CD40 and OX40 ligand are increased on stimulated asthmatic airway smooth muscle

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Background: Severe, persistent asthma is characterized by airway smooth muscle hyperplasia, inflammatory cell infiltration into the smooth muscle, and increased expression of many cytokines, including IL-4, IL-13, IL-1β, and TNF-α. These cytokines have the potential to alter the expression of surface receptors such as CD40 and OX40 ligand on the airway smooth muscle cell.

Objective: To examine whether cytokines alter expression of CD40 and OX40 ligand on airway smooth muscle cells and identify any differences in response between asthmatic and nonasthmatic airway smooth muscle cells.

Methods: We used flow cytometry and immunohistochemistry to detect CD40 and OX40 ligand on airway smooth muscle cells cultured in the presence of TNF-α, IL-1β, IL-4, or IL-13. Prostaglandin E_2 levels were assessed by ELISA.

Results: TNF-α increased expression of both CD40 and OX40 ligand on both asthmatic and nonasthmatic airway smooth muscle cells. The level of expression was significantly greater on the asthmatic cells. IL-1β alone had no effect, but it attenuated the TNF-induced expression of both CD40 and OX40 ligand. The mechanism of inhibition was COX-dependent for CD40 and was COX-independent but cyclic AMP–dependent for OX40 ligand. IL-4 and IL-13 had no effect.

Conclusion: Our study has demonstrated that TNF-α and IL-1β have the potential to modulate differentially the interactions between cells present in the inflamed airways of a patient with asthma and therefore to contribute to the regulation of airway inflammation and remodeling. (J Allergy Clin Immunol 2005;115:302-8.)

Key words: *Asthma, inflammation, CD40, OX40 ligand, TNF, IL-1β, prostaglandin E_2, human airway smooth muscle cells*

Abbreviations used

ASM:	Airway smooth muscle
cAMP:	Cyclic AMP
DMEM:	Dulbecco modified Eagle medium
FITC:	Fluorescein isothiocyanate
ICAM:	Intercellular adhesion molecule
MFI:	Mean fluorescence intensity
NF-κB:	Nuclear factor κB
OX40L:	OX40 ligand
PGE_2:	Prostaglandin E_2
8-Bromo–cAMP:	8-Bromoadenosine 3′:5′–cyclic AMP

Severe, persistent asthma is characterized by airway hyperresponsiveness, airway smooth muscle (ASM) hyperplasia, and infiltration of inflammatory cells into regions that undergo structural changes, referred to as *airway remodeling*. A large range of eosinophil-derived, mast cell–derived, and T-cell–derived mediators have been implicated in this process, and structural cells, such as epithelial and mesenchymal cells, are now accepted to play a role also. These events are associated with an overexpression of many cytokines, including IL-4, IL-13, IL-1β, and TNF-α, in asthmatic airways.[1-4]

IL-4 and IL-13 are important for the development of the allergic asthmatic phenotype, whereas IL-1β and TNF-α regulate the trafficking of inflammatory cells into and through the airways. They do this by inducing the production of chemokines from a variety of cell types as well as by regulating adhesion molecule expression on airway endothelial, epithelial, and smooth muscle cells.[5-8] Nonasthmatic ASM cells express the adhesion molecules intercellular adhesion molecule (ICAM)–1, vascular cell adhesion molecule 1, and CD44.[8] Expression of these molecules and inflammatory cell interactions with them are modulated by cytokines such as TNF-α.[8-10]

Interestingly, ASM cells also express another group of costimulatory molecules important for cell-cell interactions. Nonasthmatic cells express CD40,[9] and we have recently reported the presence of OX40 ligand (OX40L) on the surface of asthmatic and nonasthmatic ASM cells.[11] CD40 is expressed at a low constitutive level on nonasthmatic ASM cells[9] and is upregulated by TNF-α, and its engagement by trimerized human CD40 ligand produces increased IL-6 secretion, increased cytosolic calcium, and activation of nuclear factor κB (NF-κB).[9] OX40L engagement on ASM cells by trimerized recombinant human OX40 also produces increased IL-6 secretion and protein kinase C β2 translocation to the cell membrane.[11] The effect of TNF-α on surface expression of OX40L and of IL-1β, IL-4, and IL-13 on the expression of CD40 or OX40L on ASM cells is currently unknown.

