FINAL REPORT

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INSTITUTION: The Wistar Institute

GRANT TITLE: Role of Cell Adhesion Molecules in T Cell-Myocyte Interactions in Airway Inflammation

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OBJECTIVE: The overall objective of our investigations is to determine if interactions between infiltrating T lymphocytes and airway smooth muscle cells contribute to the pathophysiology of asthma in atopic patients. The specific aims of this study were to define the mechanisms underlying the direct contact of T lymphocytes and myocytes and to determine the potential functional consequences of T lymphocyte-myocyte interactions.

APPROACH: Primary human airway smooth muscle cells [ASM] were isolated and cultured *in vitro*. Primary human peripheral blood and bronchoalveolar lavage-derived T cells were isolated and in some cases CD4-positive T cells were isolated. ASM were either directly co-cultured with T cells or ASM and T cells were cultured on opposites sides of a membrane in dual chamber cultures. A quantitative adhesion assay was used to assess direct contact between T cells and ASM in direct co-cultures. Blocking antibodies were used to determine the role of specific costimulatory and adhesion molecules in mediating the direct contact between T cells and myocytes. The effect of T cells and T cell derived cytokines on the expression of major histocompatibility class II molecules and costimulatory (eg. CD40 and B7) and adhesion molecules (eg. ICAM, VCAM and CD44) on ASM was analysed by fluorescence activated flow cytometry. Antibodies against cell adhesion molecules and the costimulatory molecule CD40 as well as a soluble form of the ligand for CD40, CD40L, were used to determine the capacity of these cell surface proteins to transduce signals in myocytes.

ACCOMPLISHMENTS:

We are interested in the potential role of leukocyte-smooth muscle cell interactions in the context of airway inflammation. Lymphocyte extravasation into areas of inflammation involves the sequential engagement of multiple cell adhesion molecules expressed on lymphocytes and endothelial cells. In addition, the expression of cell adhesion molecules and the elaboration of matrix by subendothelial/submucosal cells may contribute to the retention and stimulation of infiltrating cells in an inflammatory lesion. We demonstrated that mitogen-activated T cells adhered to ASM via integrins and CD44 and induced DNA synthesis in airway smooth muscle cells in a contact dependent manner. Furthermore, we demonstrated that crosslinking of the antigen receptor (TCR) was sufficient to induce T cell adhesion to ASM. Moreover, in studies with bronchoalveolar

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13. ABSTRACT *(Maximum 200 words)*

Critical progress has been made in the identification and characterization of cells and mediators involved in allergic inflammation. Accumulating evidence supports the importance of cell adhesion molecule expression as an initiating process in tissue inflammation. Despite progress made to date, much is still unknown about the exact mechanisms responsible for this inflammatory response. Scientists have been working to understand the selective cell recruitment operating in allergic disease with the hope of discovering therapeutic intervention strategies that will prevent the accumulation of unwanted cells in inflamed airways. Research has been directed at developing various approaches to generate specific antagonists. Some approaches under study interupt airway inflammation in its early stages during leukocyte-endothelial interactions. Other approaches inhibit cell recruitment at the endothelial wall. Many studies have been done, both in vivo and in vitro, and the advances that have been made suggest that these therapeutic interventions may be the keys to controlling and, possibly, curing asthma and allergic reactions.

14. SUBJECT TERMS

cell adhesion, allergic rhinitis, atopic allergic conditions, cell biology

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lavage-derived T cells isolated from atopic donors following local antigen challenge, we observed adhesion to ASM.

ASM are MHC class II-negative and express low basal levels of intercellular adhesion molecule-1 (ICAM-1). We demonstrated however that activated T cell-derived cytokines upregulated expression of HLA-DR and ICAM-1 on ASM. Furthermore, we demonstrated that exposure to the inflammatory cytokine TNFα augmented the sensitivity of ASM to a variety of contractile agonists. Thus, T cells activated *in vitro* and bronchoalveolar lavage-derived T cells induced the expression of HLA-DR and markedly upregulated ICAM-1 expression. The induction of HLA-DR was completely inhibited, and the induction of ICAM-1 partially inhibited by neutralizing antibody against interferon-γ. Despite expression of ICAM-1 and HLA-DR, ASM could not present alloantigen to $CD4^+$ T cells.

Taken together, these findings suggest that the interaction of activated T cells with parenchymal cells of the lung such as airway smooth muscle affects the phenotype of myocytes and thus may have significant implications for inflammatory diseases such as asthma or transplant rejection.

To further pursue the potential role of direct contact between infiltrating lymphocytes and parenchymal cells in modulating the inflammatory response we investigated the expression of costimulatory molecules on ASM. We did not detect expression of B7 (which can provide a costimulatory signal to T cells through ligation of CD28) on the surface of unstimulated or cytokine activated ASM. However, we found that ASM express CD40.

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 of CD40 by its ligand, CD40L, can lead to upregulation 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 CD40L) with ASM, we postualted that ASM may express CD40 and that engagement of ASM CD40 may modulate smooth muscle cell function. We demonstrated that CD40 is expressed on cultured human ASM and that expression can be increased by treatment with tumor necrosis factor (TNF) or interferon-gamma (IFN-γ). Crosslinking CD40 on ASM resulted in enhanced secretion of interleukin-6 (IL-6) and an increase in intracellular calcium concentrations which was dependent on calcium influx. We showed that CD40-mediated signaling events include protein tyrosine phosphorylation and activation of the transcription factor 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 upstream 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 studies therefore delineated 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 or eosinophil interactions with smooth muscle cells may potentiate airway inflammation.

CONCLUSIONS:

These studies lead to several important conclusions. We established that in addition to the essential previously defined role of cell adhesion molecules in lymphocyte trafficking, integrins and CD44 can mediate the adhesion of infiltrating lymphocytes to parenchymal cells such as smooth muscle cells. Thus, in addition to the production of soluble inflammatory mediators, contact dependent cell-cell interactions may provide additional mechanisms by which infiltrating lymphocytes can affect parenchymal cell function. Importantly, we established that T cell adhesion induces DNA synthesis in ASM. We further demonstrated that engagement of CD40 on ASM by CD40L, expressed for example on activated T cells and eosinophils, can mediate signal transduction in myocytes leading to downstream responses such as calcium mobilization and the release of inflammatory mediators. The capacity of cell adhesion molecules to mediate signal transduction in airway smooth muscle and the impact of the amplification loops we described on the function of infiltrating lymphocytes will be the subject of future investigations.

SIGNIFICANCE:

The smooth muscle cell is one of the most important effector cells within the airway and is directly responsible for the increases in airway reactivity that characterize diseases of airway inflammation such as asthma. The mechanisms by which ASM are affected dufieng inflammation are multifactorial including the release of soluble bronchoconstrictors and cytokines. A third, less well studied, mechanism involves contact-dependent cell-to-cell interactions of ASM with activated inflammatory cells within the airway parenchyma. Our findings over the past several years have established that the role of cell adhesion molecules go beyond the trafficking of lymphocytes to sites of inflammation by mediating their adhesion to endothelial cells and transendothelial migration. We established that adhesion molecules also play and important role in mediating interactions between infiltrating lymphocytes and parenchymal cells. Furthermore, this work established that in addition to adhesion molecules, ASM can express costimulatory receptors such as CD40. Moreover, the expression of major histocompatibility class II molecules, adhesion receptors and CD40 are regulated by inflammatory mediators thus providing an amplification loop that could lead to enhanced interactions between infiltrating lymphocytes and ASM. Importantly, these studies established that T cell-myocyte interactions can mediate signal transduction and induce functional changes in the parenchymal cells that are associated with the pathophysiology of inflammation such as DNA synthesis, calcium mobilization which is required for muscle contraction and enhanced release of inflammatory mediators such as interleukin-6. In addition, such cell-cell interactions are likely to in turn regulate the function of the immune cells. Definition of immune amplification loops such as those described in this study provide potential targets for therapeutic interventions that may impact on the acute symptoms associated with diseases involving airway inflammation such as asthma and bronchiolitis obliterans. Furthermore, interruption of such amplification loops could impact on the tissue remodeling often associated with chronic inflammation.

In summary these studies provide new insight into the role of cell adhesion molecules in

airway inflammation and implicate ASM as an immunomodulatory component of airways.

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Review

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Antigen Receptor-stimulated Peripheral Blood and Bronchoalveolar Lavage-derived T Cells Induce MHC Class II and ICAM-1 Expression on Human Airway Smooth Muscle

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The current model of lymphocyte extravasation into areas of inflammation involves the sequential engagement of multiple cell adhesion molecules (CAMs) expressed on lymphocytes and endothelial cells. In addition, the expression of CAMs and the elaboration of matrix by subendothelial/submucosal cells may contribute to the retention and stimulation of infiltrating cells in an inflammatory lesion. We previously demonstrated that mitogen-activated T cells adhered to airway smooth muscle (ASM) in an integrin-dependent fashion. ASM are MHC class II-negative and expressed low basal levels of intercellular adhesion molecule-1 (ICAM-1). In this study, we demonstrate that anti-CD3-stimulated peripheral blood T cells also adhere to ASM and markedly upregulate ICAM-1 expression and induce the expression of MHC class II on ASM. The induction of HLA-DR was completely inhibited, and the induction of ICAM-1 partially inhibited, by neutralizing antibody against interferon-γ. Furthermore, in studies with bronchoalveolar lavage-derived T cells isolated from atopic donors following local antigen challenge, we observed adhesion to ASM and upregulation of ASM expression of ICAM-1 and HLA-DR similar to that seen with *in vitro*-activated T cells. Finally, we found that despite expression of ICAM-1 and HLA-DR, ASM could not present alloantigen to $CD4^+$ T cells. These findings suggest that the interaction of activated T cells with parenchymal cells of the lung such as airway smooth muscle affects the phenotype of myocytes and thus may have significant implications for inflammatory diseases such as asthma or transplant rejection. **Lazaar, A. L., H. E. Reitz, R. A. Panettieri, Jr., S. P. Peters, and E. Puré. 1997. Antigen receptor-stimulated peripheral blood and bronchoalveolar lavage-derived T cells induce MHC class II and ICAM-1 expression on human airway smooth muscle. Am. J. Respir. Cell Mol. Biol. 16:38–45.**

The classic model of T-cell activation is a two-step process requiring engagement of the T-cell receptor (TCR) with antigen and of a costimulatory receptor such as CD28 with its ligand CD80 or CD86 on an antigen-presenting cell (APC) (1, 2). This results in T-cell proliferation and enhanced T-cell effector functions such as cytokine production. T-cell activation also leads to an increased affinity of cell adhesion molecules (CAMs) such as LFA-1 and CD44 for their ligands (3). CAMs are critical to lymphocyte homing to areas of inflammation, as well as for lymphocyte interactions with interstitial cells such as smooth muscle cells and with extracellular matrix proteins. The adhesion of activated T cells via CAMs, along with T-cell-derived cytokines and growth factors, can induce changes in gene expression and cell growth in the target cell. For example, co-incubation of T cells leads to the induction of intercellular adhesion molecule-1 (ICAM-1) and HLA-DR on resting keratinocytes (4). NK cells can bind to allogeneic endothelial cells *in vitro* and upregulate expression of MHC class II antigens (5). Similarly, we have previously demonstrated that mitogen-activated T cells can adhere to airway smooth muscle (ASM) via β_1 and β_2 integrins and CD44, leading to increased smooth muscle cell DNA synthesis (6). These data suggest that T cells can have profound effects on parenchymal cells in areas of inflammation.

Antigen presentation by "professional" APCs such as dendritic cells that constitutively express high levels of MHC class II and costimulatory molecules such as B7 has been well characterized (7). Increasing evidence, however, supports the existence and function of nonprofessional APCs that inducibly express MHC class II and/or costimulatory molecules. Keratinocytes, endothelial cells, and smooth muscle cells can express MHC class II antigens following ex-

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Abbreviations: antigen presenting cell, APC; airway smooth muscle, ASM; bronchoalveolar lavage, BAL; cell adhesion molecules, CAMs; counts per minute, cpm; intercellular adhesion molecule-1, ICAM-1; interferon-γ, IFN-γ; interleukin, IL; segmental antigen challenge, SAC; T-cell receptor, TCR; tumor necrosis factor α, TNFα; vascular smooth muscle, VSM.

posure to interferon-γ (IFN-γ) (4, 8, 9). Both keratinocytes and endothelial cells have been shown to provide costimulatory signals to support T-cell proliferation, however these signals do not appear to be mediated by the CD28 ligand B7 (4, 8, 10). The potential of CAMs to transduce costimulatory signals is controversial, though there is evidence to suggest that the interaction of LFA-1, VLA-4, and possibly $\alpha_4\beta_7$ with their respective ligands can provide signals leading to T-cell activation (11–14).

Lymphocyte infiltration is prominent in many diseases characterized by smooth muscle cell hyperplasia, most notably asthma and atherosclerosis. However, the mechanisms of lymphocyte–smooth muscle cell interactions are not completely defined. In addition, the consequences of this potential interaction are not known. For example, does adhesion of activated T cells affect the phenotype or alter proliferation rates of myocytes? Alternatively, can smooth muscle cells present antigen and promote T-cell proliferation? Previous data regarding the ability of smooth muscle cells to act as APCs are contradictory. While several groups showed that vascular smooth muscle (VSM) inhibited T-cell proliferation (15, 16), others showed that VSM could induce T-cell proliferation (17). No studies have addressed whether ASM can induce T-cell proliferation. In this study we investigated whether T cells activated through the TCR by crosslinking with anti-CD3 antibodies *in vitro*, or BAL-derived T cells isolated from atopic donors following antigen challenge, adhere to ASM. In addition, we studied the effect of activated T cells on the expression of CAMs and MHC class II on ASM. Finally, we determined whether ASM could function as an APC and promote T-cell proliferation in response to alloantigen.

