EXTENDED REPORT

Involvement of programmed death-ligand 2 (PD-L2) in the development of experimental allergic conjunctivitis in mice

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Background/aim: Involvement of programmed death-1 (PD-1) and its ligands has been demonstrated in experimental allergic airway disease. Here, the authors aimed to examine whether PD-1 and its ligands are involved in the development of experimental allergic conjunctivitis (EC) in mice.

Methods: EC was induced in Balb/c mice by active immunisation with short ragweed pollen (RW) in alum. 10 days later (day 10), the mice were challenged with eye drops containing RW. 24 hours after the challenge, conjunctivas, spleens, and sera were harvested for histological analysis, cytokine assays, and measurement of RW specific Ig levels. The actively immunised mice were treated with anti-PD-1, anti-PD-L1, anti-PD-L2 antibodies (Abs), or normal rat immunoglobulin G (nrlgG) during either the induction (day 0, 2, 4, 6, and 8) or the effector (2 hours before RW challenge on day 10) phase.

Results: Ab treatment during the induction phase did not affect eosinophil infiltration although immune responses were modulated. In contrast, treatment with anti-PD-L2 Ab, but not anti-PD-1 or anti-PD-L1 Ab, during the effector phase significantly increased eosinophil infiltration into the conjunctiva without affecting systemic immune responses.

Conclusions: Similar to allergic airway inflammation, PD-L2 is involved in the development of EC during the effector phase but not the induction phase.

The severity of eosinophil infiltration into the conjunctiva parallels the clinical severity of allergic conjunctivitis (AC), because more eosinophils infiltrate into the conjunctiva in patients with severe types of AC such as vernal keratoconjunctivitis (VKC)¹ and atopic keratoconjunctivitis (AKC).² In addition to eosinophils, T cells may be involved in the development of VKC, since many CD4+ T cells infiltrate the conjunctiva of active VKC patients.^{3–4} Furthermore, the observations indicating that infiltrating T cells produce cytokines in the conjunctiva^{5–6} suggest that T cells exert their effects at the inflammatory site, conjunctiva. Mechanistic analyses using rats and mice have confirmed that Ag specific CD4+ T cells play a crucial part in the development of experimental AC (experimental immune mediated blepharoconjunctivitis, EC) in terms of eosinophil infiltration into the conjunctiva.^{7–9} Immunohistochemical analyses of conjunctivas from rats⁸ and mice⁹ developing EC demonstrated that eosinophils and T cells predominantly infiltrated into the conjunctiva, thus resembling the conjunctivas in VKC patients.^{2–4} Additional analyses using transgenic and knockout mice have confirmed that Th2 type T cells are putatively involved in the development of EC.¹⁰

Activation of naive T cells requires the signals from both the T cell receptor and co-stimulatory molecules.¹¹ Because EC is mediated by CD4+ T cells and since activation of T cells is required for EC development, we have investigated the involvement of several co-stimulatory molecules in the development of EC. With regard to proteins within the tumour necrosis factor (TNF) receptor superfamily, a forced stimulation of 4-1BB by intraperitoneal injection of agonistic anti-4-1BB Ab inhibited EC,¹² while injection of agonistic anti-OX40 Ab exacerbated EC.¹³ In contrast, blockade of B7 related protein 1 (B7RP-1), which is a member of the B7 family and the ligand of inducible co-stimulator (ICOS), did not affect the severity of EC.¹⁴ Thus, each co-stimulatory molecule is involved differently in the development of EC.