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TABLE I. Percentage cells positive for costimulatory molecules after cytokine treatment*

	CD40		OX40L		ICAM	
	A	NA	A	NA	A	NA
Unstimulated	12.13 ± 3.15	12.72 ± 6.51	7.2 ± 2.46	0.85 ± 0.4	88.79 ± 0.98	84.54 ± 4.32
TNF	60.99 ± 6.89†	59.76 ± 6.23†	59.72 ± 5.8†‡	25.04 ± 7.36†	95.9 ± 0.48†	96.85 ± 0.98†
IL-1	24.26 ± 6.04	27.52 ± 9.43	16.64 ± 7.5	3.34 ± 1.29	96.54 ± 0.91†	96.38 ± 1.59
IL-4	13.23 ± 2.77	5.71 ± 3.1	9.57 ± 4.36	0.9 ± 0.71	91.12 ± 0.53	81.66 ± 5.23
IL-13	12.58 ± 3.45	7.15 ± 1.7	8.89 ± 3.28	1.21 ± 0.79	89.69 ± 2.15	85.54 ± 2.97

*TNF and IL-1β, n = 6; IL-4 and IL-13, n = 3 for A and NA.
†Significantly different from unstimulated.
‡A significantly different from NA.

We hypothesized that, like the expression of ICAM-1, the expression of CD40 and OX40L on ASM cells in culture might be altered by the inflammatory mediators known to be increased in the airways of patients with asthma—that is, TNF-α, IL-1β, IL-4, and IL-13. Therefore, we compared the expression of CD40, OX40L, and ICAM-1 on human asthmatic and nonasthmatic ASM cells in the absence or presence of these cytokines, alone or in combination, as well as the signaling pathways involved.

METHODS

Chemicals

The following compounds were obtained: Dulbecco modified Eagle medium (DMEM) and PBS (JRH Biosciences, Melbourne, Australia); penicillin, streptomycin, amphotericin B, and trypan blue (Invitrogen, Heidelberg, Australia); EDTA (Ajax, Australia); FBS (Commonwealth Serum Laboratories, Melbourne, Australia); and BSA, prostaglandin E_2 (PGE_2), and 8-bromoadenosine 3′:5′–cyclic AMP (8-bromo–cAMP) (Sigma, St Louis, Mo).

Antibodies and recombinant proteins

Fluorescein isothiocyanate (FITC)–conjugated monoclonal anti—α–smooth muscle actin (mouse IgG_{2a} isotype), monoclonal anticalponin (mouse IgG_1), FITC-conjugated goat antimouse IgG (Sigma, St Louis, Mo), FITC-conjugated AffiniPure goat antimouse IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa), R-PE–conjugated polyclonal antimouse Ig antibody (Becton Dickinson, San Jose, Calif), phycoerythrin-conjugated $F(ab')_2$ goat antihuman IgG F(c) (Rockland, Gilbertsville, Pa), Texas Red–conjugated horse antimouse IgG (Vector Laboratories, Burlingame, Calif), and FITC-conjugated rabbit antigoat IgG (Sigma) were purchased.

MOPC21 (murine IgG_1 control), IgG_{2a} isotype control, and mouse antihuman CD40 mAb were obtained from Immunotech (Marseille, France). The goat antihuman OX40L was purchased from R&D Systems (Minneapolis, Minn) and the antihuman CD54 (ICAM-1) mAb from Pharmingen (San Jose, Calif). Soluble human recombinant OX40 (CD134):Fc was purchased from Alexis Corp (San Diego, Calif). Human Ig Fc fragment was purchased from Jackson ImmunoResearch Laboratories.