Materials and Methods

Antibodies and Reagents

The following murine monoclonal antibodies were used at 10 μg/ml of purified Ig, except where indicated: OKT3 (anti-CD3; American Type Culture Collection [ATCC], Rockville, MD); OKT8 (anti-CD8; ATCC); 93 (blocking anti-CD25; gift of Dr. G. Trinchieri, Wistar Institute, Philadelphia, PA); B159 (anti-CD56; gift of G. Trinchieri); IB4 (anti-β_2 integrin; gift of Dr. S. Wright, Rockefeller University, New York, NY [18]); RR6.5 (anti-ICAM-1; gift of Dr. R. Rothlein, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT [19]); 15/7 (β_1 integrin activation epitope; gift of Dr. T. Yednock [20]); B33.1 (anti-HLA-DR, gift of Dr. G. Trinchieri [21]); anti-HLA-ABC (Pel-Freez Biologicals, Rogers, AK). The following antibodies were used as ascites at the indicated dilutions: 9.3 (anti-CD28, 1:500; gift of Dr. P. Linsley, Bristol-Meyers Squibb Pharmaceutical Research Institute, Seattle, WA [22]); B133.5 (anti-IFN-γ, 1:1,000; gift of Dr. G. Trinchieri [23]); P5D2 (anti-β_1 integrin, 1:500; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA [24]). The rabbit polyclonal antibodies specific for TNFα (16-247, gift of Dr. B. Sherry, Picower Institute for Medical Research, Manhasset, NY) and lymphotoxin (Pepro Tech Inc., Rocky Hill, NJ) were used at a dilution of 1:100. The anti-CD44 mAb 5F12 (gift of Dr. B. Haynes, Duke University Medical Center, Durham, NC [25]) was prepared from an ammonium sulfate cut of hybridoma supernatant and used at 1:50. The cytokines used included: TNFα (1,000 U/ml; Boehringer Mannheim, Indianapolis, IN); IFN-γ (1,000 U/ml; Genzyme, Cambridge, MA); lymphotoxin (2 ng/ml, Pepro Tech Inc.).

Cell Culture

Human ASM was obtained from the trachealis muscle of lung transplant donors in accordance with the policies of the Committee on Studies involving Human Beings at the University of Pennsylvania. ASM cells were purified as previously described (26) and cultured in Ham's F12 supplemented with penicillin, streptomycin, glutamine, HEPES, and 10% FBS (fetal bovine serum). A minimum of 3 different smooth muscle cell lines were used. Characterization of the cultured smooth muscle, including staining for smooth muscle-specific actin and responsiveness to contractile agonists, has been previously described (26).

Human PBMC were obtained from healthy donors after Ficoll-Hypaque (Pharmacia LKB, Piscataway, NJ) gradient centrifugation. T cells were first enriched by rosetting with neuraminidase-treated (Sigma, St. Louis, MO) sheep red blood cells (Rockland Inc., Gilbertsville, PA). When indicated, $CD4^+$ T cells were further isolated by negative selection using magnetic beads (Dynal, Lake Success, NY) and antibodies against CD8, CD56, and HLA-DR. Cells were cultured for 2 days in RPMI 1640 supplemented with penicillin, streptomycin, gentimycin, fungizone, glutamine, and 10% FBS. For stimulation with anti-CD3 and anti-CD28, 6 well plates were coated with 15 μg/ml of OKT3 in phosphate-buffered saline for 1 h at 37°C, followed by washing. Anti-CD28 ascites was used at a 1:500 dilution. Mitogen-activated T cells were stimulated for 2 days with phorbol 12,13-dibutyrate (5 ng/ml; Sigma) and ionomycin (250 nM; Sigma).

Bronchoalveolar Lavage (BAL)

The protocols to obtain BAL were approved by the Institutional Review Board of Jefferson Medical College. Volunteers were screened for ragweed allergy and seasonal rhinitis. Ragweed sensitivity and response to whole-lung challenge with purified ragweed antigen (amb A I) were established as previously described (27). Segmental antigen challenge was also performed as previously described (28). Briefly, on day 1 the subject underwent BAL with 150 ml saline, followed by antigen instillation into a right middle lobe segmental bronchus. On day 2, the challenged segment was lavaged as above. BAL fluid was filtered through gauze and the cells were pelleted by centrifugation and counted. T cells were isolated by E-rosetting, as described above.

Adhesion Assay

Adhesion assays were performed using total T cells and confluent ASM in 24-well plates as previously described (6). Briefly, T cells were activated with anti-CD3/anti-CD28 as described above for 2 days and were labeled during the final 16 h with 2 μCi/ml [^{3}H]-thymidine (specific activity 20 Ci/mmol; New England Nuclear, Boston, MA). Where indicated, ASM was stimulated with TNFα (1,000 U/ml) overnight. T cells, 6×10^5, were added to ASM in a total vol-

ume of 0.5 ml and incubated at 37°C for 1 h. The nonadherent cells were removed by washing, and the adherent cells were lysed with detergent and harvested; radioactive counts were quantitated in a liquid scintillation counter. Results are expressed as mean counts per minute (cpm) ± SD of triplicate wells. Where indicated, T cells were pretreated with blocking antibodies for 45 min at 4°C.

Flow Cytometry

Confluent ASM in 12-well plates were incubated with media alone or with 4×10^5 $CD4^+$ T cells for 72 h. Where indicated, co-cultures contained antibodies specific for TNFα or IFN-γ. The wells were washed and single-cell suspensions were prepared using trypsin/EDTA. Cells were stained with antibodies specific for ICAM-1, HLA-DR, or HLA-ABC, followed by FITC-conjugated goat antimouse IgG (Jackson ImmunoResearch Laboratories, Westgrove, PA), and analyzed using a FACScan (Becton Dickinson, San Jose, CA) and CellQuest software. T cells were excluded from the analysis by gating on ASM cells using forward and side scatter.

Antigen Presentation

Single-cell suspensions of ASM stimulated for 3 days with 1,000 U/ml IFN-γ were prepared using trypsin/EDTA, washed, and 7,500 cells were replated in 96-well flat bottom plates and allowed to adhere for 4 h at 37°C. ASM cells were irradiated with 2,000 rads from a ^{137}Cesium source prior to the addition of T cells. Allogeneic PBMC were prepared as described above and irradiated with 4,000 rads. Varying concentrations of $CD4^+$ T cells were added in 200 μl of Ham's F12 and 10% human AB serum (Scantibodies Laboratory, Santee, CA). Where indicated, $CD4^+$ T cells were pretreated with anti-CD28 for 45 min at 4°C, washed, and added to the cultures in the presence of 10 μg/ml goat antimouse IgG to crosslink the receptor. Cultures were pulsed for the final 24 h of a 5-day incubation with 1 μCi/well [^{3}H]-thymidine, harvested onto glass fiber filters, and counted. All values are expressed as mean cpm ± SD of triplicate wells.

Statistical Analysis

Data are expressed as mean ± SD. The difference in adhesion of unstimulated and activated T cells to ASM was assessed by analysis of variance with the Bonferroni correction for multiple comparisons. Differences in thymidine incorporation in the assay of antigen presentation was assessed by an unpaired *t* test.

Results

CD3-activated T Cells Adhere to ASM via β_1 and β_2 Integrins

Increased adhesion via integrins is an early response of T cells to activation signals (3). The question remains whether different activation signals are equivalent in terms of increased integrin affinity. We compared the adhesion to ASM of T cells activated by phorbol ester and ionomycin, immobilized anti-CD3, soluble anti-CD28, or anti-CD3 plus anti-CD28. We confirmed our previous report that T cells activated by phorbol ester and ionomycin adhered to ASM via LFA-1/ICAM-1, VLA-4/VCAM-1, and CD44 (6). T cells activated by immobilized anti-CD3 alone or in combination with anti-CD28 adhered equally as well as mitogen-activated T cells to resting ASM (Figure 1). Adhesion of anti-CD3- and anti-CD3/CD28-stimulated T cells was enhanced by 52% and 89%, respectively, when the ASM were pretreated with TNFα, compared with untreated ASM. We phenotyped the adherent and nonadherent T cells and found that the ratio of $CD4^+$ and $CD8^+$ cells following adhesion to untreated or TNF-stimulated ASM corresponded to their representation in the input population of total T cells. Thus, there was no apparent selectivity for either T-cell subset in this assay. T cells incubated with soluble anti-CD28 alone adhered only slightly better than unstimulated T cells, despite the induction of the β_1 activation epitope 15/7 (20) (Figure 2). These data indicate that crosslinking the TCR is sufficient to induce adhesion to ASM. Adhesion of CD3/CD28-activated T cells to resting ASM was completely blocked by a combination of mAbs to β_1 (P5D2) and β_2 (IB4) integrins (Figure 3). However, adhesion of activated T cells to TNFα-treated ASM was only partially blocked by antibodies to β_1 and β_2 integrins, on average 80% (Figure 3). The addition of anti-CD44 antibody abrogated the integrin-independent adhesion to TNFα-treated ASM. Antibodies specific for $\alpha_4\beta_7$ had no effect on T-cell adhesion to ASM (data not shown).

CD3-activated T Cells Induce ASM Expression of CAMs and MHC Class II

ASM constitutively express only low levels of ICAM-1 and VCAM-1 and are MHC class II-negative. Therefore we examined whether co-culture with activated T cells could induce the expression of cell adhesion receptors and MHC molecules on ASM. Resting ASM was co-cultured for 3 days with activated $CD4^+$ T cells, then analyzed by flow cytometry for expression of ICAM-1, VCAM-1, CD44, HLA-ABC, and HLA-DR. Resting ASM displayed moderate staining for

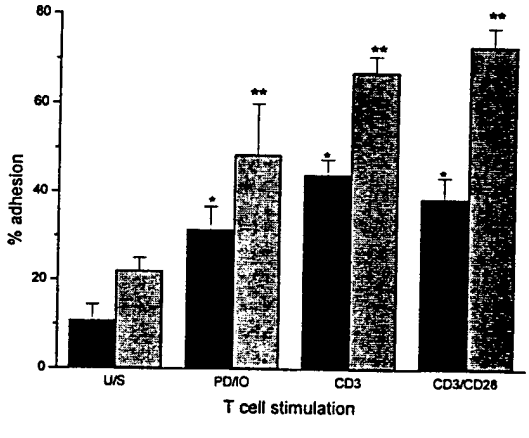

Figure 1. Anti-CD3 is sufficient to induce T-cell adhesion to ASM. Either unstimulated (u/s), phorbol ester and ionomycin (PD/IO)-, immobilized anti-CD3 (CD3)-, or anti-CD3 and soluble anti-CD28 (CD3/CD28)-activated T cells were allowed to adhere to untreated (*black bars*) or TNF-stimulated (*shaded bars*) ASM as described. Data are expressed as mean percent adhesion ± SD and are representative of 3 experiments. $^*P < 0.0001$ compared with adhesion of unstimulated T cells to untreated ASM; $^{**}P \leq 0.0002$ compared with adhesion of unstimulated T cells to TNF-stimulated ASM.

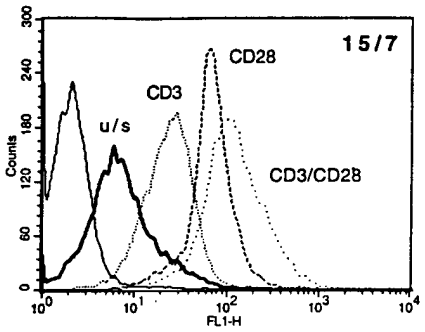

Figure 2. Soluble anti-CD28 induces the β_1-specific activation epitope 15/7 on peripheral blood T cells. Either unstimulated (u/s), immobilized anti-CD3 (CD3)-, soluble CD28 (CD28)-, or anti-CD3 and soluble anti-CD28 (CD3/CD28)-activated T cells were stained with antibodies specific for the β_1-specific activation epitope 15/7 and analyzed by flow cytometry. The data are representative of 3 experiments.

ICAM-1 and MHC class I, and no detectable expression of MHC class II (Figure 4). Following a 3-day co-culture with activated T cells, expression of ICAM-1 and class II were increased 9.7- and 6-fold, respectively, over baseline, while expression of class I was unchanged (Figure 4). VCAM-1 was minimally detected on resting ASM, and was moderately increased following co-culture with activated T cells, although to a much lesser extent than ICAM-1 (data not shown). This is in contrast to the marked upregulation of both ICAM-1 and VCAM-1 on ASM induced by TNFα (6). CD44 expression was constitutively high on resting ASM (6) and was unchanged following co-culture with activated T cells (data not shown).

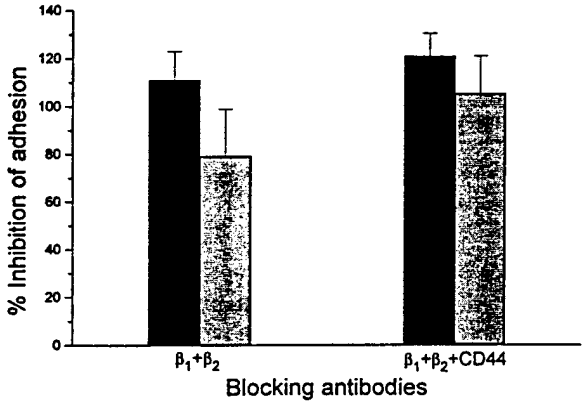

Figure 3. β_1 and β_2 integrin antibodies block adhesion of T cells to ASM. Anti-CD3/anti-CD28-stimulated T cells were allowed to adhere to untreated (*black bars*) or TNF-stimulated (*shaded bars*) ASM. Where indicated, T cells were pretreated with blocking antibodies to CD44 or β_1 and β_2 integrins. Data are expressed as mean percent inhibition of adhesion in triplicate wells and are representative of 5 experiments. Percent inhibition = (% adhesion with antibody − % adhesion of unstimulated cells)/(% adhesion without antibody − % adhesion of unstimulated cells). Inhibition greater than 100% indicates that adhesion of stimulated cells in the presence of blocking antibodies was less than that observed for unstimulated cells alone.

