

Detection and characterization of OX40 ligand expression in human airway smooth muscle cells: A possible role in asthma?

Janette K. Burgess, PhD,^{a,b} Stephen Carlin, PhD,^a Robert A. Pack, PhD,^c
Greg M. Arndt, PhD,^c Wendy W. Au, BSc(Hons),^a Peter R. A. Johnson, PhD,^{a,b}
Judith L. Black, MBBS, PhD,^{a,b,*} and Nicholas H. Hunt, PhD^{d,*} Sydney and Eveleigh,
Australia

Background: The airway smooth muscle (ASM) cell, originally thought of as a passive structural cell, is now well recognized as an active participant in the pathologic events that occur during persistent asthma. Cell-surface molecules play an important role in the development of an immune response. A number of cell-surface molecules are expressed on ASM cells, and these might contribute to the inflammatory reaction.

Objective: The purpose of this study was to determine whether OX40 ligand (OX40L), a molecule known to be involved in T-cell activation, was present on the ASM cell surface.

Methods: We used real-time RT-PCR to detect mRNA expression and flow cytometry, ELISA, and immunoprecipitation to detect the presence of cell-surface protein on ASM cells isolated from asthmatic and nonasthmatic individuals. ELISAs and Western blotting were used to determine the functional outcomes of engagement of OX40L. **Results:** OX40L was present on both asthmatic and nonasthmatic ASM cells. Engagement of OX40L with recombinant OX40:Fc resulted in a significantly greater increase in release of IL-6 from ASM cells of asthmatic patients than from ASM cells of nonasthmatic patients ($P < .01$). Ligation of OX40L resulted in a rapid translocation of protein kinase C β_2 to the cell membrane.

Conclusion: Because the receptor for OX40L, OX40, is expressed on CD4⁺ T cells within 48 hours of stimulation through the T-cell receptor, elucidation of the cross-talk between OX40 and OX40L could be very important in understanding the interaction of cells present in the inflamed

Abbreviations used

ASM: Airway smooth muscle
DMEM: Dulbecco modified Eagle medium
HRP: Horseradish peroxidase
MAP: Mitogen-activated protein
OX40L: OX40 ligand
PE: Phycoerythrin
PKC: Protein kinase C
rOX40:Fc: Recombinant OX40 [CD134]:Fc

airways of an asthmatic patient. (J Allergy Clin Immunol 2004;113:683-9.)

Key words: Asthma, cell-surface molecules, inflammation, OX40 ligand, human airway smooth muscle cells

Inflammation and airway hyperresponsiveness are key features of persistent asthma. The mechanisms that underlie these processes have not been well characterized to date but are known to involve the interaction of smooth muscle cells, infiltrating cells, and cytokines.¹ Although interactions between various airway inflammatory cells are well characterized, much less is known about those between inflammatory and mesenchymal cells. For instance, CD4⁺ T cells stimulate activated antigen-presenting cells to express OX40 ligand (OX40L), a member of the TNF receptor family.² T cells express OX40, the receptor for OX40L, on their surfaces after T-cell receptor stimulation. Bidirectional signaling occurs between OX40 and OX40L, resulting in cytosine release from the OX40⁺ T cells³ and the OX40L⁺ dendritic cells.⁴ This dual signaling is a common feature among members of the TNF family and their receptors.⁵

Activated T cells adhere to human airway smooth muscle (ASM) cells in culture through integrins and CD44 to induce ASM DNA synthesis in a contact-dependent manner.⁶ Other cell-surface molecules could play an important role in signaling between inflammatory and mesenchymal cells present in the airways.

From ^aRespiratory Research Group, Department of Pharmacology, University of Sydney; ^bWoolcock Institute of Medical Research, Sydney; ^cJohnson & Johnson Research Pty Ltd, Australian Technology Park, Eveleigh; and ^dthe Department of Pathology, University of Sydney, Sydney.

Supported by an Australian Research Council SPIRT grant and Johnson & Johnson Research Pty Ltd. J. K. Burgess is supported by a National Health and Medical Research Council Peter Doherty Fellowship no. 165722.

*Drs Black and Hunt contributed equally to this work.

Received for publication July 30, 2003; revised December 5, 2003; accepted for publication December 16, 2003.

Reprint requests: Janette K. Burgess, PhD, Respiratory Research Group, Department of Pharmacology, Bosch Building, D05, University of Sydney, Sydney, NSW, Australia, 2006.