Cell culture

Approval for all experiments with human lung was provided by the Human Ethics Committees of the University of Sydney and the Central Sydney Area Health Service. Nonasthmatic ASM was obtained from bronchial airways of 8 patients (age, 60.7 years ± 13.3 years, mean ± SD) undergoing resection for either lung transplantation or carcinoma. Asthmatic ASM was obtained from 8 patients with asthma diagnosed by a specialist physician (age, 30.7 years ± 7.9 years) who were undergoing resection for lung transplantation or deep endobronchial biopsies. The characteristics of the patients are listed in Table E1 in the Journal's Online Repository (www.mosby.com/jaci). Pure ASM bundles were dissected free and grown as explants as previously described.[12-14] ASM cell characteristics were confirmed by immunofluorescence and light microscopy. Cells were stained with antibodies against α–smooth muscle actin and calponin, and omission of the primary antibody was used as a control.[15] All experiments were performed with cells between passages 4 and 8.

Flow cytometry

Asthmatic and nonasthmatic ASM cells were seeded in 75-cm^2 flasks at 1×10^4 cells per cm^2 in 5% (vol/vol) FBS DMEM for 48 hours. Cells were equilibrated for 24 hours in 0.1% (vol/vol) FBS DMEM before incubation in the presence of TNF-α (10 ng/mL) and IL-1β (10 ng/mL), IL-4 (20 ng/mL), and IL-13 (20ng/mL) alone and in combination with TNF-α for 48 hours (concentrations chosen on the basis of previous reports in ASM[16-18]). To investigate mechanisms, in a separate series of experiments, cells were stimulated with TNF-α, IL-1β, PGE_2 (0.1 μmol/L),[19] indomethacin (2.5μmol/L), 8-bromo–cAMP (300 μmol/L),[20] TNF-α + IL-1β, TNF-α + PGE_2, TNF-α + 8-bromo–cAMP, or TNF-α + IL-1β + indomethacin for 48 hours. Adherent ASM cells were harvested from flasks by using trypsin EDTA 1:250 without Phenol Red (Thermo Electron, Melbourne, Australia) and washed with 0.1% (wt/vol) BSA in PBS.

Unstimulated and cytokine-treated cells were labeled as described previously and run through the flow-cytometer immediately or fixed in 4% (vol/vol) formalin in PBS before acquiring the data.[11,21] Full details of this method are provided in the Online Repository (www.mosby.com/jaci).

Immunohistochemistry

Asthmatic and nonasthmatic ASM cells seeded on glass coverslips were cultured and treated with TNF-α (10 ng/mL), IL-1β (10 ng/mL), or TNF-α + IL-1β as described, then fixed in 4% (vol/vol) paraformaldehyde for 20 minutes and washed in PBS. Alternatively, bronchial rings were dissected from nonasthmatic lung, treated as described, and snap-frozen in Optimal cutting temperature compound (proSci Tech, Queensland, Australia) and isopentane (Sigma). The cells and sections were stained for CD40 and OX40L. Sequential sections were stained with hematoxylin and eosin for identification of airway morphology. Full details of this method are provided in the Online Repository (www.mosby.com/jaci).

