Role of epithelial CD40 in airway inflammation.

Stacie Marie Propst

University of Alabama at Birmingham

Follow this and additional works at: https://digitalcommons.library.uab.edu/etd-collection

Recommended Citation

This content has been accepted for inclusion by an authorized administrator of the UAB Digital Commons, and is provided as a free open access item. All inquiries regarding this item or the UAB Digital Commons should be directed to the UAB Libraries Office of Scholarly Communication.
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
ROLE OF EPITHELIAL CD40 IN AIRWAY INFLAMMATION

by

STACIE M. PROPST

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Doctor of Physiology

BIRMINGHAM, ALABAMA

2000
CD40 is a transmembrane receptor that was initially discovered on hematopoietic cells and was later described on nonhematopoietic cells. The natural ligand for CD40 (CD40L) is also a transmembrane receptor and is found primarily on activated T cells, but can be expressed by mast cells, eosinophils, basophils, dendritic cells, and monocytes. CD40-CD40L interactions modulate a wide variety of cellular activities such as proliferation, differentiation, programmed cell death (apoptosis), and gene regulation. In this dissertation, we examined the expression of CD40 on the surface of airway epithelia and investigated whether triggering CD40 on these cells modulated cellular gene expression. If so, CD40 could potentially play a role in activating airway epithelial cells during an inflammatory response. CD40 cross-linking by soluble CD40L (sCD40L) up-regulates the expression of proinflammatory cytokines such as chemokines and adhesion molecules in various cell systems, including endothelium, B lymphocytes, and smooth muscle. We demonstrated that airway epithelial cells express CD40 on their surface and that these cells increased their production of inflammatory mediators when CD40 was triggered by sCD40L. Specifically, cross-linking CD40 with sCD40L induced the expression of the chemokines regulated on RANTES, IL-8, and monocyte chemoattractant protein-1 (MCP-1). This chemokine expression could be modified through costimulation with IL-1β and/or TNF-α. We also showed that CD40
engagement increased the expression of an adhesion molecule, intercellular adhesion molecule-1 (ICAM-1), on airway epithelial cells, but neither IL-1β nor TNF-α modified this expression. Next, we investigated the mechanism by which CD40 alters production of the chemokine, RANTES, in airway epithelial cells.

As a member of the TNF receptor (TNFR) family, CD40 lacks intrinsic enzymatic activity and, therefore, employs signaling adapter molecules called TNF receptor (TNFR)-associated factors (TRAFs). When CD40 is triggered and receptor oligomerization occurs, TRAFs are recruited to the membrane where they bind to specific motifs in the cytoplasmic domain of CD40. Subsequently, a cascade of downstream kinases activate transcription factors that can modulate gene expression in the nucleus. We have demonstrated that, in airway epithelial cells, the transcription factor, nuclear factor-κB (NFκB), is activated upon CD40 engagement and likely participates in the up-regulation of RANTES. These studies suggest that CD40 expressed by epithelial cells in the lung can respond to CD40L⁺ cells trafficking into or out of the airway during inflammation. Such interactions could initiate and/or amplify an inflammatory response resulting from infection or injury by activating epithelial cells to express proinflammatory mediators such as chemokines and adhesion molecules. Chronic inflammation is a hallmark of many airway diseases, and understanding the role of CD40 expression by epithelia may provide a novel approach to efficacious treatments in humans.
DEDICATION

This is dedicated to my mother, Brenda Ford, who loves all of her children unconditionally and would make each of our dreams come true if it were in her power to do so. Thanks Mom.
ACKNOWLEDGEMENTS

Most importantly, I want to thank Dr. Lisa M. Schwiebert for her excellent guidance and her friendship. I could not have asked for a better mentor, and I take special pride in being her first student. I am always impressed by her focus, and I hope that I can achieve as many things as she has. I would also like to thank my committee members, Drs. Etty Benveniste, Erik Schwiebert, Dan Bullard, Robert Jackson, and Robert Carter, for their various contributions to my project. Special thanks to the Chairman of Physiology, Dr. Dale Benos, for asking me to give science another chance and for introducing me to Lisa.

