

Regulatory role of DC-derived osteopontin in systemic allergen sensitization

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Osteopontin (OPN) is a secreted phosphoglycoprotein with a wide range of functions, and is involved in various pathophysiological conditions. However, the role of OPN in IgE and Th2-associated allergic responses remains incompletely defined. The aim of this study was to elucidate the role of OPN in systemic allergen sensitization in mice. When compared with OPN^{+/+} mice, significantly increased levels of OVA-induced IgE were found in OPN^{-/-} mice. OPN^{-/-} DC demonstrated an increased capacity to enhance Th2 cytokine production in CD4⁺ T cells from sensitized OPN^{+/+} mice. Furthermore, significantly reduced levels of IL-12p70 expression were seen in LPS-stimulated OPN^{-/-} DC as compared with the WT DC, and the reduction was reversible by the addition of recombinant OPN (rOPN). rOPN was able to suppress OVA-induced IL-13 production in the cultures of CD4 and OPN^{-/-} DC, but this inhibitory activity was neutralized by the addition of anti-IL-12 Ab. In addition, administration of rOPN *in vivo* suppressed OVA-specific IgE production; however, this suppressive effect was abrogated in IL-12-deficient mice. These results indicate that DC-derived OPN plays a regulatory role in the development of systemic allergen sensitization, which is mediated, at least in part, through the production of endogenous IL-12.

Key words: Bone marrow-derived DC • IgE • Osteopontin • Th2 cytokines

Introduction

Osteopontin (OPN), also known as Eta-1 (early T-lymphocyte activation 1), is a phosphorylated acidic glycoprotein that has been implicated in a number of physiological and patholo-

gical events. Although the mechanistic aspect of OPN function remains to be defined, it appears that OPN plays a role in T-cell-mediated immunity [1–4]. Studies using OPN-deficient mouse models and neutralizing Ab have demonstrated the importance of OPN in the development of several inflammatory diseases, particularly those involving Th1-associated immune responses [5–8]. In humans, high levels of OPN

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have been observed in several Th1-associated diseases, such as sarcoidosis [9], tuberculosis [10], rheumatoid arthritis [11], Crohn's disease [12] and multiple sclerosis [13], although other studies have contradicted these observations [14, 15].

OPN's potential involvement in Th2-associated allergic responses has been investigated recently, particularly in eosinophilic airway inflammation [16, 17] and in mast cell degranulation [18]. However, its role in systemic allergen sensitization via the production of allergen-specific IgE is still unclear. Recently, as a part of our genomics study toward identification of regulatory pathways for successful allergen-specific immunotherapy, we have identified a significant increase of OPN expression in subjects who successfully completed venom immunotherapy and showed decrease of venom-specific IgE responses, when compared with those who were hypersensitive to allergens and had not received immunotherapy [19, 20], suggesting its potential involvement in the regulatory pathway of allergic responses. Moreover, the fact that IFN- γ , a Th1 cytokine, is able to induce OPN [21], whereas Th2 cytokines (IL-4 and IL-13) suppress it in human monocytes and monocyte-derived DC as shown in our recent reports [22], would further suggest the potential role of monocyte- or DC-derived OPN in the regulation of allergen-specific IgE responses. The critical role of DC in the regulation of T-cell response has been established [23]. DC are capable of inducing both Th1- and Th2-cell responses [24, 25] and are known to express high levels of OPN [22, 26, 27].

In this communication, we provide evidence supporting the role of DC-derived OPN in the regulation of allergen-induced systemic IgE and Th2 cytokine responses in mice.