0091-6749/\$30.00

© 2004 American Academy of Allergy, Asthma and Immunology

doi:10.1016/j.jaci.2003.12.311

TABLE I. Characteristics of patients

Patient no.	Age (y)	Sex	Disease	Type of sample	Atopic
1	Unknown	Unknown	Donor	Autopsy	No
2	38	M	Cystic fibrosis	Explanted lungs	No
3	52	F	Emphysema	Explanted lungs	No
4	61	M	Emphysema	Explanted lungs	No
5	60	M	Emphysema	Explanted lungs	No
6	40	M	Bronchiectasis	Explanted lungs	No
7	50	M	α_1 -Antitrypsin deficiency	Explanted lungs	Yes
8	55	F	Emphysema	Explanted lungs	Yes
9	39	F	Cystic fibrosis	Explanted lungs	Yes
10	73	F	Carcinoma	Resection	Yes
11	62	F	Carcinoma	Resection	Not tested
12	53	M	Emphysema	Explanted lungs	Yes
13	30	M	Normal	Endobronchial biopsy	Not tested
14	57	M	Pulmonary plaques	Explanted lungs	No
15	33	M	Primary pulmonary hypertension and asthma	Explanted lungs	Yes
16	15	M	Asthma (status asthmaticus)	Autopsy	Yes
17	44	M	Emphysema and asthma	Explanted lungs	Yes
18	25	M	Asthma	Endobronchial biopsy	Yes
19	56	M	Asthma	Endobronchial biopsy	Yes
20	58	M	Asthma	Endobronchial biopsy	Not tested

To date, there are no reports of the expression or interaction of OX40 or OX40L on ASM cells. We hypothesized that ASM cells express OX40L, which, when engaged through contact with infiltrating cells, might act to stimulate the expression of cytokines that in turn can influence the pathology of the airway. We examined the expression of OX40L on ASM cells from both nonasthmatic and asthmatic patients and analyzed the functional consequences of ligand engagement by OX40 by measuring cytosine secretion and downstream signaling events.

METHODS

Chemicals

The following compounds were obtained from the following sources: Dulbecco modified Eagle medium (DMEM), PBS, penicillin, streptomycin, amphotericin B, and trypan blue were obtained from Invitrogen (Heidelberg, Australia); EDTA was purchased from Ajax (Auburn, Australia); FBS was obtained from Commonwealth Serum Laboratories (Melbourne, Australia); and BSA was acquired from Sigma (St Louis, Mo).

Antibodies and recombinant proteins

FITC-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa) R-phycoerythrin (PE)-labeled polyclonal anti-mouse Ig Ab (Becton Dickinson, San Jose, Calif), PE-labeled F(ab')₂ goat anti-human IgG F(c) (Rockland, Gilbertsville, Pa), and streptavidin-horseradish peroxidase (HRP; Amersham, Buckinghamshire, United Kingdom) were purchased as indicated.

All mAbs were of the IgG class. MOPC21 (murine IgG₁ control), IgG_{2a} isotype control, monoclonal rat anti-human IL-6, and biotinylated monoclonal rat anti-human IL-6 were purchased from PharMingen (San Jose, Calif). Mouse anti-human CD40 and CD40L mAbs were obtained from Immunotech (Marseille, France), and mouse anti-human HLA-DP, HLA-DQ, and HLA-DR mAbs and anti-human HLA class I mAb were purchased from DAKO (Glostrup, Denmark). Monoclonal anti-human CD134 (with or without PE) was

from Ancell Corp (Bayport, Minn). 5A8, a monoclonal mouse anti-human gp34 (OX40L) antibody, was a generous gift from Professor Yuetsu Tanaka (Okinawa, Japan). Rabbit anti-human anti-protein kinase C (PKC) antibodies was purchased from Santa Cruz Biotechnology, and the goat anti-rabbit secondary (HRP-conjugated) antibody was obtained from Sigma.

A soluble recombinant fusion protein of human OX40 (amino acids 1-220) trimer and the Fc portion of human IgG (rOX40:Fc) was purchased from Alexis Corporation (San Diego, Calif). Human Ig Fc fragment was purchased from Jackson ImmunoResearch Laboratories.

Cell culture

We obtained human ASM cells from 14 nonasthmatic (age, 51.54 ± 10.5 years [mean ± SD]) and 6 asthmatic (age, 38.5 ± 17.2 years [mean ± SD]) patients by using methods adapted from those previously described.^{7,8} The characteristics of the patients are listed in Table I. Approval for all experiments with human lung tissue was provided by the Human Ethics Committee of the University of Sydney and the Central Sydney Area Health Service. ASM cell characteristics were determined by means of immunofluorescence and light microscopy, as previously described.⁹ All experiments were performed with cells between passages 4 and 8.

Real-time RT-PCR

Total RNA was extracted from the cells after 7 days in 10% FBS by using the RNeasy Mini Kit (Qiagen, Clifton Hill, Australia), and the RNA concentration was determined spectrophotometrically. After extraction, samples were stored at -80°C until use.

OX40L forward primer (5'-TCACCTACATCTGCCTGCACCTT-3'), reverse primer (5'-GAAACCTTTCTCTTCTTATATTCGGTA-3'), and internal probe (FAM-5'-TGCTCTTCAGGTATCACATCGGTATCCTCG-3'-TAMRA) were designed by Primer Express (ABI Prism) and synthesized by PE Applied Biosystems (Foster City, Calif). The primers were selected to span 2 adjacent exons of the gene to avoid amplification of genomic DNA. Real-time RT-PCR with 300 nmol/L OX40L forward primer, 300 nmol/L OX40L reverse primer, and 100 nmol/L OX40L probe was performed as described previously.¹⁰ Negative controls were included in every real-time RT-PCR.