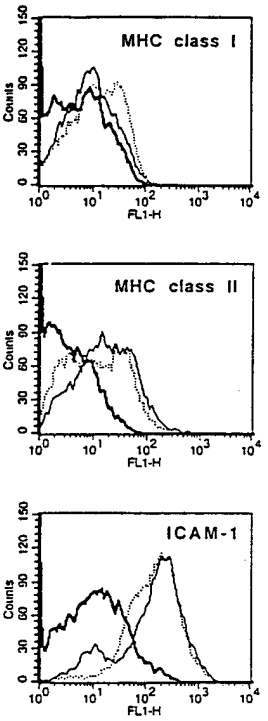

Figure 4. T cell-induced ASM expression of MHC class II and ICAM-1 is contact-independent. ASM was incubated with media alone (*bold line*), with activated CD4$^+$ T cells in direct contact (*thin line*), or with activated CD4$^+$ T cells separated by a polycarbonate membrane in the upper chamber of a Transwell (*dotted line*). After 3 days, ASM was stained with antibodies for MHC class I, MHC class II, and ICAM-1, and analyzed by flow cytometry. The data are representative of 4 experiments.

To determine the kinetics of upregulated expression, confluent ASM was incubated with activated T cells and changes in the expression of ICAM-1 and HLA-DR were determined by flow cytometry on days 1, 2, and 3. Maximal ICAM-1 expression was detectable by 24 h and was sustained over the next 2 days (Figure 5). In contrast, HLA-DR was not detectable on day 1 but gradually increased over days 2 and 3 (Figure 5). Unstimulated T cells induced low levels of ICAM-1 expression on ASM but had no effect on HLA-DR expression (data not shown).

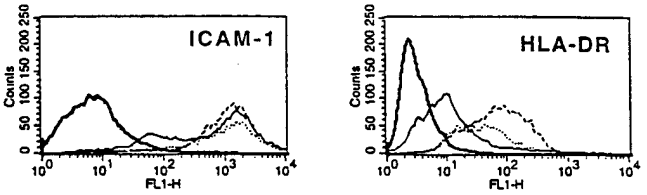

Figure 5. Kinetics of ICAM-1 and HLA-DR induction. ASM was incubated in media alone (*bold line*) or with activated CD4$^+$ T cells for 1 (*thin line*), 2 (*dotted line*), or 3 (*broken line*) days, then stained with antibodies to ICAM-1 or HLA-DR as described. The data are representative of 2 experiments.

IFN-γ Mediates T Cell-induced ASM Expression of ICAM-1 and MHC Class II

To determine whether the effect of T cells on the phenotype of ASM was due to a soluble mediator or to direct cell contact, experiments were performed using activated $CD4^+$ T cells placed in the upper chamber of dual chamber Transwells (Costar, Cambridge, MA) in which ASM were grown in the lower chamber. ASM expression of ICAM-1 and HLA-DR was comparably increased when ASM was cultured in the same well or in a separate chamber from the T cells (Figure 4), suggesting that a soluble mediator(s) secreted by the T cells induced expression. It is well known that ICAM-1 and VCAM-1 expression can be upregulated by treatment with TNFα or IFN-γ on many cell types. In addition, IFN-γ is known to induce expression of MHC class II on some MHC class II-negative cells. We therefore performed similar experiments in the presence of neutralizing Abs to TNFα and/or IFN-γ. A mAb to IFN-γ completely blocked the T cell-induced expression of MHC class II on ASM (Figure 6A). Neutralizing polyclonal Ab to TNFα had no effect on ICAM-1 expression, while mAb to IFN-γ partially blocked the upregulation of ICAM-1 (Figure 6B). The average inhibition of anti-IFN-γ was 68.3 ± 13.2% (range 49.4–88%, $n = 8$). Higher doses of antibodies had no further effect on the expression of ICAM-1 and HLA-DR (data not shown). In addition, the antibodies were able to block the effects of exogenously added cytokine, up to 1,000 U/ml (data not shown), suggesting that the lack or partial effect on T cell-induced changes in ASM phenotype likely was not due to lack of activity or dose of the antibody. We examined whether lymphotoxin, another TH_1 cytokine that is secreted by activated T cells, affects ASM expression of ICAM-1. Purified lymphotoxin markedly upregulated expression of ICAM-1 on ASM, which was blocked by neutralizing antibodies specific for lymphotoxin (Figure 6C). In addition, antilymphotoxin modestly inhibited T cell-induced ICAM-1, although the combination of antilymphotoxin and anti-IFN-γ was not additive (Figure 6C). This suggests that lymphotoxin may be secreted by T cells in limiting amounts but can play a role in inducing ICAM-1 on ASM. Finally, antibodies specific for IL-1β, IL-4, IL-5, and IL-6 were also unable to block the upregulation of ICAM-1 (data not shown). Taken together, these data indicate for the first time that upregulation of HLA-DR on ASM by activated T cells can be attributed solely to the effects of IFN-γ. In contrast, the regulation of ICAM-1 expression appears to be multifactorial and includes IFN-γ and possibly lymphotoxin, but does not involve TNFα.

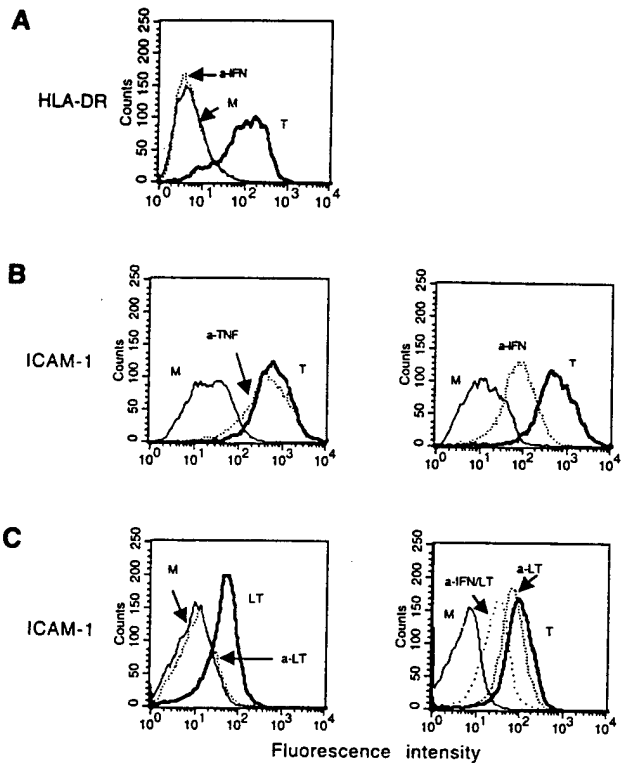

Figure 6. Antibody to IFN-γ but not TNFα inhibits T cell-induced expression of ICAM-1 and HLA-DR on ASM. (*A*) ASM was incubated for three days with media alone (M) or with anti-CD3/anti-CD28-activated $CD4^+$ T cells in Transwells in the absence (T) or presence (a-IFN) of neutralizing mAb to IFN-γ. Cells were then stained with antibodies specific for HLA-DR as described (representative of 5 experiments). (*B*) ASM was incubated with activated T cells in Transwells as above for one day in the absence (T) or presence of neutralizing antibodies to TNFα (a-TNF) or IFN-γ (a-IFN) and stained for ICAM-1 (representative of 3 experiments). (*C, left*) ASM was incubated for one day with purified lymphotoxin (2 ng/ml) in the absence (LT) or presence (a-LT) of anti-lymphotoxin antibody and stained for ICAM-1 (representative of 3 experiments). (*C, right*) ASM was incubated for one day with activated T cell supernatant in the absence (T) or presence of neutralizing antibodies to lymphotoxin (a-LT) alone or in combination with anti-IFN-γ (a-IFN/LT). Cells were then stained for ICAM-1 (representative of 2 experiments).

BAL-derived T Cells from Atopic Donors Adhere to and Induce ICAM-1 and HLA-DR Expression on ASM

Increased expression of CAMs has been found in bronchial biopsies of atopic patients with asthma (29, 30). We therefore studied the ability of T cells activated by allergen *in vivo* to induce phenotypic changes in ASM. We performed coculture experiments using BAL cells derived from allergic asthmatic patients with ragweed sensitivity. These patients underwent segmental antigen challenge (SAC) with purified ragweed antigen; BAL fluid was obtained prior to and 24 h after SAC. Adhesion to unstimulated ASM ranged from 28.1 to 32.7%; adhesion to TNF-stimulated ASM ranged from 33.1 to 44% (data not shown). Thus, adhesion of T cells activated *in vivo* following SAC was comparable to that seen with peripheral blood T cells from normal donors stimulated with mitogen or anti-CD3 antibodies.

BAL-derived T cells obtained 24 h after antigen challenge dramatically upregulated ICAM-1 expression, similar to purified peripheral blood T cells activated with anti-CD3/CD28 (Figure 7). In contrast, BAL T cells obtained prior to antigen challenge induced moderate expression of ICAM-1 on ASM, comparable to that seen with unstimulated peripheral blood T cells (Figure 7). After antigen challenge BAL-derived T cells also induced high levels of expression of HLA-DR, albeit not as great as that induced by anti-CD3/CD28-stimulated peripheral blood T cells. Prechallenge

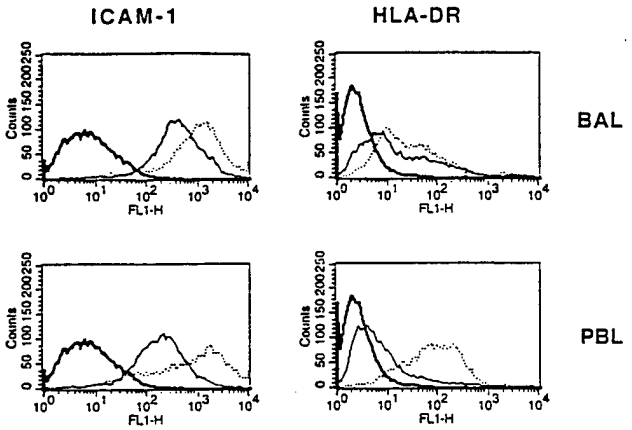

Figure 7. BAL-derived T cells induce ASM expression of ICAM-1 and HLA-DR. *Top panels*: ASM was incubated with BAL-derived T cells obtained either before (*thin line*) or 24 h after (*dotted line*) segmental antigen challenge with purified ragweed antigen. *Bottom panels*: ASM was incubated with unstimulated (*thin line*) or anti-CD3/anti-CD28-stimulated (*dotted line*) peripheral blood $CD4^+$ T cells (PBL). After 3 days, ASM was stained with antibodies specific for ICAM-1 (*left*) or HLA-DR (*right*) and analyzed by flow cytometry. The *bold line* in all panels represents staining of ASM incubated with control media alone. Data are representative of 3 experiments.

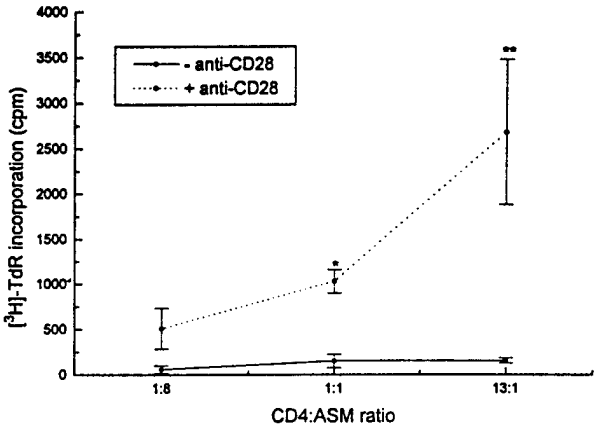

Figure 8. ASM does not present alloantigen. IFN-γ-treated irradiated ASM was incubated with resting $CD4^+$ T cells for 5 days, with (*dotted line*) or without (*solid line*) crosslinking of CD28 on the T cells. T-cell proliferation was measured as described. Data are expressed as mean cpm $\pm$ SD of triplicate wells and are representative of 2 experiments. The maximum incorporation of [^{3}H]-thymidine of the control conditions was as follows: irradiated ASM, 101 $\pm$ 45; irradiated PBMC, 91 $\pm$ 11; $CD4^+$ T cells alone, 40 $\pm$ 2; $CD4^+$ T cells + anti-CD28, 66 $\pm$ 13; $CD4^+$ T cells + irradiated PBMC, 78,302 $\pm$ 13,708. $^*P = 0.0005$, $^{**}P = 0.005$.

BAL-derived T cells induced low levels of HLA-DR expression on ASM; this appeared significant since no increase in HLA-DR was observed on ASM cultured with unstimulated peripheral blood T cells, and suggests that even without intentional antigen challenge there are low levels of cytokine-producing effector T cells in the lungs of asthmatics. These data also suggest that the production of inflammatory mediators by infiltrating T lymphocytes may contribute to the up-regulated expression of CAMs in the airways of asthmatics, and that our findings with *in vitro* stimulated T cells are physiologically relevant to *in vivo* phenomena.