ELISA

The levels of PGE_2 were tested by using commercial ELISA kits according to the manufacturer's instructions (Cayman Chemicals, Ann Arbor, Mich)

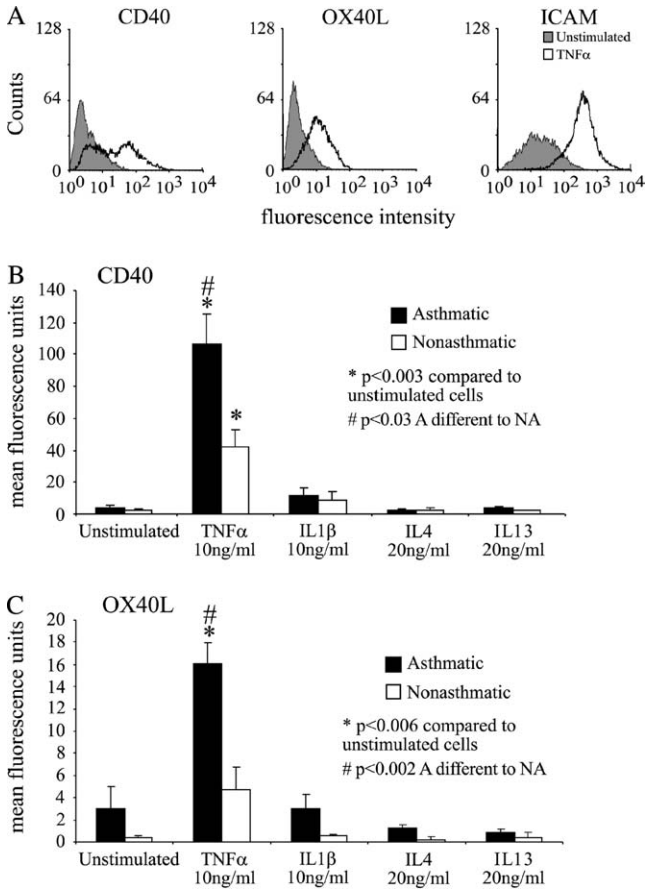

FIG 1. Induction of cell surface expression of CD40, OX40L and ICAM after stimulation with TNF-α, IL-1β, IL-4, or IL-13. **A,** Representative flow cytometric histograms from a person without asthma. **B,** MFI of CD40 expression. **C,** MFI of OX40L. TNF-α and IL-1β, n = 6; IL-4 and IL-13, n = 3 for asthmatic *(A)* and nonasthmatic *(NA)*.

Analysis of data

The fold increase in mean fluorescence intensity (MFI) over its isotype control was calculated for each treatment in each cell line. The average fluorescence intensity for each treatment for all asthmatic or nonasthmatic cell lines was then calculated. The average was designated as the MFI. Where expression levels were compared with TNF-α–induced expression, the data were expressed as percentage of TNF-α–induced expression. The data were subjected to ANOVA by using repeated measures, ANOVA factorial and the Fisher protected least-squares difference posttest. In all cases, a P value <.05 was considered significant.

RESULTS

Effect of individual cytokine treatments on expression of costimulatory molecules

The percentage of ASM cells expressing the costimulatory molecules CD40, OX40L, and ICAM (included as a positive control) was determined by using flow cytometry. TNF-α increased the percentage of cells expressing CD40, OX40L, and ICAM in the asthmatic and the nonasthmatic cells (Table I). There were significantly more asthmatic cells positive for OX40L than nonasthmatic cells. IL-1β significantly increased the percentage of cells expressing ICAM but was without effect on CD40 or OX40L (Table I). IL-4 and IL-13 had no effect.

We also examined the level of expression of the costimulatory molecules on each cell. The level of constitutive expression of CD40 and OX40L was low in unstimulated asthmatic and nonasthmatic ASM cells, but ICAM was highly expressed (Fig 1, *A*). TNF-α significantly upregulated all three molecules in both cell types (Fig 1, *A*). There was a significantly greater level of TNF-α–induced cell surface expression of CD40 (Fig 1, *B*) and OX40L (Fig 1, *C*) in the asthmatic cells compared with the nonasthmatic cells, but there was no difference in its effect on ICAM in the two cell types (see Table E2 in the Online Repository at www.mosby.com/jaci).

