

Original article

CD40 and OX40 ligand are differentially regulated on asthmatic airway smooth muscle

Background: CD40 and OX40 Ligand (OX40L) are cell-surface molecules expressed on airway smooth muscle (ASM) that can enhance inflammatory cell activation and survival. The aim of this study was to examine the effect of tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) on ASM CD40 and OX40L expression.

Methods: CD40 and OX40L expression on human ASM cells from asthmatic and nonasthmatic donors following stimulation with TNF- α and/or IFN- γ was measured using cell-surface enzyme-linked immunosorbent assay (ELISA) and flow cytometry. Involvement of signalling pathway was investigated with pharmacological inhibitors. Soluble TNF receptor levels were quantified by ELISA.

Results: Interferon- γ and TNF- α synergistically increased CD40 expression to a greater extent on asthmatic than on nonasthmatic ASM. In contrast, IFN- γ reduced TNF- α -induced OX40L expression to a similar extent in both cell types. TNF- α and IFN- γ induced CD40 via nuclear factor- κ B (NF- κ B) and signal transducer and activator of transcription-3 in both cell types and modulated OX40L via NF- κ B and c-Jun N terminal kinase in nonasthmatic cells. Similar effects on the induction of OX40L in asthmatic cells were seen with NF- κ B, but these were not statistically significant. The reduced OX40L expression with TNF- α and IFN- γ involved extracellular regulated kinase 1/2 activation.

Conclusion: Asthmatic ASM may modulate airway inflammation locally by increasing CD40 and OX40L expression in response to cytokines. IFN- γ may regulate ASM pro-inflammatory actions by differentially modulating ASM CD40 and OX40L expression.

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Asthma is a chronic disease of the airways associated with reversible airway obstruction, airway smooth muscle (ASM) cell hyperplasia, airway hyper-responsiveness and airway inflammation (1). Airway smooth muscle cells release cytokines and chemokines as well as expressing cell-surface molecules that can bind to and modulate cells of the immune system. Specifically, ASM cells can bind activated T cells, which results in increased cellular proliferation (2).

The recent discovery of the presence of two cell-surface molecules CD40 and OX40 Ligand (OX40L) on human ASM has highlighted the immunomodulatory role of this cell (3, 4). We have noted several important differences in the properties of ASM cells derived from asthmatic and nonasthmatic patients and amongst these is the observation that the expression of CD40 and OX40L on

asthmatic ASM is modulated by cytokines, which are relevant to asthma, viz. tumour necrosis factor (TNF) α and interleukin 1 β (IL1 β) (3). These co-stimulatory cell-surface molecules bind to CD154 and OX40, respectively, on activated CD4+ T cells. CD154 is expressed on CD4+ T cells immediately after their activation and provides a crucial 'second signal' for T-cell activation (5). This is followed by the binding of OX40L to OX40 on CD4+ T cells, which activates a strong signalling pathway that augments cytokine secretion and cellular proliferation (6). Importantly, this interaction maintains long-term survival of CD4+ T cells (7).

The expression of the cytokines TNF- α and interferon- γ (IFN- γ) is increased in asthmatic airways (8, 9). TNF- α facilitates inflammatory cell migration and increases the expression of intercellular adhesion molecule-1, CD40 and

OX40L on ASM (3, 4). Interferon- γ also upregulates the expression of cell-surface signalling molecules on ASM (3, 4). Attributable anti-inflammatory effects of IFN- γ in the airways are not always demonstrable but recent studies have implicated IFN- γ in asthma pathogenesis (10).

Tumour necrosis factor- α and IFN- γ have previously been reported both to enhance and inhibit each other's actions. The combination of IFN- γ and TNF- α synergistically increases the expression of CD38 and the chemokine CCL5 (regulated upon activation normal T-cell expressed and secreted (RANTES)) on ASM (11). This combination also has inhibitory effects, whereby activation of nuclear factor- κ B (NF- κ B) is attenuated (12).

TNF- α interacts with two receptors, TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2), both of which are expressed on human ASM cells (13, 14). Binding of cell-surface TNF-R1 on ASM by TNF- α leads to the activation of c-Jun N-terminal kinase (JNK) (15), p38 and extracellular regulated kinase (ERK) 1/2 (13), mitogen-activated protein kinase (MAPK) signalling pathways, as well as the transcription factor NF- κ B (14). These pathways may regulate the expression of CD40 and OX40L.

In this study, we examined the effects of TNF- α and IFN- γ on CD40 and OX40L expression on asthmatic and nonasthmatic ASM and identified the signalling pathways involved. We investigated whether TNF- α and IFN- γ also induced TNF-R1 and TNF-R2 shedding.