Flow cytometry

Adherent ASM cells were harvested from flasks with 0.05% trypsin and 0.53 mmol/L EDTA and washed in 0.2% (wt/vol) BSA in PBS at the time points indicated. Cell suspensions were prepared as previously described.¹¹ Flow cytometry was performed on a FACSort by using Cell Quest software (Becton Dickinson, Mountain View, Calif.).

ELISA

OX40L cell-surface expression. We analyzed cells grown for 7 days in 10% FBS by using the ELISA method, as described by Hicks et al,¹² with minor modifications. All mAbs were used at a final concentration of 2 μ g/mL, except 5A8, which was used at a final concentration of 10 μ g/mL.

Cytokine release. For measurement of release of cytokines, cells were grown in 6-well plates for 7 days in 10% FBS-DMEM and then equilibrated for 24 hours in 1% FBS. rOX40:Fc or human Fc was added to cells at 1 μ g/mL in 1% FBS for 24 hours, and the supernatants were collected for analysis.

The quantities of the following cytokines were tested by using commercial ELISA kits: IL-1 β , IL-4, IL-8, IL-10, IL-12, IFN- γ , leukocyte inhibitory factor, and TGF- β ₁ (R&D Systems, Minneapolis, Minn, and Amersham Pharmacia Biotech). The amount of IL-6 was measured by using an IL-6 ELISA developed in our laboratory (with anti-IL-6 antibody pairs optimized for ELISA from PharMingen) by using the method previously described.¹³

For further analysis of IL-6, supernatants were also collected from cells grown for 4 or 10 days for flow cytometric analysis or from cells grown as described above but treated for durations of between 15 minutes and 24 hours.

Immunohistochemistry

We performed immunohistochemistry by using rOX40:Fc, followed by an anti-human Ig Fc fragment-PE-conjugated secondary antibody on fixed ASM cells grown for 7 days on glass cover slips in 10% FBS-DMEM, as described previously.¹⁴ Negative controls of secondary antibody alone and the human IgG Fc fragment were included to ensure the specificity of the reaction.

Biotinylation and immunoprecipitation

ASM cells grown for 7 days in 10% FBS-DMEM were washed on ice with PBS and then surface biotinylated by means of incubation with 0.5 mg/mL sulfo-N-hydroxysuccinimide-biotin (Pierce, Rockford, Ill) in PBS on ice for 15 minutes. The cell lysate was briefly incubated with protein G agarose and then centrifuged. The supernatant was incubated with mAb 5A8 overnight (4°C) and then protein G agarose for 3 hours. The agarose beads were extensively washed and extracted with electrophoresis buffer. Samples were boiled for 5 minutes and separated by means of electrophoresis on 15% polyacrylamide gels. Membranes were blocked and incubated with streptavidin-HRP conjugate for 1 hour. Biotinylated proteins were visualized by using enhanced chemiluminescence (supersignal west dura extended duration substrate kit from Pierce), and bands were analyzed by using a 440F Kodak imaging system and software.

Western blotting

ASM cells were grown to confluence for 7 days in 10% FBS-DMEM and then equilibrated for 24 hours in 1% FBS-DMEM. rOX40:Fc or the Fc fragment control were added to the cells at 1 μ g/mL for durations from 5 minutes to 24 hours. Cells were washed in ice-cold PBS and then lysed in extraction buffer (30 mmol/L Tris [pH 7.4], 1 mmol/L EDTA, 2 mmol/L benzimidazole, and 0.5 mmol/L phenylmethylsulfonyl fluoride) for 30 minutes on ice. Cell debris was removed by means of centrifugation (5000g for 5 minutes at 4°C), and

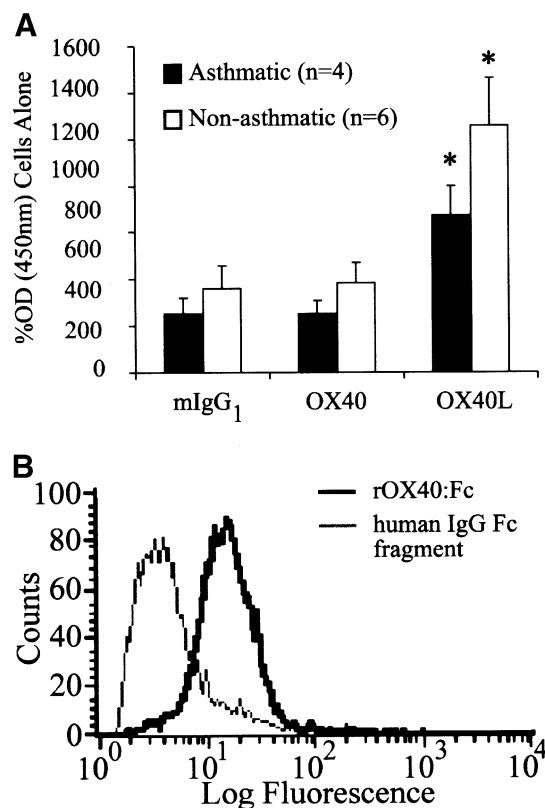


FIG 1. A, OX40L cell-surface expression measured by means of ELISA (mean $\pm$ SEM). *Greater than mIgG₁ control, $P < .05$. **B,** OX40L cell-surface expression measured by means of flow cytometry. Traces shown are from a nonasthmatic patient and are representative of results seen with 8 patients (both non-asthmatic and asthmatic patients).