The effects of IL-1β, IL-4, and IL-13 on expression of CD40 and OX40L did not follow the same pattern as TNF-α. Treatment with IL-1β (10 ng/mL), IL-4 (20 ng/mL), or IL-13 (20 ng/mL) did not alter the level of expression of CD40 or OX40L on either cell type (Fig 1, *B and C*). As expected, ICAM expression was significantly increased by IL-1β compared with unstimulated cells on both asthmatic and nonasthmatic cells, but treatment with

IL-4 or IL-13 had no effect (see Table E2 in the Online Repository at www.mosby.com/jaci).

Effect of combined cytokine treatments on expression of cell surface molecules

We examined the effect of the addition of IL-1β, IL-4, or IL-13 in combination with TNF-α on expression of CD40 or OX40L. The addition of IL-1β to TNF-α reduced the number of cells expressing OX40L in both the asthmatic and the nonasthmatic populations (19.01 cells ± 7.05 cells and 3.63 cells ± 0.78 cells expressing OX40L, respectively). This effect was not observed with CD40 or ICAM (see Table E3 in the Online Repository at www.mosby.com/jaci). The addition of IL-4 or IL-13 to TNF-α did not further affect the number of cells expressing CD40, OX40L, or ICAM.

The addition of IL-4 or IL-13 did not alter the TNF-α–induced increase in the level of expression of CD40 (Fig 2, *A*), OX40L (Fig 2, *B*), or ICAM (data not shown) in either cell type. The addition of IL-1β to TNF-α significantly abrogated the TNF-α–induced cell surface expression of CD40 and OX40L in the asthmatic cells. Significant inhibition was not observed in the nonasthmatic cells (Fig 2, *A and B*; see Table E4 in the Online Repository at www.mosby.com/jaci). The combination of IL-1β and TNF-α did not significantly alter the expression of ICAM in either cell type (see Table E4 in the Online Repository at www.mosby.com/jaci).

Immunohistochemical detection of CD40 and OX40L cell surface expression

CD40 and OX40L were colocalized on asthmatic and nonasthmatic cells and in tissue sections treated with TNF-α (Fig 3). IL-1β downregulated TNF-α–induced CD40 and OX40L expression in both cells and tissue sections. IL-1β alone did not induce significant expression of CD40 or OX40L in either the asthmatic or the nonasthmatic cells or the tissue sections.

Role of PGE_2

We investigated whether the inhibition by IL-1β of TNF-α–induced CD40 and OX40L cell surface expression was via PGE_2. As has been reported previously,[22,23] ASM cells released PGE_2 in the presence of IL-1β. TNF-α alone did not induce the release of PGE_2 but significantly potentiated IL-1β–induced release. Indomethacin inhibited PGE_2 released in response to IL-1β and TNF-α in both cell types (see Fig E1 in the Online Repository at www.mosby.com/jaci).

The addition of PGE_2 and 8-bromo–cAMP (an analogue of cyclic AMP [cAMP] with increased stability) to TNF-α significantly reduced the percentage of cells positive for CD40 and OX40L, reflecting the result obtained with IL-1β + TNF-α (see Table E5 in the Online Repository at www.mosby.com/jaci). Indomethacin reversed the IL-1β–induced reduction for CD40 in the asthmatic but not the nonasthmatic cells but did not reverse the inhibition for OX40L in either cell type

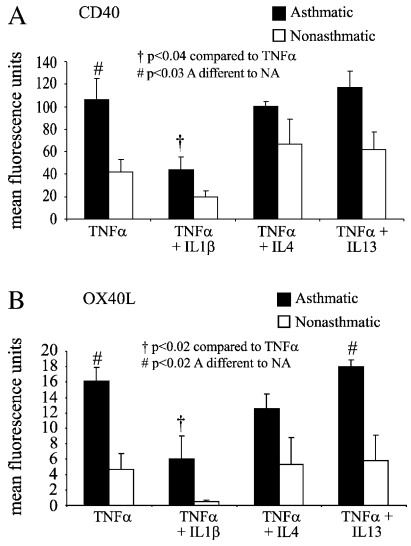

FIG 2. Induction of cell surface expression of CD40 **(A)** and OX40L **(B)** after stimulation with TNF-α with or without IL-1β, IL-4, or IL-13. The data presented in Fig 1 for TNF-α treatment are included here for comparative purposes. TNF-α and TNF-α + IL-1β, n = 6; TNF-α + IL-4 and TNF-α + IL-13, n = 3 for asthmatic *(A)* and nonasthmatic *(NA)*.