Programmed death-1 (PD-1) is a member of an immunoglobulin (Ig) superfamily member and is related to CD28

and cytotoxic T lymphocyte associated antigen-4 (CTLA-4).¹⁵ PD-1 is expressed on activated, but not resting, CD4+ and CD8+ T cells, on B cells and on myeloid cells.^{16–17} Initially, PD-1 was identified as being involved in programmed cell death of a T cell hybridoma.¹⁶ However, subsequent studies have not supported a direct role in cell death. For example, a recent study indicated that PD-1 ligand induced PD-1 expressing cells to arrest the cell cycle in G0/G1.¹⁸ Two members of the B7 family, PD-L1 (B7-H1) and PD-L2 (B7-DC), have been identified to be the ligands for PD-1.¹⁹ PD-L1-Ig fusion protein in vitro inhibits proliferation and cytokine production of both resting and previously activated T cells.²⁰ Furthermore, proliferation of PD-1 deficient T cells was not inhibited by PD-L1-Ig fusion proteins, indicating that stimulation through PD-1 provides a negative signal to T cell activation.²⁰ Thus, it may be that PD-1 is a negative regulator of the development of EC, which is mediated by CD4+ T cells. In this study, we investigated the involvement of PD-1 and its ligands in the development of EC.

MATERIALS AND METHODS

Mice

Inbred Balb/c mice (Japan SLC Inc, Hamamatsu, Shizuoka, Japan) were kept in specific pathogen free conditions at the animal facility of Kochi Medical School; 6–12 weeks old female mice were used in these studies. All research adhered to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

Abbreviations: Abs, antibodies; AC, allergic conjunctivitis; AKC, atopic keratoconjunctivitis; ALP, alkaline phosphatase; B7RP-1, B7 related protein 1; CTLA, cytotoxic T lymphocyte associated antigen; EC, experimental allergic conjunctivitis; ICOS, inducible co-stimulator; nrlgG, normal rat immunoglobulin G; PBS, phosphate buffered saline; PD, programmed death; PD-L2, programmed death-ligand 2; RW, ragweed pollen; TNF, tumour necrosis factor; VKC, vernal

Figure 1 Treatment with anti-PD-1, anti-PD-L1, or anti-PD-L2 Ab during the induction phase did not affect eosinophil infiltration into the conjunctiva. Balb/c mice were immunised with RW in alum. Ten days later (day 10), the mice were challenged with RW in eye drops and their conjunctivas were harvested 24 hours later for histological analysis by Giemsa staining. The mice were intraperitoneally injected with nrlgG, anti-PD-1, anti-PD-L1, or anti-PD-L2 Ab during the induction phase (200 µg per injection on days 0, 2, 4, 6, and 8). The infiltrating eosinophils were counted and the data are expressed as eosinophils per mm² (n = 10 per group).

Reagents

Short ragweed pollen (RW) was purchased from Polysciences, Inc (Warrington, PA, USA). RW extract was obtained from LSL Co Ltd (Tokyo, Japan). Aluminium hydroxide (alum) was purchased from Sigma (St Louis, MO, USA). Antagonistic anti-PD-1 mAb (RMP1-14, rat IgG2a),²¹ anti-PD-L1 mAb (MIH6, rat IgG2a),^{21–23} and anti-PD-L2 mAb (TY25, rat IgG2a)^{21,22} Abs were prepared as described previously and were purified from ascitic fluid using a Protein G column. The preparations contained less than 100 pg endotoxin/ml. Normal rat IgG (nrlgG) was purchased from MP Biomedicals Inc (Aurora, OH, USA).

EC induction by active immunisation and treatment with Abs

RW adsorbed on alum was injected into the left hind footpad and at the base of the tail. A volume of 50 µl of the emulsion (50 µg of RW and 2 mg of alum) was injected into each site. The mice were injected intraperitoneally with 200 µg of purified anti-PD-1, anti-PD-L1, anti-PD-L2 Ab, or control rat IgG (n = 10 per group) on days 0, 2, 4, 6, and 8 after RW immunisation. On day 10, the eyes of the immunised mice were challenged with RW in PBS (2 mg in 10 µl per eye). In a separate experiment, the actively immunised mice (n = 10 per group) were injected intraperitoneally with 200 µg of each Ab on day 10 only, 2 hours before RW challenge. Twenty four hours after the RW challenge, the eyes, sera, and spleens were harvested for histological analysis, measurement of RW specific Ig levels, and cytokine assays, respectively.