Results

Altered systemic allergen-specific IgE production in OPN^{-/-} mice

To investigate the involvement of OPN in the regulation of systemic allergen sensitization, the levels of allergen-induced Ab were first examined in OPN^{-/-} mice. As shown in Fig. 1, titration experiments of pooled sera from the same group of mice showed that the titers of specific IgE and IgG1 Ab were significantly higher in OPN^{-/-} mice when compared with those seen in WT mice (Fig. 1A). Also, when individual samples were analyzed, significantly higher levels of OVA-specific IgE were noted in OPN^{-/-} mice. Moreover, no significant difference in the relative levels of specific IgG2a Ab (Fig. 1B) was found between sensitized OPN^{+/+} and OPN^{-/-} mice.

Modulation of Th2 responses by OPN in vitro

To investigate the underlying mechanisms for altered IgE response seen in OPN^{-/-} mice, the functional capacity of OPN^{-/-} DC, a major cell type expressing OPN [21], in the induction of T-cell cytokine expression was examined and compared with that of OPN^{+/+} mice. CD4⁺ T cells isolated from the spleens of sensitized WT and OPN^{-/-} mice were stimulated with medium alone or OVA (25 μ g/mL) in the presence of DC from either OPN^{+/+} or OPN^{-/-} mice. As shown in Fig. 2A, while, as expected, the T cells from OPN^{+/+} mice showed upregulated levels of OVA-induced IL-13 expression in the presence of OPN^{+/+} DC, further enhancement of the IL-13 level was observed

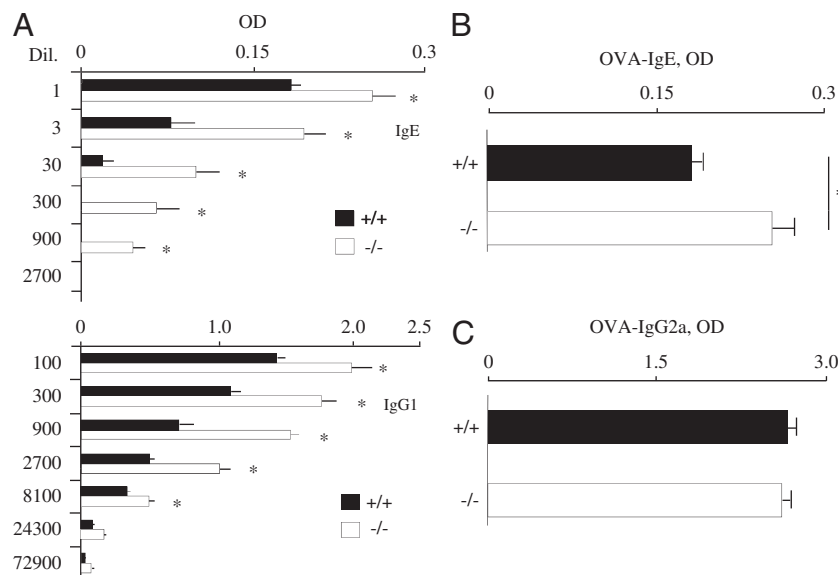


Figure 1. The relative levels of serum specific Ab in sensitized OPN^{-/-} and OPN^{+/+} mice. (A) Serum titration analysis of specific IgE and IgG1 levels in pooled sera, and (B) the mean levels of specific IgE in individual sample, from each group. (C) The relative levels of specific IgG2a in each group. Data are expressed as mean $\pm$ SEM. * p < 0.05 (N = 9–12 mice per group, Mann–Whitney U-test).