(see Table E5 in the Online Repository at www.mosby.com/jaci).

Prostaglandin E_2, 8-bromo–cAMP, and IL-1β each significantly inhibited the TNF-α–induced increase in the level of CD40 expression in both cell types (Fig 4, *A*; see Table E6 in the Online Repository at www.mosby.com/jaci). In the presence of indomethacin, IL-1β did not reduce TNF-α–induced expression (Fig 4, *A*). PGE_2, indomethacin, or 8-bromo–cAMP alone had no effect on the expression of CD40 (see Fig E2 in the Online Repository at www.mosby.com/jaci).

The mechanism of IL-1β–induced inhibition of the TNF-α–induced expression of OX40L was different from that seen with CD40 in that indomethacin did not reverse the inhibition of OX40L expression mediated by IL-1β (Fig 4, *B*). As observed with CD40, the addition of PGE_2, 8-bromo–cAMP, or IL-1β to TNF-α significantly inhibited TNF-α–induced OX40L expression (Fig 4, *B*). PGE_2, indomethacin, or 8-bromo–cAMP alone had no effect on the expression of OX40L (see Fig E2 in the Online Repository at www.mosby.com/jaci).

DISCUSSION

In this study, we have shown that the inflammatory mediators TNF-α and IL-1β, but not IL-4 or IL-13, influence the expression of the costimulatory molecules CD40 and OX40L on the surface of asthmatic and nonasthmatic ASM cells. TNF-α increases the level of expression of both CD40 and OX40L and increases the number of cells that express these molecules to a significantly greater extent on asthmatic than nonasthmatic ASM cells. IL-1β alone had little effect on the expression

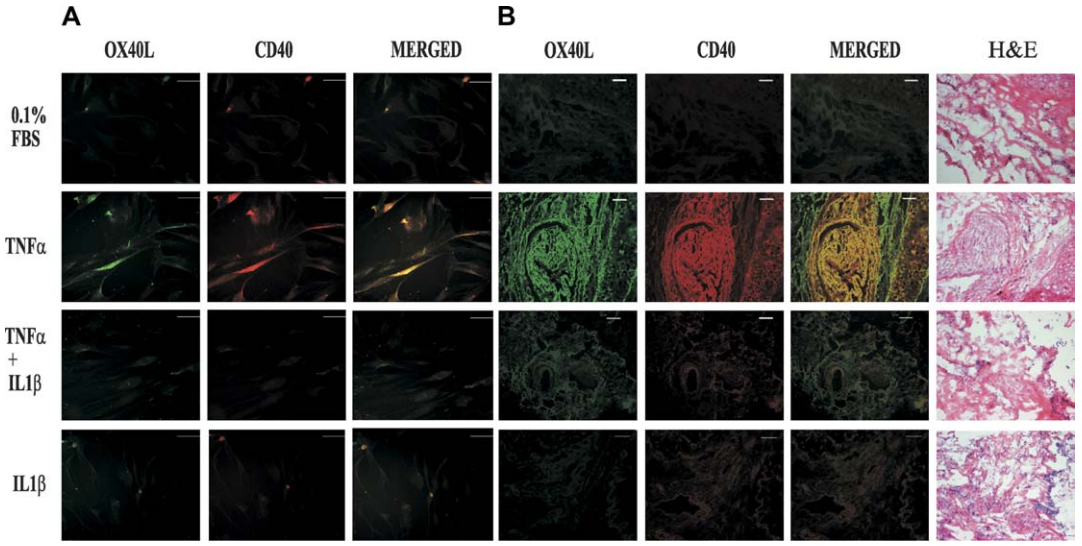

FIG 3. Immunohistochemical detection and colocalization of CD40 *(red)* and OX40L *(green)* after stimulation with TNF-α and IL-1β alone or in combination in **(A)** nonasthmatic cells in culture and **(B)** tissue sections from a patient without asthma. *H&E,* Hematoxylin and eosin.