Methods

Chemicals

The following compounds were obtained from the companies identified in parentheses: Dulbecco's Modified Eagle's Medium (DMEM), dimethyl sulphoxide (DMSO), bovine serum albumin, actinomycin D, dimethyl fumarate, wortmannin (Sigma, St Louis, MI, USA), phosphate-buffered saline, penicillin, streptomycin, amphotericin B (Invitrogen, Carlsbad, CA, USA), fetal bovine

serum (FBS) (JRH Biosciences, Melbourne, Australia), cocktail lysis buffer (III), PD98059, BMS-345541, pyridone-6, LY294002, LY303511, SB203580, SB202474 (Calbiochem, San Diego, CA, USA), SP600125 (A.G. Scientific, San Diego, CA, USA), U0126 (Promega, Madison, WI, USA) and cycloheximide (BioMol, Plymouth Meeting, PA, USA).

Antibodies and recombinant proteins

Mouse anti-human CD40 mAb (Immunotec, Marseille, France), goat anti-human OX40L (R&D Systems, Minneapolis, MN, USA), mouse anti-human TNF-R1 mAb and mouse anti-human TNF-R2 mAb (R&D Systems), mouse IgG₁ (R&D Systems), goat IgG (Jackson, West Grove, PA, USA), polyclonal rabbit anti-goat-horse radish peroxidase (HRP) (Dako, Glostrup, Denmark), goat anti-mouse-HRP (Dako), biotinylated goat anti-human OX40L (R&D Systems), rabbit anti-goat-fluorescein isothiocyanate (FITC) (Sigma), goat anti-mouse-phycoerythrin (Becton Dickinson, Mountain View, CA, USA), recombinant human IFN- γ (Pierce, Rockford, IL, USA), human TNF- α (Endogen, Rockford, IL, USA) and human OX40L (R&D Systems) were purchased from the companies mentioned above. A soluble recombinant fusion protein of human OX40 (aa: 1–220) trimer and the Fc portion of human IgG (rOX40:Fc) was purchased from Alexis Corporation (San Diego, CA, USA). Human Ig Fc fragment was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Cell culture

Approval for all experiments with human lung was provided by the Human Ethics Committees of the University of Sydney and the Sydney South West Area Health Service. Nonasthmatic ASM was obtained from bronchial airways of 18 patients (age: 53.7 ± 17.5 years, mean $\pm$ SD) undergoing resection for either lung transplantation or carcinoma and from individuals who volunteered for a deep endobronchial biopsy. Asthmatic ASM was obtained from 17 patients (age: 35.9 ± 13.8 years, mean $\pm$ SD) with asthma, diagnosed by a specialist physician and who volunteered for a deep endobronchial biopsy. Details of the patients are provided in Table 1.

Pure ASM bundles were dissected free and grown as previously described (16). Airway smooth muscle cell characteristics were confirmed by immunofluorescence and light microscopy. All

Table 1. Effect of the inhibitors of the transcription factors NF- κ B (BMS-345541), STAT-1/3 (pyridone 6), MAPKs ERK (PD98059) and JNK (SP600125) on cell-surface expression of CD40 and OX40L

	Unstimulated				TNF- α stimulated			
	BMS-345541	Pyridone 6	PD98059	SP600125	BMS-345541	Pyridone 6	PD98059	SP600125
Asthmatic								
CD40	91.66 $\pm$ 4.0	87.26 $\pm$ 4.9*	103.2 $\pm$ 4.3	92.58 $\pm$ 7.3	89.46 $\pm$ 1.8*	102.0 $\pm$ 3.3	91.88 $\pm$ 6.7	110.1 $\pm$ 7.3
OX40L	98.62 $\pm$ 1.9	90.1 $\pm$ 2.3*	104.8 $\pm$ 5.7	93.23 $\pm$ 9.2	81.48 $\pm$ 6.5*	84.0 $\pm$ 7.9	122.5 $\pm$ 5.8*	87.48 $\pm$ 5.1
Nonasthmatic								
CD40	99.57 $\pm$ 2.2	99.88 $\pm$ 3.9	105.0 $\pm$ 8.1	102.3 $\pm$ 4.8	104.5 $\pm$ 1.9*	103.8 $\pm$ 4.4	90.51 $\pm$ 4.9	120.0 $\pm$ 9.8
OX40L	91.74 $\pm$ 2.8	87.45 $\pm$ 3.8*	105.5 $\pm$ 3.3	101.8 $\pm$ 3.4	72.94 $\pm$ 5.2*	78.58 $\pm$ 4.4*	116.2 $\pm$ 1.9*	63.09 $\pm$ 6.2*

TNF- α , tumour necrosis factor; NF- κ B, nuclear factor κ B; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular regulated kinase; STAT, xxxxxxx.

Data are expressed as % of their respective vehicle control.

* $P < 0.05$ compared to vehicle control.

CD40 asthmatic BMS-345541, $n = 10$; pyridone 6, $n = 8$; PD98059, $n = 10$; SP600125, $n = 8$; nonasthmatic, $n = 10$.