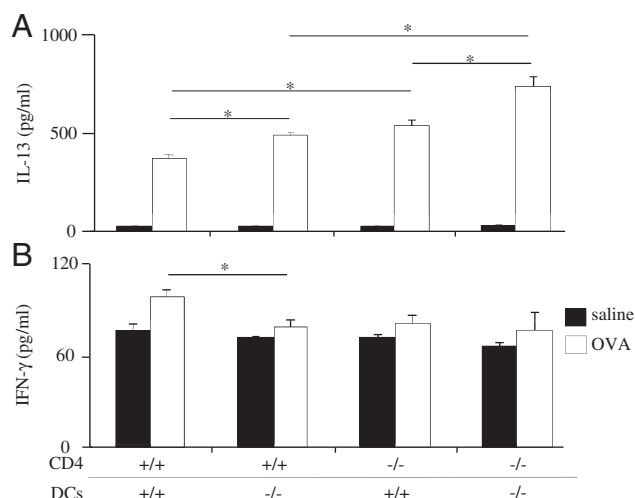


Figure 2. Analysis of T-cell cytokines. The levels of (A) IL-13 and (B) IFN- γ secretion in splenic CD4⁺ T cells from OVA-sensitized mice. The cells were cultured in the presence of DC from OPN^{-/-} or OPN^{+/+} mice and stimulated with medium alone or OVA. Data are expressed as mean $\pm$ SEM from three independent experiments. * p < 0.05.

when the T cells were co-cultured with DC from OPN^{-/-} mice. In fact, T cells from sensitized OPN^{-/-} mice produced the highest levels of IL-13 when DC from OPN^{-/-} mice were used as the Ag-presenting cells. Increased levels of IL-13 in OPN^{-/-} T cells were also noted as compared with those seen in OPN^{+/+} T cells. In contrast, the level of IFN- γ was reduced in OPN^{+/+} T cells in the presence of OPN^{-/-} DC when compared with that of the co-culture with OPN^{+/+} DC (Fig. 2B), whereas no statistical difference was noted in the level of IFN- γ in OPN^{-/-} CD4⁺ T cells from sensitized OPN^{-/-} mice when co-cultured with OPN^{+/+} DC. In this experimental setting using the C57BL/6 (B6) strain of mice, known to produce low IL-4, the levels of IL-4 in the culture supernatants were low (<15 pg/mL) in all groups of mice.

Adoptive transfer of OPN^{-/-} DC upregulates Th2 responses

In order to further confirm the regulatory effects of OPN^{-/-} DC, adoptive transfer experiments were performed, in which DC (1×10^6) derived from naïve OPN^{+/+} or OPN^{-/-} mice were transferred once weekly for 2 wk into OPN^{+/+} mice, and mice were then sensitized with PBS or Ag 1 day after each cell transfer. On day 22 after the first Ag sensitization, splenocytes were isolated and stimulated *in vitro* with OVA (25 μ g/mL), and cytokine levels were analyzed. Consistent with the *in vitro* findings, significantly increased levels of IL-13 were found, while reduced levels of IFN- γ were seen, in cells from OPN^{-/-} DC-transferred mice when compared with those from OPN^{+/+} DC-transferred mice (Fig. 3A). Moreover, the levels of serum specific IgE, but not IgG2a, were significantly increased in OPN^{-/-} DC-transferred mice as compared with those seen in mice receiving OPN^{+/+} DC (Fig. 3B). These results thus suggest a modulatory

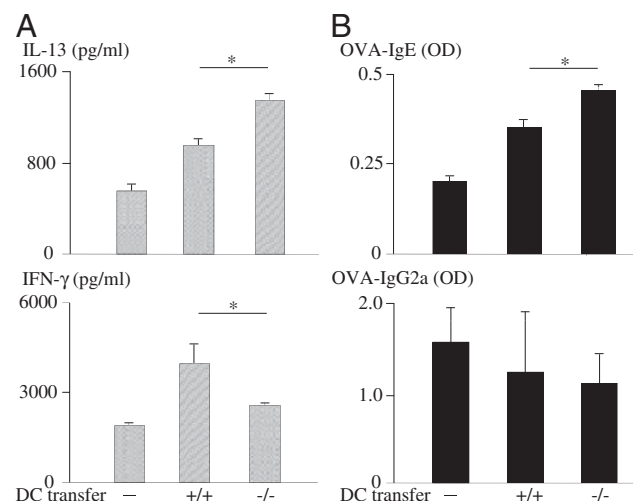


Figure 3. Effects of DC transfer on T-cell cytokines and OVA-IgE, IgG2a. DC were transferred intravenously once weekly for 2 wk. One day after each DC transfer, mice were sensitized with Ag. On day 22, splenocytes were stimulated with OVA, and (A) cytokine levels were analyzed. (B) OVA-specific IgE and IgG2a were also measured. Data are expressed as mean $\pm$ SEM. * p < 0.05 (N = 6 mice per group).

effect of DC-derived OPN on the induction of IgE and Th2 responses.