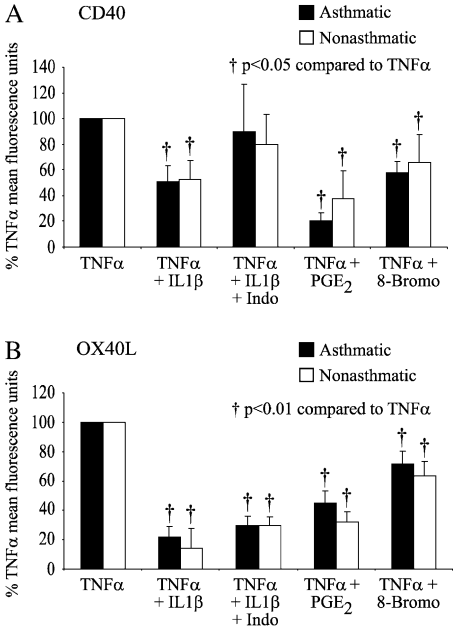

FIG 4. Inhibition of cell surface expression of CD40 **(A)** and OX40L **(B)** after treatment with TNF-α with IL-1β ± indomethacin, PGE_2, or 8-bromo–cAMP. The data presented in Fig 2 for TNF-α with or without IL-1β treatment are included here for comparative purposes. TNF-α and TNF-α + IL-1β, n = 6; all other treatments, n = 3 for asthmatic and nonasthmatic. *8-Bromo,* 8-Bromo-cAMP; *Indo,* indomethacin.

profiles, but it significantly attenuated the TNF-α–induced expression on both asthmatic and nonasthmatic ASM cells. The mechanism by which this attenuation of expression occurred was COX-dependent for CD40 and COX-independent but cAMP-dependent for OX40L.

One way in which the ASM and the inflammatory cells interact is through contact-dependent associations via the adhesion molecules expressed on the surface of the ASM cells.[9] These and other interactions induce ASM DNA synthesis, further cytokine production, and proliferation in a contact-dependent manner.[8-10] Although there are OX40L and CD40 molecules on the ASM, it is not known whether T cells adhere to human ASM cells also via these molecules. The recent characterization of CD40[9] and OX40L[11] on ASM cells highlights the possibility that these molecules may have a role in inducing the functional consequences of ASM cell inflammatory cell interactions, as occurs in T-cell–antigen-presenting cell interactions.

In this study, we have shown that the inflammatory mediators TNF-α and IL-1β influence the expression of CD40 and OX40L. As previously,[9] TNF-α increased expression of CD40, and we have demonstrated for the first time that it also increased the expression of OX40L. The number of cells expressing CD40 and OX40L also was greater after TNF-α stimulation. The expression of both of these surface molecules was significantly greater in the asthmatic ASM cells, indicating a greater responsiveness of these cells to TNF-α. IL-1β alone did not influence the expression of CD40 or OX40L, but it attenuated the TNF-α–induced increase in expression. The degree of inhibition induced by IL-1β was similar in both cell types. The induction of CD40 and OX40L expression by TNF-α, and modulation by IL-1β, was also observed in ASM cells in bronchial rings freshly isolated from human lung samples. This observation demonstrates that our findings in the cell culture model are unlikely to be the result of a culture artifact but rather reflect the events occurring in the ASM cells *in vivo*.