then the supernatants were fractionated by means of centrifugation (14,000g for 60 minutes at 4°C). For membrane fractions, the supernatants were removed, and the pellet was dissolved in extraction buffer containing 0.5% Triton X-100. Western blots were then performed as previously described.^{7,8}

Statistics

Repeated-measures ANOVA, factorial ANOVA, and the Fisher protected least-squares difference posttest were performed where appropriate on the results for real-time RT-PCR, ELISA, and Western blots. In all cases a P value of less than .05 was considered significant.

RESULTS

RT-PCR to detect OX40L mRNA expression

Expression of OX40L was normalized to the level of 18S rRNA in each reaction. OX40L mRNA was expressed equally in cells from both nonasthmatic and asthmatic patients (data not shown).

Immunodetection of OX40L on ASM cells

OX40 and OX40L presence on ASM cells measured by means of ELISA. Both nonasthmatic and asthmatic ASM cells express OX40L, measured with 5A8, on the cell surface (Fig 1, A). The results are expressed as a percentage of absorbance (450 nm) of cells alone, which

TABLE II. Characterization of ASM cell-surface molecules

	OX40L	OX40	CD40	CD40L	MHC I	MHC II
Nonasthmatic						
1	++	—	++	—	+++	—
2	++	—	+	—	+++	—
3	+++	—	++	NT	+++	+
4	+	—	++	—	+++	—
5	+++	—	++	—	+++	—
6	++	—	++	—	+++	—
7	+	—	+	—	+++	—
8	+	—	+	NT	+++	—
9	+	—	+	—	+++	—
10	+	—	+	—	+++	—
11	++	—	+	—	+++	—
12	+++	—	+	—	+++	—
13	+++	—	++	—	+++	—
Asthmatic						
15	++	—	++	—	+++	—
16	++	—	+	—	+++	—
17	++	—	++	—	+++	—
18	++	—	+	—	+++	—
19	+	—	+	—	+++	—

The presence of the molecules was classified according to the percentage of fluorescence relative to the isotype control cells gated at 50% expressed as follows: —, less than 55%; +, 55% to 70%; ++, 71% to 85%; or +++, greater than 85%. Patient numbers refer to Table I.

NT, Not tested.

was set at 100%. The absorbance in the presence of 5A8 was significantly greater than the IgG₁ isotype control for both cell types ($P < .05$), but no difference was detected between the asthmatic and nonasthmatic expression levels. Surface expression of OX40 was not detected in this assay on either cell type.

Flow cytometric detection of OX40L. Surface expression of OX40L on ASM cells was detected by means of flow cytometry with 2 separate epitope recognition strategies, antibody 5A8 (data not shown) and the recombinant human rOX40:Fc construct (mean fluorescence intensity, 12.97 ± 2.69 for asthmatic patients [$n = 5$] and 19.59 ± 3.72 for nonasthmatic patients [$n = 12$]; Fig 1, B). Human IgG Fc fragment did not bind to the surface of the ASM cells, indicating that the rOX40:Fc binding was specific for the OX40-OX40L interaction (data not shown). No difference was observed between the binding of the secondary antibody alone or the human IgG Fc fragment.

Immunoprecipitation of OX40L on human cultured ASMs. Immunoprecipitation of the biotinylated ASM whole cell or membrane fraction proteins with 5A8 isolated a band in cells from 5 nonasthmatic and 2 asthmatic patients of the same molecular weight as the OX40L immunoprecipitated from CD4⁺ T cells after intercellular transfer, as described by Baba et al (see Fig E1 in the Online Repository).¹⁵ In the absence of 5A8, the specific band was not immunoprecipitated.

OX40L immunohistochemistry on human cultured ASMs. Immunohistochemistry with rOX40:Fc followed by an anti-human Ig Fc fragment—PE-conjugated secondary antibody incubated with fixed ASM cells grown

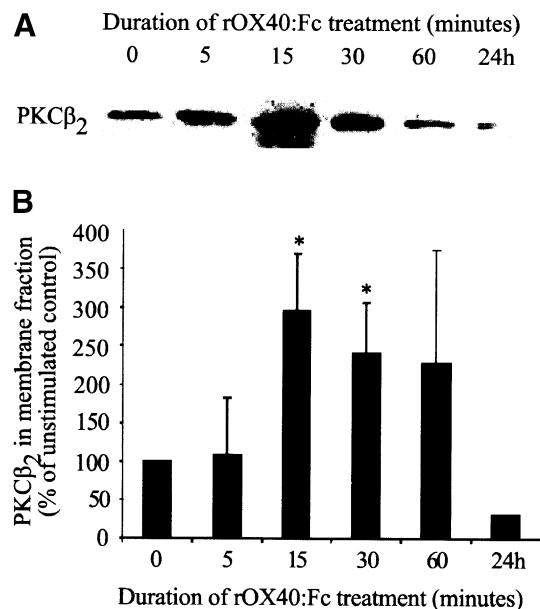


FIG 2. A, Expression of PKC β_2 in the cell-membrane fraction after addition of rOX40:Fc. Representative Western blot is shown (nonasthmatic patient). B, Means $\pm$ SEMs from 3 separate patients (24-hour time point $n = 1$). *Increase in densitometric value compared with time 0, $P < .05$.

for 7 days to confluence on glass cover slips demonstrated that OX40L was expressed on ASM, although there was no discernible difference in the immunofluorescence pattern between ASM cells from nonasthmatic and asthmatic patients (see Fig E2 in the Online Repository).

