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CD40-Mediated Signal Transduction in Human Airway Smooth Muscle

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CD40 is a member of the TNF receptor family that was initially described on the surface of B cells. Recently, CD40 has also been described on mesenchymal cells, such as endothelial cells and fibroblasts, where engagement by its ligand CD40 ligand can lead to up-regulation of costimulatory and cell adhesion molecules, as well as secretion of proinflammatory cytokines. Since airway inflammation potentially involves cell-cell interactions of T cells and eosinophils (which express CD40 ligand) with airway smooth muscle (ASM) cells, we postulated that ASM may express CD40 and that engagement of ASM CD40 may modulate smooth muscle cell function. We demonstrate that CD40 is expressed on cultured human ASM and that expression can be increased by treatment with TNF-α or IFN-γ. Cross-linking of CD40 on ASM resulted in enhanced IL-6 secretion and an increase in intracellular calcium concentrations, which were dependent on calcium influx. We show that CD40-mediated signaling events include protein tyrosine phosphorylation and activation of NF-κB. Pretreatment of ASM with the tyrosine kinase inhibitors genistein or herbimycin inhibited the rapid mobilization of calcium induced via CD40, suggesting that calcium mobilization was coupled to activation of protein tyrosine kinases. In addition, inhibition of calcium influx inhibited both CD40-mediated NF-κB activation and enhancement of IL-6 secretion. These results delineate a potentially important CD40-mediated signal-transduction pathway in ASM, involving protein tyrosine kinase-dependent calcium mobilization, NF-κB activation, and IL-6 production. Together, these results suggest a mechanism whereby T cell/smooth muscle cell interactions may potentiate airway inflammation. The Journal of Immunology, 1998, 161: 3120–3127.

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3 Abbreviations used in this paper: CD40L, CD40 ligand; ASM, airway smooth muscle; CD40LT, CD40 ligand trimer; PTK, protein tyrosine kinase.

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One interesting feature of CD40-mediated signaling is that it is contact dependent, requiring adhesion of the CD40L-positive activated T cell to the surface of the CD40-positive target cells. Because engagement of CD40 on endothelial cells and fibroblasts resulted in a number of changes that are relevant to inflammation, we hypothesized that ASM might also express CD40 and that this molecule might serve as an important signal-transduction molecule with regard to activated lymphocyte-ASM interactions. Accordingly, we analyzed the expression of CD40 on human airway smooth muscle cells and its regulation by cytokines, examined the functional consequences of receptor engagement by CD40L or anti-CD40 mAb by measuring ASM cytosolic calcium and cytokine secretion, and identified several early signaling events induced by ligation of CD40 on ASM.

Materials and Methods

Reagents

SC3 (anti-human CD40) was purchased from PharMingen (San Diego, CA); trimerized human CD40L (CD40LT) was produced as previously described (34); 4G10 (anti-phosphotyrosine) was purchased from Upstate Biotechnology (Lake Placid, NY). TNF-α was purchased from Boehringer Mannheim (Indianapolis, IN); IFN-γ was purchased from PharMingen.

Cell culture

Human airway smooth muscle was obtained from the trachealis muscle of lung transplant donors in accordance with the policies of the Committee on Studies Involving Human Beings at University of Pennsylvania (Philadelphia, PA). ASM cells were purified as previously described (35) and cultured in Ham’s F12 supplemented with penicillin, streptomycin, glutamine, HEPES, and 10% heat-inactivated FBS. The results reported are representative of those obtained with a minimum of three different smooth muscle cell lines. Characterization of the cultured smooth muscle, including staining for smooth muscle-specific actin and responsiveness to contractile agonists, has been previously described (35).

Flow cytometry

Confluent ASM in 12-well plates were incubated with media alone or with cytokines for 24 to 72 h. The monolayers were washed, and then single cell suspensions were prepared using 5 mM EDTA. Cells were stained with Abs specific for human CD40, followed by FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), and analyzed using a FACScan (Becton Dickinson, San Jose, CA) and CellQuest software.

IL-6 determination

Confluent ASM were treated with either TNF-α or IFN-γ. After 72 h, the monolayers were washed and supplied with fresh media, and soluble CD40LT (10 μg/ml) was then added to the indicated wells. The plates were incubated for an additional 24 h, at which time the supernatants were collected and frozen at −80°C until cytokine assays were performed. Human IL-6 was quantitated using a commercial ELISA (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions. Statistical differences in IL-6 production were calculated using a nonparametric matched-pair analysis. Values of p < 0.05 were considered statistically significant.

