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Expression of B7-H1 and B7-DC on the airway epithelium is enhanced by double-stranded RNA

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Abstract

Viral infection in the airway provokes various immune responses, including Th1 and Th2 responses, which are partly initiated by double-stranded RNA (dsRNA), a viral product for its replication. B7-H1 (PD-L1) and B7-DC (PD-L2) are B7-family molecules that bind to programmed death-1 (PD-1) on lymphocytes and are implicated in peripheral tolerance. We investigated the effect of dsRNA on the expression of B7-H1 and B7-DC on airway epithelial cell lines. B7-H1 and B7-DC were constitutively expressed on the cells, and their expression was profoundly upregulated by stimulation with an analog of viral dsRNA, polyinosinic–polycytidylic acid. B7-H1 and B7-DC were also upregulated by stimulation with IFN-γ, IL-13, and the supernatant from T cell clones. A relatively high concentration of dexamethasone (1 μM) was required to suppress the upregulation of B7-H1 or B7-DC. These results suggest that epithelial B7-H1 and B7-DC play a role in virus-associated immune responses in the airways.

Keywords: Innate immunity; Double-stranded RNA; IFN-γ; IL-13; Programmed death-1; BEAS-2B; T cell clone; Glucocorticoid

The elucidation of epithelial cell-to-virus interactions, particularly, in the context of immune regulation, has been an important research target in the area of airway diseases [1]. The rhinovirus, respiratory syncytial virus (RSV), influenza virus, and parainfluenza virus are known to exacerbate asthma and chronic obstructive pulmonary diseases (COPD). These viruses synthesize double-stranded RNA (dsRNA) for their replication. Recently, dsRNA was identified as a natural ligand for Toll-like receptor 3 (TLR3) [2], and it was shown that virus-infected epithelial cells secrete several chemokines in response to dsRNA via TLR3, dsRNA-dependent protein kinase (PKR), and mitogen-activated protein kinases [3–5] Although these responses are associated with innate immunity against viral invasion, the following process may frequently lead to the exacerbation of underlying diseases.

Virus-infected cells express the virus-associated antigens on the major histocompatibility complex (MHC) class I and await cytolysis by cytotoxic/killer T cells, a mechanism which prevents the further spread of the virus [6,7]. On the other hand, viral species have developed escape mechanisms from the host immune system [8], including interruptions of the infected cell-to-T cell responses. When antigen-specific T cells recognize the MHC-class I/II-antigen peptide complex on their counterparts, the following T cell responses are critically affected by the simultaneous signaling operated through costimulatory molecules. B7-1 (CD80) and B7-2 (CD86)

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are well-characterized costimulatory molecules expressed on professional antigen-presenting cells (APCs). These molecules are upregulated by various stimuli, such as LPS and dsRNA [9]. During the last decade, an array of molecules with homologous sequences to B7-1 and B7-2 have been identified, including B7-H1 (PD-L1), B7-H2 (inducible costimulator ligand; ICOSL), B7-H3, B7-H4 (B7S1, $B7_X$), and B7-DC (PD-L2).

B7-H1 and B7-DC are ligands for programmed death-1 (PD-1), which is expressed on activated T and B cells [10–13]. Colligation of PD-1 and the T cell receptor (TCR) leads to rapid phosphorylation of SHP-2, a phosphatase suggested to inhibit T cell activation. Indeed, various experimental disease models using PD-1-deficient mice or neutralizing antibodies against B7-H1, B7-DC, or PD-1 suggested that B7-H1 and B7-DC have a crucial role in the regulation of immune responses in the periphery [14–18]. More interestingly, B7-H1 and B7-DC are expressed not only on professional APCs but also on tissue structural cells, including endothelial cells, keratinocytes, and several tumor cells [11,19–21]. These findings imply that structural cells might actively contribute to the maintenance of various immune responses. Thus, we sought to assess the expression of B7-H1 and B7-DC on human airway epithelial cells and investigated whether virus-associated compounds affect their expression.