Panel of cell-surface molecule expression detected by means of flow cytometry. Consistent with other studies,^{6,16} the ASM cells expressed CD40 and MHC I on their surfaces (Table II). OX40L was observed on the surface of all the cells tested. OX40, CD40L, and MHC II were not observed on the cells. The degree of expression of OX40L and CD40 was variable in both the nonasthmatic and the asthmatic cell populations. There was no correlation between the expression of CD40 and OX40L on the resting ASM cells. No difference was observed in the surface expression pattern between nonasthmatic and asthmatic patients.

OX40 addition to ASM cells

Signal transduction experiments. An initial screen of 6 PKC isoforms and mitogen-activated protein (MAP) kinase showed that PKC β_2 was translocated to the membrane in response to rOX40:Fc (Fig 2, A). PKC β_2 was maximally activated at 15 minutes (3-fold increase in membrane fraction) in 2 nonasthmatic patients and 1 asthmatic patient (Fig 2, B), whereas other PKC isoforms, namely PKC α and PKC β_1 , were not activated, and ERK 1 and 2 phosphorylation was not altered (data not shown). There was no response to incubation with the Fc fragment control (data not shown).

Cytokine release. Of the panel of cytokines tested, only IL-6 release was altered by incubation of the cells with

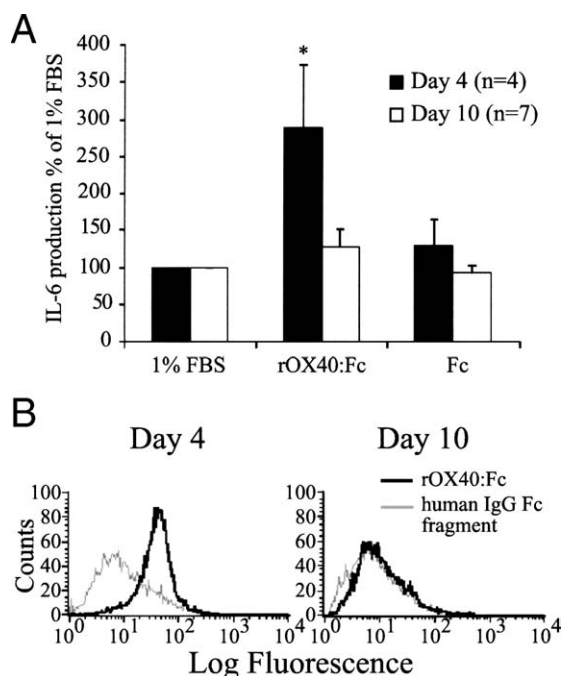


FIG 3. A, IL-6 released by ASM cells exposed to 1% FBS with or without human IgG Fc fragment or rOX40:Fc. Data are expressed as a percentage of IL-6 released in 1% FBS (means $\pm$ SEMs). *Increase in IL-6 release compared with day 0, $P < .01$. B, Cell-surface expression of OX40L. Traces from the nonasthmatic patient are representative of all patients assessed for IL-6 release.

rOX40:Fc. In all patients examined (both nonasthmatic and asthmatic patients), incubation of the ASM cells with rOX40:Fc caused a significant increase in the release of IL-6 ($P < .01$). In contrast, incubation of the cells with human IgG Fc fragment did not increase the amount of IL-6 released. IL-6 release in response to rOX40:Fc was greater in subconfluent cells (day 4) than in postconfluent cells (day 10) from the same patient (Fig 3, A). When the level of surface expression of OX40L detected by means of flow cytometry was high (day 4; Fig 3, B), the amount of IL-6 released was also high. On day 10, the level of surface expression and the amount of IL-6 released had decreased.

An increase in IL-6 release was detected after 4 hours of exposure to rOX40:Fc in cells from both the nonasthmatic and asthmatic patients (Fig 4). Significantly increased IL-6 levels were seen at 8 and 24 hours in response to rOX40:Fc treatment in cells from both the nonasthmatic and the asthmatic patients ($P < .01$). In both cell types the response to OX40:Fc was greater than the response seen with the control Fc fragment ($P < .01$). The overall release of IL-6 from the cells of asthmatic patients was significantly greater than that from the cells of nonasthmatic patients ($P < .02$).

