by anti-Jagged1 Ab treatment and IL-12 production was significantly increased by anti-Jagged1 or anti-Jagged2 Ab treatment, as compared to the effect of nhlgG treatment (Fig. 3B). In addition, splenocyte transfer experiments demonstrated that Ab treatment during the effector phase did not affect the EC-inducing capability of splenocytes (Fig. 3C). Moreover, the levels of total serum IgE and IgG1 were not significantly affected by treatment with any Abs relative to the effect of nhlgG treatment (Fig. 3D).

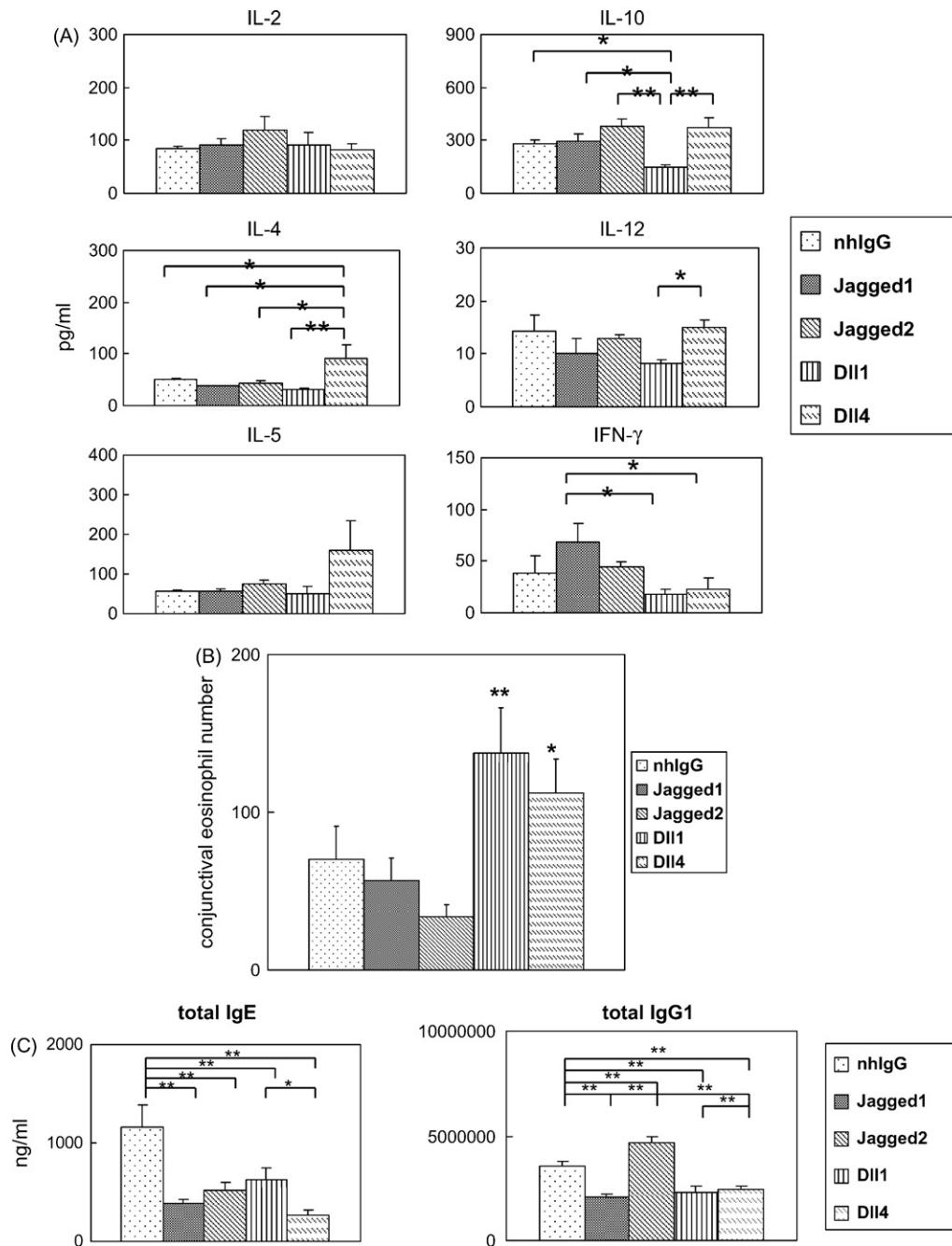


Fig. 2. Effects of anti-DII4 Ab treatment during the induction phase on immune responses. At the time the conjunctivas were harvested as described in the legend to Fig. 1, the spleens and blood were harvested for splenocyte cytokine production analysis (A) or splenocyte transfer (B) and measurement of serum IgE and IgG1 levels (C), respectively. (A) Cytokine production