Materials and methods

Culture of airway epithelial cells. The BEAS-2B and the 16HBE, both of which are SV40-transformed human bronchial epithelial cell lines, and the A549, a human type II alveolar epithelial cell line, were cultured in DMEM/F12 containing 10% FBS, 100 U/ml penicillin, and 100 ng/ml streptomycin (Invitrogen, Tokyo, Japan) at 37 °C with 5% CO_2 in humidified air.

Stimulation of airway epithelial cells. When cells reached 90% confluence, they were stimulated with various concentrations of polyinosinic–polycytidylic acid (referred to as dsRNA; Sigma–Aldrich, St. Louis, MO), polycytidylic acid (referred to as single-stranded RNA, ssRNA; Sigma–Aldrich), human recombinant IFN-γ (Techne, Minneapolis, MN), IL-13 (Daiichi Pharmaceutical, Tokyo, Japan) or both of the cytokines for 24 h. In the study of dsRNA, several samples were treated with 7.5 μg/ml of rabbit anti-human IFN-β antibody (Chemicon International, Temecula, CA) for neutralization of IFN-β. Some dsRNA was pretreated with an excessive amount of RNase (Qiagen, Tokyo, Japan) for 30 min to determine whether the biological effect of dsRNA in this study is derived from its ribonucleic acid structure. Several samples were incubated with ranging concentrations of dexamethasone (Banyu Pharmaceutical, Tokyo, Japan) 30 min before stimulation. After stimulation, the supernatants of the cell culture were collected for the quantification of chemokines by ELISA. Remaining cells were treated with 0.02% trypsin/EDTA (Sigma–Aldrich) in HBSS (Invitrogen), washed three times with Ca^{2+}–Mg^{2+}-free HBSS, and further treated with Ca^{2+}–Mg^{2+}-free HBSS containing 0.02% EDTA without trypsin for 20 min. Cells were harvested by repeated pipetting and then processed for flow cytometry.

Stimulation of airway epithelial cells with culture supernatant of T cell clones. Der f 2-specific human T cell clones were generated from peripheral blood mononuclear cells (PBMCs) of atopic asthmatic donors by antigenic stimulation, which was followed by the limiting dilution methods as described previously [22]. Briefly, PBMCs (2×10^6/ml) were cultured in an AIM-V medium (Life Technologies, Gaithersburg, MD) with 1 μg/ml recombinant Der f 2 protein (Asahi Food and Healthcare, Tokyo, Japan). After 10 days of culture, 10^2 to 10^4 live non-adherent cells were cultured in 96-well round-bottomed culture plates (Nunc, Roskilde, Denmark) with antigen and 2500 rad-irradiated autologous PBMCs (5×10^4 cells). A fresh medium containing 10 U/ml recombinant IL-2 (kindly provided by Shionogi Pharmaceutical, Osaka, Japan) was added once per week. When fewer than 1 of 10 wells contained proliferating cells, the resulting cell lines were considered to have originated from a single clone. To ensure their clonality, these T cells were further subcloned by limiting the dilution using irradiated autologous PBMCs and antigen. After 10–14 days, expanding cultures were transferred to 24-well culture plates (BD Biosciences, San Diego, CA). These clones were maintained by antigenic stimulation with irradiated autologous PBMCs (2×10^6/ml) and antigen every 2–3 week.

T cell clones were harvested at least 10 days after the last antigenic stimulation and suspended in an RPMI-1640 medium with 10% FBS and penicillin/streptomycin (Invitrogen). Cells (5×10^6/ml) were cultured with or without 10-μg/ml plate-bound anti-human CD3 mAb (OKT3, Ortho, Raritan, NJ) in 6-well culture plates (Greiner Japan, Tokyo, Japan) for 36 h, and their supernatants were then collected. An aliquot of the supernatant was used for the quantification of IFN-γ, IL-5, IL-13, and TNF-α by ELISA. When BEAS-2B cells reached 90% confluence, their culture medium was carefully replaced with the supernatant of a T cell clone, incubated for 24 h, and then processed for flow cytometry.