DISCUSSION

OX40L is a 34-kd protein expressed on the surface of activated B lymphocytes,¹⁷ activated dendritic cells,⁴

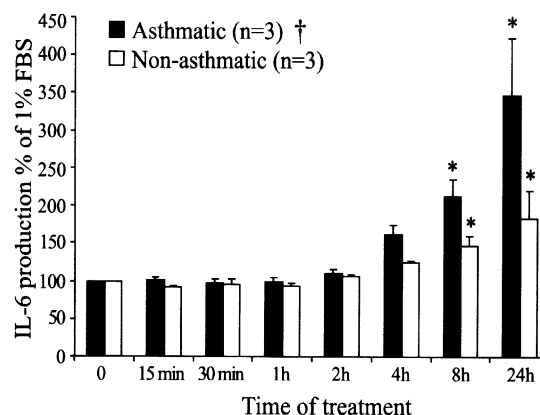


FIG 4. IL-6 released by ASM cells exposed to rOX40:Fc. Data are expressed as a percentage of IL-6 released in 1% FBS (means $\pm$ SEMs). *Increase in IL-6 release compared with time 0, $P < .01$; † curve analysis showing a significant difference between asthmatic and nonasthmatic patients, $P < .02$ (factorial ANOVA).

microglial cells,¹⁸ and vascular endothelial cells.¹⁹ This, to our knowledge, is the first report of the expression of OX40L on the surface of human ASM cells. In this study we have demonstrated that stimulation of OX40L with rOX40:Fc mediates a signal transduction pathway involving PKC β_2 and release of IL-6. These observations support the importance of interactions between ASM cells and infiltrating inflammatory cells in the development of an allergic asthmatic exacerbation.

The ASM cell releases a number of cytokines²⁰ and expresses adhesion molecules on the cell surface.⁶ Activated T cells and other inflammatory cells adhere to ASM cells through integrins and CD44 expressed on the cell surface, and they are able to induce ASM DNA synthesis, further cytokine production, and further proliferation in a contact-dependent manner.^{6,16,21} Cross-linking of CD40 on the surface of ASM cells with recombinant CD40L results in downstream signaling events and release of IL-6 from the cells.¹⁶ Activated T cells or activated ASM cells can activate the reciprocal cell, as measured by surface expression of CD25.²² The presence of OX40L on the surface of the ASM cells provides another link in the signaling cascade that is engaged during the interaction between the ASM and infiltrating inflammatory cells.

The sequential expression of CD40-CD40L and OX40-OX40L during T cell-B cell/antigen-presenting cell interactions has been well characterized,^{2,23} and some researchers suggest that the OX40-OX40L interaction is involved in T-cell priming rather than the initial stimulatory signal.^{2,24,25}

Recently, in a murine model of asthma, OX40-deficient mice primed with ovalbumin showed a reduced T_H2 response to aerosolized antigen.²⁶ These mice produced less IL-4 and IL-5 and had less lung inflammation. The authors suggested that OX40 on T cells plays an important role in the initial development of allergic asthma. In a second recent murine study, Salek-Ardakani et al²⁷

demonstrated a critical role for OX40-OX40L interactions in regulating the recall responses to inhaled allergen of memory T_H2 cells that mediate lung inflammation. Our finding of the presence of OX40L on ASM cells provides a previously unreported mechanism for T-cell priming, thereby supporting the importance of OX40 on T cells in the development of asthma. The role of OX40L in a murine model of allergic inflammation was recently examined.^{28,29} After ovalbumin sensitization and challenge, the OX40L^{-/-} mice had significantly reduced total serum IgE levels, bronchoalveolar lavage eosinophilia, levels of T_H1- and T_H2-type cytokines in the lavage fluid, and airway hyperresponsiveness in response to allergen. Administration of a neutralizing anti-OX40L mAb in wild-type mice during the sensitization stage also inhibited these parameters. These studies demonstrate the potential importance of OX40L in the development of asthma, but the ASM was not examined.

We have previously shown that PKC and MAP kinase signaling are important effectors in ASM cells.^{30,31} Very little is known about the signaling events after OX40L stimulation. In a mouse epithelial cell line treatment with rOX40 resulted in induction of the downstream products of MAP kinase signaling,³² c-jun and c-fos.³³ We have elucidated some preliminary downstream signaling events after rOX40:Fc stimulation in the ASM. PKC β_2 was consistently and rapidly translocated to the cell membrane after engagement of OX40L. There are no previous reports of PKC β_2 upregulation in response to OX40L. PKC β_2 is activated by calcium and diacylglycerol and is implicated in the control of structural proteins. It is associated with the microtubule cytoskeleton in resting cells³⁴ and with the actin cytoskeleton.³⁵ PKC β_2 also acts on structural proteins in its role in cell-cycle progression.³⁶ In this well-characterized function, PKC β_2 translocates to the nuclear membrane, where it phosphorylates lamin B, allowing nuclear lamina disassembly and entry into mitosis.