OX40L asthmatic, $n = 6$; nonasthmatic, $n = 6$.

experiments were performed with cells between passages 4 and 8. Asthmatic and nonasthmatic ASM cells at these passages retain the typical smooth muscle 'hill and valley' pattern of growth and contain similar levels of α -smooth muscle actin [asthmatic ($n = 5$) and nonasthmatic ($n = 4$) cell mean fluorescence intensities $\pm$ SEM: 50.8 ± 21.6 and 51.36 ± 13.1 , respectively] and h-calponin (56.9 ± 7.5 and 68.1 ± 12.5) when assessed using flow cytometry (A. Qawasmeh – personal communication).

Flow cytometry

Asthmatic and nonasthmatic ASM cells were seeded in 75 cm² flasks at 1×10^4 cells per cm² in 5% (vol/vol) FBS/antibiotics/DMEM for 48 h. Cells were then equilibrated for 24 h in 0.1% (vol/vol) FBS/antibiotics/DMEM before incubation in the presence of TNF- α (10 ng/ml) and IFN- γ (10 ng/ml) alone and in combination. The cell-surface expression of CD40 and OX40L after 24 and 48 h was analysed by flow cytometry using mouse anti-human CD40 and goat anti-human OX40L antibodies at 1 μ g/ml as described previously (3). Immunostaining for OX40L was carried out using OX40 (CD134):Fc together with phycoerythrin (PE) F(ab')₂ fragment anti-hIgG Fc (17) or goat anti-human OX40L with rabbit anti-goat FITC (3) as described previously. Full details of this method are provided in the Online Repository.

ELISA

The cell-surface expression of CD40 and OX40L was also analysed by enzyme-linked immunosorbent assay (ELISA) using mouse anti-human CD40 and goat anti-human OX40L antibodies at 1 μ g/ml. Full details of this method are provided in the Online Repository. A soluble OX40L ELISA assay was developed using 1 μ g/ml goat anti-human OX40L (R&D Systems) antibody as capture antibody with 2 μ g/ml biotinylated goat anti-human OX40L (R&D Systems) as detection antibody. Full details of this method are provided in the Online Repository. Cell-surface expression of TNF-R1 and TNF-R2 was analysed by ELISA using mouse anti-human TNF-R1 and TNF-R2 antibodies (R&D Systems) at 1 μ g/ml. Full details of this method are provided in the Online Repository. The levels of soluble TNF-R1 and soluble TNF-R2 were measured using commercial ELISA kits according to the manufacturer's instructions (Biosource, Camarillo, CA, USA).

Signalling inhibition

Airway smooth muscle cells were seeded in 96-well plates and incubated for 72 h in 5% FBS/antibiotics/DMEM at a density of 1×10^4 /cm². Cells were then equilibrated for 24 h in 0.1% (vol/vol) FBS/antibiotics/DMEM before incubation in the presence of SP600125 (10 μ M), PD98059 (10 μ M), LY294002 (3 μ M), LY303511 (3 μ M), BMS-345541 (30 μ M), Pyridone-6 (65 nM), wortmannin (100 nM), SB203580 (10 μ M) or SB202474 (10 μ M) in appropriate concentrations of DMSO in 0.1% FBS/antibiotics/DMEM. After 1 h, the medium was aspirated before the addition of TNF- α (10 ng/ml) and IFN- γ (10 ng/ml) alone or in combination in the presence of inhibitors for 48 h.

Data analysis

CD40 and OX40L expression in asthmatic and nonasthmatic ASM following cytokine stimulation was analysed using two-way ANOVA. Comparisons between inhibitors of signalling molecules and DMSO vehicle controls were made using paired Student's *t*-tests. All

analyses were performed using GRAPHPAD PRISM 5.0 software (GraphPad Software, La Jolla, CA, USA). Differences were considered significant when $P \leq 0.05$.

Results

IFN- γ -potentiated TNF- α -induced CD40 expression but decreased TNF- α -induced OX40L expression

Cell-surface CD40 expression was significantly increased by 24 h stimulation with the combination of TNF- α and IFN- γ compared with the individual cytokine alone, on both asthmatic and nonasthmatic ASM cells (Fig. 1A). Furthermore, CD40 expression was augmented to a greater extent on asthmatic ASM than on nonasthmatic ASM following 48 h combined cytokine stimulation (Fig. 1B). In contrast, the low basal expression of OX40L was increased after 24 h stimulation with TNF- α but the addition of IFN- γ attenuated this effect of TNF- α on OX40L expression (Fig. 1C). The pattern of OX40L expression was similar after 48 h cytokine stimulation (Fig. 1D). These response patterns were also seen when CD40 and OX40L expression was measured using flow cytometry at the same time point (Fig. 2). As previously described (3), TNF- α significantly increased CD40 and OX40L expression in both cell types. Soluble/shed OX40L was not detectable in the supernatant of ASM from either cell type ($n = 4, 4$) (data not shown).