In our laboratory we have an unusual comraderie, and I would like to thank the people who have been a part of that. First and foremost, I must thank Kim Estell, who helped me in so many ways that I cannot enumerate them here. Suffice it to say that Kim keeps our laboratory running. Thanks to Raquia Denson and Emily Rothstein, who each had a hand in the development of this dissertation. Thanks also go to Dr. Jim Collawn for constructive criticisms of my writing and data presentation. Finally, I would like to thank my lifelong friend, Julie Byers Moss, for being my touchstone.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Airway epithelial cells</td>
<td>1</td>
</tr>
<tr>
<td>CD40-CD40L</td>
<td>4</td>
</tr>
<tr>
<td>CD40 Signaling Mechanisms</td>
<td>11</td>
</tr>
<tr>
<td>Proinflammatory Cytokines</td>
<td>18</td>
</tr>
<tr>
<td>TNF-α</td>
<td>18</td>
</tr>
<tr>
<td>IL-1β</td>
<td>21</td>
</tr>
<tr>
<td>Leukocytic Chemotaxis and Transmigration</td>
<td>23</td>
</tr>
<tr>
<td>Chemokines: RANTES, IL-8, MCP-1</td>
<td>27</td>
</tr>
<tr>
<td>Adhesion Molecules: ICAM-1 and VCAM-1</td>
<td>30</td>
</tr>
<tr>
<td>Th1/Th2 Immunity and CD40</td>
<td>32</td>
</tr>
<tr>
<td>Dissertation Objectives</td>
<td>34</td>
</tr>
<tr>
<td>PROINFLAMMATORY AND TH2-DERIVED CYTOKINES MODULATE CD40-MEDIATED EXPRESSION OF INFLAMMATORY MEDIATORS IN AIRWAY EPITHELIA: IMPLICATIONS FOR THE ROLE OF EPITHELIAL CD40 IN AIRWAY INFLAMMATION</td>
<td>36</td>
</tr>
<tr>
<td>TRAF3 ENHANCES CD40-MEDIATED SIGNALING IN AIRWAY EPITHELIAL CELLS</td>
<td>65</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>95</td>
</tr>
<tr>
<td>Summary of Results</td>
<td>95</td>
</tr>
<tr>
<td>Future Studies</td>
<td>102</td>
</tr>
</tbody>
</table>

**Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.**
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS (Continued)</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>GENERAL REFERENCES ...................................................................</td>
<td>107</td>
</tr>
<tr>
<td>APPENDIX: ANIMAL USE APPROVAL FORM</td>
<td>121</td>
</tr>
</tbody>
</table>

viii
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

INTRODUCTION

1 Examples of chemokines and the cells that they attract ........................................28
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Schematic representation of members of the TNFR family</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Schematic representation of members of the TNF family</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Putative signaling pathways for CD40 in a B lymphocyte</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Putative signaling pathways for CD40 in a B lymphocyte</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>Schematic representation of TRAF molecules</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>Predicted association of TRAFs 2, 3, 5, and 6 with CD40</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>Model of leukocyte chemotaxis and migration</td>
<td>25</td>
</tr>
</tbody>
</table>