Flow cytometric analysis. The mAbs used for flow cytometry were fluorescein isothiocyanate (FITC)-labeled anti-human B7-1 mAb (L307.4, BD Biosciences), FITC-labeled anti-human B7-2 mAb (2331, BD Biosciences), biotinylated anti-human B7-H1 mAb (MIH1), and biotinylated anti-human B7-DC mAb (MIH18). MIH1 and MIH18 were generated as described previously [23]. For each analysis, 5×10^5 BEAS-2B cells were incubated in 100 μl of phosphate-buffered saline (PBS) with 0.5% BSA and 0.02% NaN_3 (Sigma–Aldrich) containing each mAb at room temperature for 30 min. The samples with biotinylated mAbs were washed and suspended in phycoerythrin (PE)-labeled streptavidin (BD Biosciences) for 20 min. After washing with PBS/0.5% BSA/0.02% NaN_3, the cells were fixed with 4% paraformaldehyde (medium A; Caltag Laboratories, Burlingame, CA) for 20 min. Fixed cells were washed again and then processed for flow cytometric analysis using a FACSCalibur flow cytometer and CELLQuest software (BD Biosciences). Ten thousand events were acquired in a list mode with debris excluded by the forward-scatter threshold. The mean fluorescence intensity (MFI) was compared with control staining using an irrelevant isotype-matched mouse mAb.

Quantification of chemokine and cytokine. The levels of eotaxin, regulated on activation, normal T cells expressed and secreted (RANTES), IFN-γ, IL-5, IL-13, and TNF-α in the culture supernatant were measured using an ELISA kit (Biosource International, Camarillo, CA). The level of IFN-β in the supernatant was measured using an ELISA kit (Biomedical laboratories, Picataway, NJ).

Data analysis. Values were expressed as means ± SEM. The data of flow cytometry are shown by histograms or MFI. Differences among groups were analyzed using unpaired t tests or an ANOVA together with a post hoc Bonferroni analysis. A value of $p < 0.05$ was considered significant.

Results

Expression of B7-H1 and B7-DC on unstimulated cells

The flow cytometric study showed that BEAS-2B cells spontaneously expressed B7-H1 and B7-DC on

their cell surface (Fig. 1A). The MFI of B7-H1 was 156 ± 5, while that of B7-DC was 16 ± 2, suggesting dominant expression of B7-H1 compared with B7-DC. The unstimulated BEAS-2B cells did not express B7-1 or B7-2. The incubation of cells with 10^{-8} to 10^{-6} M dexamethasone had no significant effect on the baseline expression of B7-H1 or B7-DC (Fig. 1B). 16HBE cells (Fig. 1C) and A549 cells (Fig. 1D) also expressed B7-H1 and B7-DC constitutively, whereas their expression on A549 cells was to a much lesser degree.