Exogenous OPN enhances LPS-induced IL-12p70 production

To explore the potential effector mechanism, the DC were activated by LPS and the subsequent release of two known immune modulators, IL-10 and IL-12p70, was examined. Although the levels of LPS-induced IL-10 were similar, significantly reduced levels of IL-12p70 expression were seen in LPS-stimulated OPN^{-/-} DC as compared with the WT DC (Fig. 4). Significantly, the addition of exogenous OPN was able to reverse, in a dose-dependent manner, the reduction in the level of LPS-induced IL-12p70 to a level similar to that of the WT DC (Fig. 4), suggesting that the expression of IL-12p70 is dependent, in part, on the expression of OPN. Note that consistent with our previous report [28], the addition of OPN alone without a concomitant LPS stimulation was incapable of inducing IL-12p70 expression.

To further confirm the inhibitory effect of OPN on the expression of Th2 cytokines and its dependence on the expression of IL-12, the levels of Ag-induced IL-13 were analyzed in co-cultures of OPN^{+/+} CD4⁺ T cells from OVA-sensitized mice and DC from either OPN^{+/+} or OPN^{-/-} mice in the presence or absence of rOPN (10 μ g/mL), neutralizing anti-IL-12 (0.1 μ g/mL) or isotype control (0.1 μ g/mL) Ab. As shown in Fig. 5, exogenous OPN was able to suppress OVA-induced IL-13 production in the cultures of CD4⁺ T cells and OPN^{-/-} DC, but this inhibitory activity was neutralized by the addition of anti-IL-12 Ab when compared with that seen in cultures with the addition of an

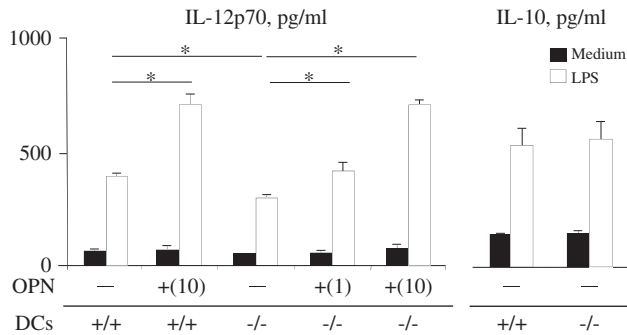


Figure 4. Effect of OPN on the DC cytokine response. Analysis of LPS (1 ng/mL)-induced IL-12p70 and IL-10 expression in DC from OPN^{-/-} or OPN^{+/+} mice in the presence or absence of rOPN (1 or 10 µg/mL), with the level of IL-12p70 and IL-10 analyzed by ELISA. Data are expressed as mean±SEM from three independent experiments. **p*<0.05.