We also examined the functional consequences of stimulating ASM cells with rOX40:Fc. OX40L engagement induced a significant increase in the release of IL-6, and the ASM cells of asthmatic patients produced significantly greater amounts of IL-6 than the ASM cells of nonasthmatic patients. These results suggest that the cells of asthmatic patients have an enhanced response to the signaling events after OX40L stimulation. IL-6 has a number of proinflammatory properties that might be important in airway inflammation during asthma, including the differentiation of B cells into antibody-producing cells,³⁷ upregulation of IL-4-dependent IgE production,³⁸ and stimulation of T-cell proliferation.³⁹

The signaling cascade from CD40 to OX40L and the release of IL-6 provide further evidence for the active role the ASM cell plays in the maintenance of the localized inflammatory response within the asthmatic airway. Future studies are needed to examine other important consequences of OX40L engagement on the ASM and to understand the molecular mechanisms involved in regulating signaling from OX40L. Elucidation of the interaction between CD40-CD40L and OX40-

OX40L could be very important in understanding the interaction between smooth muscle and inflammatory cells in asthma.

We acknowledge the collaborative effort of the cardiopulmonary transplant team and the pathologists at St Vincent's hospital, Sydney, and the thoracic physicians and pathologists at Royal Prince Alfred Hospital, Concord Repatriation Hospital, and Strathfield Private Hospital and Rhodes Pathology, Sydney. We also acknowledge the contribution of Dr Gregory King at the Woolcock Institute of Medical Research for supplying the asthmatic biopsy specimens.

REFERENCES

- Bousquet J, Vignola AM, Chanez P, Campbell AM, Bonsignore G, Michel FB. Airways remodelling in asthma: no doubt, no more? *Int Arch Allergy Immunol* 1995;107:211-4.
- Murata K, Ishii N, Takano H, Miura S, Ndhlovu LC, Nose M, et al. Impairment of antigen-presenting cell function in mice lacking expression of OX40 ligand. *J Exp Med* 2000;191:365-74.
- Flynn S, Toellner KM, Raykundalia C, Goodall M, Lane P. CD4 T cell cytosine differentiation: the B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, Blnr-1. *J Exp Med* 1998;188:297-304.
- Ohshima Y, Tanaka Y, Tozawa H, Takahashi Y, Maliszewski C, Delespesse G. Expression and function of OX40 ligand on human dendritic cells. *J Immunol* 1997;159:3838-48.
- Watts AD, Hunt NH, Wanigasekara Y, Bloomfield G, Wallach D, Roufogalis BD. A casein kinase I motif present in the cytoplasmic domain of members of the tumour necrosis factor ligand family is implicated in "reverse signalling." *EMBO J* 1999;18:2119-26.
- Lazaar AL, Albelda SM, Pilewski JM, Brennan B, Pure E, Panetti eri RA, Jr. T lymphocytes adhere to airway smooth muscle cells via integrins and CD44 and induce smooth muscle cell DNA synthesis. *J Exp Med* 1994;180:807-16.
- Johnson PR, Armour CL, Carey D, Black JL. Heparin and PGE2 inhibit DNA synthesis in human airway smooth muscle cells in culture. *Am J Physiol Lung Cell Mol Physiol* 1995;269:L514-9.
- Carlin S, Yang KX, Donnelly R, Black JL. Protein kinase C isoforms in human airway smooth muscle cells: activation of PKC-zeta during proliferation. *Am J Physiol Lung Cell Mol Physiol* 1999;276:L506-12.
- Durand-Arczynska W, Marmy N, Durand J. Caldesmon, calponin and alpha-smooth muscle actin expression in subcultured smooth muscle cells from human airways. *Histochem J* 1993;100:465-71.
- Burgess JK, Johnson PR, Ge Q, Au WW, Poniris MH, McParland BE, et al. Expression of connective tissue growth factor in asthmatic airway smooth muscle cells. *Am J Respir Crit Care Med* 2003;167:71-7.
- Burgess JK, Lopez JA, Berndt MC, Dawes I, Chesterman CN, Chong BH. Quinine-dependent antibodies bind a restricted set of epitopes on the glycoprotein Ib-IX complex: characterization of the epitopes. *Blood* 1998;92:2366-73.
- Hicks C, Sleight M, Chesterman C. Quantitation of platelet derived growth factor receptors on cells from human arterial segments. *J Immunol Methods* 1994;170:83-92.
- Sukkar MB, Hughes JM, Johnson PR, Armour CL. GM-CSF production from human airway smooth muscle cells is potentiated by human serum. *Mediators Inflamm* 2000;9:161-8.
- Triantafyllou M, Wilson KM, Triantafyllou K. Identification of Echovirus 1 and coxsackievirus A9 receptor molecules via a novel flow cytometric quantification method. *Cytometry* 2001;43:279-89.
- Baba E, Takahashi Y, Lichtenfeld J, Tanaka R, Yoshida A, Sugamura K, et al. Functional CD4 T cells after intercellular molecular transfer of OX40 ligand. *J Immunol* 2001;167:875-83.
- Lazaar AL, Amrani Y, Hsu J, Panettieri RA, Jr, Fanslow WC, Albelda SM, et al. CD40-mediated signal transduction in human airway smooth muscle. *J Immunol* 1998;161:3120-7.
- Calderhead DM, Buhlmann JE, van den Eertwegh AJ, Claassen E, Noelle RJ, Fell HP. Cloning of mouse Ox40: a T cell activation