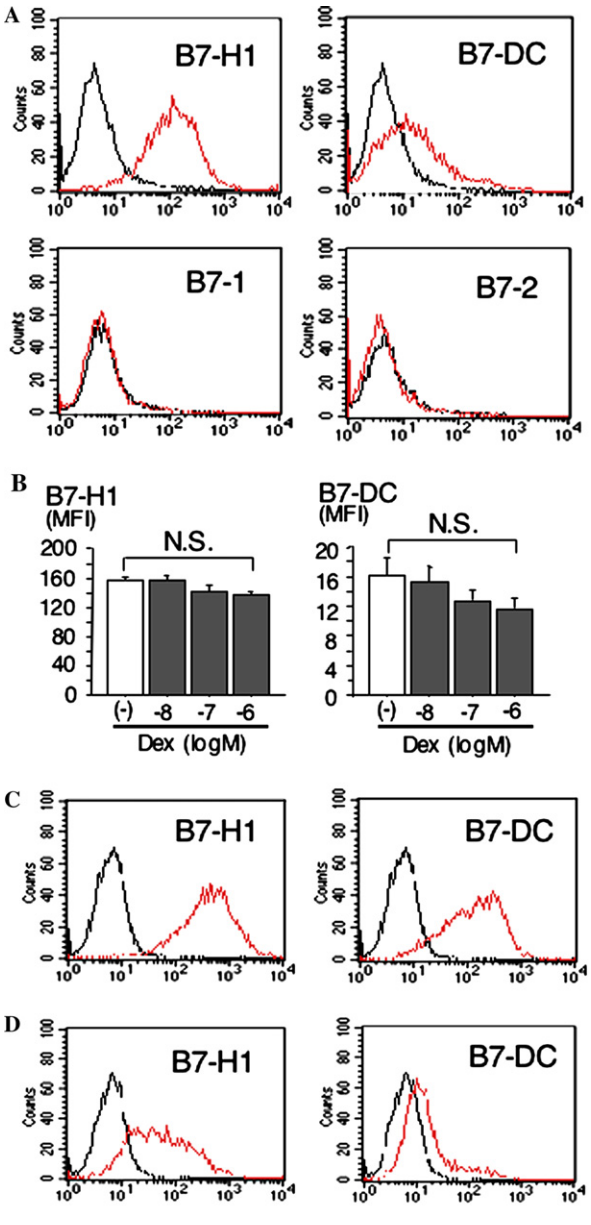

Fig. 1. Expression of B7-H1 and B7-DC on unstimulated cells. BEAS-2B, 16HBE, and A549 cells were stained with biotinylated anti-B7-H1 or anti-B7-DC mAbs followed by PE-streptavidin or with FITC-anti-B7-1 and PE-anti-B7-2 mAbs. Samples were analyzed by flow cytometry. (A) Representative histograms (shaded histograms) with the isotype controls (dotted lines) of BEAS2B cells. (B) BEAS-2B cells were incubated with dexamethasone (Dex) for 24 h. (C) Representative histograms (shaded histograms) with the isotype controls (dotted lines) of 16HBE cells. (D) Representative histograms (shaded histograms) with the isotype controls (dotted lines) of A549 cells. Data are evaluated as mean fluorescence intensity (MFI) and shown as means ± SEM of five experiments. NS, not significant. (NOTE: For the color figure in the web version, red lines and black lines correspond to shaded histograms and dotted lines, respectively.)

Effect of dsRNA on B7-H1 and B7-DC expression

The expression of B7-H1 and B7-DC on BEAS-2B cells was profoundly upregulated by stimulation with dsRNA in a dose-dependent manner (Figs. 2A and B). This upregulation was not found for samples treated with denatured dsRNA (Fig. 2A). In addition, the stimulation with ssRNA did not affect the expression of B7-H1 and B7-DC (data not shown). The expression of B7-H1 and B7-DC on 16HBE cells, but not on A549 cells, was also upregulated by dsRNA (data not shown). The upregulation of B7-H1 was not affected by dexamethasone ranging from 10^{-8} to 10^{-6} M, whereas the upregulation of B7-DC by dsRNA was suppressed only by 10^{-6} M dexamethasone (Fig. 2C). To evaluate the suppressive effect of dexamethasone on B7-H1 and B7-DC expression, the effect of dexamethasone on cytokine-induced chemokine production from BEAS-2B cells was examined by ELISA (Fig. 3). IFN-γ-induced RANTES production was significantly suppressed by 10^{-7} M dexamethasone, and IL-13-induced eotaxin production was significantly suppressed by 10^{-8} M dexamethasone. The stimulation of cells by dsRNA at the concentration inducing maximal expression of B7-H1 and B7-DC did not induce the expression of B7-1 or B7-2 (data not shown).

Effect of IFN-β neutralization

Viral infection induces innate immune responses including the production of type I interferons, IFN-α/β. Thus, we measured IFN-β concentration in the supernatant of BEAS-2B cells (Fig. 4). The concentration of IFN-β was markedly increased in the supernatant of dsRNA-stimulated cells up to 220 pg/ml, compared with that of unstimulated cells. Although the pretreatment of cell culture with excessive amount of anti-IFN-β antibody completely neutralized IFN-β in the supernatant, it failed to suppress the dsRNA-induced upregulation of B7-H1 and B7-DC.