Immunoblotting

Confluent ASM cells were rendered quiescent by culturing in serum-free media for 24 h, then stimulated with 2 μg/ml anti-CD40 for the times indicated. Cells were lysed in buffer containing 50 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.1% deoxycholate, 100 μg/ml leupeptin, 1 mM PMSF, 10 μg/ml aprotinin, 5 mM EDTA, 50 mM NaF, 40 mM β-glycerophosphate, and 1 mM Na3VO4 for 10 min at 4°C. Postnuclear extracts were obtained by centrifugation of lysates at 14,000 x g for 10 min. Equivalent amounts of protein, as determined by the bicinchoninic acid (BCA) assay, was added to each lane. The resolved proteins were transferred onto nitrocellulose and blotted with Abs specific for human CD40, followed by rabbit anti-mouse IgG (Jackson ImmunoResearch). Bound Ab was detected using 125I-labeled protein A (DuPont NEN, Boston, MA) and visualized by autoradiography.

Cytosolic calcium measurements

ASM cells were plated at low density onto 15-mm coverslips 3 to 5 days before the experiments were performed. All experiments were performed using subconfluent cells between third and fifth passage. Cells were loaded with 2.5 mM fura-2/AM (in HEPES buffer containing 137.5 mM NaCl, 1.25 mM CaCl2, 1.25 mM MgCl2, 0.4 mM NaH2PO4, 6 mM KCl, 3.6 mM glucose, supplemented with 1 mg/ml BSA) for 30 min at 37°C, and washed in HEPES-buffered saline. Cells were then placed in a thermostatically controlled cell chamber on a Nikon inverted microscope (Diaphot). Cells were imaged using a ×40 (oil) fluorescence objective lens. Excitation energy was switched between 340 and 380 nm wavelength using a 75 W xenon lamp source and a fura-2 dichroic mirror (Chroma Technology, Brattleboro, VT). The emitted fluorescence (510 nm) was diverted to an image-intensified CCD camera (Hamamatsu, Hamamatsu City, Japan) attached to the video analogue-to-port digital conversion board (Maatrox). Image analysis of individual cells was accomplished using the Image-1 AT/Fluor program (Universal Imaging, West Chester, PA). The 340/380 ratio was converted to an estimate of cytosolic free calcium using previously described methods (36, 37). Calibration measurements were made by treatment of cells with ionomycin (10 nM) in the presence of 12 mM calcium to measure Rmax, or by adding a stoichiometric excess of EGTA to achieve Rmin. Values used for the calibration equation were Rmax = 0.3 and Rmin = 0.6, Kd = 224 and r380max/r380min = 5. Abs or soluble ligand were added directly to the bath. Bradykinin (1 nM; Sigma, St. Louis, MO) was added to compare the magnitude of bradykinin-induced calcium transients with those evoked by CD40L. NiCl2 (4 mM; Fisher Scientific, Springfield, NJ) or MnCl2 (200 μM; Fisher) was added to distinguish release of calcium from intracellular stores from calcium influx. Where indicated, cells were pretreated with either genistein (5 μM; Life Technologies, Grand Island, NY) for 1 h or herbimycin (10 μM; Sigma) for 4 h at 37°C before stimulation with anti-CD40.

Nuclear extracts and mobility shift assay

Nuclear extracts were prepared according to the method of Anderson and Faull (38). Confluent ASM were treated with IFN-γ (500 U/ml) for 72 h, then stimulated with CD40LT (10 μg/ml) for the indicated times. Cells were harvested by scraping into cold PBS. Nuclei were isolated by treatment with hypotonic lysis buffer A containing 10 mM HEPES, 10 mM KCI, 1.5 mM MgCl2, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, and 0.5% Nonidet P-40. The nuclear pellet was resuspended in buffer B (420 mM NaCl, 20 mM HEPES, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, and 10 μg/ml leupeptin) for 20 min on ice and clarified by centrifugation at 13,000 x g. The resulting supemantants contained 1 to 2 mg/ml protein by the bicinchoninic acid (BCA) assay. Nuclear extracts were stored at −80°C.

A double-stranded oligonucleotide probe containing the NF-κB consensus sequence (Promega, Madison, WI) was end labeled with [γ-32P]ATP using T4 kinase (Life Technologies). A total of 8 μg of nuclear extract was incubated with 1 μg poly(dI-dC) and radiolabeled probe for 30 min at room temperature. DNA-protein complexes were resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel at 200 V. The gels were then dried and exposed to x-ray film for autoradiography. In supershift experiments, nuclear extracts were preincubated with polyclonal goat Abs specific for p50, p65, or c-Rel (Santa Cruz Biotechnology, Santa Cruz, CA) for 15 min on ice before the addition of 32P-labeled probe.

