Expression and Function of the Inducible Costimulator Ligand B7-H2 in Human Airway Smooth Muscle Cells

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ABSTRACT

Background: B7-H2 is a ligand for the inducible costimulator (ICOS). The aim of this study was to examine the expression and function of B7-H2 in human airway smooth muscle (ASM) cells and compare them with those of CD40 or OX40 ligand (OX40L).

Methods: Expression of B7-H2, CD40 and OX40L in ASM cells and their respective counterparts in T cells was analyzed by RT-PCR or flow cytometry. The modulating effect of polyinosinic-polycytidylic acid (poly I:C) on expression of B7-H2, CD40 and OX40L was also examined. The function of these three molecules was evaluated by virtue of adhesion of anti-CD3-activated T cells, IL-6 and IL-8 production and DNA synthesis.

Results: ASM cells constitutively expressed B7-H2, CD40 and OX40L that mediated adhesion of activated T cells expressing ICOS, CD40L and OX40. ASM cells responded to poly I:C with upregulated expression of B7-H2, CD40 and OX40L and displayed enhanced adhesion of activated T cells. Functional analysis performed on untreated ASM cells showed that engagement of B7-H2 with ICOS-Ig clearly induced DNA synthesis, whereas that of CD40 or OX40L with trimeric CD40L or OX40-Ig greatly increased IL-6 and IL-8 production. These responses were enhanced in poly I:C-treated ASM cells.

Conclusions: The data demonstrate that ASM cells express functionally active B7-H2, CD40 and OX40L and suggest that B7-H2-dependent signaling may play an active role in a proliferative response rather than in cytokine and chemokine production. In addition, the modulation of B7-H2, CD40 and OX40L expression and function by poly I:C may have important implications for the function of virus-infected ASM cells.

KEY WORDS

airway inflammation, airway smooth muscle cells, B7-H2, DNA synthesis, inducible costimulator

INTRODUCTION

The airway inflammatory response that characterizes the asthmatic phenotype involves the interaction of structural cells, infiltrating cells, cytokines, chemokines and growth factors.1,2 Airway smooth muscle (ASM) cells are a rich source of different inflammatory mediators and play an important interactive role with inflammatory and structural cells in the pathogenesis of asthma.1,3,4 Many cytokines and growth factors regulate the activity and proliferation of ASM cells via autocrine and paracrine actions. ASM cell proliferation is also inducible by contact with mitogen-activated T cells.5 ASM cells express adhesion molecules such as intercellular adhesion molecule-1 and CD44 and costimulatory molecules such as CD40 and OX40 ligand (OX40L).5,8 These adhesion and costimulatory molecules interact with their respective counterparts expressed on other cell types. ASM cells also express several members of the Toll-like receptor (TLR) family, including TLR3,9,12 Members of the family recognize pathogen-associated molecular patterns and activate the innate immune responses. In addition, ASM cells may have
the capacity to act as antigen-presenting cells because they can express major histocompatibility complex class II molecules.13 This potential capacity suggests that ASM cells may participate in the adaptive immune responses under certain conditions.

The B7 family, which is expressed by professional antigen-presenting cells, including dendritic cells, macrophages and B cells, forms another group of costimulatory or coinhibitory molecules crucial for interaction with antigen-primed T cells.14,16 The individual members of the family play important roles in regulating T cell responses. For example, B7-1 (CD80) and B7-2 (CD86) are ligands for the costimulatory CD28 and coinhibitory cytotoxic T-lymphocyte antigen-4. B7-H1 (CD274) and B7-DC (CD273) are ligands for the programmed death-1 that delivers a coinhibitory signal to T cells. B7-H2 (CD275) is a ligand for the inducible costimulator (ICOS) that delivers a costimulatory signal to T cells in a fashion different from CD28. B7-H3 (CD276) enhances the induction of cytotoxic T cells, although its receptor remains to be identified. B7 homologs can signal into the antigen-presenting cell on which they are expressed.17 Some of the B7 homologs are also demonstrated on tissue cells such as nasal, bronchial and alveolar epithelial cells.18–21 With regard to B7-1 and B7-2 expression on epithelial cells, however, conflicting results are reported.18,22 Although there is a report showing that ASM cells express low constitutive levels of B7-1 and B7-2,23 little is known about the expression of B7 homologs by ASM cells.