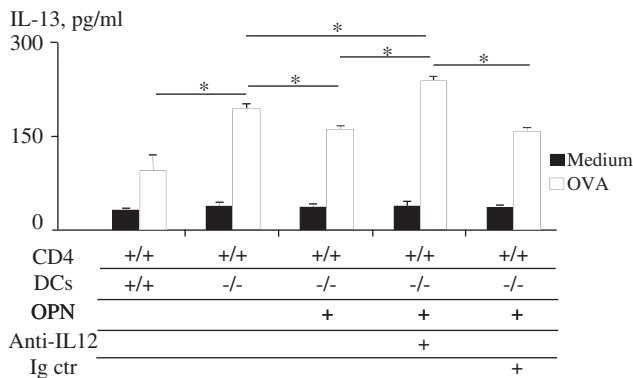


Figure 5. Analysis of OVA-induced IL-13 levels in splenic CD4⁺ T cells from OVA-sensitized OPN^{+/+} mice in the presence of DC from OPN^{+/+} or OPN^{-/-} mice, exogenous rOPN (10 µg/mL), neutralizing anti-IL-12 (anti-IL-12, 0.1 µg/mL) or Ig isotype control (Ig Ctr, 0.1 µg/mL) Ab. Data are expressed as mean±SEM from three independent experiments. **p*<0.05.

isotype control Ab. It is noteworthy that in the presence of neutralizing anti-IL12 Ab and OPN^{-/-} DC, CD4⁺ T cells expressed higher levels of IL-13 after OVA stimulation than those seen in the absence of neutralizing Ab. No significant difference was seen in the levels of class II MHC, CD80 and CD86 between OPN^{-/-} and OPN^{+/+} DC, and the viability also remained similar during the culture time period (data not shown).

Suppression of allergen-specific IgE production by rOPN *in vivo*

To examine the modulatory effect of exogenous OPN *in vivo*, mice received Ag (OVA) sensitizations, and i.p. injections of either rOPN (25 µg/mouse) or PBS before, during and after each Ag sensitization (see Fig. 6 for schematic presentation). Results showed that while mice receiving PBS alone revealed a significant level of OVA-specific IgE production, concurrent treatment of Ag-sensitized mice with rOPN produced a significantly lower level of OVA-specific IgE (Fig. 6A and B). The levels of OVA-specific

IgG2a were similar in each group of mice. Of significance is the finding that the suppressive effect of OPN on Ag-induced OVA-specific IgE response *in vivo* was attenuated in IL-12-deficient mice (Fig. 6C), where no significant difference was seen in the relative levels of OVA-specific IgE between OPN treated and untreated mice. These results suggest, therefore, that OPN may exert its effect at the level of Ag priming and/or sensitization phase, and that this suppressive effect is dependent, in part, on its ability to enhance the level of IL-12.

Discussion

In this study, we identified a significant regulatory effect of OPN on systemic allergen sensitization in mice. Increased levels of allergen (OVA)-induced IgE and systemic Th2 responses were found in OPN^{-/-} mice, while treatment with rOPN significantly suppressed OVA-specific IgE level. We also demonstrated that modulation of Ag-induced IgE and Th2 response by DC-derived OPN is associated with the ability of OPN to enhance the production of IL-12p70, a known Th2 regulator.

Although other potential regulatory mechanisms cannot be ruled out at this time, it is tempting to speculate that the induction of IL-12p70 is the key factor in mediating the OPN effect. In support of this hypothesis, in our study of the OVA-sensitized, IL-12 gene-deficient mice, the loss of OPN's suppressive activity on OVA-induced IgE production was noted (Fig. 6C). It is worth noting that a recent publication by Shinohara *et al.* [29] demonstrated that plasmacytoid DC-derived intracellular OPN is required for the IFN-α production induced by various stimuli. Therefore, it is possible that increased expression of OPN may upregulate IL-12 and IFN-α in myeloid DC and plasmacytoid DC respectively, which in turn may modulate the IgE and Th2 responses. These results are relevant to our recent reports showing that significant increase of OPN expression is found in subjects who have successfully completed allergen immunotherapy [19, 20], suggesting that OPN may play a role in the efficacy of allergen immunotherapy.