Results

CD40 is expressed on cultured ASM and up-regulated by TNF-α and IFN-γ

To determine whether the interaction between activated T cells and airway smooth muscle could potentially involve CD40-CD40L binding, we examined whether human ASM expressed CD40. Unstimulated, cultured human ASM cells were stained with anti-CD40 Abs and analyzed by flow cytometry. Low constitutive expression of CD40 was detected compared with control ASM reacted with an isotype-matched control mAb (Fig. 1A). Treatment of ASM with either TNF-α or IFN-γ induced a two- to fourfold increase in CD40 expression (Fig. 1A), and the effect of TNF-α and IFN-γ together was at least additive, inducing a sevenfold increase in CD40 expression (data not shown). In contrast, IL-4

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had no effect or resulted in a slight decrease in CD40 expression compared with baseline expression (Fig. 1A), but had no effect on TNF-α or IFN-γ-induced up-regulation of CD40 (data not shown). Maximal expression of CD40 occurred on day 2 following treatment with TNF-α, and on day 3 following IFN-γ stimulation (Fig. 1B, top) and was dose dependent (Fig. 1B, bottom).

Ligation of CD40 enhances IL-6 production by ASM

We examined whether CD40 triggered cytokine production by ASM by measuring IL-6 in response to stimulation with soluble CD40LT. Unstimulated ASM produced low basal levels of IL-6, while treatment with CD40LT increased IL-6 secretion by approximately 38 ± 9% (Fig. 2). Stimulation with TNF-α increased IL-6 secretion by approximately 2.7-fold over baseline values; CD40LT increased TNF-α-induced IL-6 production by 26 ± 10%. IFN-γ alone had minimal effects on IL-6 production by ASM, but acted synergistically with CD40LT, increasing IFN-γ-induced IL-6 secretion by approximately 73 ± 12% compared with IFN-γ alone (Fig. 2). These data suggest that CD40-CD40L interactions can transduce a costimulatory activation signal leading to the augmented release of inflammatory mediators by ASM.

CD40 mediates an increase in intracellular calcium in cultured ASM

Although the results have been contradictory, there is evidence to suggest that CD40 can mediate a rise in intracellular calcium concentrations in B cells (17). We therefore measured CD40-induced changes in cytosolic calcium in human ASM. After the addition of CD40LT to fura-2-loaded ASM cells, there was a gradual and protracted increase in cytosolic calcium (Fig. 3A). The average increase in cytosolic calcium evoked by CD40LT was 141 ± 15 nM (n = 15–25 cells). This increase was abrogated by pretreatment with blocking anti-CD40LT Abs (Fig. 3B). Similar results were obtained when cells were stimulated with anti-CD40 (Fig. 3C), in which the average increase in cytosolic calcium evoked was 103 ± 17 nM (n = 33 cells). The calcium response to CD40LT or anti-CD40 stimulation differed from that seen in response to smooth muscle cell contractile agonists such as bradykinin, which was rapid, with a maximum peak increase to 268 ± 38 nM (Fig. 3C). The specificity of the response is evidenced by the lack of effect of an isotype-matched binding anti-ICAM-1 control Ab (Fig. 3D). The addition of NiCl₂ before Ab cross-linking abolished the CD40-induced signal (Fig. 4), suggesting that the increase in intracellular calcium was dependent on an influx through transmembrane calcium channels. Similar effects were seen using another divalent cation MnCl₂ (data not shown). This differs from the response to bradykinin (Fig. 4), which was shown previously to be due to release of intracellular calcium stores (39, 40). Finally, pretreatment of the ASM with the tyrosine kinase

![Figure 1](http://www.jimmunol.org/Downloadedfrom)  
**FIGURE 1.** Cytokine induction of CD40 on ASM. A, ASM were incubated in media alone (thin line), or stimulated with TNF-α (1000 U/ml, bold line), IFN-γ (1000 U/ml, dotted line), or IL-4 (20 ng/ml, broken line). After 48 h, cells were stained with Abs to CD40 and analyzed by flow cytometry. B (top), ASM were stimulated with TNF-α (1000 U/ml) for 1 to 3 days and stained with anti-CD40, as described. Bottom, ASM were stimulated with increasing doses of TNF-α for 48 h, then stained with anti-CD40.