In the present study, we analyzed the expression profiles of B7-1, B7-2, B7-H1 and B7-H2 in primary cultures of human ASM cells and describe the cell surface expression of B7-H1 and B7-H2, but not of B7-1 or B7-2. To explore the significance of the presence of B7-H2 on ASM cells, we examined the regulation of B7-H2 expression by a synthetic ligand for TLR3, the ability of B7-H2 to mediate adhesion of anti-CD3 antibody-activated T cells and the functional consequences of engagement of B7-H2 with ICOS-Ig, a soluble chimeric protein, by measuring cytokine and chemokine production and DNA synthesis. In addition, we compared the expression and function of B7-H2 with those of CD40 or OX40L in ASM cells.

METHODS

CELLS

Primary cultures of normal human ASM cells from five different donors were obtained from Clonetics (San Diego, CA, USA). ASM cells were grown in smooth muscle cell basal medium (Clonetics) containing 5% fetal bovine serum, 1 ng/ml epidermal growth factor, 2 ng/ml fibroblast growth factor, 10 μg/ml insulin, 50 μg/ml gentamicin and 50 ng/ml amphotericin B, according to the manufacturer’s instructions. Confluent ASM cells from passages 3–5 were starved of serum and growth factors for 24 hours and used for all experiments. The airway epithelial cell line BEAS-2B was obtained from American Type Culture Collection (Rockville, MD, USA) and maintained in Ham’s F12/Dulbecco’s modified Eagle’s medium (Life Technologies, Gaithersburg, MD, USA) containing 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-sodium metrizoate (Organon Teknika, Durham, NC, USA) density gradient centrifugation from heparinized venous blood of three different healthy adults who had given informed consent to the blood donation. The study was approved by the Ethics Committee of Sagamihara National Hospital. T cells were negatively isolated from PBMC by an indirect magnetic labeling system using mouse monoclonal anti-human CD14, CD19, CD36 and CD56 antibodies (Dynal, Oslo, Norway), according to the manufacturer’s instructions. Purified T cells contained >98% CD3+ cells as determined by flow cytometry and were suspended in RPMI1640 medium (Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin.

REAGENTS

Recombinant human ICOS-Ig (homodimer), CD28-Ig (homodimer) and trimeric CD40L were obtained from R&D Systems (Minneapolis, MI, USA), and recombinant human OX40-Ig (homotrimer) was obtained from Alexis (San Diego, CA, USA). Blocking mouse monoclonal antibodies to human ICOS, CD40L and OX40 were obtained from Ancell (Bayport, MI, USA). Culture plates uncoated or coated with mouse monoclonal anti-human CD3 antibody were obtained from BD Biosciences (Bedford, MA, USA). Polynosinic-polycytidylic acid (poly I:C), a synthetic ligand for TLR3, was obtained from InvivoGen (San Diego, CA, USA), and [3H]thymidine ([3H]TdR) was obtained from Perkin Elmer Life Sciences (Boston, MA, USA). N-hydrosuccinimido-biotin was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