Thus far, it remains unclear as to the exact role of OPN in the induction of cytokines in DC, a cell type critical in the regulation of T-cell response [26] and known to express high levels of OPN [21]. OPN has been suggested to promote a Th1 response via direct induction of monocyte- or DC-derived IL-12 [30–32]. Contrary to these findings, other recent studies have provided evidence that OPN alone is incapable of inducing IL-12p70. Using endotoxin-depleted recombinant as well as native forms of OPN preparations, we have previously demonstrated that the ability of commercially available human OPN to induce several proinflammatory cytokines was solely due to contaminating endotoxin, providing no evidence of the ability of OPN itself to induce proinflammatory cytokines in human cells *in vitro* [28]. Further studies are warranted to elucidate the mechanistic basis of how OPN regulates the expression of IL-12.

It is noted that the alteration in the levels of IgE and Th2 cytokines was relatively modest in OPN^{-/-} mice, with an average of 30–40% increase as compared with those seen in the OPN^{+/+}

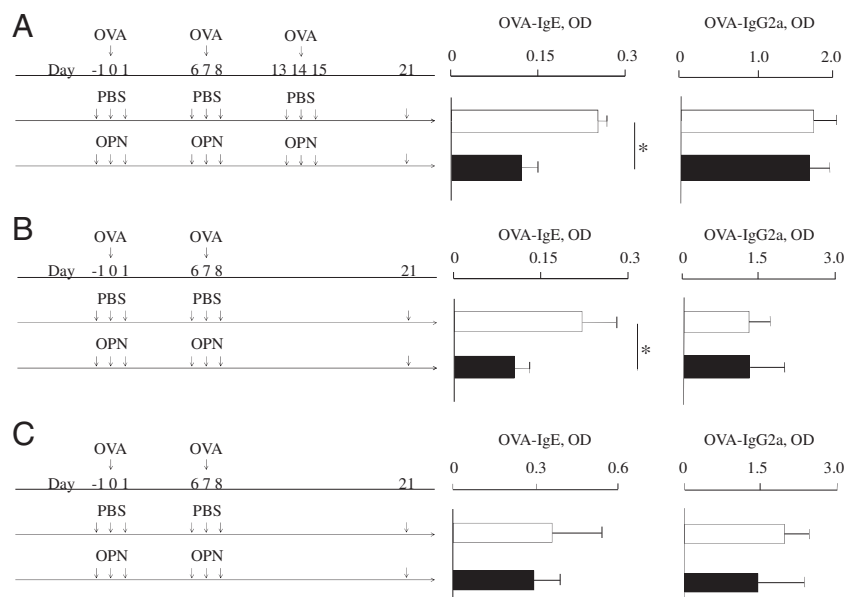


Figure 6. Suppression of IgE by OPN. (A) OPN-deficient mice (C57BL/6), (B) WT mice (BALB/c) and (C) IL-12-deficient mice (BALB/c) were treated with rOPN (25 µg/mouse) or PBS control mixture containing BSA and a trace amount of contaminating endotoxin at indicated time periods. On day 21, the levels of serum total and specific IgE and IgG2a Ab, respectively, were analyzed. Data are expressed as mean ± SEM. * $p < 0.05$ ($N = 6$ –9 mice per group).

mice. Further, the expression of LPS-induced and basal IL-12p70 was not completely dependent on the presence of OPN (Fig. 4). These results would suggest that like many other cytokines, OPN is able to modify the degree of IgE and Th2 responses. Also, it is likely that OPN is operative in concert with additional cytokines/factors in a redundant regulatory network.

It is worth noting that, in a recent finding of Abel *et al.* [33], while no dramatic difference in the frequency of IFN- γ -expressing T cells was found when OPN $^{-/-}$ DC were present in the culture, the frequency of IL-4-expressing T cells increased from 0.9% in the presence of WT DC to 2.8% in the presence of OPN $^{-/-}$ DC. Interestingly, in the same cultures, the frequency of IFN- γ -expressing cells dropped from 83 to 75%. Although cytokine secretion was not measured in the study of Abel *et al.* [33], this modest difference found in *in vitro* culture may have significant impact in the development and modulation of immune responses *in vivo*, considering that the expression of OPN is both constitutive and inducible in many inflammatory conditions. Its long-term, accumulative effect could be substantial.