**PROINFLAMMATORY AND TH2-DERIVED CYTOKINES MODULATE CD40-MEDIATED EXPRESSION IN AIRWAY EPITHELIA: IMPLICATIONS FOR THE ROLE OF EPITHELIAL CD40 IN AIRWAY INFLAMMATION**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Airway epithelial cells express CD40 in vivo</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Airway epithelial cells express CD40 in vivo</td>
<td>44</td>
</tr>
<tr>
<td>2</td>
<td>Airway epithelial cells express CD40 surface protein</td>
<td>46</td>
</tr>
<tr>
<td>3</td>
<td>CD40 engagement induces chemokine expression in airway epithelial cells</td>
<td>48</td>
</tr>
<tr>
<td>4</td>
<td>CD40 engagement induces adhesion molecule expression in airway epithelial cells</td>
<td>49</td>
</tr>
<tr>
<td>5</td>
<td>TNF-α and/or IL-1β enhance CD40-mediated effects on chemokine expression</td>
<td>52</td>
</tr>
<tr>
<td>6</td>
<td>CD40 ligation enhances the sensitivity of airway epithelial cells to the effects of TNF-α and IL-1β with regard to IL-8 expression</td>
<td>54</td>
</tr>
<tr>
<td>7</td>
<td>Ligation of CD40 does not prime airway epithelial cells to respond to TNF-α or IL-1β</td>
<td>55</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>TNF-α, IL-1β, IL-4, and IL-13 have no effect on CD40-mediated adhesion molecule expression</td>
<td>56</td>
</tr>
<tr>
<td>9</td>
<td>CD40 ligation enhances the sensitivity of, but does not prime, airway epithelial cells to the effects of TNF-α with regard to VCAM-1 expression</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td><strong>TRAF3 ENHANCES CD40-MEDIATED SIGNALING IN AIRWAY EPITHELIAL CELLS</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Maps of the TRAF and RANTES-luc promoter constructs</td>
<td>72</td>
</tr>
<tr>
<td>2</td>
<td>Airway epithelial cells express TRAF2 and TRAF3</td>
<td>75</td>
</tr>
<tr>
<td>3</td>
<td>TRAF molecules modulate CD40-mediated RANTES promoter activation in airway epithelial cells</td>
<td>77</td>
</tr>
<tr>
<td>4</td>
<td>Ligation of CD40 activates the RANTES promoter via an NF-κB-dependent mechanism</td>
<td>79</td>
</tr>
<tr>
<td>5</td>
<td>IKK-α is necessary for CD40 activation of the RANTES promoter</td>
<td>81</td>
</tr>
<tr>
<td>6</td>
<td>IKK-β is necessary for CD40 activation of the RANTES promoter</td>
<td>82</td>
</tr>
<tr>
<td>7</td>
<td>CD40 ligation on airway epithelial cells enhances phosphorylation of IκB-α</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td><strong>SUMMARY</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Potential role of epithelial CD40 in airway inflammatory responses</td>
<td>97</td>
</tr>
<tr>
<td>2</td>
<td>Model of CD40-mediated signaling pathways and potential cross-talk with cytokine receptors</td>
<td>106</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

Ab      antibody
APC     antigen-presenting cell
ARDS    acute respiratory syndrome
BAL     bronchoalveolar lavage fluid
BALT    bronchus-associated lymphoid tissue
ERK     extracellular signal-regulated kinase
GAS     IFN-γ-activated sequence
GM-CSF  granulocyte-macrophage-colony stimulating factor
HEK     human embryonic kidney
HIGM    hyper-IgM syndrome
Ig      immunoglobulin
IkB     inhibitor of NF-κB
IKK     IkB kinase
ICAM    intercellular adhesion molecule
IEL     intraepithelial lymphocyte
IFN-γ   interferon-gamma
IL-      interleukin
ISRE    IFN-γ-stimulated regulatory element
Jak     Janus kinase
JNK     cJun N-terminal kinase

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAD</td>
<td>leukocyte adhesion deficiency</td>
</tr>
<tr>
<td>LFA</td>
<td>lymphocyte function-associated antigen</td>
</tr>
<tr>
<td>LNFRG</td>
<td>low-affinity nerve factor growth receptor</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>lymphotoxin</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP</td>
<td>monocyte chemoattractant protein</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-kappa B</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB-inducing kinase</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>PTK</td>
<td>protein tyrosine kinase</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated on activation, normal T expressed and secreted</td>
</tr>
<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
</tr>
<tr>
<td>sCD40L</td>
<td>soluble CD40 ligand</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>tumor necrosis factor receptor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>TRAF</td>
<td>tumor necrosis factor receptor-associated factor</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
</tr>
</tbody>
</table>
INTRODUCTION

The research presented herein focuses on the idea that epithelial cells lining the airway participate actively in lung immunity. In recent years, the immunogenic potential of epithelial cells has been recognized, and this project investigated the expression and function of a transmembrane receptor, CD40, in airway epithelial cells. CD40 was first identified as a B cell receptor (1, 2) and was later discovered to play a critical role in the development of immune responses (3-5). Consequently, the majority of studies involving CD40 have focused on its functions during immune cell interactions. However, in the airway, epithelial cells are the most abundant cells as well as the first point of contact for many inhaled pathogens. As a consequence, their ability to express immunomodulatory molecules such as CD40 can drastically influence the progression of infection and inflammation in the lung. The goal of this body of work is to elucidate the functional significance of CD40 being expressed on the surface of airway epithelial cells.