Effect of IFN-γ and IL-13 on B7-H1 and B7-DC expression

Viral infection generally induces T cell responses, and those responses are partly mediated by Th1 and Th2 cytokines. The expression of B7-H1 and B7-DC was upregulated on BEAS-2B cells by stimulation with Th1 cytokine, IFN-γ, in a dose-dependent manner (Figs. 5A and C). The upregulation of B7-H1 and B7-DC was not

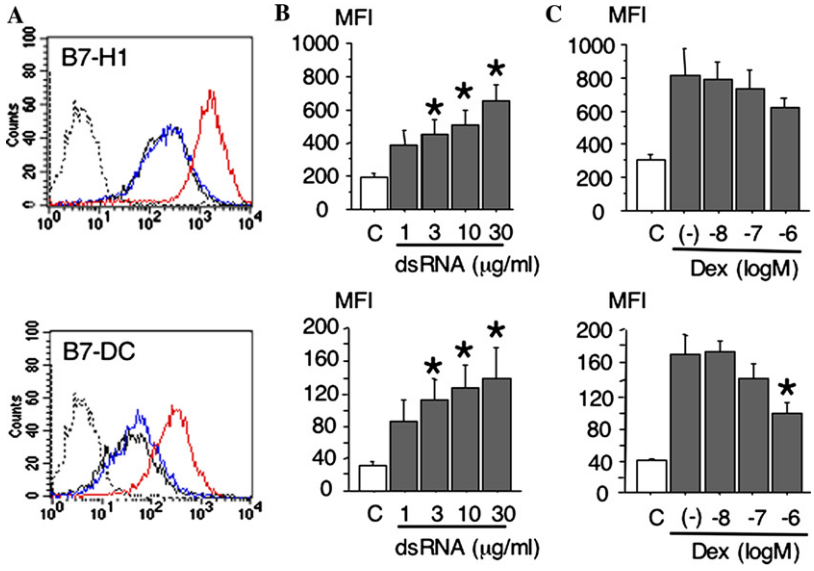

Fig. 2. Effect of double-stranded RNA on B7-H1 and B7-DC expression. BEAS-2B cells were stimulated with dsRNA for 24 h and then processed for flow cytometric analysis of B7-H1 (upper panel) and B7-DC (lower panel). (A) Representative histograms showing the data stimulated with dsRNA (30 μg/ml) (arrowheads), those with RNase-pretreated dsRNA (shaded histograms), those of the unstimulated control (arrows), and those of the isotype control (dotted lines). (B) The effect of various concentrations of dsRNA was evaluated. *$p < 0.05$ compared with the unstimulated control. (C) The effect of dexamethasone was evaluated using cells stimulated with dsRNA (30 μg/ml). *$p < 0.05$ compared with the dsRNA-stimulated and dexamethasone-free control. Data are shown as means ± SEM of five experiments. (NOTE: For the color figure in the web version, red lines, blue lines, and black lines correspond to arrowheads, shaded histograms, and arrows, respectively.)

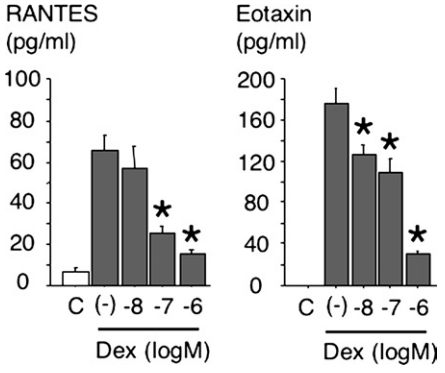

Fig. 3. Effect of dexamethasone on chemokine production. BEAS-2B cells were stimulated with IFN-γ (100 ng/ml) or IL-13 (50 ng/ml) in the presence of dexamethasone for 24 h; then, RANTES or eotaxin in the supernatant was quantified by ELISA. Data are shown as means ± SEM of five experiments. *$p < 0.05$ compared with the unstimulated control.