by RW-stimulated splenocytes. The data of 3 different samples in each group are presented as mean concentrations (pg/ml) $\pm$ SEM. * P < 0.05 and ** P < 0.01. (B) *In vitro*-stimulated RW-primed splenocytes from Ab-treated mice were transferred into syngeneic WT mice. Four days later, the recipient mice were challenged with RW in eye drops and 24 h later, the conjunctivas were harvested for histological analysis to evaluate conjunctival eosinophil infiltration. Conjunctival eosinophil numbers of 8 mice in each group are shown as means $\pm$ SEM. * P < 0.05 and ** P < 0.01 as compared to nhlgG group. (C) Total IgE and IgG1 levels in serum. The data of 10 mice in each group are shown as mean concentrations (ng/ml) $\pm$ SEM. * P < 0.05 and ** P < 0.01.

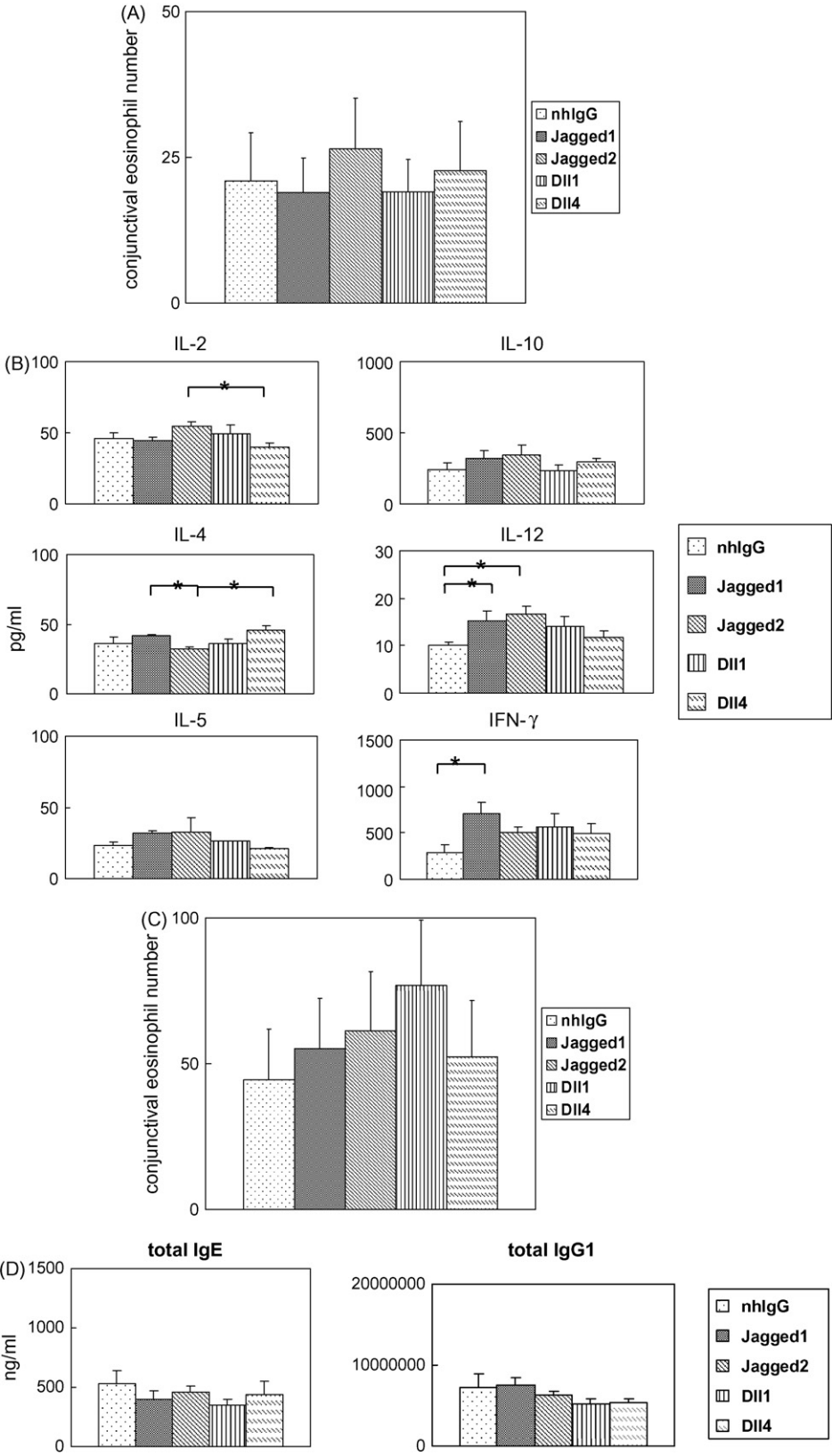
3.3. Treatment with anti-Notch ligand Abs during the induction phase does not affect the severity of EC in GKO mice