Both TNF-α and IL-1β are increased in the airways of patients with asthma.[3,4] Monocytes and dendritic cells are the cellular sources of IL-1β,[4] whereas mast cells,

monocytes, and epithelial cells are the sources of TNF-α.[3,4] Importantly, the number of mast cells within the ASM bundles in an asthmatic airway is increased.[24,25] This potentially would result in a greater concentration of TNF-α within the vicinity of the ASM bundle and hence would lead to even greater expression of both CD40 and OX40L on the ASM cells.

IL-1β induces the release of PGE_2 from a variety of cell types, including ASM cells,[22,23,26-28] and this is enhanced by TNF-α.[22,23,28] Expression of CD40 can be regulated by PGE_2, as demonstrated by Vancheri et al,[26] who showed that PGE_2 released from human lung fibroblasts down-regulated CD40 expressed on human blood monocytes. Similarly, in our study, although the difference between the cells stimulated with TNF-α in the presence of IL-1β with or without indomethacin was not significant, our results are consistent with PGE_2 released from the ASM cells themselves mediating the inhibition of TNF-α–induced CD40 expression in both asthmatic and non-asthmatic ASM cells. This inhibition is partially COX-dependent, because the addition of indomethacin blocked, albeit incompletely, the inhibition of CD40 expression. Furthermore, exogenous PGE_2 and 8-bromo–cAMP (a biologically stable analogue of cAMP) were equally effective at inhibiting CD40 expression in both cell types.

However, the mechanism of IL-1β attenuation of TNF-α–induced expression of OX40L differed from that of CD40. Whereas exogenous PGE_2 and 8-bromo–cAMP were both effective at inhibiting OX40L expression, intriguingly, the addition of indomethacin to IL-1β and TNF-α did not reverse the inhibition. This observation demonstrates that a cAMP-dependent but COX-independent pathway is involved in the inhibition of OX40L expression. We do not have any further information about this pathway at this stage.

Very little is known about the pathways involved in TNF-α induction of CD40 and OX40L. The OX40L promoter contains two NF-κB–like elements.[29] Although the signaling events after TNF-α stimulation have not been reported, when the OX40L gene was transcriptionally activated by the Tax oncoprotein of human T-cell leukemia virus type I, both NF-κB–like elements were able to bind to NF-κB. However, the NF-κB–like elements of the OX40L promoter are unique because, unlike the promoters of many Tax-activated genes, they are not activated by tissue plasminogen activator treatment in Jurkat cells, despite complex formation with the NF-κB–like elements.[29] In addition, a region upstream of the NF-κB–like elements was found to reduce profoundly the basic promoter activity.[29] Exactly how this region functions to suppress promoter activity is currently unknown. It is possible that the IL-1β–induced COX-independent inhibition of OX40L that we have observed in our study may involve a factor that interacts with this region of the OX40L promoter. Similarly, the CD40 promoter contains four putative NF-κB binding sites. In macrophages, TNF-α induction of CD40 expression is dependent on activation of NF-κB.[30] The presence of an upstream regulator sequence in the CD40 promoter has not been examined, but this may represent a difference between the two promoters.

Our observation of significantly greater expression of CD40 and OX40L on asthmatic ASM cells after stimulation with TNF-α is yet another difference that we have noted between asthmatic and nonasthmatic ASM cells. We have reported previously that asthmatic ASM cells differ from nonasthmatic cells in proliferation rate,[13] release of growth factors,[14] production of extracellular matrix proteins,[31] release of PGE_2 after specific stimuli,[32] and cell surface receptor numbers for PGE_2.[33] This study shows differences in expression of costimulatory molecules involved in ASM/inflammatory cell interactions modulated by relevant cytokines in the asthmatic airway. Overall, these observations indicate that the ASM cell has an enhanced responsiveness to relevant stimuli present in the asthmatic airway and has the potential to contribute actively to the inflammatory process.

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