- marker that may mediate T-B cell interactions. *J Immunol* 1993;151:5261-71.
18. Weinberg AD, Wegmann KW, Funatake C, Whitham RH. Blocking OX-40/OX-40 ligand interaction in vitro and in vivo leads to decreased T cell function and amelioration of experimental allergic encephalomyelitis. *J Immunol* 1999;162:1818-26.
 19. Matsumura Y, Imura A, Hori T, Uchiyama T, Imamura S. Localization of OX40/gp34 in inflammatory skin diseases: a clue to elucidate the interaction between activated T cells and endothelial cells in infiltration. *Arch Dermatol Res* 1997;289:653-6.
 20. Hirst SJ. Airway smooth muscle as a target in asthma. *Clin Exp Allergy* 2000;30:54-9.
 21. Hughes JM, Arthur CA, Baracho S, Carlin SM, Hawker KM, Johnson PR, et al. Human eosinophil-airway smooth muscle cell interactions. *Mediators Inflamm* 2000;9:93-9.
 22. Hakonarson H, Kim C, Whelan R, Campbell D, Grunstein MM. Bi-directional activation between human airway smooth muscle cells and T lymphocytes: role in induction of altered airway responsiveness. *J Immunol* 2001;166:293-303.
 23. Gramaglia I, Weinberg AD, Lemon M, Croft M. Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J Immunol* 1998;161:6510-7.
 24. Chen AI, McAdam AJ, Buhlmann JE, Scott S, Lupher ML, Jr, Greenfield EA, et al. Ox40-ligand has a critical costimulatory role in dendritic cell:T cell interactions. *Immunity* 1999;11:689-98.
 25. Lane P. Role of OX40 signals in coordinating CD4 T cell selection, migration, and cytosine differentiation in T helper (Th)1 and Th2 cells. *J Exp Med* 2000;191:201-6.
 26. Jember AG, Zuberi R, Liu FT, Croft M. Development of allergic inflammation in a murine model of asthma is dependent on the costimulatory receptor OX40. *J Exp Med* 2001;193:387-92.
 27. Salek-Ardakani S, Song J, Halteman BS, Jember AG, Akiba H, Yagita H, et al. OX40 (CD134) Controls memory T helper 2 cells that drive lung inflammation. *J Exp Med* 2003;198:315-24.
 28. Arestides RS, He H, Westlake RM, Chen AI, Sharpe AH, Perkins DL, et al. Costimulatory molecule OX40L is critical for both Th1 and Th2 responses in allergic inflammation. *Eur J Immunol* 2002;32:2874-80.
 29. Hoshino A, Tanaka Y, Akiba H, Asakura Y, Mita Y, Sakurai T, et al. Critical role for OX40 ligand in the development of pathogenic Th2 cells in a murine model of asthma. *Eur J Immunol* 2003;33:861-9.
 30. Lee JH, Johnson PR, Roth M, Hunt NH, Black JL. ERK activation and mitogenesis in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L1019-29.
 31. Carlin S, Poronnik P, Cook DI, Carpenter L, Biden TJ, Johnson PR, et al. An antisense of protein kinase C-zeta inhibits proliferation of human airway smooth muscle cells. *Am J Respir Cell Mol Biol* 2000;23:555-9.
 32. Black JL, Roth M, Lee JH, Carlin SC, Johnson PRA. Mechanisms of airway remodeling. Airway smooth muscle. *Am J Respir Crit Care Med* 2001;164:63S-6.
 33. Matsumura Y, Hori T, Kawamata S, Imura A, Uchiyama T. Intracellular signaling of gp34, the OX40 ligand: induction of c-jun and c-fos mRNA expression through gp34 upon binding of its receptor, OX40. *J Immunol* 1999;163:3007-11.
 34. Kiley SC, Parker PJ. Differential localization of protein kinase C isozymes in U937 cells—evidence for distinct isozyme functions during monocyte differentiation. *J Cell Sci* 1995;108:1003-16.
 35. Faux MC, Scott JD. More on target with protein phosphorylation—conferring specificity by location. *Trends Biochem Sci* 1996;21:312-5.
 36. Gokmenpolar Y, Fields AP. Mapping of a molecular determinant for protein kinase C beta(ii) isozyme function. *J Biol Chem* 1998;273:20261-6.
 37. Muraguchi A, Hirano T, Tang B, Matsuda T, Horii Y, Nakajima K, et al. The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the terminal differentiation of B cells. *J Exp Med* 1988;167:332-44.
 38. Sanchez-Guerrero I, Herrero N, Muro M, Vegara R, Campos M, Garcia-Alonso A, et al. Co-stimulation of cultured peripheral blood mononuclear cells from intrinsic asthmatics with exogenous recombinant IL-6 produce high levels of IL-4-dependent IgE. *Eur Respir J* 1997;10:2091-6.
 39. Uyttenhove C, Coulie P, Van Snick J. T cell growth and differentiation induced by interleukin-HP1/IL-6, the murine hybridoma/plasmacytoma growth factor. *J Exp Med* 1988;167:1417-27.