RT-PCR

Extraction of total cellular RNA and cDNA synthesis by reverse transcriptase were performed as described previously.12 The resultant cDNA was amplified on a thermal cycler using a Pyrobest DNA polymerase kit (TaKaRa, Tokyo, Japan) by PCR using the following sense and antisense primers: B7-1, 5’-TGGAGAGGTTA-3’; B7-2, 5’-ATTCTCTTTGTGATGGGTCTTTCTCACTTCTGTT-3’ and 5’-GGATCACAAUGUAAACGTGGCC-3’; the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5’-TGAGCTCCGGTCATCTTCTGCAAT-3’; B7-H1, 5’-ACTGGCATTGTTGCTAGTGCCCT-3’ and 5’-GCCCTGCATCCTGCAAT-3’; B7-H2, 5’-CTGGGTACTGGACAAAGCCG-3’ and 5’-TGAGCTCCGGTCAAAACGTGGCC-3’; the housekeeping gene glyceralde-
dehydrate 3-phosphate dehydrogenase (GAPDH), 5′-TG AAGTGCGAGTCAACGGATTTGTT3′ and 5′-CATC TGGGCCATGAGGTCACCAC-3′. The sequences of these primer sets and the PCR conditions were the same as described elsewhere.12,18,19,23 The amplified products were subjected to electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. The detection of mRNA expression of B7-1, B7-2, B7-H1 and B7-H2 in CD40L-stimulated PBMC was confirmed by PCR amplification using primers for these four B7 homologs (data not shown).

FLOW CYTOMETRY

The following mouse monoclonal antibodies were used for flow cytometry: anti-human B7-1 and B7-2 (PharMingen, San Diego, CA, USA), anti-human B7-H1 (eBioscience, San Diego, CA, USA), anti-human B7-H2 (Lab Vision, Fremont, CA, USA), anti-human OX40L (R&D Systems) and phycoerythrin-labeled anti-human CD40, CD40L, OX40 and ICOS (Ancell). IgG1 isotype control (MOPC21) was obtained from Immunotech (Marseille, France), and IgG2b isotype control was from R&D Systems. Anti-OX40L antibody was biotinylated by the standard technique using N-hydrosuccinimido-biotin. Cells (1 × 10^6/ml) were incubated in the presence of 1 mg/ml human IgG (PharMingen) with the appropriate concentrations of unlabeled or labeled antibodies for 1 hour at 4°C. B7-1, B7-2, B7-H1 and B7-H2 expression was assessed by staining with a fluorescein isothiocyanate-labeled secondary goat F(ab′)2 anti-mouse Ig (BioSource, Camarillo, CA, USA), and OX40L expression was determined by staining with phycoerythrin-labeled streptavidin (PharMingen). In each assay, isotype control antibody was used for negative staining. Samples were analyzed on a FACSCalibur using CellQuest software (Becton Dickinson, San Jose, CA, USA). Expression of immunoreactive proteins was expressed as a ratio of the mean fluorescence intensity (MFI) obtained with isotype control antibody, as described previously.12

QUANTITATIVE ADHESION ASSAY

This assay was performed according to some modifications of the procedure described elsewhere.5 Briefly, T cells purified from PBMC were activated with immobilized anti-human CD3 antibody for 42 hours at 37°C and pulsed with [3H]TdR (37 kBq/ml) for the final 16-hour incubation. [3H]TdR-labeled activated T cells were pretreated with or without the indicated blocking antibodies for 1 hour at 4°C, added to ASM cells and allowed to adhere for 1 hour at 37°C. After nonadherent cells were removed by washing, adherent cells were recovered by using trypsin and harvested onto glass fibre strips with a cell harvester. Retained radioactivity was counted in a Wallac MicroBeta Jet liquid scintillation counter (Perkin Elmer Life Sciences) and expressed as the counts per minute (cpm).

MEASUREMENT OF IL-6 AND IL-8

The concentrations of IL-6 and IL-8 in the culture supernatants from ASM cells stimulated with or without the indicated recombinant proteins for 24 hours at 37°C were measured by commercially available ELISA kits (BioSource), according to the manufacturer’s instructions.

DNA SYNTHESIS

DNA synthesis in ASM was measured by incorporation of [3H]TdR. Cells were stimulated with or without the indicated recombinant proteins at 37°C for 1 to 7 days, and [3H]TdR (37 kBq/ml) was added for the final 12-hour incubation. After incubation, cells were harvested, and [3H]TdR incorporation was measured in a liquid scintillation counter. The stimulation index was calculated as a ratio of the cpm recovered from stimulated cells/unstimulated cells.

STATISTICAL ANALYSIS

Data were expressed as means ± SEM. Statistical significance of differences was determined with unpaired or paired Student’s t test. Differences were considered significant at p < 0.05.