Histological analysis

The eyes, including the conjunctivas, were harvested along with the lid margin by cutting with razor blades and scissors, and then fixed in 10% buffered formalin. Vertical 2 µm thick sections were cut and stained with Giemsa. Infiltrating eosinophils in the lamina propria mucosae of the tarsal and bulbar conjunctivas in the entire section were counted by two observers given blind samples. The sections counted were those from the central portion of the eye, which included the pupil and optic nerve head. Data are expressed as infiltrating

eosinophil numbers per section. Since the counts vary depending on the severity of inflammation (when inflammation is severe, the thickness of lamina propria mucosae increases), the cell count data are expressed as infiltrating eosinophil numbers divided by area (mm²) as measured by Scion Image (Scion Corporation, Frederick, MD, USA). The data were presented as an average (SEM) of all the mice examined.

Measurement of cytokines in the culture supernatants

RBC depleted splenocytes (10⁷ cells/ml) were cultured for 48 hours with RW extract (25 µg/ml) in 96 well flat bottom plates in a final volume of 0.2 ml RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, ICN Biomedical Japan Co, Tokyo, Japan), 2-mercaptoethanol (2-ME, 5 × 10⁻⁵ M), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml). The levels of IL-4, IL-5, IL-10, IL-13, and IFN-γ in the culture supernatants were measured with commercially available ELISA kits (Duoset; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendations.

Measurement of RW specific Igs in serum

Twenty four hours after RW challenge, blood was collected and serum was prepared. RW specific Ig levels in serum were assessed by ELISA. Briefly, EIA plates (Costar, Corning, NY, USA) were coated with RW extract (1 µg/ml) at 4°C overnight. After blocking with 1% bovine serum albumin in phosphate buffered saline (PBS), serum samples were added and incubated for 2 hours at room temperature or overnight at 4°C. As the control, the quantified RW specific IgE and pooled hyperimmune serum were used. The plates were washed with PBS plus 0.05% Tween 20 (PBS/T) (Wako, Osaka, Japan) and incubated for 2 hours at room temperature with alkaline phosphatase (ALP) conjugated goat Ab specific for IgG₁ or IgG_{2a} (Zymed, San Francisco, CA, USA). In the case of IgE, biotin conjugated rat anti-mouse IgE (BD Biosciences, Franklin Lakes, NJ, USA) was added to each well for 2 hours at room temperature. After washing, avidin-ALP (Sigma) was added to each well for 1 hour. After washing with PBS/T, p-nitrophenyl phosphate (p-nitrophenyl phosphate liquid substrate system, Sigma) was added to each well and allowed to develop for 15 minutes. Absorbance was measured at 405 nm. IgG1 and IgG2a concentrations were expressed as units (U)/ml relative to the pooled hyperimmune serum, while IgE concentrations were shown as ng/ml.

Immunohistochemistry

Sections were prepared for immunohistochemistry by following the previously described method.⁸ In brief, the eyes from naive or actively immunised mice that were developing EC without Ab treatment were immediately frozen in 3% carboxymethyl cellulose (CMC) gel. Sections 4 µm thick were prepared and fixed in methanol. Endogenous peroxidase activity was inhibited by incubation with 0.1% NaN₃ and 0.3% H₂O₂ in distilled water for 10 minutes at room temperature. The samples were then exposed to anti-PD-L2 (TY25) for 30 minutes and subsequently to biotinylated anti-rat Abs for another 30 minutes. As a negative control, samples were incubated with secondary Abs (biotinylated anti-rat Abs) without exposing to anti-PD-L2 Ab. All slides were subjected to an avidin-biotin-complex kit (Vector laboratories Inc, USA) and then developed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO, USA).