ASM Does Not Stimulate an Allogeneic T-Cell Response

In order to address whether cytokine-activated ASM exerts effects on T-cell function, we investigated whether ASM cells present antigen to resting peripheral blood $CD4^+$ T-cells. We found no increase in [^{3}H]-thymidine incorporation by allogeneic T cells cultured with IFN-γ-treated ASM alone (Figure 8). In addition, we were unable to detect either constitutive or inducible expression of B7 on ASM following incubation with TNFα or IFN-γ (data not shown). However, when costimulation was provided by crosslinking CD28 on the T cells co-cultured with ASM, there was a 17-fold increase in T-cell thymidine incorporation at T cell:ASM ratio of 13:1 (Figure 8). In contrast, allogeneic PBMC induced a 30-fold greater increase in thymidine incorporation in a mixed leukocyte reaction (Figure 8).

Studies with VSM suggested that myocytes can activate both allogeneic and HLA-matched T cells as evidenced by expression of IL-2R and secretion of IL-2, but that they are arrested in G_1 of the cell cycle and thus could not be detected by [^{3}H]-thymidine incorporation (15, 16). We therefore studied expression of IL-2R on the surface of T cells following culture with allogeneic ASM. In contrast to the results reported for VSM, incubation of T cells with allogeneic ASM alone did not increase surface expression of IL-2R (data not shown). Taken together, these data suggest that ASM lacks the necessary costimulatory molecules to process and present antigen to $CD4^+$ T cells and/or delivers a negative signal.

Discussion

The activation of adhesion receptors is associated with defined signaling mechanisms in lymphocytes. Our studies demonstrated that engagement of CD3 was sufficient to induce β_1- and β_2-mediated T-cell adhesion to ASM. We found that the addition of soluble anti-CD28 antibody alone had no effect on adhesion of resting T cells to ASM, in spite of inducing expression of an epitope of VLA-4 that is associated with activation of this receptor (20). This implies that expression of this epitope is not sufficient to define the activated state of VLA-4. In addition, changes in β_1 integrin activity might not alter T cell–ASM adhesion, depending on the hierarchy of adhesion molecule usage. Our findings differ from those of Shimizu and colleagues (31), who reported that CD28 crosslinking induced adhesion of $CD4^+$ T cells to fibronectin and ICAM-1, comparable to that induced by CD3 crosslinking. There are several possible explanations for this apparent difference. First, secondary antibody or immobilized anti-CD28 may be required to induce sufficient crosslinking to activate the integrins. Furthermore, their study was restricted to $CD4^+$ T cells monitored for adhesion to purified ligand coated on plastic, where we used a mixed population of T cells and assayed adhesion to a physiologic matrix elaborated by ASM cells.

We also observed that CD44-mediated adhesion by anti-CD3/CD28-activated T cells, similar to mitogen-activated T cells, was evident only upon interaction with TNFα-treated ASM. Whether TNFα has any direct effect on the activation state of CD44 is currently unknown. However, Camp and associates (32) showed that CD44-negative leukocytes can accumulate normally in draining lymph nodes but are not induced to infiltrate into the site of a cutaneous DTH (delayed type hypersensitivity) reaction. Our *in vitro* data are consistent with the view that CD44 is pivotal only in homing to areas of inflammation rather than in normal lymphocyte trafficking.

Previously, we found that several cytokines, including TNFα (6) and IFN-γ (unpublished results) induced upregulation of ICAM-1 expression on ASM. In addition, we found that the MHC class II antigen HLA-DR is expressed by ASM in response to treatment with IFN-γ (unpublished results). We now demonstrate that TCR-activated T cells, when cocultured with ASM, can induce ASM expression of ICAM-1 and HLA-DR. We observed similar effects using either peripheral blood lymphocytes activated *in vitro* by antireceptor antibodies or BAL-derived lymphocytes activated *in vivo* by SAC. We found that BAL-derived lymphocytes obtained prior to SAC also induced significant upregulation of ICAM-1 and HLA-DR on ASM. This is not surprising because one would expect effector/memory CD45RO$^+$ T cells, not resting cells, to enter the lung.

We could attribute T cell-induced HLA-DR expression on ASM to the presence of IFN-γ, as anti-IFN-γ completely blocked the induction of HLA-DR. In contrast, anti-IFN-γ only partially inhibited the upregulation of ICAM-1. In addition, anti-TNFα had no effect on T cell-induced expression of either HLA-DR or ICAM-1 by ASM, indicating that these T cells did not produce TNFα under the conditions used. Lymphotoxin appeared to be produced in low concentrations by activated T cells and contributed, albeit modestly, to the upregulation of ICAM-1 expression. These data suggest that TH_0- or TH_1-, as well as the previously implicated TH_2-like T cells may be important in airway inflammation. It is also interesting that neutralizing antibodies specific for IL-β, IL-4, IL-5, and IL-6 were unable to inhibit upregulation of ICAM-1 by anti-CD3/anti-CD28-activated T cells (data not shown). This may mean that these factors are present but are not active in upregulating ICAM-1 expression on ASM, or that under our *in vitro* culture conditions TH_2 cytokines are not produced in significant levels. Further work will be necessary in order to define any additional factors that contribute to the T cell-mediated upregulation of ICAM-1 on ASM. IL-4 may still be of particular interest in the upregulation of ICAM-1 expression induced by BAL-derived T cells from allergic asthmatic patients, because studies have shown that these T cells are predominantly of the TH_2-like phenotype, which can produce IL-4 (33).

Increasing evidence supports the idea that parenchymal smooth muscle cells can contribute to the inflammatory response, both by the synthesis of cytokines such as IL-1 (34, 35) and chemokines such as MCP-1 (36), ENA-78 (37), and IL-8 (Lazaar and Noble, unpublished results), and by acting as potential APCs by virtue of their ability to express MHC class II products (9, 38, 39). Antigen presentation by non-professional APCs has been demonstrated in several systems. For example, there are data demonstrating the induction of expression of ICAM-1 and HLA-DR on keratinocytes that can present superantigen to both autologous and allogeneic T cells (4). Similarly, endothelial cells can induce upregulation of IL-2R and induce DNA synthesis by allogeneic CD4$^+$ T cells (8). However, the findings using smooth muscle as an APC have not been conclusive. Work by Fabry and colleagues (40) suggested that VSM derived from murine brain microvessels presented antigen to TH_1 but not TH_2 clones. In contrast, VSM derived from murine mesenteric arteries inhibited the progression of activated HLA-matched T-cell clones through the cell cycle, despite an increase in expression of IL-2R and secretion of IL-2 (16). This inhibitory effect appeared to be due to a labile, soluble mediator released from the VSM. Similar results have been obtained using human VSM and allogeneic CD4$^+$ T cells (15). Interestingly, this did not result in the induction of T-cell anergy by the smooth muscle cells, as the cells recovered their proliferative capability when re-cultured in the absence of VSM.

Finally, we investigated whether ASM had the ability to present alloantigen and promote T-cell proliferation. We found that IFN-γ-treated ASM which expresses HLA-DR did not induce an allogeneic response of CD4$^+$ T cells. This may be due to an inhibitory activity or a lack of appropriate costimulatory molecules on ASM, despite the presence of ICAM-1, which can act as a costimulatory molecule for T-cell activation (11). The latter hypothesis is supported by our finding that crosslinking CD28 on the T cell was able to reconstitute a suboptimal allogeneic response. However, Nickoloff and coworkers (4) demonstrated that the presentation of bacterial superantigen by keratinocytes and the resultant T-cell proliferation was blocked by anti-ICAM-1 or anti-CD18, but not by anti-HLA-DR or anti-B7 (4), suggesting that B7 is not necessary for successful antigen presentation. Our data are consistent with those of Murray and colleagues (15), where VSM was unable to stimulate CD4$^+$ T cells in response to alloantigen. Further studies are necessary to determine whether the observed lack of T-cell activation by ASM is due to active inhibition, perhaps through the release of cytokines.

In summary, we have shown that activated T cells can adhere to ASM and induce the expression of ICAM-1 and HLA-DR on the myocytes. Importantly, *in vitro*-activated cells behaved similarly to BAL-derived cells activated *in vivo* by antigen challenge. ASM was unable to support the proliferation of resting CD4$^+$ T cells in response to alloantigen, which may be due to active inhibition by a soluble mediator released by the ASM. Taken together, these data provide additional evidence for the role of parenchymal cells in sustaining a local inflammatory response in the lung.

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Regulation of the type II oncostatin M receptor expression in lung-derived epithelial cells

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Abstract **Oncostatin M (OSM) is a potent modulator of human lung-derived epithelial cell function. This cytokine binds two distinct receptor complexes: type I OSM receptor which is also a functional receptor for leukemia inhibitory factor (LIF), and type II OSM-specific receptor. The role of these two distinct receptors in mediating the response of individual cell types to OSM has not been delineated. In contrast to LIF, OSM induces synthesis of α_1-antichymotrypsin and α_1-antiproteinase inhibitor in lung-derived epithelial cells. The differential responsiveness to LIF and OSM suggested that the response of lung epithelial cells to OSM may be mediated by the OSM-specific receptor. Therefore, we characterized lung-derived epithelial cells for the expression of type II OSM receptor mRNAs, and the regulation of the mRNAs encoding the components of the OSM-specific receptor by cytokines and dexamethasone.**

Key words: Interleukin-6; Leukemia inhibitory factor; Transforming growth factor β1; Bronchial epithelium

1. Introduction

Oncostatin M (OSM) is a multifunctional cytokine synthesized by hematopoietic cells as well as activated T lymphocytes and monocytes [1–3]. OSM is functionally related to the interleukin-6 family of cytokines, including IL-6, leukemia inhibitory factor (LIF), IL-11, ciliary neurotrophic factor and cardiotrophin-1 [4,5]. Members of this family mediate their biological effects by inducing homodimerization of gp130 (IL-6, IL-11) or heterodimerization of gp130 and other signaling (β) receptor subunits (other members). Some of these cytokines, including IL-6, also require a ligand-binding subunit that determines receptor specificity but is not directly involved in signaling. Receptor dimerization results in the activation of cytoplasmic tyrosine kinases followed by the phosphorylation and nuclear translocation of the STAT family of transcription factors [4–6].

Biologically active OSM receptor consists of a heterodimer of gp130 and LIFRβ. This gp130·LIFRβ complex, described as type I OSM receptor, is also a functional receptor for LIF. Recently, a second OSM-specific receptor complex has been identified in the human system, consisting of gp130 heterodimerized with an OSM-specific subunit OSMRβ, which forms the type II OSM receptor [7]. Although type I and II OSM receptors are relatively broadly distributed on a variety of cell types [7], the role of these two distinct receptors in mediating the response of individual cell types to OSM has not been delineated.

In addition to bioactivities shared with other members of the IL-6 family of cytokines, OSM exhibits some unique activities [8]. For example, OSM but not LIF or IL-6 upregulates α_1-antichymotrypsin (ACH) and α_1-antiproteinase inhibitor (α_1-PI) levels in lung-derived epithelial cells whereas both LIF and OSM mediate regulation of both these genes in hepatocyte-derived HepG2 cells [9–12]. OSM was found to be one of the most potent 'proinflammatory' inducers of α_1-PI and ACH synthesis in lung-derived epithelial cells [9–12] and cells of epithelial origin, including lung epithelium, have been shown to bind significant amounts of OSM [13]. Thus the role of OSM in the lung is of considerable interest. Although the overlapping spectrum of biological activities of IL-6, OSM and LIF is well explained [5], much less is known about the basis for differences in the response to these cytokines. The specific response to OSM of lung-derived epithelial cells, including normal bronchial epithelial cells (NBEC) and two cell lines, HTB55 and HTB58 (adenocarcinoma and squamous carcinoma respectively), may indicate the utilization of the type II OSM receptor. Lung-derived epithelial cells may thus provide a good model to study potential differences in the levels of expression of the signaling receptor subunits or distinct signal transduction pathways, both of which are likely to account for differences in biological activities between IL-6, OSM and LIF.

In this study we demonstrate that epithelial cells derived from lung express type II OSM receptor mRNAs, with OSMRβ levels most likely being more abundant relative to gp130. Furthermore, we show that the expression of OSMRβ and to a lesser extent gp130 is regulated in these cells by specific inflammatory mediators, including OSM.

2. Materials and methods

2.1. Stimulating factors

Human recombinant OSM and human recombinant transforming growth factor β1 (TGF-β) were purchased from R&D Systems (Minneapolis, MN). Human recombinant IL-1β was obtained from Genzyme (Cambridge, MA). Human LIF from conditioned medium of Chinese hamster ovary cells, containing recombinant LIF at 10^5 U/ml, was a generous gift of Dr. H. Baumann (Buffalo, NY). Dexamethasone (DEX) was purchased from Sigma (St. Louis, MO).

2.2. Cell culture

HTB58 human lung squamous carcinoma, HTB55 human lung adenocarcinoma and HepG2 human hepatoma cell lines were obtained from the American Type Culture Collection (Rockville, MD). Normal human bronchial epithelial cells were purchased from Clonetics (San

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Abbreviations: OSM, oncostatin M; LIF, leukemia inhibitory factor; IL-6, interleukin-6; IL-1, interleukin-1; FBS, fetal bovine serum; MEM, minimal essential medium

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Diego, CA). Cells were cultured in Eagle's MEM (Biowhittaker, Walkersville, MD) supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin G, 100 μg/ml streptomycin (all from Gibco, Grand Island, NY) and 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA). Bronchial cells were cultured in serum-free bronchial epithelial cell basal medium (Clonetics) containing 0.5 ng/ml human epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone and epinephrine, 10 μg/ml transferrin, 0.5 ng/ml triiodothyronine and 0.4% v/v bovine pituitary extract (all from Clonetics). Cells were plated, allowed to grow to confluency and then treated with various stimulating factors.