We induced EC in GKO mice and treated the mice with anti-Notch ligand Abs or nhlgG to investigate whether IFN- γ plays a role in the anti-DII4 Ab-mediated augmentation of EC in WT mice. Unlike in WT mice, while treatment with anti-DII4 Ab slightly

attenuated the severity of EC in GKO mice, this effect was not statistically significant (Fig. 4).

3.4. Anti-DII4 Ab treatment *in vitro* of RW-primed splenocytes does not affect cytokine production and EC-inducing capability

Finally, to investigate whether anti-DII4 Ab directly affects immune cells, RW-primed splenocytes were cultured *in vitro* with



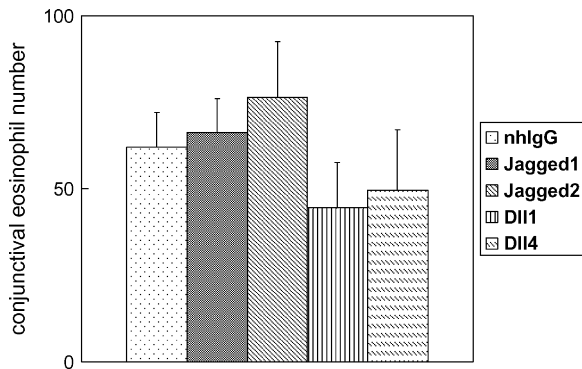


Fig. 4. The augmenting effect of anti-Dll4 Ab treatment during the induction phase is absent in GKO mice. GKO mice were actively immunized with RW and intraperitoneally injected 5 times during the induction phase with 200 μ g of nhlgG, anti-Jagged1 Ab, anti-Jagged2 Ab, anti-Dll1 Ab or anti-Dll4 Ab ($n = 5$ mice per group), as described in the legend to Fig. 1. Twenty-four hours after RW challenge, the conjunctivas were harvested for histological analysis. Conjunctival eosinophil numbers are presented as means $\pm$ SEM.

RW extract in the presence of either anti-Dll4 Ab or nhlgG. Culture supernatants were subjected to cytokine assays and collected splenocytes were transferred into naïve recipient mice that were later challenged with RW in eye drops to induce EC. Conjunctival eosinophil numbers did not differ between the mice received of anti-Dll4 Ab- and nhlgG-treated splenocytes (Fig. 5A). With regard to cytokine production, the levels of tested cytokines were not different between the two groups (Fig. 5B). Additionally, flow cytometric analysis demonstrated that immune cell compartment (such as CD3⁺ cells) of cultured splenocytes did not differ between the two groups (data not shown).

4. Discussion

The Notch ligands Dll and Jagged are expressed on dendritic cells and can provide activation signals that promote the development of Th1 or Th2 cells, respectively [11,12]. With regard to experimental airway inflammation, lymphocytes from animals treated with anti-Dll4 Ab *in vivo* exhibited increased IL-5 and IL-13 production compared to the effect of the control Ab [17]. More recently, allergen-sensitized and allergen-challenged mice treated with Delta1-Fc exhibited decreased airway hyperresponsiveness and airway inflammation [13]. This effect was accompanied by higher levels of IFN- γ in bronchoalveolar lavage fluid [13]. These results suggest that Notch ligands can participate in the development of allergic diseases.