Airway epithelial cells

The lining of the lung is composed of epithelial cells with unique structural interactions that form a continuous barrier to the environment and preserve the integrity of the airway. Although the epithelium is continuous, it is not homogeneous (6, 7). Generally, the upper or proximal airway is composed of ciliated cells interspersed by basal cells and a few goblet cells that are secretory. They form a layer that is characterized as pseudostratified, with the ciliated cells and goblet cells at the air...
compartment surface and the basal cells lying underneath in contact with the basement membrane. Distally, the epithelium begins to thin, and Clara cells increase in number, presumably replacing ciliated cells. Beyond the respiratory bronchioles is the alveolar space where gas exchange occurs. At the alveolar level, the epithelium loses its columnar appearance and becomes sheetlike. Alveolar cells (Type I and Type II) are squamous and are in direct contact with the vasculature for optimal gas exchange. For the purposes of this dissertation, further discussion of epithelia refer to cells found proximal to the alveolar space.

Epithelial cells form a confluent layer and have connections near their apex that are referred to as tight junctions; hence, the cells are polarized and display two distinct surfaces. The apical or lumenal (mucosal) surface is exposed to the environment, and the basolateral surface is adjacent to the underlying tissue. This design forms a selectively permeable barrier that allows for the exchange of fluid and ions while excluding foreign particles. Clearance of unwanted materials occurs by a mechanism referred to as the mucociliary escalator. Mucous is secreted by specialized cells called goblet cells in addition to mucous and serous cells found in submucosal glands. This mucous bathes the apical surface of the epithelium and traps foreign particles. Synchronized beating of the cilia can then push the mucous coated inhaled material back up the airway tract where it can be expelled (7). Normally, the physical barrier of the epithelium and the mucociliary escalator are sufficient to maintain the integrity of the lung. However, when these innate defenses fail, an adaptive immune response is initiated, and inflammation follows.

The mucosal surface of the airway epithelium is a critical point of contact for pathogens. The airway is continually exposed to potential toxins and antigens from the
environment. The epithelium is therefore the first line of defense against infectious or injurious agents. Until recently, epithelia were considered to be primarily a mechanical barrier, but epithelia are now appreciated as effector cells that play a significant role in airway immunity (8). Epithelial cells act as sensors that communicate with inflammatory cells as well as with cells that underlie the mucosa such as smooth muscle cells and vascular endothelium (9). Pulmonary epithelial cells have the capacity to recruit immune cells to the airway via release of chemoattractant molecules called chemokines and to facilitate trafficking of immune cells across the epithelium via expression of cellular adhesion molecules (6). In addition, epithelial cells can initiate or amplify inflammation in the airway by secretion of cytokines. It is generally agreed that epithelial cells can synthesize several proinflammatory molecules including IL-6, IL-8, IL-11, GM-CSF, ICAM-1, RANTES, and MCP-1 (8).

It is still a matter of debate whether epithelial cells function as antigen-presenting cells. Airway epithelial cells do express MHC class II antigens as well as HLA-DR molecules (reviewed in Ref. 8). The expression of these molecules on the surface of airway epithelial cells makes it feasible for epithelial cells to present antigen to lymphocytes. In vitro experiments have demonstrated that airway epithelial cells can stimulate the proliferation of CD4⁺ and CD8⁺ T cells in mixed lymphocyte cultures (10) and that epithelial cells treated with IFN-γ can present antigens to autologous T cells in an HLA-DR restricted manner (11). A recent report by Nguyen et al. asserts that virus-infected intestinal epithelial cells effectively present viral antigens and are successfully lysed by CD8⁺ cytotoxic T cells (12). Since many viruses infect epithelial cells at mucosal surfaces, the ability of those cells to present antigen to resident lymphocytes is
an attractive concept. Research exploring the role of pulmonary epithelial cells as immune effector cells in the airway is ongoing. In particular, a receptor found on antigen-presenting cells (APCs), CD40, has recently been described on a number of epithelial cell types, including keratinocytes, thymic, kidney, retinal, and airway epithelia (13-18). The importance of CD40 on cells of lymphoid origin is well established. CD40 plays a central role in humoral and cell-mediated immunity (3, 4), and the role of CD40 on airway epithelial cells may provide important information regarding the development of airway inflammation.