CD40 and OX40L expression following cytokine stimulation involves different signalling pathways

Signal transducer and activator of transcription (STAT)-1/3 partly mediated the basal cell-surface expression of both CD40 and OX40L in asthmatic cells and that of OX40L in nonasthmatic cells (Table 1). As expected, inhibiting NF- κ B had no effect on basal CD40 and OX40L expression but inhibited TNF- α -induced CD40 in asthmatic ASM cells and induced it in nonasthmatic ASM cells (Table 1). The induction of OX40L expression by TNF- α was only inhibited by BMS-345541 (NF- κ B) in the asthmatic cells but by both BMS-345541 and pyridone-6 (STAT-1/3) in nonasthmatic cells.

c-Jun N-terminal kinase and ERK had contrasting effects on OX40L expression. TNF- α -induced OX40L was significantly increased by inhibiting ERK in both cell types, whereas it was inhibited by blocking JNK in nonasthmatic but not asthmatic cells (Table 1). Inhibition of p38 had no effect on CD40 or OX40L expression (data not shown).

The combination of TNF- α and IFN- γ led to different signalling patterns from those of the cytokines independently. Only CD40 expression was mediated by NF- κ B in the cytokine-treated asthmatic cells (Fig. 3A). In contrast, in nonasthmatic cells, CD40 and OX40L expression was mediated by NF- κ B (Fig. 3B), as seen with TNF- α

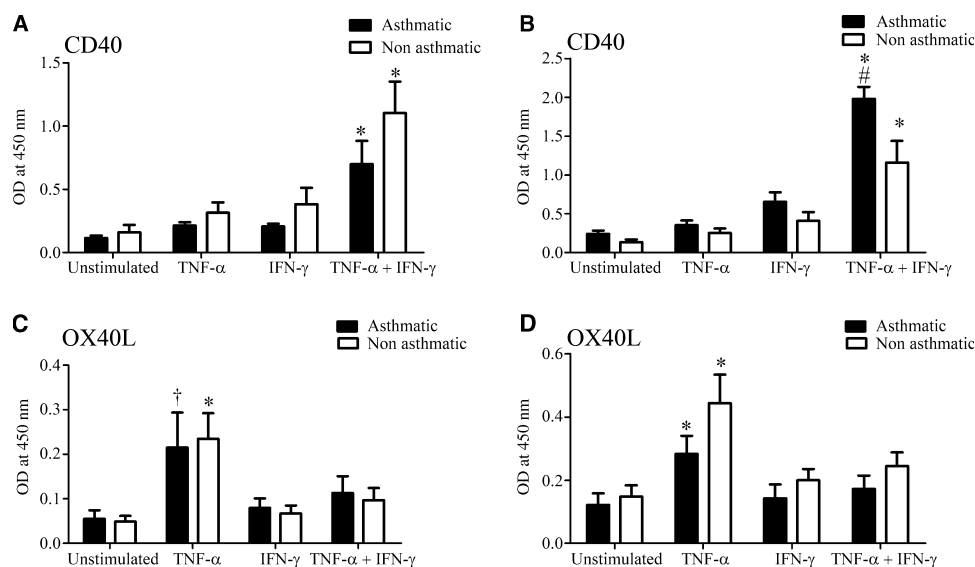


Figure 1. Induction of cell-surface expression of CD40 and OX40L by tumour necrosis factor (TNF)- α and interferon (IFN)- γ . Cell-surface expression of CD40 after (A) 24 h and (B) 48 h and OX40L after (C) 24 h and (D) 48 h on airway smooth muscle cells stimulated with TNF- α and IFN- γ alone or in combination was measured using ELISA (mean + SEM). $n = 9$ for 24 h asthmatic (AS) and nonasthmatic (NA); $n = 6$ for 48 h A and NA. * $P < 0.05$ compared to all treatments, † $P < 0.05$ compared to unstimulated and IFN- γ , # $P < 0.001$ AS compared to NA.