RESULTS

ANALYSIS OF B7 HOMOLOG EXPRESSION IN ASM CELLS AND BEAS-2B CELLS

We first examined whether human ASM cells constitutively express B7 homologs, including B7-H2. RT-PCR analysis showed that ASM cells were positive for B7-1, B7-2, B7-H1 and B7-H2 mRNAs (Fig.1A, left lane). Flow cytometric analysis showed that expression of B7-H1 and B7-H2, but not B7-1 or B7-2, was detectable on ASM cells (Fig.1B, upper panels). Our failure to detect B7-1 and B7-2 expression on ASM cells was not caused by analytical errors, since the antibodies used reacted with B7-1 and B7-2 expressed on untreated or B7-2 mRNA (Fig.1A, right lane), and cell surface expression of B7-H1 and B7-H2, but not B7-1 or B7-2, was detectable (Fig.1B, lower panels). The observed expression profiles of mRNA and protein for these four B7 homologs in BEAS-2B cells were in keeping with previous reports.18,20,24 Flow cytometry revealed that the expression level of B7-H1 and B7-H2 was significantly higher in BEAS-2B cells than in ASM cells (Fig. 1C). Our data demonstrated that B7-H2, as well as B7-H1, was expressed on both ASM cells and BEAS-2B cells.

EFFECT OF Poly I:C ON B7-H2, CD40 AND OX40L EXPRESSION ON ASM CELLS

ASM cells are known to express low levels of CD40
Fig. 1  Expression profiles of B7 homologs in ASM cells and BEAS-2B cells.  

A: RT-PCR analysis of B7-1, B7-2, B7-H1 and B7-H2 mRNA expression in ASM cells from one representative donor and in BEAS-2B cells is shown.  

B: Flow cytometric analysis of B7-1, B7-2, B7-H1 and B7-H2 expression on ASM cells from one representative donor and on BEAS-2B cells is shown. The dotted tracing in each panel indicates background staining.  

C: The MFI for B7-1, B7-2, B7-H1 and B7-H2 expression was determined. Results are presented as means ± SEM of five different donors (ASM cells) and three independent experiments (BEAS-2B cells). **p < 0.01.

and OX40L. In addition, BEAS-2B cells are shown to respond to poly I:C via TLR3 with increased B7 homolog expression. Since ASM cells also express TLR3, we next examined whether poly I:C can modulate B7-H2, CD40 and OX40L expression on ASM cells. Treatment of ASM cells with poly I:C (25 μg/ml) for 24 hours resulted in upregulated expression of these three molecules, as analyzed by flow cytometry (Fig. 2A), and their upregulation was significant (Fig. 2B). Under our experimental conditions, however, B7-1 and B7-2 were undetectable on ASM cells even after poly I:C treatment (data not shown).

INDUCTION OF ICOS, CD40L AND OX40 EXPRESSION ON T CELLS BY ANTI-CD3 ANTIBODY
To determine expression of counter molecules for B7-H2, CD40 and OX40L by T cells, peripheral blood T cells were activated with immobilized anti-CD3 antibody for 42 hours and analyzed for expression of ICOS, CD40L and OX40 by flow cytometry. As expected, high expression of these three molecules was induced on the surface of anti-CD3-activated T cells (Fig. 3A). We observed that the induced expression level of CD40L or OX40 was very high compared with that of ICOS (Fig. 3B).

COSTIMULATORY MOLECULE-DEPENDENT ADHESION OF T CELLS TO ASM CELLS
Based on the observations that B7-H2, CD40 and OX40L were detected on ASM cells and upregulated after poly I:C treatment and that ICOS, CD40L and OX40 were expressed at high levels on anti-CD3-activated T cells, we examined the capacity of T cells to adhere to ASM cells. Microscopic examination showed that compared with resting T cells (Fig. 4A), a large number of anti-CD3-activated T cells adhered to untreated ASM cells (Fig. 4B). Moreover, enhanced adhesion of anti-CD3-activated T cells to poly I:C-treated ASM cells was observed (Fig. 4C). Since
Expression of B7-H2 in ASM Cells

**Fig. 2** Effect of poly I:C on B7-H2, CD40 and OX40L expression on ASM cells. **A**: ASM cells were treated with or without poly I:C (25 μg/ml) for 24 hours and analyzed for expression of B7-H2, CD40 and OX40L by flow cytometry. The dotted tracing in each panel indicates background staining. Data from one representative donor are shown. **B**: The MFI for B7-H2, CD40 and OX40L expression was determined. Results are presented as means ± SEM of five different donors. *p < 0.05, **p < 0.01.