Statistical analysis

Significant differences in serum Ig levels, cytokine production, and infiltrating eosinophil numbers between the Ab

Figure 2 Treatment with anti-PD-1, anti-PD-L1, or anti-PD-L2 Ab during induction phase affected systemic immune responses. Spleens and sera were collected at the time of harvesting the conjunctivas from actively immunised mice treated with nrlgG, anti-PD-1, anti-PD-L1, or anti-PD-L2 Ab during the induction phase as described in the legend to figure 1. The IL-4, IL-5, IL-10, IL-13, and IFN- γ levels in the culture supernatants of splenocytes stimulated with RW were measured by ELISA (A). RW specific IgE, IgG1, and IgG2a levels were evaluated by ELISA (B). * $p < 0.05$, ** $p < 0.01$, compared with nrlgG treated group.

treated and nrlgG treated groups were determined by Student's *t* test. *p* Values less than 0.05 were considered significant.

RESULTS

Treatment with anti-PD-1, anti-PD-L1, or anti-PD-L2 Ab during the induction phase did not affect the infiltration of eosinophils into the conjunctiva

To investigate whether PD-1, PD-L1, or PD-L2 is involved in the development of EC during the induction phase, actively immunised mice were intraperitoneally injected with each Ab every other day from days 0 to 8. There were no significant differences in the numbers of infiltrating eosinophils into the conjunctiva among the four groups (fig 1).

Treatment with anti-PD-1, anti-PD-L1, or anti-PD-L2 Ab during the induction phase affected both cellular and humoral immune responses

Since Ab treatment did not affect eosinophil infiltration into the conjunctiva, compared to the nrlgG treatment, we

conducted control studies to exclude the possibility that the treatment with Abs exerted no effects. We evaluated both cellular and humoral immune responses. Treatment with anti-PD-1, anti-PD-L1 or anti-PD-L2 Abs significantly increased IL-4, IL-5, IL-10, and IL-13 production by splenocytes in response to the RW stimulation *in vitro*, especially when anti-PD-L2 Ab was injected (fig 2A). RW specific IgE levels in serum were not significantly different among the four groups, while RW specific IgG1 and IgG2a levels were significantly upregulated by treatment with anti-PD-L2 Ab (fig 2B). Thus, the treatment with anti-PD-1, anti-PD-L1, or anti-PD-L2 Ab significantly affected systemic immune responses.

Treatment with anti-PD-L2 Ab during the effector phase increased eosinophil infiltration into the conjunctiva without significantly affecting systemic immune responses

To investigate whether PD-1, PD-L1, or PD-L2 is involved in the development of EC during the effector phase, actively

Figure 3 Treatment with anti-PD-L2 Ab during the effector phase increased eosinophil infiltration into the conjunctiva. Balb/c mice were immunised with RW in alum. Ten days later (day 10), the mice were challenged with RW in eye drops and their conjunctivas were harvested 24 hours later for histological analysis by Giemsa staining. The mice were intraperitoneally injected with 200 µg of nrlgG, anti-PD-1, anti-PD-L1, or anti-PD-L2 Ab during the effector phase on day 10, 2 hours before RW challenge. The infiltrating eosinophils were counted and the data are expressed as eosinophils per mm² (n = 10 per group). *p < 0.05, compared with nrlgG treated group.

immunised mice were intraperitoneally injected with each Ab on day 10, 2 hours before RW challenge. Anti-PD-L2 Ab treatment significantly increased eosinophil infiltration (fig 3). Analyses of RW specific cytokine production (fig 4A) and RW specific Ig levels (fig 4B) demonstrated that no significant differences were observed among the four groups.

PD-L2 expression in the conjunctiva was upregulated by the induction of EC

Finally, to confirm the expression of PD-L2 in the conjunctiva, conjunctivas from naive and EC developing mice were harvested for immunohistochemical analysis. A small number of PD-L2 expressing cells were detected in the conjunctiva of naive mice (fig 5A). In contrast, PD-L2 expressing cells were increased by the induction of EC (fig 5B). Negative controls did not show any positive signals (data not shown).