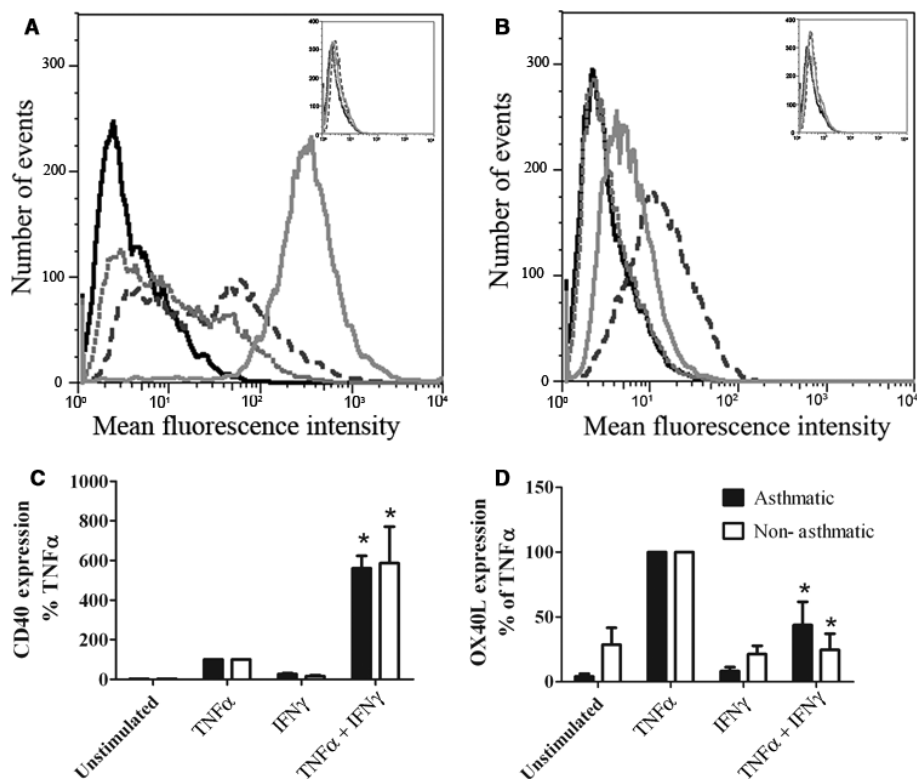


Figure 2. Flow cytometric analysis of CD40 and OX40L expression after stimulation with tumour necrosis factor (TNF)- α and interferon (IFN)- γ . Representative fluorescence histograms of (A) CD40 and (B) OX40L illustrating peaks for unstimulated (black solid line), TNF- α (black dashed line), IFN- γ (grey dotted line) and TNF- α and IFN- γ (grey solid line). The isotype controls are shown in the inserts. (C) CD40 expression (% of TNF- α) (mean + SEM). (D) OX40L expression (% of TNF- α) (mean + SEM). CD40, $n = 6$ for asthmatic (A) and nonasthmatic (NA), OX40L, $n = 5$ for asthmatic and nonasthmatic. * $P < 0.001$ compared to TNF- α alone.

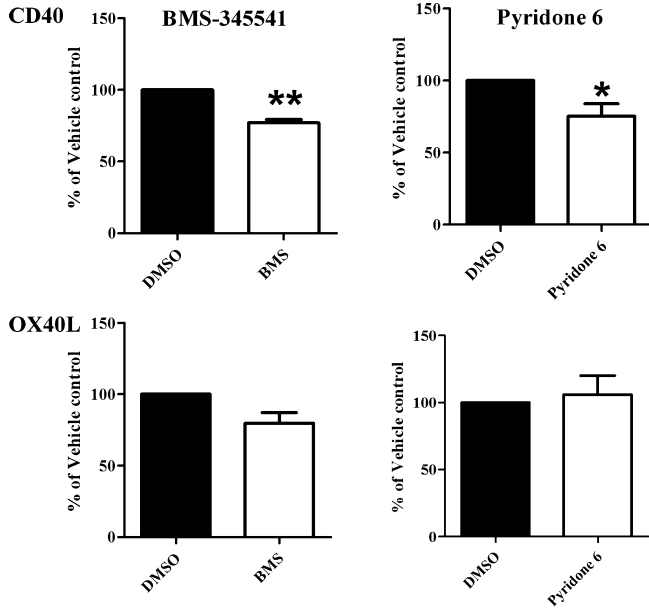
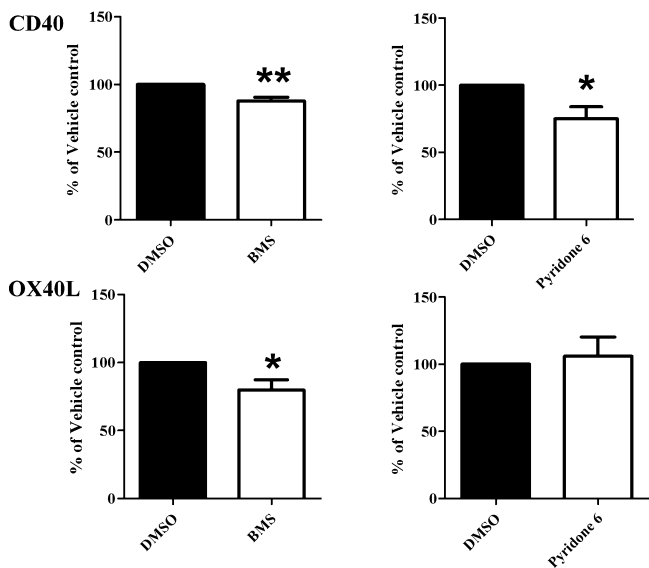
A Asthmatic**B Nonasthmatic**