2.3. Northern blot analysis

Total RNA was isolated as previously described [14,15]. Northern blot analysis was carried out by electrophoresis of RNA samples in 1% agarose gels containing 2.2 M formaldehyde, followed by capillary transfer [16] to Hybond-N membranes (Amersham, Arlington Heights, IL). Filters were hybridized with the plasmid containing human OSMRβ cDNA (a generous gift of Dr. B. Mosley, Immunex Corporation, Seattle, WA), human gp130 cDNA (a generous gift of Dr. T. Kishimoto, Osaka University, Osaka, Japan) and *SmaI-Bam*HI restriction fragment containing the complete coding sequence of human LIFRβ (a generous gift of Dr. D. Gearing, Immunex Corporation, Seattle, WA). The probes were labeled using the Megaprime Labeling Kit (Amersham). The hybridization was carried out at 65°C in 0.5 M phosphate buffer pH 7.0 containing 7% SDS, 1 mM EDTA, 10 mg/ml BSA and 100 μg/ml of herring DNA. Non-specifically bound radioactivity was removed by three washes at 65°C in 40 mM phosphate buffer pH 7.0 containing 1% SDS, 1 mM EDTA and 0.5% BSA, followed by three washes at 65°C in the same buffer without BSA. After probing blots were analyzed using a PhosphorImager (Molecular Dynamics).

3. Results

3.1. Normal bronchial epithelial cells and lung-derived epithelial cell lines express OSMRβ and gp130 mRNA

Northern blot analysis of RNA extracted from lung-derived epithelial cells revealed significant levels of OSMRβ and gp130 mRNAs (Fig. 1). The amounts of both transcripts were compared to that expressed by HepG2 cells. In HepG2

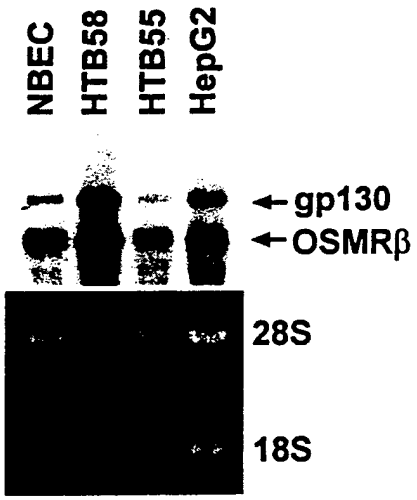

Fig. 1. Comparison of OSMRβ and gp130 mRNA levels in NBEC, HTB55, HTB58 and HepG2 cells. Aliquots of total RNA (5 μg) isolated from the indicated cell types were resolved by gel electrophoresis, transferred to a nylon membrane, and the ethidium bromide-impregnated blot was photographed to demonstrate comparable loading (bands of 28S rRNA and 18S rRNA are indicated). The blot was then hybridized with ^{32}P-labeled gp130 and OSMRβ probes. The blot is representative of three independent experiments.

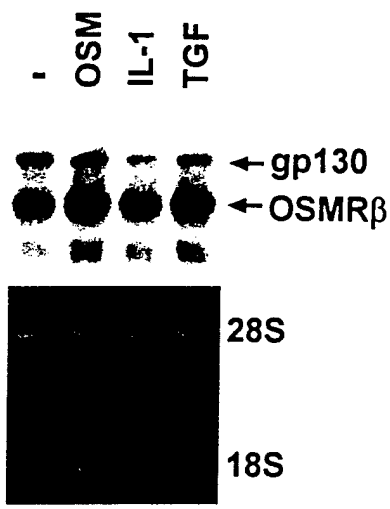

Fig. 2. Regulation of expression of the type II OSM receptor components in NBEC. Cells were incubated for 24 h with 50 ng/ml OSM, 5 U/ml IL-1 or 10 ng/ml TGFβ. Total RNA was then isolated and subjected to Northern blot analysis. Similar amounts of ethidium bromide-stained 28S and 18S rRNA are visualized in the bottom panel. In the upper panel the positions of the gp130- and OSMRβ-specific bands are indicated. The blot is representative of three independent experiments.

cells which are responsive to IL-6 and to OSM, synthesis of both gp130 and OSMRβ has been reported previously [7]. In agreement with this study HepG2 were found to express high levels of OSMRβ and gp130 mRNA and the signal was greater for OSMRβ than for gp130 (Fig. 1, Table 1). In comparison to HepG2 cells, NBEC appeared to express higher amounts of OSMRβ message but less gp130 message (Fig. 1, Table 1). In HTB55 cells levels of both OSMRβ and gp130 mRNAs were lower than in HepG2 cells and the ratio of OSMRβ to gp130 was even greater than in HepG2 cells (Table 1). A similar ratio of OSMRβ to gp130 to that observed in HepG2 cells was noted in HTB58 cells, although in this case both transcripts were much more abundant (Fig. 1, Table 1).

3.2. Expression of OSMRβ and gp130 in lung-derived epithelial cells is regulated by cytokines and dexamethasone

Factors which modulate function of lung epithelial cells were then studied as potential regulators of type II OSM receptor components in NBEC including, OSM, IL-1 [9–12] and TGF-β [17]. OSM and TGFβ caused significant increases in OSMRβ mRNA levels; IL-1, however, had no effect (Fig. 2). OSM was also effective in upregulating gp130 mRNA

Table 1
Relative quantitation of OSMRβ and gp130 mRNA levels in lung-derived epithelial cells

	OSMRβ	gp130	OSMRβ/gp130
NBEC	28.5	10.1	2.8
HTB58	53.9	36.6	1.5
HTB55	19.8	4.3	4.6
HepG2	26.5	16.5	1.6

The Northern blot presented in Fig. 1 was exposed to a PhosphorImager screen, and the resulting image was scanned. Arbitrary numbers indicate the relative amounts of OSMRβ- and gp130-specific mRNAs in NBEC, HTB58, HTB55 and HepG2 cells.

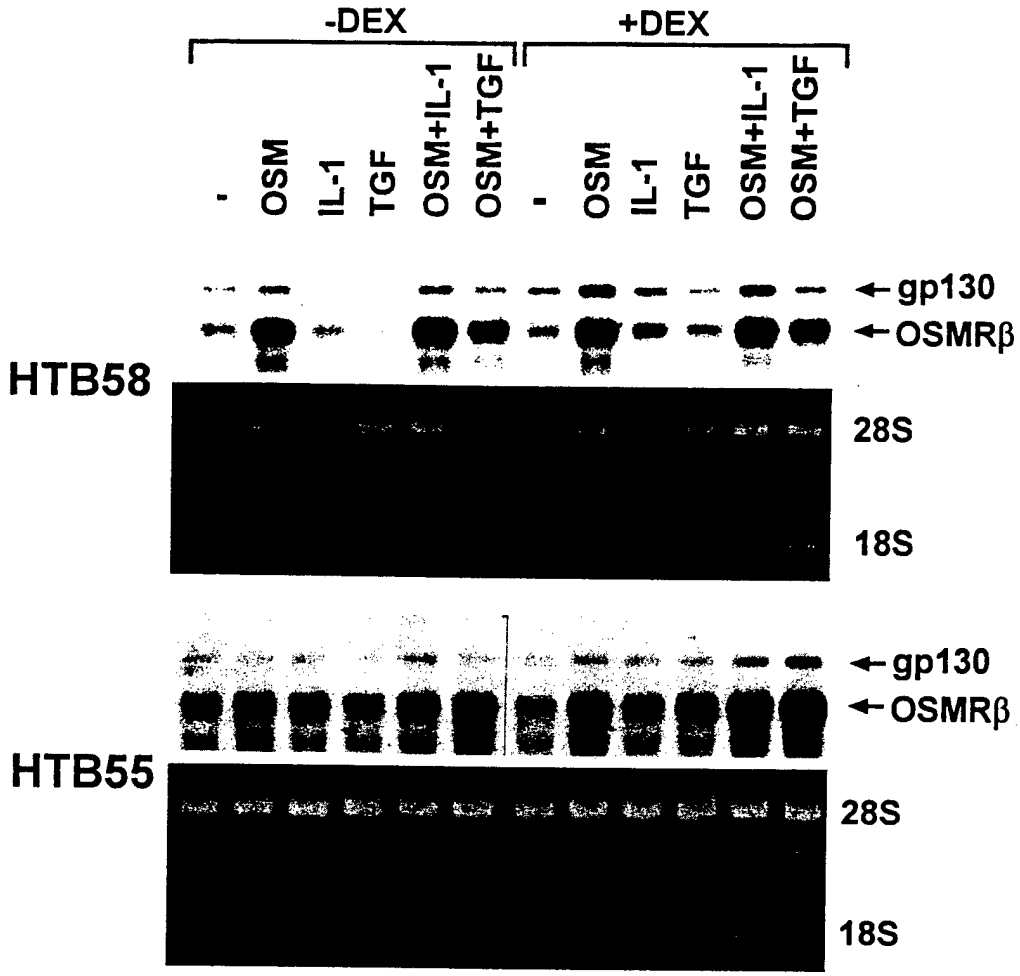

Fig. 3. Regulation of gp130 and OSMRβ mRNA levels in HTB58 and HTB55 cells. Cells were incubated for 24 h with 50 ng/ml OSM, 5 U/ml IL-1, 10 ng/ml TGFβ and/or 10^{-6} M DEX. The levels of gp130 and OSMRβ were then determined by Northern blot analysis. The blot was photographed to demonstrate equal loading (bands of 28S RNA and 18S rRNA are shown) and then hybridized to ^{32}P-labeled gp130 and OSMRβ probes. The blots are representative of three independent experiments.

levels, whereas both TGFβ and IL-1 reduced the amount of gp130 transcripts.

In order to compare the regulation of OSMR in NBEC to other lung-derived epithelial cells which expressed different ratios of OSMRβ to gp130 mRNA, we extended our study to HTB58 and HTB55 cells. Treatment of both cell types with OSM led to stimulation of OSMRβ and gp130 expression, although in the case of HTB55 cells the effect of OSM was more evident in the presence of the glucocorticoid analog dexamethasone (DEX), a factor capable of intensifying the effect of cytokines on these cells [9–12]. DEX given alone significantly upregulated gp130 levels in HTB58 cells. When given in combination with cytokines, DEX generally increased the levels of gp130 mRNA and, to a lesser extent, OSMRβ mRNA in both cell types. IL-1 alone appeared not to affect the expression of OSMR but given in combination with OSM, or more markedly OSM and DEX, IL-1 further enhanced the upregulation of the mRNA levels for both components of the receptor in HTB58 and HTB55 cells. In contrast, the effect of TGFβ was found to be cell-specific. TGFβ generally acted as a negative regulator of OSMR expression in HTB58 cells and as a stimulator in HTB55 cells. Interestingly, in the HTB58 cells, the most substantial increase in OSMRβ and gp130 mRNA levels was noted when the cells were treated with a combination of TGFβ, OSM and DEX.

3.3. *OSM and LIF exert different effects on expression of type I and II receptors*

Since OSM significantly increased type II OSM receptor levels it was interesting to determine if it had any effect on LIFRβ and ultimately type I receptor. We found that treatment with OSM caused a reduction in the levels of LIFRβ transcripts in HTB58 cells (Fig. 3). We previously demonstrated that HTB58 cells have the potential to utilize type I receptor since LIF treatment induced tyrosine phosphorylation of gp130 and LIFRβ [12].

We therefore examined the effect of LIF on type I and II receptors in these cells. In the presence of DEX, LIF similarly to OSM, stimulated OSMRβ and to a lesser extent gp130 mRNA levels, but, in contrast to OSM, did not reduce LIFRβ mRNA levels (Fig. 4). The increase in OSMRβ and gp130 levels by LIF was also observed in NBEC (data not shown).

4. Discussion

Many of the overlapping biological effects of IL-6 related

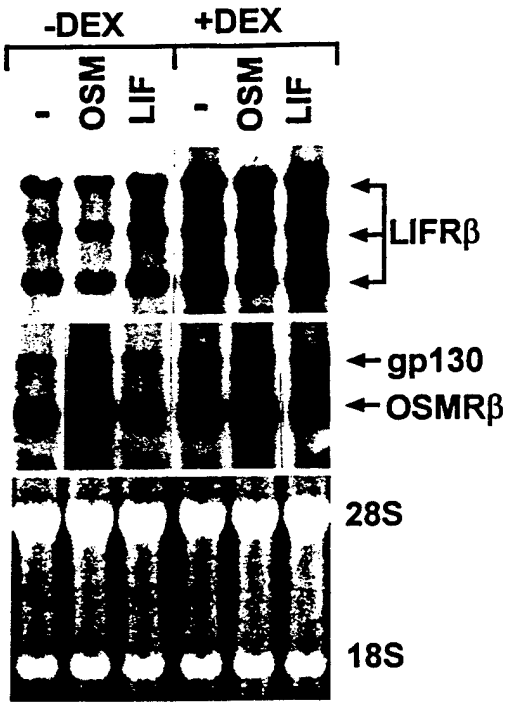

Fig. 4. Effect of OSM and LIF on expression of type I and II OSM receptors in HTB58 cells. Cells were incubated for 24 h with 50 ng/ml OSM, 10 U/ml LIF and/or 10^{-6} M DEX. The levels of LIFRβ, gp130 and OSMRβ were then determined by Northern blot analysis. The blot was photographed to demonstrate equal loading (bands of 28S RNA and 18S rRNA are shown) and then hybridized to ^{32}P-labeled LIFRβ probe. The same samples of RNA were used for parallel hybridization with gp130 and OSMRβ probes.

cytokines can be attributed to the presence of a common receptor subunit, gp130, or in the case of OSM and LIF, to binding of the same receptor consisting of two components, gp130 and LIFRβ [5]. However, significant functional differences between LIF and OSM have also been described and, some cell types are incapable of binding LIF in spite of expressing high affinity functional OSM receptors [8]. Taken together, these findings suggested the existence of an OSM-specific receptor. Recently, an alternative subunit, OSMRβ, of an OSM receptor complex was cloned [7]. This subunit associates with the low affinity OSM·gp130 complex to form a high affinity heterodimeric receptor that is capable of transducing OSM-specific signaling events. Although the signal transduction pathways that mediate OSM-specific responses have not yet been fully delineated, OSM can activate pathways linked to MAP kinase [8], *src*-related kinases [18] and more notably a JAK-STAT pathway that includes STAT3, STAT1 and STAT5 [5,19,20]. Interestingly, activation of STAT5 has been reported to be mediated by the type II OSM receptor [19]. The biological activities of OSM, some of which are OSM-specific and some of which are shared by LIF, can be explained by utilization of a dual receptor system. However, although a dual receptor system is evident in the human system, in mice OSM apparently utilizes only its specific receptor complex [21].