![Figure 2](http://www.jimmunol.org/Downloadedfrom)  
**FIGURE 2.** CD40 enhances IL-6 production by ASM. ASM were incubated in media alone or stimulated with TNF-α (250 U/ml) or IFN-γ (500 U/ml). After 72 h, cells were washed and replaced with fresh media with (black bars) or without (gray bars) the addition of CD40LT (10 μg/ml). Supernatants were collected after 24 h, and IL-6 was determined by ELISA. The data are expressed as mean IL-6 concentrations from duplicate wells and are representative of five experiments. For those conditions in which the SE bars are not apparent, the errors were less than 3%. *p < 0.01 compared with media alone; **p < 0.05 compared with TNF-α alone; ***p < 0.005 compared with IFN-γ alone.
inhibitors genistein (Fig. 5) or herbimycin (data not shown) completely inhibited the early CD40-induced calcium response, suggesting that calcium mobilization induced by engagement of CD40 is coupled to activation of PTK. Genistein had no effect, however, on the bradykinin-evoked calcium response (Fig. 5).

FIGURE 3. CD40-induced cytosolic calcium response of human ASM cells. A, The kinetics of cytosolic calcium in response to CD40LT (20 μg/ml). Each cell, represented by a single tracing, demonstrates a gradual and sustained rise in intracellular calcium. A subsequent addition of bradykinin (1 mM) evokes a rapid and large calcium transient (n = 2). B, The calcium response to CD40LT is abrogated by pretreatment with anti-CD40LT (10 μg/ml). C, The time course and magnitude of cytosolic calcium in response to anti-CD40 (10 μg/ml) are similar to that seen following stimulation with CD40LT (n = 4). D, In response to an isotype-matched binding (ICAM-1) control Ab, there is no change in intracellular free calcium (n = 4).

Ligation of CD40 induces protein tyrosine phosphorylation in cultured ASM

We next attempted to identify some of the receptor-proximal signaling events that are activated by CD40 cross-linking. It was demonstrated previously that engagement of CD40 induced tyrosine phosphorylation in transformed or activated, but not resting, B cells (9, 41, 42). In addition, the effect of genistein on the calcium

FIGURE 4. Extracellular calcium is required for CD40-induced calcium transients. The addition of NiCl2 (4 mM) alone does not cause a change in intracellular free calcium. The subsequent addition of anti-CD40 (10 μg/ml) fails to effect a calcium response. The addition of bradykinin (1 mM) evokes a large calcium transient (n = 2).

FIGURE 5. Inhibition of PTK activation blocks CD40-, but not bradykinin-induced increases in cytosolic calcium. ASM was pretreated with genistein (5 μM) for 1 h, and then treated with anti-CD40 (10 μg/ml), followed by bradykinin (1 mM) (n = 3).
response described above indicated that CD40 may be coupled to PTK signal-transduction pathways in ASM. To directly assess whether CD40 mediated activation of PTK, cells were made quiescent by culturing in serum-free media for 24 h, then stimulated with anti-CD40 mAb for the indicated times. Protein tyrosine phosphorylation of cellular proteins was detected by immunoblotting with anti-phosphotyrosine Abs. Tyrosine-phosphorylated species with Mr of 40 to 50 kDa were detected at 10 to 15 min following stimulation (Fig. 6). Phosphorylation of these proteins was not seen when smooth muscle cells were incubated with an isotype-matched control Ab (data not shown). Similar to the CD40-mediated increase in intracellular calcium, CD40-induced tyrosine phosphorylation did not require pretreatment of the cells with cytokine.

**CD40 induces NF-κB activation in ASM**

The transcription factor NF-κB is important for maximal transcription of many cellular products involved in inflammatory responses, such as TNF-α, IL-1β, IL-6, and IL-8. To test whether CD40 could induce activation of NF-κB in ASM, cells were pretreated with IFN-γ for 72 h, then stimulated with soluble CD40LT. Two NF-κB/DNA-binding complexes were observed (Fig. 7A). An inducible NF-κB activity was seen as early as 15 min following exposure to CD40LT, which peaked at approximately 30 min, then decreased. In comparison, TNF-α induced a comigrating NF-κB complex more rapidly, and the presence of this complex was sustained over the same time course (data not shown). A second complex was constitutively present and unaffected by treatment with either CD40LT or TNF-α. Formation of the inducible complex was inhibited by excess cold oligonucleotide (Fig. 7B). Cells that were stimulated with CD40L, in the absence of IFN-γ pretreatment, exhibited the constitutive, but not the inducible NF-κB complex (data not shown). To determine the subunit composition of these complexes, nuclear extracts were incubated with specific subunit Abs. Anti-p65/RelA abrogated formation of the inducible complex, but had no effect on the constitutive complex (Fig. 7B). Anti-p50 had a partial inhibitory effect on formation of the inducible NF-κB complex (Fig. 7B). No supershift or inhibition of either complex was seen with anti-c-Rel, suggesting that these effects were specific. Thus, it appears that CD40 activates the formation of an NF-κB heterodimer, consisting of at least p50 and p65/RelA. In addition, this effect was dependent on priming of the cells by IFN-γ, which alone had no effect.