Figure 3. Cell-surface expression of CD40 and OX40L following inhibition of nuclear factor- κ B or STAT-1/3. Cell-surface expression of CD40 and OX40L on airway smooth muscle from asthmatic (A) or nonasthmatic (B) donors, detected using enzyme-linked immunosorbent assay following 48 h stimulation with tumour necrosis factor- α and interferon- γ in the presence of BMS-345411, Pyridone 6 or dimethyl sulfoxide (DMSO, vehicle control). Columns represent % of vehicle control (mean + SEM), $n = 6$ for asthmatic and nonasthmatic cells, * $P < 0.05$, ** $P < 0.005$ compared to matched vehicle control.

alone. STAT-1/3 signalling led to the expression of CD40, but not OX40L, in both asthmatic and nonasthmatic cells treated with TNF- α and IFN γ (Fig. 3). The CD40 and OX40L expression in the asthmatic cells in the presence of the MAPK inhibitors was different to that in the

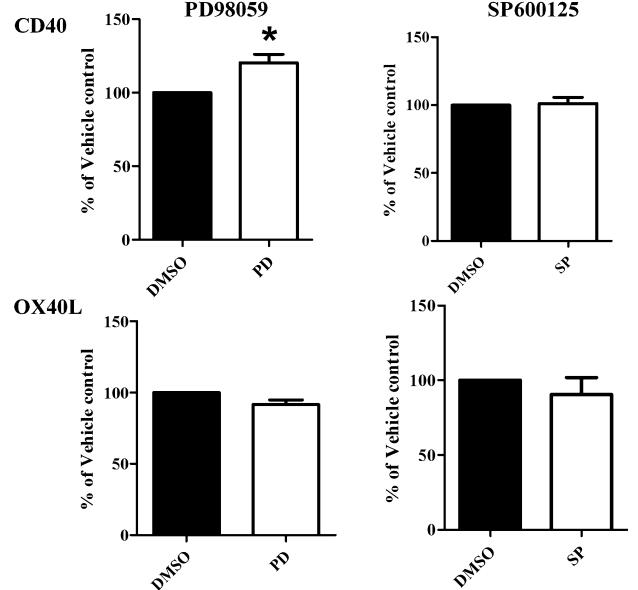
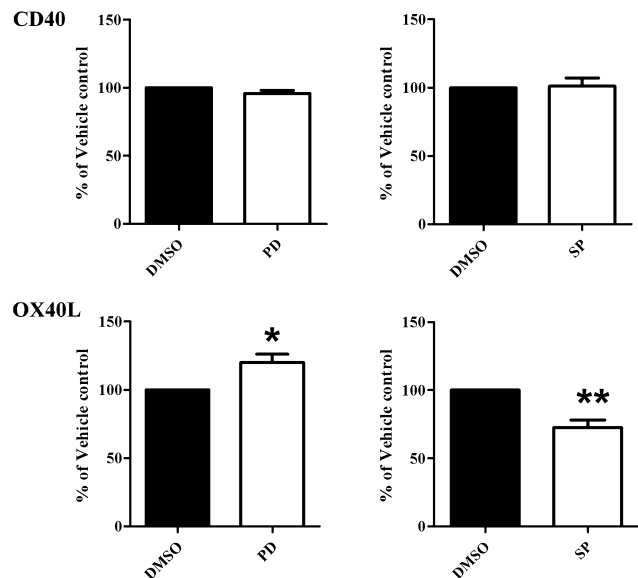
A Asthmatic**B Nonasthmatic**

Figure 4. Cell-surface expression of CD40 and OX40L following inhibition of mitogen-activated protein kinase extracellular regulated kinase or c-Jun N-terminal kinase. Cell-surface expression of CD40 and OX40L on airway smooth muscle from asthmatic (A) or nonasthmatic (B) donors, detected using enzyme-linked immunosorbent assay following 48 h stimulation with tumour necrosis factor- α and interferon- γ in the presence of PD98059 (PD), SP600125 (SP) or dimethyl sulfoxide (DMSO; Vehicle control). Columns represent percent of vehicle control (mean + SEM), $n = 6$ for asthmatic and nonasthmatic cells, * $P < 0.05$, ** $P < 0.005$ compared to matched vehicle control.

nonasthmatic cells. Only cytokine-induced CD40 expression was dependent on ERK signalling while the other expression profiles were not changed (Fig. 4A). However,