We previously demonstrated in the human system that several genes are specifically regulated by OSM but not other members of the IL-6 family of cytokines [9–12]. Our understanding of the specificity of the response to OSM and LIF will depend on identifying the role of the two potential receptors in mediating these responses. There are several major findings in this study: (i) lung-derived epithelial cells express both subunits of type II OSM receptor, i.e. gp130 and OSMRβ. (ii) OSMRβ mRNA levels appeared to be higher than gp130 in these cells, suggesting that the levels of gp130 may be the limiting factor in the formation of the type I and type II receptors. (iii) there is no consistent difference in the ratio of OSMRβ to gp130 between HepG2 (responsive to OSM, LIF and IL-6) and lung-derived epithelial cells (responsive only to OSM). (iv) OSMRβ and gp130 mRNA levels are regulated by specific mediators. (v) type II receptor is upregulated by either OSM or LIF whereas type I receptor is downregulated by OSM in HTB58 cells. The expression of OSMRβ and gp130 in all lung-derived epithelial cell types examined indicates that the type II OSM-specific receptor may mediate the effects of OSM on lung-derived epithelial cells. Furthermore, based on the high ratio of OSMRβ mRNA relative to gp130 mRNA levels, it is likely that there is an excess of OSMRβ produced that can successfully compete to form dimers with the gp130 subunit even if the gp130 is expressed in limited amounts and in the presence of LIFRβ.

The expression of OSM type II receptor in lung-derived epithelial cells was found to be regulated by cytokines, of which OSM was the most potent stimulator. The cytokines that regulated the steady state mRNA levels of the components of the OSM type II receptor are produced by activated monocytes and T cells and can be produced by infiltrating cells at sites of inflammation. Importantly, lung-derived epithelial cells are stimulated by OSM and IL-1 for the production of serine proteinase inhibitors, proteins which have been postulated to prevent tissue damage associated with inflammation [9–12]. The additive effects of OSM and IL-1 on synthesis of these inhibitors can be explained by the present study showing the role of both cytokines in upregulation of OSMR expression. Moreover, since TGFβ was also found to regulate OSMR levels it may also act as a modulator of OSM effects in lung-derived epithelial cells. However, such a role of TGFβ remains to be determined.

The finding that LIF was capable of modulating type II receptor levels in HTB58 and NBEC further supports the concept that the inability of LIF to induce α_1-PI and ACH synthesis in these cells is not due to the lack of functional type I receptors. Different effects of OSM and LIF resulting in downregulation of LIFRβ by OSM and upregulation of OSMRβ by both cytokines may suggest that in the presence of both OSM and LIF preference will be given to OSM due to the preferential increase in type II OSM receptors.

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CD40-mediated Signal Transduction in Human Airway Smooth Muscle[1]

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Running Title: CD40 mediated signal transduction in smooth muscle

ABSTRACT

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 CD40L can lead to upregulation of costimulatory and cell adhesion molecules, as well as secretion of pro-inflammatory cytokines. Since airway inflammation potentially involves cell-cell interactions of T cells and eosinophils (which express CD40L) 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 interferon-γ. Cross-linking CD40 on ASM resulted in enhanced IL-6 secretion and an increase in intracellular calcium concentrations, which was 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.

CD40 is a 50 kDa glycoprotein that is a member of the TNF receptor family, that also includes the TNF-receptor, nerve growth factor-receptor and Fas. CD40 was initially identified on the surface of B cells (reviewed in (1)), where it binds to a surface-expressed ligand (CD40L) on activated CD4+ T lymphocytes (2-5) and plays a critical role in B cell activation and isotype switching (1). Induction of several signaling events has been described in B cells following crosslinking of CD40, including the demonstration that treatment of B cells with anti-CD40 antibodies resulted in the phosphorylation of intracellular proteins on tyrosine. Since CD40 itself does not have intrinsic kinase activity (6), these results indicated that the receptor may be linked via adaptor proteins to cytoplasmic protein tyrosine kinases (PTK)[3]. Subsequent studies demonstrated that CD40 cross-linking induced increased kinase activity of the src family PTK, lyn (7;8). In addition, ligation of CD40 induced activation of the serine/threonine kinases cJun kinase (9-13) and ERK (9;14), as well as PI3-kinase (7;15;16) and ras (15). Ligation of CD40 on murine B cells also resulted in an increase in intracellular calcium concentration, which was sensitive to FK506, an immunosuppressive agent which inhibits calcineurin (17).

CD40 is also constitutively expressed on a variety of other leukocytes such as macrophages (18), dendritic cells (19) and eosinophils (20), and on the surface of cells of mesenchymal and epithelial origin such as endothelial cells (21-23), fibroblasts (24;25), keratinocytes (26;27) and vascular smooth muscle cells (28). On mesenchymal cells, where CD40 is inducible by cytokines such as IFNγ, engagement of CD40 has been reported to lead

to upregulation of costimulatory and cell adhesion molecules (22-24), secretion of pro-inflammatory cytokines (24;25;27-29) and effects on cellular proliferation (24;26), although the mechanisms of such signaling are currently unknown. These data support a widespread role for CD40 in the inflammatory response.

We are interested in the potential role of leukocyte-smooth muscle cell interactions in the context of airway inflammation. We previously demonstrated that activated T cell-derived cytokines upregulated expression of HLA-DR and ICAM-1 on ASM (30) and that the exposure to the inflammatory cytokine TNFα augmented the sensitivity of ASM to a variety of contractile agonists (31;32). In addition to soluble mediators, however, we previously showed that activated T cells adhered to ASM via integrins and CD44 and induced ASM DNA synthesis in a contact dependent manner (33). These data suggest that T cell-ASM interactions likely play a potentially important role in cellular recruitment and airway inflammation in diseases such as asthma.

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: 5C3 (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 Inc. (Lake Placid, NY). TNFα was purchased from Boehringer Mannheim (Indianapolis, IN); IFNγ 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 the University of Pennsylvania. 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 24-72 hours. The monolayers were washed and then single cell suspensions were prepared using 5 mM EDTA. Cells were stained with antibodies specific for human CD40, followed by FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Westgrove, PA), and analyzed using a FACScan (Becton Dickinson, San Jose, CA) and CellQuest software.

IL-6 determination: Confluent ASM were treated with either TNFα or IFNγ. After 72 hours, 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 hours, 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 hours, 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, 1 mM Na_3VO_4 for 10 minutes at 4°C. Post-nuclear extracts were obtained by centrifugation of lysates at 14,000xg for 10 minutes. Equivalent amounts of protein, as determined by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL), were resolved on an 8% SDS-polyacrylamide gel under reducing conditions, transferred to polyvinylidene difluoride membranes and blocked in a solution containing 30 mM Tris pH 7.6, 75 mM NaCl and 3% BSA. Membranes were immunoblotted with anti-phosphotyrosine for 1 hour at $4^{o}C$, followed by rabbit anti-mouse IgG (Jackson ImmunoResearch). Bound antibody was detected using ^{125}I-protein A (DuPont NEN,

Boston, MA) and visualized by autoradiography.

Cytosolic calcium measurements. ASM cells were plated at low density onto 15 mm coverslips 3-5 days before the experiments were performed. All experiments were performed using subconfluent cells between 3rd-5th passage. Cells were loaded with 2.5 mM fura-2/AM (in HEPES buffer containing 137.5 mM NaCl, 1.25 mM $CaCl_2$, 1.25 mM $MgCl_2$, 0.4 mM $NaHPO_4$, 6 mM KCl, 5.6 mM glucose, supplemented with 1 mg/ml BSA) for 30 minutes 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 40X (oil) fluorescence objective lens. Excitation energy was switched between 340 and 380 nm wavelength using a 75 watt 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 analog-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 treating cells with ionomycin (10 mM) in the presence of 12 mM calcium to measure R_{max}, or by adding a stoichiometric excess of EGTA to achieve R_{min}. Values used for the calibration equation were R_{min}=0.3 and R_{max}=0.6, K_d=224 and $f380_{min}/f380_{max}$=5. Antibodies or soluble ligand were added directly to the bath. Bradykinin (1 mM, Sigma Chemical Co., St. Louis,

MO) was added to compare the magnitude of bradykinin-induced calcium transients with those evoked by CD40. $NiCl_2$ (4 mM, Fisher Scientific, Springfield, NJ) or $MnCl_2$ (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, Gibco BRL, Grand Island, NY) for 1 hour or herbimycin (10 μM, Sigma) for 4 hours at 37°C prior to stimulation with anti-CD40.

Nuclear extracts and mobility shift assay. Nuclear extracts were prepared according to the method of Andrews and Faller (38). Confluent ASM were treated with IFNγ (500 U/ml) for 72 hours, then stimulated with CD40LT (10 μg/ml) for the indicated times. Cells were harvested by scraping into cold PBS. Nuclei were isolated by treatement with hypotonic lysis buffer A containing 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin and 0.5% NP-40. The nuclear pellet was resuspended in buffer B (420 mM NaCl, 20 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin) for 20 minutes on ice and clarified by centrifugation at 13,000xg. The resulting supernatants contained 1-2 mg/ml protein by the BCA assay. Nuclear extracts were stored at -80°C.

A double-stranded oligonucleotide probe containing the NF-κB consensus sequence (Promega Corp., Madison, WI) was end-labeled with γ-^{32}P-ATP using T4 kinase (Gibco BRL). Eight μg of nuclear extract were incubated with 1 μg poly(dI-dC) and radiolabeled probe for 30 minutes at room temperature. DNA-protein complexes were resolved by

electrophoresis on a 4% non-denaturing polyacrylamide gel at 200V. The gels were then dried and exposed to X-ray film for autoradiography. In supershift experiments, nuclear extracts were preincubated with polyclonal goat antibodies specific for p50, p65 or c-Rel (Santa Cruz Biotechnology, Santa Cruz, CA) for 15 minutes on ice prior to the addition of ^{32}P-labeled probe.

RESULTS

CD40 is expressed on cultured ASM and upregulated by TNFα and IFNγ.

To determine if 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 antibodies and analyzed by flow cytometry. Low constitutive expression of CD40 was detected compared to control ASM reacted with an isotype matched control mAb (Figure 1A). Treatment of ASM with either TNFα or IFNγ induced a 2-4-fold increase in CD40 expression (Figure 1A) and the effect of TNFα and IFNγ together was at least additive, inducing a 7-fold increase in CD40 expression (data not shown). In contrast, IL-4 had no effect or resulted in a slight decrease in CD40 expression compared to baseline expression (Figure 1A) but had no effect on TNFα or IFNγ-induced upregulation of CD40 (data not shown). Maximal expression of CD40 occurred on day 2 following treatment with TNFα and on day 3 following IFNγ stimulation (Figure 1B, top) and was dose dependent (Figure 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% (Figure 2). Stimulation with TNFα increased IL-6 secretion by approximately 2.7-fold over baseline values; CD40LT increased TNFα-induced IL-6 production by 26±10%. IFNγ alone had minimal effects on IL-6 production by ASM, but acted synergistically with CD40LT, increasing IFNγ-induced IL-6 secretion by approximately 73±12% compared to IFNγ alone (Figure 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 (Figure 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 antibodies (Figure 3B). Similar results were obtained when cells were stimulated with anti-CD40 (Figure 3C), where 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 (Figure 3C). The

specificity of the response is evidenced by the lack of effect of an isotype-matched binding anti-ICAM-1 control antibody (Figure 3D). The addition of $NiCl_2$ prior to antibody cross-linking abolished the CD40-induced signal (Figure 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 $MnCl_2$ (data not shown). This differs from the response to bradykinin (Figure 4), which was previously shown to be due to release of intracellular calcium stores (39;40). Finally, pretreatment of the ASM with the tyrosine kinase inhibitors genistein (Figure 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 protein tyrosine kinases. Genistein had no effect, however, on the bradykinin-evoked calcium response (Figure 5).