Notch ligands are composed of Dll and Jagged groups. Despite strong domain homology, it appears that the signals transmitted by Dll and Jagged ligands affect the target cell differently [18]. Different effects are also observed within the same group. For example, the T-

cell development induced by Dll1 but not Dll4 is associated with an increase in the number of ectopically developing double-positive T cells [19]. Thus, it is conceivable that each Notch ligand plays a distinct role in the development of allergic diseases. Therefore, we here investigated the roles of four different Notch ligands in the development of EC.

Regardless of whether they were administered in the induction or effector phase of EC, the Abs against Jagged1, Jagged2 or Dll1 did not significantly affect the severity of EC. This may be consistent with no essential role for Jagged2 in Th2 development [20]. In contrast, anti-Dll4 Ab treatment during the induction phase, but not the effector phase, significantly augmented the severity of EC. Moreover, induction phase treatment with anti-Dll4 Ab significantly upregulated IL-4 production but did not significantly affect IFN- γ production. This finding is in agreement with the observation made by using genetically manipulated mice that Notch is required for Th2-, but not Th1-cell development [21]. Since EC can be induced by the transfer of Ag-specific Th2 cells, but not Th1 cells, it may be that anti-Dll4 Ab treatment relatively upregulates Th2 immune responses and this effect may explain its ability to exacerbate EC. To prove that Dll4 ligand inhibits development of EC, it is necessary to perform a reciprocal set of experiments by using agonistic Dll4 fusion protein.

In GKO mice, IFN- γ deficiency leads to the relative upregulation of Th2 immune responses. Since the anti-Dll4 Ab upregulated Th2 cytokine production in WT mice, it would be reasonable to expect that this Ab would further upregulate Th2 immune responses and therefore exacerbate EC in GKO mice. However, we found that the anti-Dll4 Ab had no effect on the severity of EC in GKO mice. This indicates that IFN- γ plays a role, at least in part, in the augmentation of EC by anti-Dll4 Ab treatment. We [22] and others [23] have shown that IFN- γ participates in the development of EC in the BALB/c background. This may be why anti-Dll4 Ab treatment did not exert a significant effect on EC in GKO mice.

To investigate how anti-Dll4 Ab affects immune cells, RW-primed splenocytes were stimulated *in vitro* with RW extract in the presence of either anti-Dll4 Ab or nhlgG. Anti-Dll4 Ab did not affect cytokine production and EC-inducing capability of splenocytes. In this experiment, anti-Dll4 Ab treatment was conducted on already primed splenocytes and therefore, it could be considered that the data were similar to those of effector phase treatment (Fig. 3). Alternatively, anti-Dll4 Ab may have affected not only immune cells but also other types of cells.

In summary, the Notch ligand Dll4 appears to suppress the development of EC, an experimental allergic disease. The mechanism how Dll4 suppresses EC may be that Dll4 provides a negative signal for Th2 development during the induction phase, as recently reported [24,25]. Therefore, agonistic Dll4 or some strategy to induce Dll4 expression may be useful to suppress the development of allergic diseases such as AC.

Fig. 3. Treatment with Abs against Notch ligands during the effector phase does not affect the severity of EC and immune responses. Two hours prior to RW challenge, the actively immunized WT mice were intraperitoneally injected once with 200 μ g of nhlgG, anti-Jagged1 Ab, anti-Jagged2 Ab, anti-Dll1 Ab or anti-Dll4 Ab ($n = 10$ mice per group). Twenty-four hours after RW challenge, the conjunctivas, spleens and blood were harvested for histological analysis (A), cytokine production (B) or splenocyte transfer (C) and measurement of serum total IgE and IgG1 levels (D), respectively. (A) The conjunctival eosinophils were counted. The data of 10 mice in each group are presented as means $\pm$ SEM. (B) Cytokine production by RW-stimulated splenocytes. The data of 3 different samples in each group are presented as mean concentrations (pg/ml) $\pm$ SEM. * $P < 0.05$. (C) *In vitro*-stimulated RW-primed splenocytes from Ab-treated mice were transferred into syngeneic WT mice. Four days later, the recipient mice were challenged with RW in eye drops and 24 h later, the conjunctivas were harvested for histological analysis to evaluate conjunctival eosinophil infiltration. Conjunctival eosinophil numbers of 8 mice in each group are shown as means $\pm$ SEM. (D) Total IgE and IgG1 levels in the serum. The data of 10 mice in each group are shown as mean concentrations (ng/ml) $\pm$ SEM.