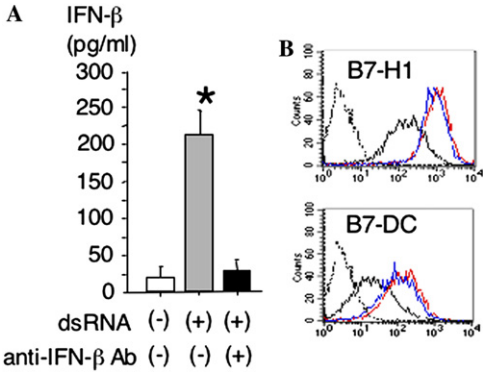

Fig. 4. Effect of IFN-β neutralization on B7-H1 and B7-DC expression. (A) BEAS-2B cells were stimulated with dsRNA (30 μg/ml) for 24 h in the presence or absence of anti-IFN-β antibody (7.5 μg/ml); then, IFN-β in the supernatant was quantified by ELISA. Data are shown as means ± SEM of five experiments. *$p < 0.05$ compared with the unstimulated control. (B) Representative histograms showing the data stimulated with dsRNA (arrowheads), those with dsRNA in the presence of anti-IFN-β antibody (shaded histograms), those of the unstimulated control (arrows), and those of the isotype control (dotted lines). (NOTE: For the color figure in the web version, red lines, blue lines, and black lines correspond to arrowheads, shaded histograms, and arrows, respectively.)

affected by dexamethasone ranging from 10^{-8} to 10^{-6} M. The expression of B7-H1 and B7-DC was also upregulated by stimulation with Th2 cytokine, IL-13, in a dose-dependent manner (Figs. 5B and D). The upregulation of B7-DC, but not that of B7-H1, was significantly suppressed by 10^{-6} M dexamethasone. The combined administration of IFN-γ and IL-13 further increased the expression of B7-H1 and B7-DC (Fig. 5E). The stimulation of cells by IFN-γ or IL-13 at concentrations of inducing maximal expression of B7-H1 and B7-DC did not induce the expression of B7-1 or B7-2 (data not shown).

Effects of T cell clone-derived supernatant on B7-H1 and B7-DC expression

Five T cell clones were established and used for the study. The expression of both B7-H1 and B7-DC was markedly upregulated by the incubation of BEAS-2B cells in the supernatant from anti-CD3 mAb-stimulated