The results obtained demonstrated the active participation of activated T cells in their adhesion to ASM cells, a quantitative adhesion assay was performed using [3H]TdR-labeled activated T cells. The participation of costimulatory molecules in T cell adhesion to ASM cells was determined using blocking antibodies. Preliminary experiments showed that pretreatment of activated T cells with a mixture of anti-ICOS, anti-CD40L and anti-OX40 antibodies markedly decreased T cell adhesion to untreated ASM cells; retained radioactivities in the adherent T cells pretreated with medium alone, control antibody (MOPC21) at 30 μg/ml and a combination of anti-ICOS, anti-CD40L and anti-OX40 antibodies at 10 μg/ml were 4537 ± 1604, 4180 ± 1179 and 678 ± 152 cpm, respectively (means ± SEM in three different experiments). In keeping with microscopic examination, compared with untreated ASM cells, poly I:C-treated ASM cells displayed approximately a two-fold increase in T cell binding (Fig. 5). We observed significant inhibition of binding to both untreated and poly I:C-treated ASM cells when activated T cells were pretreated with 10 μg/ml each of anti-ICOS, anti-CD40L or anti-OX40 antibody. Although inhibition by these three antibodies was incomplete, no significant differences among them with respect to blocking activity were seen, suggesting that B7-H2, CD40 and OX40L on ASM cells contribute equally to adhesion of activated T cells expressing ICOS, CD40L and OX40.

**IL-6 AND IL-8 PRODUCTION BY ASM CELLS**
To examine the functional consequences of engagement of B7-H2, CD40 and OX40L on ASM cell, we evaluated cytokine and chemokine production by measuring IL-6 and IL-8. To that end, ASM cells that had been treated with or without poly I:C (25 μg/ml)
Fig. 3 Induction of ICOS, CD40L and OX40 expression on T cells by anti-CD3 antibody. A: T cells were cultured with or without immobilized anti-CD3 antibody for 42 hours and analyzed for expression of ICOS, CD40L and OX40 by flow cytometry. The dotted tracing in each panel indicates background staining. Data from one representative donor are shown. B: The MFI for ICOS, CD40L and OX40 expression was determined. Results are presented as means ± SEM of three different donors. "p < 0.01, ***p < 0.001.

for 24 hours were washed, rested for 12 hours and then stimulated with or without 10 μg/ml each of ICOS-Ig, CD40L or OX40-Ig for 24 hours. ICOS-Ig had a tendency to increase IL-6 and IL-8 production by untreated and poly I:C-treated cells, while CD40L and OX40-Ig greatly increased IL-6 and IL-8 production by both cells (Fig. 6). Although poly I:C-treated cells produced much higher levels of IL-6 and IL-8 than untreated cells, this was due to poly I:C induction of IL-6 and IL-8 production, as reported previously. ICOS-Ig, CD40L and OX40-Ig induced a two- to three-fold increase in IL-6 and IL-8 production by untreated and poly I:C-treated cells. Moreover, CD40L and OX40-Ig substantially enhanced IL-6 and IL-8 production by poly I:C-treated cells. Consistent with the lack of surface expression of B7-1 and B7-2, CD28-Ig (10 μg/ml) had no effect on IL-6 and IL-8 production by untreated and poly I:C-treated cells.