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 crosslinking. It was previously demonstrated 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 response described above indicated that CD40 may be coupled to protein tyrosine kinase signal transduction pathways in ASM. To directly assess whether CD40 mediated activation of PTKs, cells were made quiescent by culturing in serum-free media for 24 hours, then stimulated with anti-CD40 mAb

for the indicated times. Protein tyrosine phosphorylation of cellular proteins was detected by immunoblotting with anti-phosphotyrosine antibodies. Tyrosine phosphorylated species with Mr of 40-50kD were detected at 10-15 minutes following stimulation (Figure 6). Phosphorylation of these proteins was not seen when smooth muscle cells were incubated with an isotype-matched control antibody (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 TNFα, IL-1β, IL-6 and IL-8. To test whether CD40 could induce activation of NF-κB in ASM, cells were pretreated with IFNγ for 72 hours, then stimulated with soluble CD40LT. Two NF-κB-DNA binding complexes were observed (Figure 7A). An inducible NF-κB activity was seen as early as 15 minutes following exposure to CD40LT, which peaked at approximately 30 minutes, then decreased. In comparison, TNFα-induced a co-migrating NF-κB complex more rapidly and the presence of this complex was sustained over the same timecourse (data not shown). A second complex was constitutively present and unaffected by treatment with either CD40LT or TNFα. Formation of the inducible complex was inhibited by excess cold oligonucleotide (Figure 7B) Cells that were stimulated with CD40L, in the absence of IFN-γ pretreatment,

exhibited the constitutive but not the inducible NF-kB complex (data not shown). To determine the subunit composition of these complexes, nuclear extracts were incubated with specfic subunit antibodies. Anti-p65/RelA abrogated formation of the inducible complex, but had no effect on the constitutive complex (Figure 7B). Anti-p50 had a partial inhibitory effect on formation of the inducible NF-kB complex (Figure 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 pro-inflammatory effects of CD40, including activation of NF-κB and IL-6. ASM cells were treated with IFN-γ for 72 hours, then placed in serum free media containing 2 mM calcium. The cells were treated for 15 minutes with 4 mM $NiCl_2$ prior to being stimulated with CD40LT. Pretreatment with $NiCl_2$ completely abrogated the ability of CD40LT to activate the inducible NF-κB complex, but had no effect on the constitutive complex (Figure 8A). $NiCl_2$ alone had no demonstrable effect on NF-κB activation. In addition, pretreatment with $NiCl_2$ inhibited

CD40-induced increases in IL-6 secretion by ASM, but had minimal effects on basal secretion (Figure 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 has also been recognized that inflammatory cytokines such as TNFα, 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 recently reported the upregulation of the cell adhesion molecules ICAM-1 and VCAM-1 on TNFα-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 which 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 pleiotropic inflammatory cytokine IL-6. CD40LT induced a significant enhancement of IL-6 secretion by both unstimulated and TNFα- and IFNγ-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 upregulate 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 physiological and pathophysiologic significance since intracellular calcium is an essential second messenger regulating smooth muscle cell contractility (reviewed in (46)). Previous data regarding CD40-induced calcium mobilization in B cells has been variable and has not been investigated in other cell types that we are aware of. 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 (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 $NiCl_2$ or $MnCl_2$, 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 T cell-receptor-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 JNK 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 completely inhibited 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 completely abolished 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 protein tyrosine kinase pathways.

Data derived from transformed B cell lines or resting or activated normal B cells suggests that engagement of CD40 leads to both PTK and serine/threonine kinase activation (7;8;47;48;56). Little is known, however, about CD40 coupling to PTK signaling pathways in non-B cells. Gaspari et al. (27) described tyrosine phosphorylation of a single 50kD species in keratinocytes stimulated with anti-CD40 antibodies. **We now demonostrate 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 IFNγ. 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 functionally expressed 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 physiological important consequences of CD40 engagement, and in understanding the molecular events that regulate signaling by CD40.

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FOOTNOTES

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

4. KEY WORDS: inflammation, stromal cells, signal transduction

FIGURE LEGENDS

1. *Cytokine induction of CD40 on ASM.* A) ASM were incubated in media alone (thin line), or stimulated with TNFα (1000 U/ml, bold line), IFNγ (1000 U/ml, dotted line) or IL-4 (20 ng/ml, broken line). After 48 hours, cells were stained with antibodies to CD40 and analyzed by flow cytometry. 1B. (top) ASM were stimulated with TNFα (1000 U/ml) for 1-3 days and stained with anti-CD40 as described. (bottom) ASM were stimulated with increasing doses of TNFα for 48 hours then stained with anti-CD40.

2. *CD40 enhances IL-6 production by ASM.* ASM were incubated in media alone or stimulated with TNFα (250 U/ml) or IFNγ (500 U/ml). After 72 hours, 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 hours 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 standard error bars are not apparent, the errors were less than 3%. *p<.01 compared to media alone, **p<.05 compared to TNFα alone, ***p<.005 compared to IFNγ alone.**

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) is similar to that seen following stimulation with CD40LT (n=4). D) In response to an isotype-matched binding (ICAM-1) control antibody, there is no change in intracellular free calcium (n=4).

4. *Extracellular calcium is required for CD40-induced calcium transients.* The addition of $NiCl_2$ (4 mM) alone does not cause a change in intracellular free calcium. The subsequent additon 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).

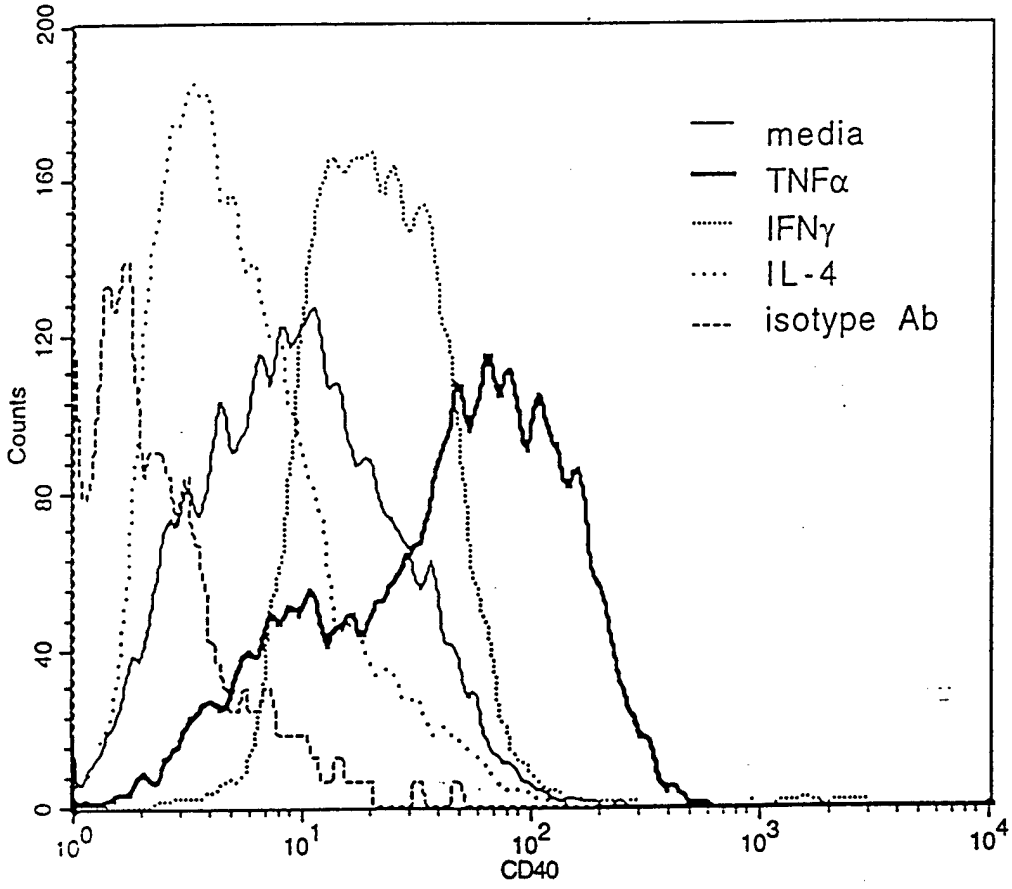
5. *Inhibition of PTK activation blocks CD40-, but not bradykinin-induced increases in cytosolic calcium.* ASM was pretreated with genistein (5 μM) for 1 hour, and then treated with anti-CD40 (10 μg/ml) followed by bradykinin (1 mM) (n=3).

6. *CD40-induced tyrosine phosphorylation.* ASM cells were treated with anti-CD40 mAb (2 μg/ml) for the indicated times. Cell lysates (20 μg/lane) were electrophoresed by SDS-PAGE, transferred to PVDF membrane and analyzed by immunoblotting with anti-phosphotyrosine as described. Arrowheads indicate phosphorylated proteins. These results

are representative of 6 experiments.

7. *CD40 activates NF-κB in ASM.* A) Kinetics of NF-κB activation. ASM cells were pretreated with IFN-γ (500 U/ml) for 72 hours then stimulated with media alone or with CD40LT (10 μg/ml) for the indicated times. Nuclear extracts were prepared as described in the Materials and Methods (n=4). B) Competition and supershift analysis. Nuclear extracts prepared from CD40LT-treated (10 μg/ml, 30 minutes) ASM were incubated with the indicated molar excess of unlabeled competitor oligonucleotide, or with antibodies specific for p50, p65 or c-Rel prior to the assay for NF-κB binding activity.

8. *Extracellular calcium is required for NF-κB activation and IL-6 secretion.* A) ASM cells were pretreated with IFN-γ (500 U/ml) for 72 hours then placed in HBSS containing 1% BSA and 2 mM calcium. Cells were pretreated with 4mM $NiCl_2$ for 15 minutes prior to stimulation with CD40LT. Nuclear extracts were prepared after 30 minutes of stimulation. B) ASM cells were treated as above with IFN-γ (500 U/ml), then pretreated with 2mM $NiCl_2$ for 15 minutes in HBSS containing 1% BSA. Cells were then stimulated with CD40LT (10 μg/ml) in the presence of $NiCl_2$ and culture supernatants were collected after 24 hours. The data shown are representative of two similar experiments.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160
120
80
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IFNγ
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10^4
CD40

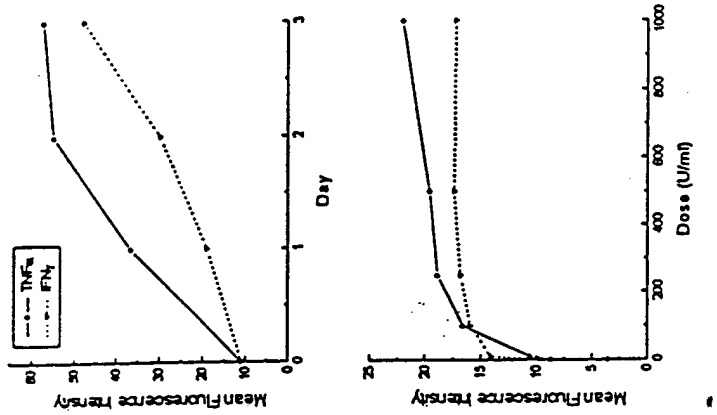
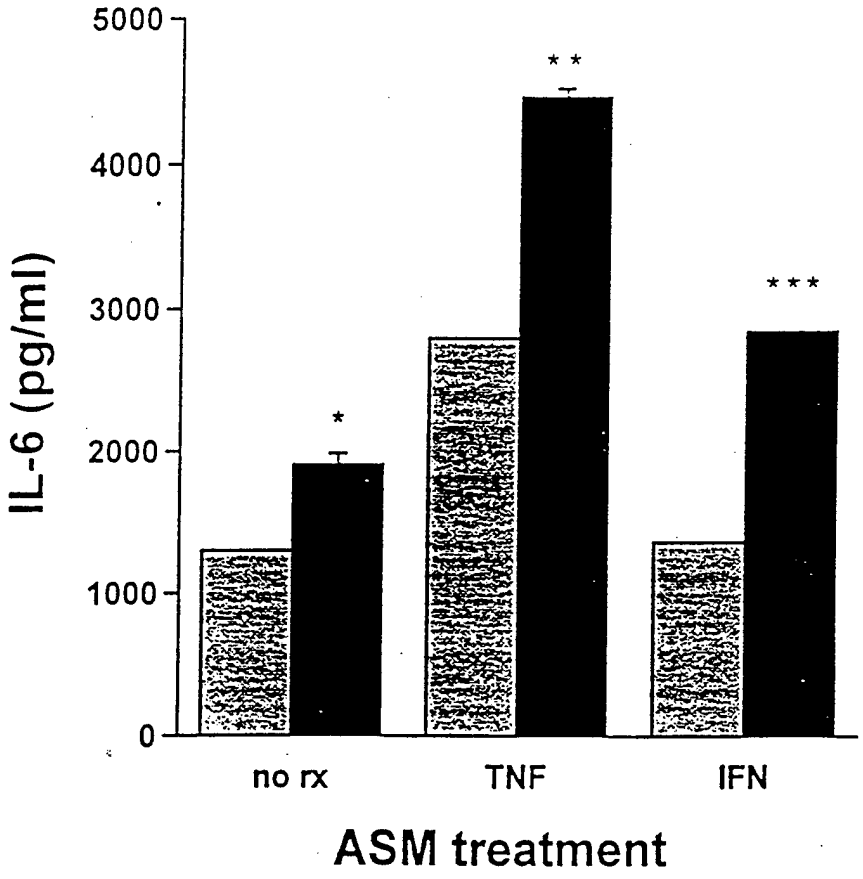

Mean Fluorescence Intensity
Day
TNFα
IFNγ
Mean Fluorescence Intensity
Dose (U/ml)