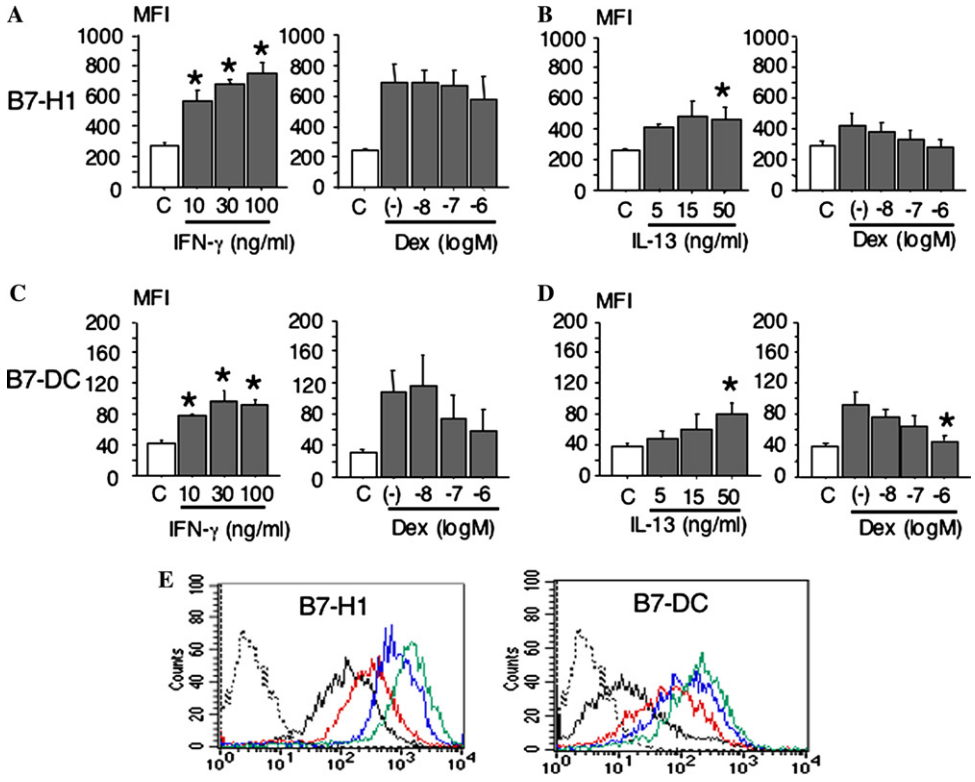

Fig. 5. Effect of IFN-γ and IL-13 on B7-H1 and B7-DC expression. BEAS-2B cells were stimulated with IFN-γ (A,C) or IL-13 (B,D) for 24 h and then processed for flow cytometric analysis of B7-H1 (upper panels) and B7-DC (lower panels). The effect of various concentrations of cytokine was evaluated. *$p < 0.05$ compared with the unstimulated control. The effect of dexamethasone was evaluated using cells stimulated with IFN-γ (100 ng/ml) or IL-13 (50 ng/ml). *$p < 0.05$ compared with the cytokine-stimulated and dexamethasone-free control. (E) Representative histograms showing the data stimulated with IFN-γ (100 ng/ml) (shaded histograms), those with IL-13 (50 ng/ml) (dotted arrows), those with both IFN-γ (100 ng/ml) and IL-13 (50 ng/ml) (arrowheads), those of the unstimulated control (arrows), and those of the isotype control (dotted lines). Data are shown means ± SEM of five experiments. (NOTE: For the color figure in the web version, blue lines correspond to shaded histograms, red lines correspond to dotted arrows, green lines correspond to arrowheads, and black lines correspond to arrows.)

T cell clones but not in the supernatant from unstimulated clones (Fig. 6A). The supernatants of anti-CD3 mAb-stimulated clones contained a substantial amount of IFN-γ, IL-5, IL-13, and TNF-α compared with those of unstimulated T cell clones (Fig. 6B). The concentration of IL-13 was particularly high among the four cytokines, suggesting that B7-H1 and B7-DC may be upregulated in Th2-dominant milieu.

Discussion

The present study demonstrated that dsRNA profoundly upregulates the expression of B7-H1 and B7-DC on airway epithelial cell lines, a phenomenon which was not observed for cells treated with denatured dsRNA or ssRNA. This finding suggests that the effect of dsRNA on airway epithelial cells may be derived from its double-stranded structure. dsRNA is a natural ligand for TLR3 and BEAS-2B cells constitutively express mRNA and protein of TLR3 [4,5,24]. Recent studies have shown that dsRNA stimulation to BEAS-2B cells induces an array of genes including TLR3 itself [4,24] and causes the secretion of cytokines through the activation of TLR3 [4]. These findings favor the idea that TLR3-dependent pathway associates with dsRNA-induced upregulation of B7-H1 and B7-DC. However, in addition to TLR3-mediated pathway, PKR and retinoic acid inducible gene 1 (RIG-1) are known to recognize dsRNA [3,25]. Previous studies reported the contribution of PKR on the mechanism that airway epithelial cells recognize dsRNA and elicit anti-viral responses including the secretion of cytokines and the upregulation of NOS2 genes [3,26].

The induction of type I interferon constitutes a primary defense system for virus infection. dsRNA is known to activate airway epithelial cells to produce IFN-β [4,27]. IFN-β strongly upregulates the expression of B7-H1 on monocytes and dendritic cells [28]. In the present study, the supernatant of dsRNA-stimulated BEAS-2B cells contained substantial amount of IFN-β. However, the neutralization of IFN-β did not block the upregulation of B7-H1 and B7-DC, suggesting that their upregulation on BEAS-2B cells may not depend on the induction of IFN-β.