**DNA SYNTHESIS IN ASM CELLS**

We also explored whether ICOS-Ig, CD40L and OX40-Ig have the ability to induce DNA synthesis in ASM cells by measuring uptake of [3H]TdR. This measurement was performed at 3, 5 and 7 days after stimulation with or without 10 μg/ml each of ICOS-Ig, CD40L or OX40-Ig, since 1-day stimulation by these recombinant proteins did not promote DNA synthesis in untreated and poly I:C-treated cells (data not shown). As shown in Figure 7, ICOS-Ig could induce DNA synthesis in untreated and poly I:C-treated cells, reaching a peak at 5 days after stimulation and declining thereafter. However, CD40L and OX40-Ig had only minimal effects on DNA synthesis even after 5-day stimulation. Interestingly, poly I:C-treated cells showed enhanced DNA synthesis in response to ICOS-Ig, but not to CD40L or OX40-Ig. As expected, CD28-Ig (10 μg/ml) did not induce DNA synthesis in untreated and poly I:C-treated cells.
Expression of B7-H2 in ASM Cells

Fig. 4  Adhesion of T cells to ASM cells. T cells were cultured with or without immobilized anti-CD3 antibody for 42 hours. ASM cells were treated with or without poly I:C (25 μg/ml) for 24 hours. Resting (A) or anti-CD3-activated (B) T cells were added to untreated ASM cells. Anti-CD3-activated T cells were added to poly I:C-treated ASM cells (C). After incubation for 1 hour, nonadherent T cells were removed, and T cell adhesion to ASM cells was examined with a phase-contrast microscopy (×400). Open and closed arrows indicate T cells and ASM cells, respectively. Results are representative of three experiments using T cells from one donor and ASM cells from three different donors.

Fig. 5  Effect of blocking antibodies on adhesion of anti-CD3-activated T cells to ASM cells. [3H]TdR-labeled activated T cells were pretreated with 10 μg/ml of control antibody (MOPC21) or with 10 μg/ml each of anti-ICOS, anti-CD40L or anti-OX40 antibody for 1 hour, added to ASM cells that had been treated with or without poly I:C (25 μg/ml) for 24 hours and allowed to adhere for 1 hour. Retained radioactivity in adherent T cells was counted. Results are presented as means ± SEM of five experiments using T cells from one donor and ASM cells from five different donors. *p < 0.05, **p < 0.01, ***p < 0.001. Similar results were obtained when T cells from two other donors were used.

DISCUSSION
This study showed that cultured human ASM cells express costimulatory molecules capable of binding anti-CD3-activated T cells. In addition to CD40 and OX40L, B7-H2, as well as B7-H1, was also demonstrated on ASM cells. B7-1 and B7-2, however, were undetectable on ASM cells under our experimental conditions. We observed that B7-H2, CD40 and OX40L expression on ASM cells could be upregulated by poly I:C and was accompanied by enhanced adhesion of activated T cells where high expression of ICOS, CD40L and OX40 was induced. Adhesion was inhibited proportionally to the same extent by antibodies directed against ICOS, CD40L and OX40 on T cells, suggesting that B7-H2, CD40 and OX40L on ASM cells contribute equally to T cell binding. Functional analysis performed on ASM cells showed that engagement of B7-H2 with ICOS-Ig clearly induced DNA synthesis, whereas that of CD40L or OX40L with CD40L or OX40-Ig greatly increased IL-6 and IL-8 production. Thus, B7-H2 on ASM cells appears to play a role in a proliferative response rather than in cytokine and chemokine production by binding ICOS expressed on activated T cells.