Fig 1B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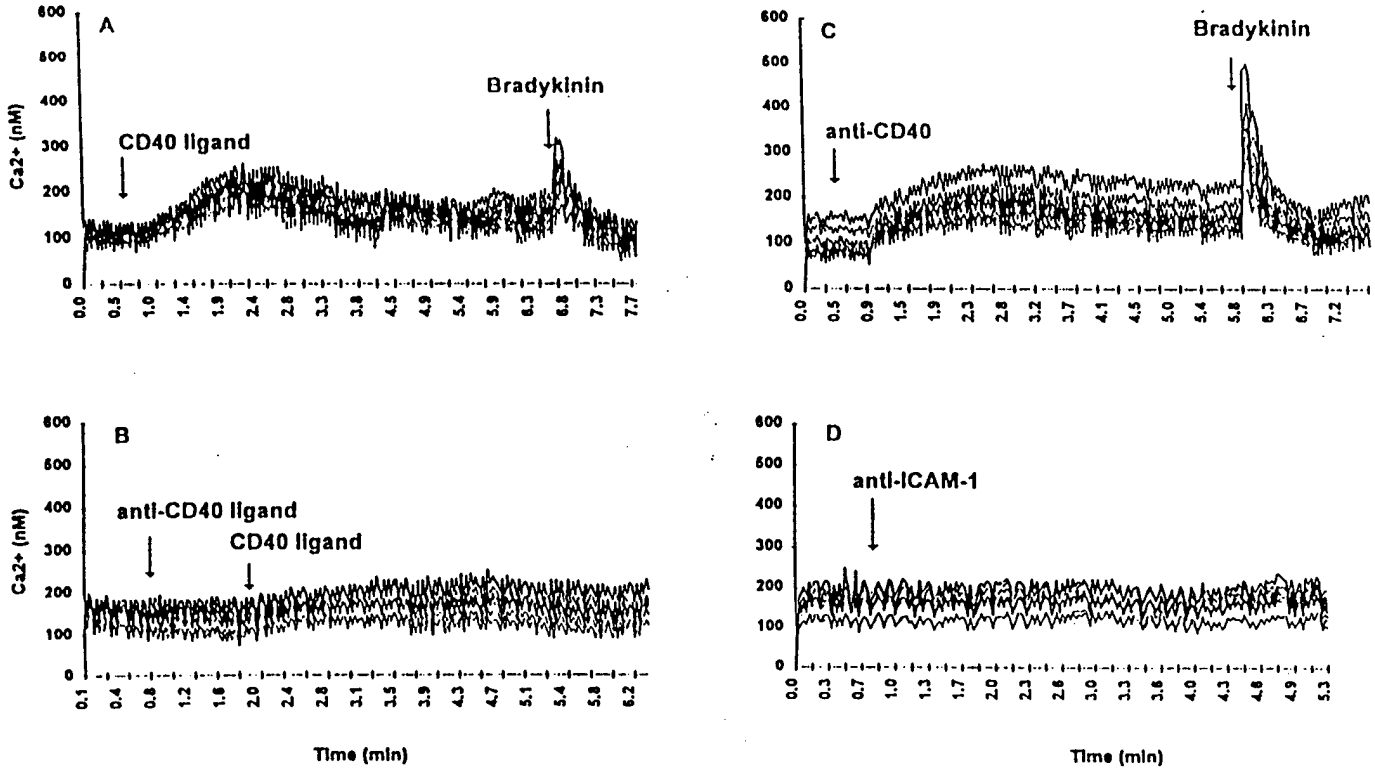
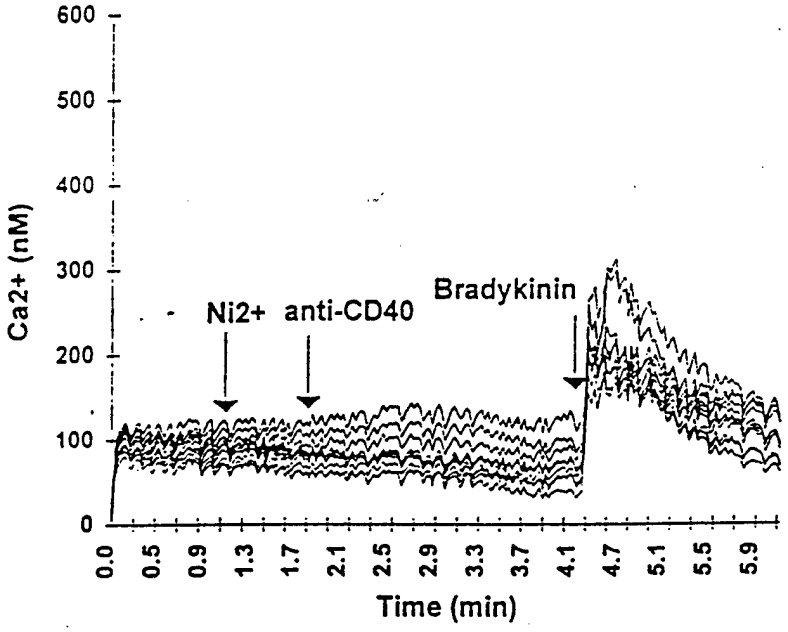
4000
3000
2000
1000
0
IL-6 (pg/ml)
*
* *
* * *
no rx
TNF
IFN
ASM treatment


A
CD40 ligand
Bradykinin
Ca2+ (nM)
B
anti-CD40 ligand
CD40 ligand
Time (min)
C
anti-CD40
Bradykinin
D
anti-ICAM-1
Time (min)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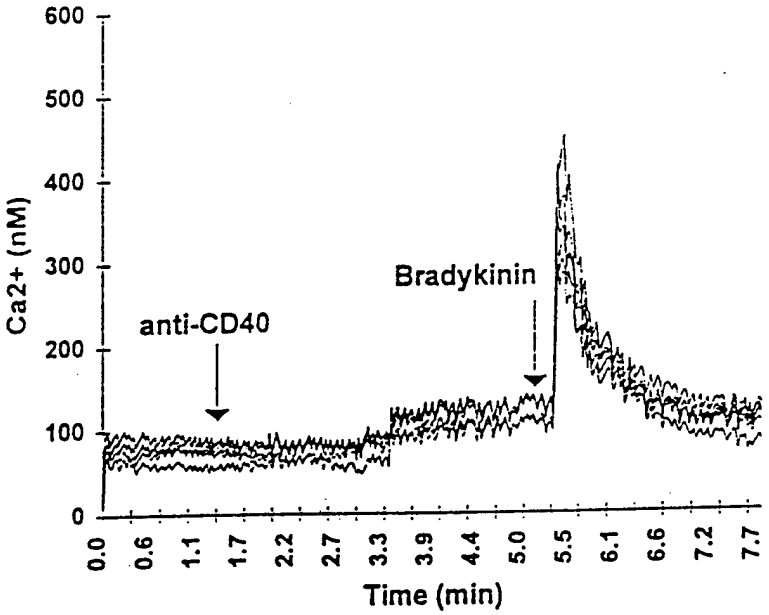
500
400
300
200
100
0
Ca2+ (nM)
Ni2+
anti-CD40
Bradykinin
0.0
0.5
0.9
1.3
1.7
2.1
2.5
2.9
3.3
3.7
4.1
4.7
5.1
5.5
5.9
Time (min)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500
400
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200
100
0
Ca2+ (nM)
anti-CD40
Bradykinin
0.0
0.6
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1.7
2.2
2.7
3.3
3.9
4.4
5.0
5.5
6.1
6.6
7.2
7.7
Time (min)

Fig 5

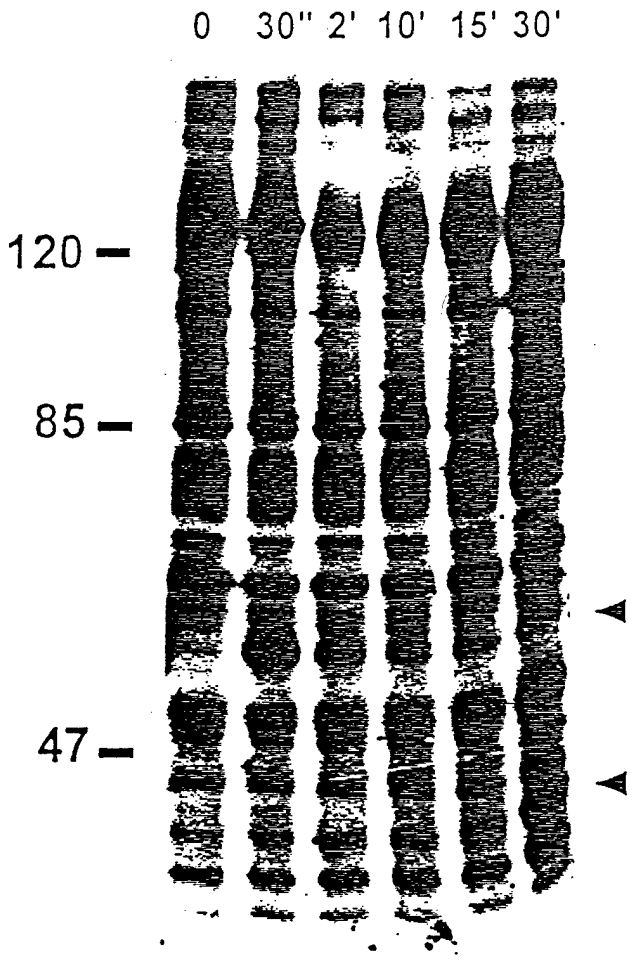


Fig 6

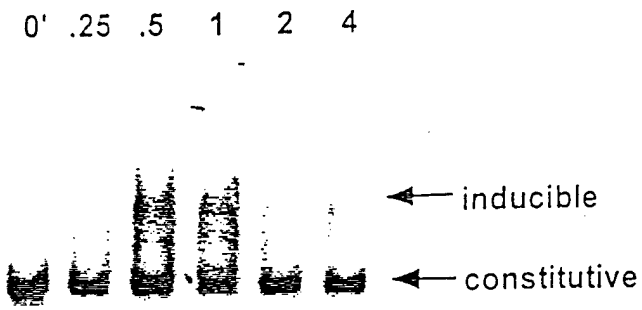
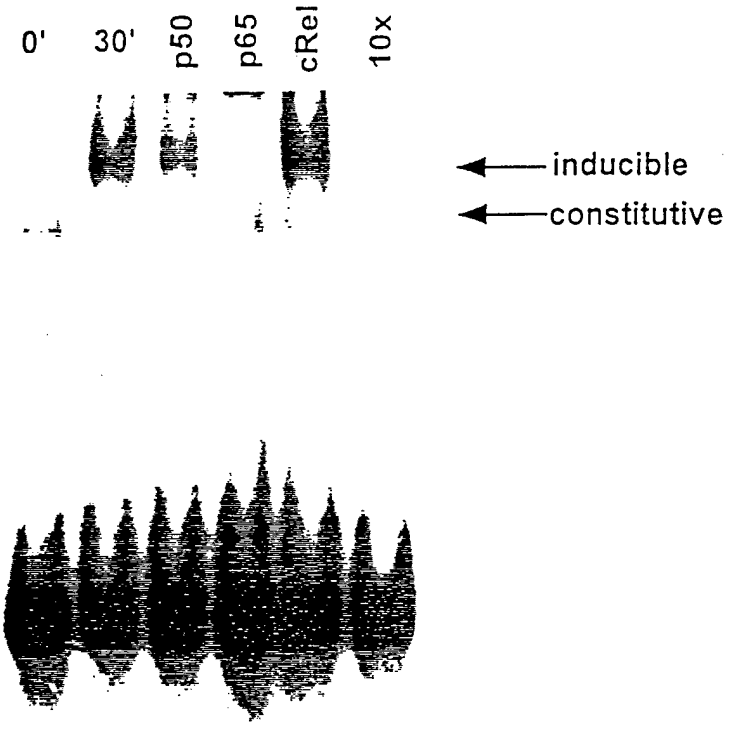
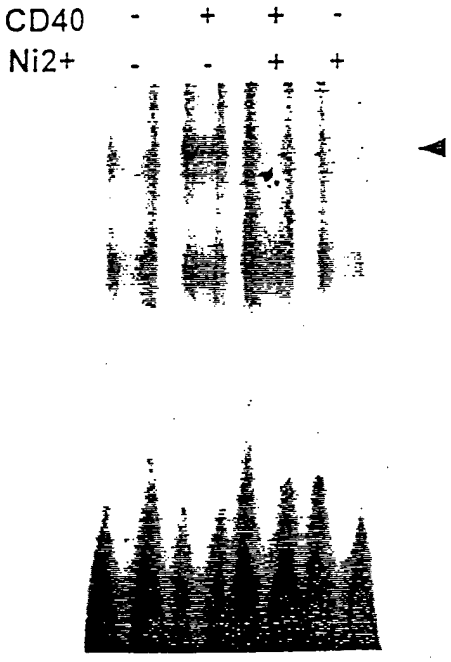


Fig 7A


0'
30'
p50
p65
cRel
10x
inducible
constitutive


CD40 - + + -
Ni2+ - - + +

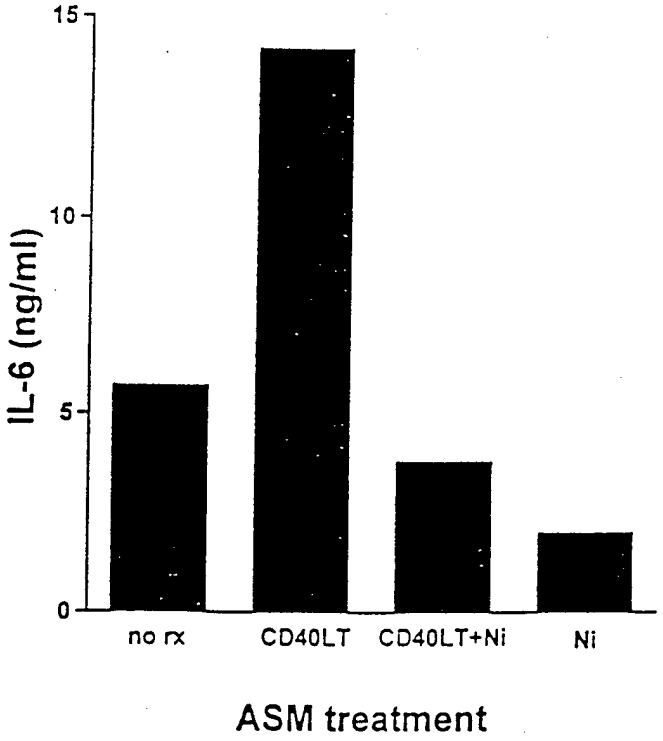


Fig 8B