CD40-CD40L

CD40 is a 50-kDa type I transmembrane glycoprotein originally identified in B lymphocytes and bladder carcinoma cell lines (1, 2). In 1989, CD40 cDNA was cloned and sequenced from a human B cell expression library (19). Stamenkovic and coworkers noted that CD40 displayed structural relatedness to the low-affinity nerve growth factor receptor (LNGFR) which is a member of the TNFR superfamily. This family also includes TNF Type I (p55) receptor (TNFRI), TNF Type II (p75) receptor (TNFRII), FAS (CD95), and CD30 (Fig. 1) (5). The extracellular domains of the TNFR family members all display cysteine-rich modules that form tertiary folds and confer ligand specificity (20). The cytoplasmic domain of CD40 does not display homology to any other known receptor. CD40 is expressed by all cells that are traditionally considered APCs such as B cells, dendritic cells, and monocytes/macrophages. A number of non-hematopoietic cells also express CD40, including vascular endothelia, smooth muscle cells, fibroblasts, and epithelia (3).
FIGURE 1. Schematic representation of members of the TNFR family.
The ligand for CD40 (CD40L) is a 33-kDa type II membrane-bound TNF-like molecule that was originally identified on activated T lymphocytes (21-23). Other members of this superfamily include the ligands for members of the TNFR family such as TNF-α, CD30L, and FASL (Fig. 2). CD40L is expressed on activated CD4+ T cells, activated CD8+ T cells, eosinophils, mast cells, basophils, NK cells, monocytes, macrophages, and activated dendritic cells (24). CD40L is tightly regulated on T cells and is expressed as soon as 5-15 min after activation. This immediate expression does not require de novo protein synthesis. A second wave of expression occurs at 1-2 h after activation and is maximal at 6-8 h. After 24-48 h, expression returns to resting levels (25). This transient expression allows for a quick immune response and a limited time course for interaction between CD40-expressing cells and CD40L+/-activated cells.

Many lymphoid cells reside in the lung (intraepithelial lymphocytes (IELs), alveolar macrophages, bronchus-associated lymphoid tissue (BALT)), as well as traffic through the lung microvasculature and interstitium. These cells can be induced to express CD40L by lectins such as phytohemagglutinin and concanavalin A, phorbol esters, and the proinflammatory cytokines TNF-α and IL-1β (5, 26). In addition, T cells in the lung can be stimulated to express CD40L in a transient fashion through cross-linking of the TCR, CD3, and a costimulatory molecule, CD28 (27). In the lung, there are CD4+ and CD8+ T cells interspersed throughout the epithelium of the upper (proximal) airway that are in constant and direct contact with CD40-bearing epithelial cells. There are also alveolar macrophages, capable of expressing CD40L, in the alveolar space that also make direct contact with the airway epithelium.
FIGURE 2. Schematic representation of members of the TNF family.
The importance of CD40-CD40L interactions in human physiology was first highlighted by a severe form of human immunodeficiency known as hyper-IgM syndrome (HIGM) (28). This disease is characterized by normal or elevated levels of IgM and IgD, but low or absent levels of IgA, IgE, and IgG, and by the absence of germinal centers in lymphoid tissue (29). Most cases of HIGM are caused by point mutations or deletions in the gene encoding CD40L, which results in the inability of T cells to express functional CD40L on their surface. HIGM patients have normal B cells that can respond appropriately in vitro to CD40L*-activated T cells. The clinical symptoms of HIGM patients result from the inability of their B cells to switch from IgM to other Ig isotypes and a lack of B cell memory that results in recurrent infections (30). The critical nature of CD40-CD40L interaction for generation of T cell-dependent B cell responses was subsequently confirmed by the generation of CD40 and CD40L knockout mice (31-33). These mice display deficiencies in humoral immunity and do not develop germinal centers in response to T cell-dependent antigens. However, neither CD40 nor CD40L knockout mice display altered antibody responses to T cell-independent antigens which require IgM. Taken together, these data corroborate the initial suggestion that CD40-CD40L interactions are essential costimulatory molecules in T cell-dependent B cell activity.