B7-H2, which provides ICOS-dependent T cell
costimulation, is expressed on several types of professional antigen-presenting cells. Its expression is also demonstrated on airway, alveolar and renal tubular epithelial cells. We found that B7-H2, as well as B7-H1, was expressed on ASM cells, suggesting their potential interaction with activated T cells expressing ICOS. The significance of the B7-H2/ICOS-mediated costimulation pathway in T cell activation is well documented in various experimental systems. For example, blocking the B7-H2/ICOS interaction attenuates CD4+ T cell expansion and Th2 cytokine production, resulting in decreased Th2 responses. B7-1 and B7-2, which provide CD28-dependent T cell costimulation, are expressed on activated B cells and other antigen-presenting cells. Despite a report that B7-1 and B7-2 are expressed at low levels on ASM cells, we failed to detect surface expression of B7-1 and B7-2. This result seems to be in support of the previous finding that ASM cells expressing HLA-DR could not present alloantigen to CD4+ T cells. We observed that the expression profiles of B7 homologs in ASM cells were quite similar to those in BEAS-2B cells, an airway epithelial cell line. Indeed, BEAS-2B cells also failed to express B7-1 and B7-2 on their cell surface. This failure is consistent with previous studies which showed that both BEAS-2B cells and primary bronchial epithelial cells lacked surface expression of B7-1 and B7-2. Our data, showing that ASM cells express B7-H2, but not B7-1 or B7-2, suggest that B7-H2 may provide a primary costimulatory signal on ASM cells.

ASM cells displayed upregulated expression of B7-H2, as well as of CD40 or OX40L, following treatment with poly I:C. It has been shown that TNF-α, a potent pro-inflammatory cytokine, also upregulates CD40 and OX40L expression on ASM cells and B7-H2 expression on B cells, monocytes and A549 cells, a type II alveolar epithelial cell line, whereas it downregulates B7-H2 expression on dendritic cells, bronchial epithelial cells and BEAS-2B cells. Therefore, the modulating effect of TNF-α on B7-H2 expression differs among cell types. Treatment of ASM cells with poly I:C led to enhanced adhesion of anti-CD3-activated T cells, suggesting that production of double-stranded RNA by virus-infected ASM cells may bring about a similar effect on T cell binding. The observed adhesion was inhibited by pretreatment of activated T cells with antibodies directed against ICOS, CD40L and OX40. Since inhibition by these antibodies was incomplete, additional adhesion pathways might be involved with adhesion to ASM cells. In this regard, mitogen-activated T cells have been shown to adhere to ASM cells through integrins and CD44. Although the contribution of such adhesion molecules to adhesion of anti-CD3-activated T cells to ASM cells was not examined in the present study, intercellular adhesion molecule-1 expression on ASM cells was also upregulated by poly I:C (data not shown). This upregulation might be accompanied by enhanced adhesion of anti-CD3-activated T cells. Integrin receptors expressed on ASM cells have also been shown to mediate eosinophil adhesion.

**Fig. 6** IL-6 and IL-8 production by ASM cells. ASM cells were treated with or without poly I:C (25 μg/ml) for 24 hours, washed, rested for 12 hours and then stimulated with or without 10 μg/ml each of ICOS-Ig, CD40L, OX40-Ig or CD28-Ig for 24 hours. The concentrations of IL-6 and IL-8 in the supernatants were quantified by ELISA procedures. Results are presented as means ± SEM of five different donors. *p < 0.05, **p < 0.01.

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Expression of B7-H2 in ASM Cells

Fig. 7 DNA synthesis in ASM cells. ASM cells were treated with or without poly I:C (25 μg/ml) for 24 hours, washed, rested for 12 hours and then stimulated with or without 10 μg/ml each of ICOS-Ig, CD40L, OX40-Ig or CD28-Ig for 3 to 7 days, and [3H]Tdr was added for the final 12-hour incubation. DNA synthesis was measured by uptake of [3H]Tdr. The basal values in untreated cells were 228 ± 69, 270 ± 86 and 292 ± 71 cpm on days 3, 5 and 7, respectively, and those in poly I:C-treated cells were 375 ± 84, 398 ± 96 and 371 ± 98 cpm on days 3, 5 and 7, respectively (five experiments with different donors). The stimulation index was calculated as a ratio of the cpm recovered from recombinant protein-stimulated cells/unstimulated cells. Results are presented as means ± SEM of five different donors. *p < 0.05, **p < 0.01.