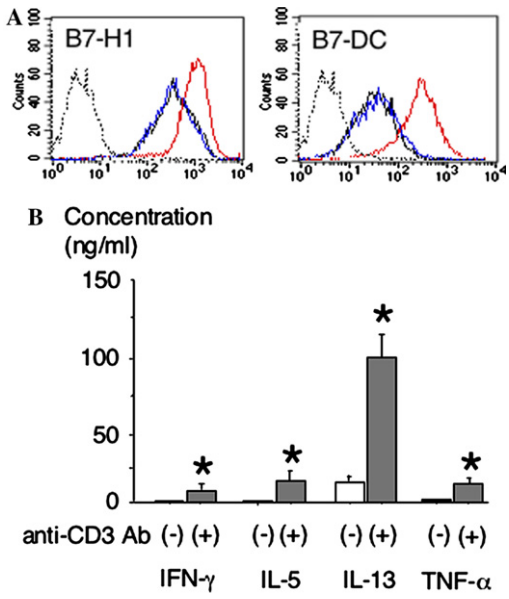

Fig. 6. Effects of T cell supernatant on B7-H1 and B7-DC expression. BEAS-2B cells were incubated for 24 h in the supernatant of T cell clones that had been activated with or without anti-CD3 mAb (10 μg/ml) for 24 h. Epithelial cells were then processed for flow cytometric analysis of B7-H1 and B7-DC. (A) Representative histograms showing the data with the supernatant from the anti-CD3 mAb-stimulated clone (arrowheads), with that from unstimulated clone (shaded histograms), and with medium alone (arrows). Samples incubated with isotype IgG are shown as dotted lines. (B) Concentrations of IFN-γ, IL-5, IL-13, and TNF-α in the supernatant from T cell clones. Data are shown as means ± SEM of five experiments. $*p < 0.05$ compared with the unstimulated control. (NOTE: For the color figure in the web version, red lines, blue lines, and black lines correspond to arrowheads, shaded histograms, and arrows, respectively.)

dsRNA was reported to upregulate B7-1 and B7-2 on professional APCs via TLR3, suggesting that TLR3 mediated innate immunity link to the development of adaptive immunity against viral invasion, partly through the upregulation of costimulatory molecules [9]. This linkage may also be relevant for airway epithelial cells, in which virus-derived antigens are presented onto MHC-class I molecules and then recognized by antigen-specific $CD8^+$T cells. However, the expression of either B7-1 or B7-2 on airway epithelial cells was not found even after maximal dsRNA stimulation. Given that PD-1 ligation delivers an inhibitory signal to T cell activity, the upregulation of B7-H1 and B7-DC by dsRNA may be associated with an escape mechanism of viral species from the host immune system [8,29]. Otherwise, their upregulation might result in the enhancement of anti-viral immunity. Growing evidence has indicated that B7-H1/B7-DC could "positively" costimulate T cells by the yet unidentified second receptor [10,13,18,30,31].

Viral infection leads the host immune system to induce IFN-γ production from T cells and the macrophage/monocyte lineage. In addition, several viruses, such as RSV, induce Th2 cytokine production, which has been implicated as the causal mechanism of asthmatic exacerbation in RSV-infected children [32]. In the present study, the stimulation of airway epithelial cells with IFN-γ or IL-13 upregulated the expression of B7-H1 and B7-DC in a dose-dependent manner. The culture supernatant of allergen-specific T cell clones, which contained a variety of cytokines, particularly IL-13, markedly enhanced B7-H1 and B7-DC expression. These findings suggest that both Th1 and Th2 cytokines, produced in the natural course of viral infection in the airway, may also augment the expression of B7-H1 and B7-DC.

Because glucocorticoids are used worldwide for the treatment of asthma and COPD, it is important to determine whether glucocorticoid critically alters the expression profiles of B7-H1 and B7-DC on airway epithelial cells. Previous studies have reported that the production of IL-8 and eotaxin from cytokine-stimulated BEAS-2B cells was markedly inhibited by 10^{-9} to 10^{-7} M dexamethasone [33,34]. In the present study, 10^{-8} to 10^{-7} M dexamethasone significantly suppressed IFN-γ-induced RANTES production and IL-13-induced eotaxin production but failed to suppress the dsRNA-induced or cytokine-induced upregulation of B7-H1 and B7-DC. Significant suppression by 10^{-6} M dexamethasone was found only in dsRNA-induced and IL-13-induced upregulation of B7-DC. These results indicate that the suppressive effect of dexamethasone on B7-H1 and B7-DC expression was substantially weaker than that on chemokine production.

The enhanced expression of B7-H1 and B7-DC by dsRNA, cytokines, and activated T cell supernatant suggests that airway epithelial cells may utilize costimulatory molecules in response to viral infection. Further elucidation of their profiles and regulatory mechanisms may provide a novel perspective for the treatment of airway inflammatory diseases.

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