It is also noteworthy that in the presence of neutralizing anti-IL12 Ab and OPN $^{-/-}$ DC, CD4 $^{+}$ T cells expressed higher levels of IL-13 after OVA stimulation than those found in the absence of neutralizing Ab. Although the precise underlying mechanisms are unclear, it is likely that the enhancement of the IL-13 expression under these conditions is the result of the neutralizing effect of anti-IL-12 Ab on both baseline and OPN-dependent IL-12. As shown in Fig. 4, DC from OPN $^{-/-}$ mice showed low, but detectable, levels of IL-12.

At present, the role of OPN seems to be complex, because contradictory reports have been published regarding the contribution of OPN in several inflammatory conditions [8, 14, 15] and

dual roles of this molecule in a single disease have also been reported [16, 17, 34, 35]. Xanthou *et al.* recently showed that after Ag sensitization and pulmonary challenge, the levels of Ag-specific IgE were higher in OPN $^{-/-}$ mice when compared with OPN $^{+/+}$ mice, while administration of anti-OPN Ab during Ag sensitization suppressed Ag-specific IgE production [16]. It is difficult, at present, to reconcile these discrepancies, which may be due, in part, to the multi-functional nature of OPN in the regulation of cytokine generation and inflammatory cell migration through different functional domains in OPN. It is noted that Xanthou *et al.* measured OVA-specific IgE levels after the OVA challenge followed by i.p. OVA injection [16], which is different from our study in that OVA-specific IgE was measured without OVA challenge. To better understand the actual effects of OPN on systemic allergen sensitization in mouse models, our strategy, which measured OVA-IgE levels before OVA challenge, might be more representative of a systemic response, particularly considering the multi-functional nature of OPN. Furthermore, our results showed that the suppressive effects of endogenous OPN on Th2 responses can be reproduced by exogenous OPN, thus suggesting the direct involvement of OPN in systemic allergen sensitization. Additional detailed analyses are clearly required in order to ultimately establish the functional role of OPN in the regulation of systemic immune responses.

Although we emphasize the involvement of DC-derived OPN on the regulation of allergen-specific systemic sensitization, OPN secreted from other cells, including T cells, might be also important for regulation of allergic response. In fact, various inflammatory cells, including CD4 $^{+}$ T cells [36], reportedly express and secrete OPN, and the current results (Fig. 2A)

indicate that thin OPN secreted from CD4⁺ T cells is also involved in the regulation of Th2 response.

In summary, our current results suggest a role of DC-derived OPN in the regulation of IgE and Th2 cytokine responses. Many animal model studies and human data strongly suggest that DC function is critical to the development and regulation of the atopic state. It is likely, therefore, that a highly controlled release of OPN could be one mechanism to improve protective immunity, while limiting the extent of the allergic response. How DC and T cells integrate OPN signal to limit the extent of the Th2 and IgE responses may thus represent an important and novel mechanism for controlling allergic responses. The results of our current studies would provide an important basis for an in-depth investigation.

Materials and methods

Mice and immunization

C57BL/6 (B6) and BALB/c (6–8 wk old) mice were purchased from the Jackson Laboratory or Sankyo (Tokyo, Japan). IL-12-deficient mice of BALB/c background were purchased from the Jackson Laboratory. OPN^{-/-} [37] mice backcrossed eleven times with B6 mice were used. All mice were maintained under specific pathogen-free conditions. All mouse experiments were approved by the Ethics Committee on Animal Research of Johns Hopkins University of Medicine, Showa University, Hokkaido University and conformed to the institutional and National Institute of Health guidelines. Mice were sensitized with PBS or OVA, 50 µg (grade V; Sigma, St. Louis, MO, USA) along with 1 mg alum (Imject Alum; Pierce, Rockford, IL, USA) by i.p. injection on days 0, 7 and 14. On day 21, the sera were obtained and the levels of OVA-specific IgE, IgG1 and IgG2a were determined by ELISA [38, 39]. In some cases, titration experiments of pooled sera were performed to determine the relative levels of IgE and IgG1 Ab and the upper/lower limits of detection. Based on the results, optimal dilutions (1:3 dilution for IgE and 1:1000 for IgG2a measurement) of serum samples were selected for the analysis of the relative levels of Ab in each mouse sample.