Adhesion of inflammatory cells to ASM cells has recently been shown to be mediated in part by tumor suppressor in lung cancer-1, an Ig-like cell adhesion molecule. Thus, at least three types of inflammatory cells adhere to ASM cells through adhesion molecules. Our observation of costimulatory molecule-dependent adhesion of activated T cells to ASM cells, together with the known fact that B7-H2, CD40 or OX40L on antigen-presenting cells preferentially binds activated rather than resting T cells, suggests the potential for interaction of ASM cells with infiltrating T cells in the inflamed airways through the B7-H2/ICOS, CD40/CD40L or OX40L/OX40 pathway.

Adhesion of inflammatory cells to ASM cells has been shown to evoke many responses, including DNA synthesis and cytokine and chemokine production. For example, irradiated activated T cells induce ASM cell DNA synthesis in a contact-dependent manner, suggesting the possible involvement of adhesion molecules and/or costimulatory molecules. In addressing the potential consequences of engagement of B7-H2, CD40 and OX40L on ASM cells with their respective counterparts expressed on anti-CD3-activated T cells, our observations demonstrated that direct activation of B7-H2 by ICOS-Ig evoked DNA synthesis, whereas that of CD40 or OX40L by CD40L or OX40-Ig induced high production of IL-6 and IL-8.

The latter finding is in line with previous studies showing that the signal delivered by CD40 or OX40L on ASM cells led to increased IL-6 production. Although CD40L and OX40-Ig predominated over ICOS-Ig in increasing IL-6 and IL-8 production, these three recombinant proteins had no ability to induce eotaxin production (data not shown). A recent study has shown that mast cell adhesion to ASM cells can induce eotaxin production. With regard to the function of upregulated B7-H2, CD40 and OX40L in poly I:C-treated ASM cells, each of them was also functionally active. For example, engagement of upregulated B7-H2 with ICOS-Ig led to enhanced DNA synthesis. Although the mechanism involved in ICOS-Ig induction of DNA synthesis remains to be defined, ASM cells have the capacity to produce growth factors such as platelet-derived growth factor and insulin-like growth factor. Given that prolonged exposure of ASM cells to ICOS-Ig was required for the induction of DNA synthesis, the possibility that this induction might be dependent on autocrine production of growth factor(s) cannot be ruled out. Since alternations in extracellular matrix proteins around the ASM have been shown to modulate mitogenic stimulus-induced DNA synthesis, the possibility that B7-H2 engagement might influence a profile of extracellular matrix proteins cannot be also ruled out. Structural
Changes in ASM cells are important factors that contribute to airway remodeling, a key feature of asthma. Part of this remodeling includes induction of hypertrophy and/or hyperplasia leading to increased ASM mass that may alter airway function. ASM cell proliferation that leads to hyperplasia has been shown to be increased in asthmatic patients. The observation that poly I:C-treated ASM cells responded to ICOS-Ig with enhanced DNA synthesis suggests a potential mechanism by which ICOS-dependent adhesion of activated T cells to virus-infected ASM cells induces an increase in proliferation. Consistent with the lack of B7-1 and B7-2 expression on ASM cells, CD28-Ig had no effect either on DNA synthesis or cytokine and chemokine production. Engagement of B7-1 and B7-2 on dendritic cells with CD28-Ig, on the other hand, has been shown to result in induction of IL-6 production. Taken together, our results demonstrate that B7-H2, as well as CD40 or OX40L, can signal into ASM cells and suggest that B7-H2-dependent signaling may play an active role in ASM cell proliferation.

In summary, human ASM cells constitutively express B7-H2, CD40 and OX40L that mediate adhesion of anti-CD3-activated T cells expressing ICOS, CD40L and OX40. Although the ability of B7-H2 engagement with ICOS-Ig to increase IL-6 and IL-8 production is inferior to that of CD40 or OX40L engagement with CD40L or OX40-Ig, B7-H2 engagement leads to induction of DNA synthesis. In addition, treatment of ASM cells with poly I:C upregulates expression of functionally active B7-H2, CD40 and OX40L. This upregulation may have important implications for the function of virus-infected ASM cells. Our data suggest that the possible B7-H2/ICOS interaction between ASM cells and activated T cells may contribute to ASM cell proliferation rather than to cytokine and chemokine production.

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