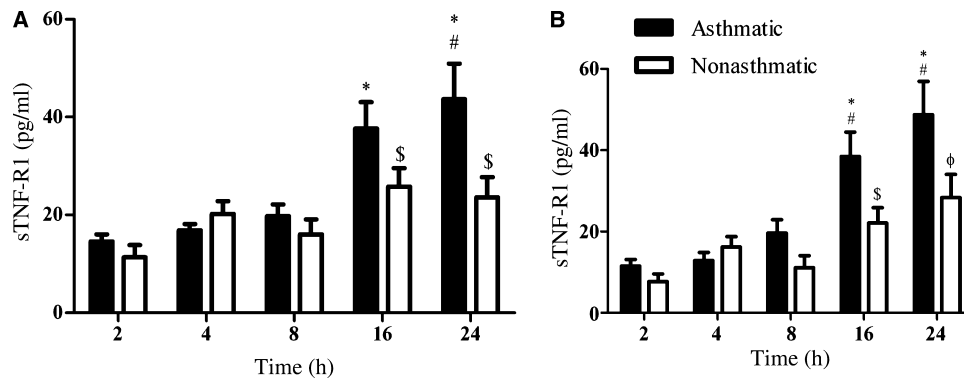


Figure 5. Detection of soluble tumour necrosis factor (sTNF)-R1 in supernatants from airway smooth muscle following stimulation with interferon (IFN)- γ alone (A) and in combination with TNF- α (B). Columns represent mean levels + SEM in pg/ml. $n = 5$ for asthmatic (AS) and nonasthmatic (NA). \$ $P < 0.05$ compared to 2 h, * $P < 0.01$ compared to 2, 4 and 8 h, $\phi P < 0.05$ AS compared to NA.

the inhibition of MAPKs in the presence of the combined cytokines in the nonasthmatic cells reflected the signalling pattern seen with the individual cytokines. CD40 expression was not changed but OX40L expression was sensitive to both ERK (which increased expression) and JNK (which decreased expression) signalling (Fig. 4B). The inhibitors of phosphoinositide 3-kinase (PI3K) did not have any effect on the cell-surface expression of CD40 and OX40L under any of the conditions examined in either cell type (data not shown).

IFN- γ alone and in combination with TNF- α increased TNF-R1 shedding from ASM

Basal TNF-R1 and TNF-R2 expression remained constant over time in both asthmatic and nonasthmatic ASM cells. When the cells were stimulated by the addition of TNF- α and IFN- γ alone or in combination, there was no significant difference in cell-surface TNF-R1 or TNF-R2 expression in either cell type (data not shown).

The concentration of soluble(s) TNF-R1 was significantly increased after 16 and 24 h stimulation with IFN- γ alone and in combination with TNF- α (Fig. 5), whereas soluble TNF (sTNF)-R2 was not detectable (data not shown). TNF- α did not induce an increase in shed sTNF-R1 (data not shown). Stimulation with TNF- α and IFN- γ in combination caused more shedding than either cytokine alone, although this increase was additive rather than synergistic. The concentration of sTNF-R1 was greater in supernatants from stimulated ASM from asthmatic donors compared with nonasthmatic (Fig. 5).

Discussion

In this study, we have demonstrated, for the first time, that stimulation of asthmatic and nonasthmatic ASM with TNF- α and IFN- γ in combination led to a decrease in cell-surface OX40L expression whilst simultaneously

causing a synergistic increase in CD40 expression. TNF- α and IFN- γ in combination induced CD40 via NF- κ B and STAT-1/3 in both cell types and induced OX40L via NF- κ B and JNK in nonasthmatic cells. The reduced OX40L expression with TNF- α and IFN- γ involved ERK activation in nonasthmatic but not asthmatic cells.

The increase in CD40 expression concomitantly with a decrease in OX40L is an important finding as decreasing OX40L expression may reduce the opportunity for ligation of OX40 on CD4+ T cells or other local inflammatory cells. Signalling through OX40 recruits TNF receptor-associated factor-2 and signals for T-cell survival in a survivin-dependent manner (18). This maintains the activation of CD4+ T cells, which have a key role in airway inflammation in asthma [as reviewed in (19)]. Decreasing OX40L expression on ASM may also attenuate OX40/OX40L-mediated protein kinase C- β 2 upregulation and IL-6 release from ASM (17).

We were not able to elucidate the mechanism underlying the reduction in OX40L cell-surface expression. However, we have demonstrated that the decreased OX40L expression was not because of increased shedding of OX40L. This finding is important as soluble OX40L may potentially maintain the activation of inflammatory cells by binding OX40 on CD4+ T cells or other inflammatory cells not in the immediate vicinity of the ASM (7). Whether the combination of TNF- α and IFN- γ can decrease cell-surface OX40L on other cell types is yet to be investigated.

We report for the first time a greater increase in CD40 expression on asthmatic ASM, compared with nonasthmatic ASM, in response to the combination of TNF- α and IFN- γ . This result suggests that asthmatic ASM is more responsive to cytokine stimulation, adding to the increasing body of data, which suggest that the asthmatic ASM cell is somehow primed to respond to a variety of stimuli (3, 17, 20–23) in a pro-inflammatory manner. The induction of CD40 cell-surface expression by TNF- α and IFN- γ had previously been reported in nonasthmatic ASM (3, 4) and we have confirmed and extended these findings.

The mean age of the asthmatics and nonasthmatics from which ASM cells were derived was different in this study. However, a study in fibroblasts isolated from 150 participants showed that the age of the donor did not influence the behaviour of the cells in culture (24). As our ASM cells are also mesenchymal cells, it is reasonable to assume that the age of our donors would not alter the responsiveness of the cells. In addition, we have found that there was no difference to the statistical outcome when the proliferation pattern of asthmatic and nonasthmatic ASM cells *in vitro* was compared in a complete population, in which the mean age differed, or a subpopulation in which the mean age was not significantly different (16, 25).