DISCUSSION

In murine experimental asthma, the involvement of PD-1 and its ligands has been demonstrated.²¹ Consistent with this previous report demonstrating that treatment with anti-PD-1 Ab did not affect asthmatic responses,²¹ we show here that treatment with anti-PD-1 Ab had no effect on EC during

Figure 4 Treatment with anti-PD-1, anti-PD-L1, or anti-PD-L2 Ab during the effector phase did not significantly affect systemic immune responses. Splens and sera were collected at the time of harvesting the conjunctivas from actively immunised mice treated with nrlgG, anti-PD-1, anti-PD-L1, or anti-PD-L2 Ab during the effector phase as described in the legend to figure 3. The IL-4, IL-5, IL-10, IL-13, and IFN-γ levels in the culture supernatants of splenocytes stimulated with RW were measured by ELISA (A). RW specific IgE, IgG1, and IgG2a levels were evaluated by ELISA (B).

Figure 5 PD-L2 expression in the conjunctiva was upregulated by the induction of EC. Conjunctivas from naive (A) and EC developing mice (B) were harvested for immunohistochemical analysis using anti-PD-L2 Ab (TY25). PD-L2 expressing cells were detected in the conjunctiva of naive mice (A). By induction of EC, PD-L2 expressing cells increased in the conjunctiva (B). Bar = 20 μ m. One representative of four mice is shown. Arrows indicate Ab stained cells.

either the induction or the effector phase. In contrast, treatment with anti-PD-L2 Ab during the effector phase increased inflammation in the lung,²¹ a result in good agreement with our observation that anti-PD-L2 Ab treatment during the effector phase increased eosinophil infiltration into the conjunctiva. It has also been reported that injection of murine PD-L2-Fc fusion protein increased cell infiltration to bronchoalveolar lavage fluid.²⁴ Recent studies have shown that PD-L2-Fc binds and co-stimulates naive CD4⁺ T cells from PD-1 deficient mice.^{25–26} Thus, our data further support the possibility that PD-L2 interacts not only with PD-1 but also with some receptor other than PD-1.

Treatment with anti-PD-1, anti-PD-L1, or anti-PD-L2 Ab during the induction phase significantly affected RW specific immune responses. Particularly, treatment with anti-PD-L2 Ab markedly increased RW specific Th2 cytokine production and RW specific IgGs in serum. However, these changes in immune responses did not affect eosinophil infiltration into the conjunctiva. It remains unclear why the upregulated Th2 immune responses (IL-4, IL-5, and IL-13) did not affect the eosinophil infiltration. It is of note that IL-10, which regulates allergic reactions,²⁷ was also upregulated in the anti-PD-L2 Ab treated group and the upregulation of IL-10 might have suppressed the effector function of Th2 immune responses. Further study will be necessary to elucidate the role of IL-10 in the development of EC.

Importantly, the upregulation of eosinophil infiltration by the treatment with anti-PD-L2 Ab was noted during the effector phase but not the induction phase indicating that the interaction between PD-L2 and its putative receptor other than PD-1 is important for the recruitment of eosinophils into the conjunctiva. In fact, using immunohistochemical methods, we found that expression of PD-L2 in the conjunctiva was upregulated by the induction of EC. Although we have not examined which types of cells express PD-L2 in the conjunctiva, it was reported that expression of PD-L2 is restricted in macrophages and dendritic cells.²⁸ Thus, the interaction between PD-L2 presumably expressed in macrophages and dendritic cells and its receptor occurring in the conjunctiva is likely to be involved in eosinophil infiltration. Thus, PD-L2 may be a therapeutic candidate for the inhibition of eosinophil infiltration into the conjunctiva in severe AC.

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