**Extracellular calcium is necessary for CD40-mediated NF-κB activation and IL-6 secretion**

Engagement of the CD40 receptor results in mobilization of extracellular calcium in ASM. We examined whether extracellular calcium was necessary for the proinflammatory effects of CD40, including activation of NF-κB and IL-6. ASM cells were treated with IFN-γ for 72 h, then placed in serum-free media containing 2 mM calcium. The cells were treated for 15 min with 4 mM NiCl₂.
before being stimulated with CD40LT. Pretreatment with NiCl₂ completely abrogated the ability of CD40LT to activate the inducible NF-κB complex, but had no effect on the constitutive complex (Fig. 8A). NiCl₂ alone had no demonstrable effect on NF-κB activation. In addition, pretreatment with NiCl₂ inhibited CD40-induced increases in IL-6 secretion by ASM, but had minimal effects on basal secretion (Fig. 8B).

Discussion

Airway smooth muscle cells directly modulate the bronchial hyperresponsiveness that characterizes diseases of airway inflammation such as asthma. The mechanisms by which ASM cell function is altered with inflammation remain unknown. The most well-recognized and well-studied mechanisms involve direct stimulation of ASM by contractile agonists released during inflammation, such as histamine or leukotrienes. It also has been recognized that inflammatory cytokines such as TNF-α, that do not by themselves induce ASM contraction, can prime airway smooth muscle cells to respond at lower doses to directly acting bronchoconstrictors (31, 32). A third, but much less well-studied, mechanism involves contact-dependent cell-to-cell interactions of ASM with activated inflammatory cells within the airway. In support of this contact-dependent mechanism, we have reported recently the up-regulation of the cell adhesion molecules ICAM-1 and VCAM-1 on TNF-α-stimulated ASM cells (33). These, along with CD44, support the adhesion of activated T cells to ASM (33). Importantly, adhesion of these activated lymphocytes induces DNA synthesis in ASM cells (33). In this study, we extended our prior observations by demonstrating that an additional ligand pair (CD40-CD40L) can impart cellular signals that modulate smooth muscle cell function. We have delineated a potentially important signal-transduction pathway in ASM, involving PTK-dependent activation of calcium mobilization. This calcium response appears to be required for the further downstream activation of NF-κB and secretion of IL-6, a gene known to be regulated by NF-κB. These data provide further evidence for the role of CD40 in regulating the inflammatory response.

We initially tested the ability of CD40 engagement to affect ASM cytokine secretion focusing on the pleotropic inflammatory cytokine IL-6. CD40LT induced a significant enhancement of IL-6 secretion by both unstimulated and TNF-α- and IFN-γ-treated ASM. Similar findings have been noted in both transformed (25) as well as primary fibroblasts (24, 29), keratinocytes (27), and vascular smooth muscle cells (28). IL-6 has a number of proinflammatory effects, including its ability to stimulate T cell proliferation (43) and up-regulate IL-4-dependent IgE production (44). In addition, IL-6 has been shown to increase the phosphorylation of CD40 in B cells (45), supporting the idea of a CD40/IL-6 feedback loop. Therefore, the ability of CD40 to enhance IL-6 production by ASM has important implications for airway inflammation.

We also studied the effect of CD40 engagement on intracellular calcium levels. This response has physiologic and pathophysiologic significance since intracellular calcium is an essential second messenger regulating smooth muscle cell contractility (reviewed in Ref. 46). Previous data regarding CD40-induced calcium mobilization in B cells have been variable and have not been investigated in other cell types of which we are aware. Klaus et al. (17) directly measured intracellular calcium in murine B cells in response to anti-CD40 and observed a slow, moderate increase. In contrast, other investigators found no increase in intracellular calcium following engagement of CD40 on either human (47) or murine (48) B cells. This discrepancy may be due to both species-specific and activation state-dependent differences in CD40 signaling.