In the study of the effect of OPN treatment OPN^{+/-} mice (BALB/c) or OPN^{-/-} mice (C57BL/6) were sensitized i.p. on days 0 and 7 (14 for C57BL/6) with OVA. Sensitized mice were divided into two treatment groups: Group 1, mice received PBS i.p. once daily on days -1, 0 and 1 and from six to eight (and from 13 to 15 for C57BL/6); Group 2, mice received rOPN (25 µg/mouse; R&D Systems, Minneapolis, MN, USA) once daily on days -1, 0 and 1, from six to eight (and from 13 to 15 for C57BL/6). To control for a carrier effect and for a potential effect from a trace amount of contaminating endotoxin, an equivalent amount of BSA (Sigma, A-2058; 50 µg BSA/1 µg rOPN) and LPS (*Escherichia coli*, 0127:BB, Sigma) was added to PBS and used as a control. Serum was collected from each mouse on day 21 after the OVA sensitization, and the levels of OVA-specific IgE and IgG2a were determined by ELISA.

DC transfer and CD4⁺ T-cell stimulation and analysis of cytokines

BM-DC were prepared using a method adapted from that of Inaba *et al.* [40]. Briefly, BM cells from femurs and tibiae were cultured for 10 days in the presence of 20 ng/mL of GM-CSF (Schering-Plough, Kenilworth, NJ, USA), and surface expression of CD11c, MHC II, CD80 and CD86 was analyzed to confirm the purity (>97%) of the DC. CD4⁺ T cells were isolated from the splenocytes of sensitized OPN^{+/-} or OPN^{-/-} mice to >97% purity with positive selection by using anti-CD4⁺ magnetic beads (Miltenyi Biotec, Auburn, CA, USA). CD4⁺ T cells were co-cultured with DC from either OPN^{+/-} or OPN^{-/-} mice at a ratio of 10:1 in the presence or absence of OVA (25 µg/mL). DC from OPN^{-/-} mice were also co-cultured with CD4⁺ T cells and OVA (25 µg/mL), rOPN and anti-IL-12 Ab (R&D Systems). DC from either OPN^{+/-} or OPN^{-/-} mice were stimulated with LPS in the presence or absence of rOPN. The levels of IL-4, IL-13, IL-10, IL-12p70 and IFN-γ were measured with the use of cytokine ELISA kits (for IL-4, IL-12p70 and IFN-γ, eBioscience, San Diego, CA, USA and for IL-13, R&D Systems). For adoptive transfer of DC, DC (1 × 10⁶) derived from OPN^{+/-} or OPN^{-/-} mice were transferred intravenously once weekly for 2 wk into OPN^{+/-} mice. One day after each DC transfer, mice were sensitized with PBS or Ag. On day 22 after the first Ag sensitization, splenocytes (5 × 10⁶) from DC-transferred mice were isolated and stimulated with OVA (25 µg/mL), and cytokine levels were analyzed. Moreover, OVA-specific IgE and IgG2a in serum were measured.

Statistical analysis

All statistical analyses were performed using the Stat View 5.0, 1998 SAS Institute. All data are expressed as mean ± SEM. Differences between groups were assessed by the Kruskal–Wallis test followed by Tukey's test for multiple comparisons or Mann–Whitney *U*-test. A *p*-value of <0.05 was considered to be statistically significant.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: OPN: osteopontin · rOPN: recombinant OPN

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