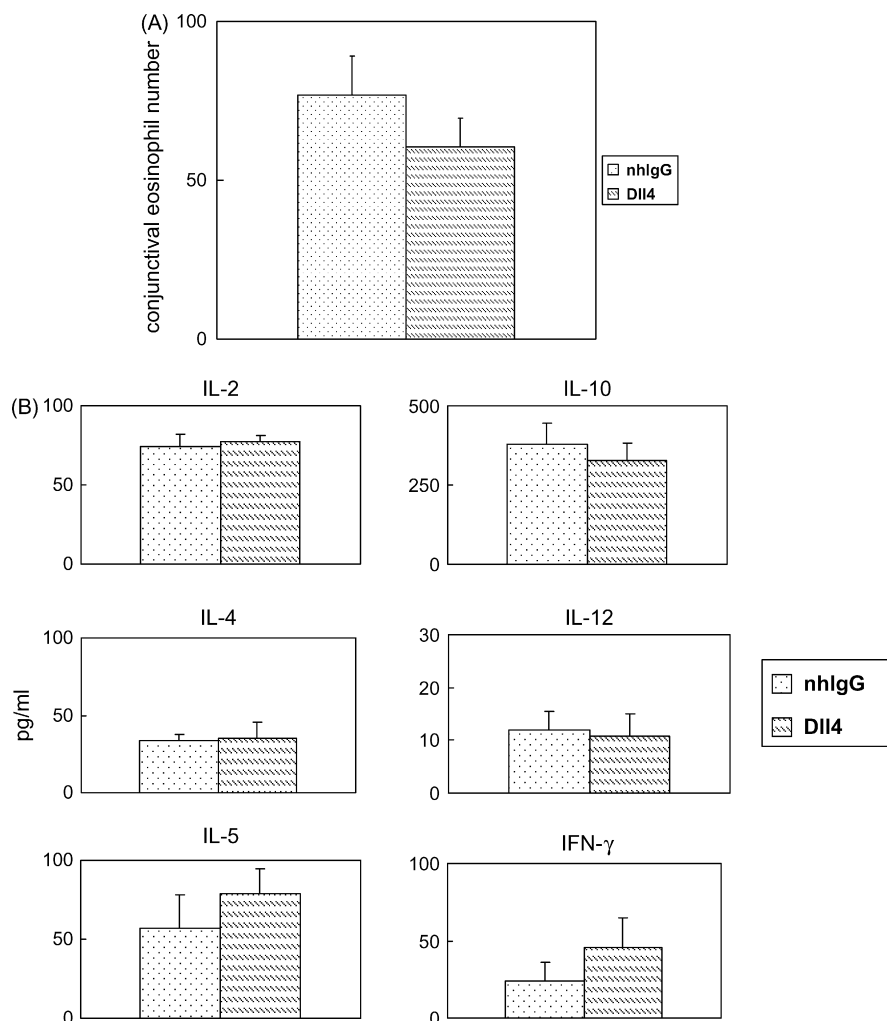


Fig. 5. Anti-Dll4 Ab treatment *in vitro* does not affect the severity of EC and cytokine production. WT mice were actively immunized with RW in alum and 10 days later, spleens were harvested. Splenocytes were prepared and cultured with 5 μ g/ml (A) or 25 μ g/ml (B) RW extract in the presence of either anti-Dll4 Ab or nhlgG (10 μ g/ml) (A). Three days after culture, splenocytes were injected intraperitoneally into naïve BALB/c mice ($n = 8$ mice per group). Four days after the transfer, the eyes of the recipient mice were challenged with RW in PBS. Twenty-four hours later, the eyes were harvested for histological analysis. Conjunctival eosinophil numbers of 8 mice in each group are shown as means $\pm$ SEM. (B) Forty-eight hours after culture, the supernatants were collected for cytokine assays. Data are shown as mean concentrations (pg/ml) $\pm$ SEM.

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