CD40 and OX40L expression is likely to be controlled by independent signalling mechanisms as they have different responses to TNF- α and IFN- γ stimulation. This study provides evidence of the direct involvement of NF- κ B in the transcription of OX40L, further adding to those of previous studies demonstrating the key role of NF- κ B-dependent gene transcription in asthmatic ASM inflammation (12). The inhibition of TNF- α -induced OX40L expression by the NF- κ B inhibitor BMS-345541 demonstrates the functionality of the NF- κ B consensus sites on the OX40L promoter (26). In the presence of TNF- α and IFN- γ , JNK and NF- κ B inhibition further reduced the expression of OX40L in nonasthmatic ASM cells. This was consistent with the OX40L expression we observed in the presence of TNF- α alone, which was inhibited by BMS-345541 (NF- κ B). Therefore, the two cytokines may signal through different pathways that both contribute to the final outcome of cell-surface expression of OX40L in the nonasthmatic cells.

The pathways identified above were not involved in the decreased OX40L expression that we observed in the presence of the combined cytokines. In nonasthmatics only, inhibition of ERK resulted in a reversal of this decrease in OX40L expression. Our findings suggest that ERK is the pathway mediating this effect. Interestingly, inhibition of ERK in the presence of TNF- α alone led to an increase in OX40L expression in both cell types suggesting that in the cytokine combination, IFN- γ has the dominant role in downregulating OX40L expression. In contrast, it is likely that NF- κ B and STAT-1/3 mediate the increase in CD40 cell-surface expression in ASM cells whether or not they are derived from asthmatics or nonasthmatics.

None of the inhibitors examined reversed the reduced expression of OX40L seen in the asthmatic ASM cells in the presence of the combined cytokines. This was despite inhibition of the NF- κ B pathway being able to reduce the expression seen in the presence of the TNF α alone. These observations may indicate that the asthmatic ASM cells have intrinsic changes that alter their responses to any given stimulus. Thus, the removal of one of the signalling pathways may be insufficient to decrease the cell-surface expression when a compensatory stimulus is present.

It was unexpected that inhibition of janus kinase (JAK)/STAT signalling had no effect on OX40L expression in

asthmatic cells but decreased expression following TNF- α and combined cytokine stimulation in nonasthmatic ASM cells. Inhibitory STAT-NF- κ B interactions (12) were prime candidates for the attenuation by IFN- γ of the OX40L expression induced by TNF- α . Other unknown pathways may be involved, although our results may reflect promiscuity of the inhibitor used. Alternatively, the lack of inhibition in the asthmatic ASM cells may reflect the generalized altered responsiveness of these cells.

We have provided evidence that OX40L and CD40 are regulated by distinct signalling pathways in ASM cells. Whilst the cascade of CD40 and OX40L expression is usually sequential, our findings indicate clear differences in the signalling pathways involved in the induction of these molecules.

A novel finding in this study was that sTNF-R1 is shed from human ASM following cytokine stimulation. This shedding was enhanced in ASM from asthmatic donors and was also increased by stimulation with IFN- γ alone, or in combination with TNF- α , but not by TNF- α alone. The increased shedding from asthmatic ASM further demonstrates the heightened responsiveness of asthmatic ASM to cytokine stimulation. This result is consistent with the finding that serum levels of sTNF-R1 are higher in asthmatics than in nonasthmatics (27) and shedding of TNF receptors may serve as a homeostatic mechanism in locally regulating inflammatory TNF- α activity in the airways (28).

This study has examined the regulation of expression of the cell-surface molecules CD40 and OX40L on ASM cells. The cytokine responsiveness of ASM from asthmatic individuals was enhanced compared with that of nonasthmatics. Greater expression of CD40 in the presence of TNF- α and IFN- γ may lead to an enhanced interaction between the ASM cells and airway inflammatory cells, possibly explaining the recent observation of a direct interaction between ASM cells, mast cells and lymphocytes in asthmatic biopsies (29). These findings further support the role of the ASM as an immunomodulatory cell, both directly through interactions with inflammatory cells but also indirectly through cytokine release.

Our demonstration that OX40L expression was decreased in the presence of the two cytokines may represent a natural control system for the regulation of the inflammatory reaction. It is possible that OX40L expression on other inflammatory cells in the asthmatic airway is able to compensate for this decreased expression but the expression on the surface of the ASM cells would play an important role in regulating the events in the immediate vicinity of the ASM. We have previously reported that OX40:OX40L engagement induces IL6 release indicating a potential direct effect, on airway inflammation, of the reduction in cell-surface OX40L on the ASM. This is the first report to separate the signalling events that lead to the expression of CD40 and OX40L and may indicate unique roles for these two members of the TNF superfamily in the asthmatic airway.

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