We found that engagement of CD40 on ASM consistently evoked an increase in intracellular calcium. The calcium mobilization induced by CD40 was slow and protracted, contrasting with the rapid and transient response induced by agonists that release inositol-3-phosphate-dependent calcium stores (49). These data suggest that CD40-mediated calcium mobilization involves a pathway distinct from the classical phospholipase C pathways, which are activated by agonists that bind to seven-transmembrane-spanning G protein-coupled receptors (reviewed in Ref. 50). The CD40-induced calcium mobilization we observed in ASM was dependent on an influx of extracellular calcium, inasmuch as the use of competitive inhibitors of calcium influx, such as NiCl₂ or MnCl₂, abrogated the response. Furthermore, we found that extracellular calcium was also required for the CD40-induced activation of NF-κB and IL-6 secretion in ASM cells. Others have shown that NF-κB is a calcium-sensitive transcriptional regulator. For example, Kanno and Siebenlist (51) demonstrated that TCR-mediated activation of NF-κB was abrogated in the presence of a calcium channel blocker. Similarly, studies from Dolmetsch et al. (52) demonstrated that both the amplitude and duration of calcium signals could modulate gene transcription. Large transient rises in
intracellular calcium were found to activate both NF-κB and INK in B lymphocytes.

The underlying mechanisms whereby CD40 activates calcium influx remain to be determined. Consistent with our data in ASM, Wijetunge et al. (53, 54) reported that voltage-operated calcium channels could be modulated by endogenous tyrosine kinases, including pp60c-src, based on the fact that the calcium channel currents were inhibited completely by tyrosine kinase inhibitors, including genistein. We observed no change in the tyrosine phosphorylation of src (unpublished observation). However, the CD40-induced calcium transient in ASM was abolished completely by genistein and herbimycin, while these inhibitors had no effect on agonist-induced calcium mobilization. Together, these results suggest that the CD40-mediated calcium response in ASM may involve the activation of voltage-operated calcium channels, which were shown to be functionally present in ASM cells (40, 55), and that this may be coupled to PTK pathways.

Data derived from transformed B cell lines or resting or activated normal B cells suggest that engagement of CD40 leads to both PTK and serine/threonine kinase activation (7, 8, 47, 48, 56). Activated normal B cells suggest that engagement of CD40 leads to calcium influx remain to be determined. Consistent with our data in ASM, Wijetunge et al. (53, 54) reported that voltage-operated calcium channels could be modulated by endogenous tyrosine kinases, including pp60c-src, based on the fact that the calcium channel currents were inhibited completely by tyrosine kinase inhibitors, including genistein. We observed no change in the tyrosine phosphorylation of src (unpublished observation). However, the CD40-induced calcium transient in ASM was abolished completely by genistein and herbimycin, while these inhibitors had no effect on agonist-induced calcium mobilization. Together, these results suggest that the CD40-mediated calcium response in ASM may involve the activation of voltage-operated calcium channels, which were shown to be functionally present in ASM cells (40, 55), and that this may be coupled to PTK pathways.

GS and FL are known, however, about CD40 coupling to PTK signaling pathways in non-B cells. Gasperi et al. (27) described tyrosine phosphorylation of a single 50-kDa species in keratinocytes stimulated with anti-CD40 Abs. We now demonstrate that in resting human ASM, CD40 cross-linking leads to increases in protein tyrosine phosphorylation. This effect, as well as the CD40-mediated increase in calcium mobilization, did not require pretreatment of the smooth muscle cells with IFN-γ. On the other hand, cytokine pretreatment enhanced CD40-mediated IL-6 secretion and was required for CD40-mediated NF-κB activation. This suggests that cytokines may prime the smooth muscle cell for certain responses, possibly through the modulation of transcription factors, as has been described in macrophages (57, 58).

In summary, we have demonstrated for the first time that CD40 is expressed functionally on the surface of human airway smooth muscle, and delineated a CD40-mediated signal-transduction pathway involving PTK-dependent calcium mobilization and calcium-dependent activation of NF-κB and IL-6 secretion. Interactions between CD40-positive ASM and CD40L-positive cells such as CD4+ T lymphocytes or eosinophils should thus be considered a potentially important component of the inflammatory response of the airways. Future studies will focus on defining the role of these molecules in in vivo models of airway inflammation, in defining other physiologic important consequences of CD40 engagement, and in understanding the molecular events that regulate signaling by CD40.

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