CD40-CD40L interactions have been found to play a role in inflammation associated with bacterial and viral infection, gene therapy, atherosclerosis, and autoimmune disorders (3). Inflammation is characterized by vasodilation of the microvasculature, increased permeability of the capillaries, swelling, and migration of granulocytes and leukocytes into targeted tissues (34). Some of the many molecules that
mediate these responses include proinflammatory cytokines (TNF-α, IL-1β, IL-6), adhesion molecules (ICAM-1, VCAM-1), and chemokines (IL-8, RANTES, MCP-1) (35). CD40-CD40L interactions in a variety of cell types have been shown to up-regulate the expression of these proinflammatory molecules (reviewed in Ref. 36). For example, engagement of CD40 on monocytes results in the production of IL-1, IL-8, TNF-α, and IL-6 (37). The cross-linking of CD40 expressed on human endothelium by a recombinant murine CD40L up-regulated the expression of the adhesion molecules, E-selectin, ICAM-1, and VCAM-1 (38). Similarly, CD40 positive kidney proximal tubular epithelial cells, cocultured with CD40L-bearing fibroblasts, expressed increased levels of the chemokines, IL-8, RANTES, and MCP-1 (15). In human keratinocytes, CD40 ligation induced the expression of ICAM-1 (39), and human retinal pigment epithelial cells increased their production of IL-6 and IL-8 when stimulated with sCD40L or CD40L-bearing cells (16). These reports make it clear that epithelial cells in various organ systems are poised to respond to proinflammatory signals as well as to summon immune cells to compromised tissues. Similar to the effects that the cytokines TNF-α, IL-1β, and IFN-γ have on airway epithelial cells, CD40L binding to CD40 expressed by epithelia can play a critical role in the modification of epithelial cell activation.

In vivo studies also indicate the involvement of CD40 in airway inflammation. Using sCD40L administered intranasally, Wiley et al. observed that CD40 knockout mice attracted 150 times fewer polymorphonuclear leukocytes (PMN) to the airway than CD40 wild-type mice (40). In an antigen-induced model using ovalbumin (OVA) sensitization and challenge, Lei and colleagues reported altered airway immune responses in CD40L knockout mice compared to wild-type controls (41). Specifically, at 72 h post-challenge,
dramatically fewer monocytes, lymphocytes, neutrophils, and eosinophils were detected in the bronchoalveolar lavage (BAL) fluid of CD40L knockout mice as compared to wild-type control mice. Additionally, the BAL of the CD40L knockout mice had reduced levels of IL-4 and TNF-α as well as decreased serum levels of OVA-specific IgE and IgG1 as compared to control mice. In studies designed to examine the impact of blocking CD40-CD40L interactions on hyperoxic lung injury, Adawi and coworkers discovered that intraperitoneal administration of a monoclonal Ab (MR1) to CD40L could significantly reduce or prevent pulmonary damage in mice (42). These authors showed by immunohistochemistry that cyclooxygenase-2, a proinflammatory enzyme responsible for prostaglandin synthesis, was abrogated in oxygen-treated mice that received MR1 pretreatment or post-treatment. In a similar study, Adawi et al. used the CD40L-blocking Ab, MR1, to protect mice against radiation induced pulmonary toxicity (43). As before, cyclooxygenase-2 production was reduced and the mouse lungs displayed a dramatically reduced influx of inflammatory cells into the lungs.

Several studies have focused on the efficacy of blocking CD40-CD40L interactions during gene transfer with adenoviral vectors. Using a murine Ab (MR1) to CD40L, researchers were able to inhibit development of a humoral response by abrogating production of neutralizing antibodies as well as increase the persistence of the transferred gene expression and allow repeated administrations of the vector (44). Finally, in a BALB/c mouse model of respiratory syncytial virus (RSV), Tripp et al. coimmunized with plasmid vectors containing CD40L and RSV antigens, and found increased production of the proinflammatory mediators TNF-α, IL-2, IFN-γ, and NO in comparison to animals treated with empty vector (45). As a result, the mice
demonstrated accelerated virus clearance and increased RSV-specific antibody responses. Based on these animal studies, the interaction of CD40 and CD40L is a critical target for possible therapies that could lessen the severity of airway inflammatory diseases and lung injury.

_CD40 Signaling Mechanisms_

The pleiotropic biological roles that are mediated by CD40 suggest multiple intracellular signaling pathways and complex regulation through association with adapter proteins. Over the last ten years, a large amount of information has been amassed regarding the signaling mechanisms of CD40 and other TNFR family members (Fig. 3). It is important to mention here that the research investigating CD40 signaling can be confusing. Collectively, it is apparent that cell type, differentiation stage, triggering stimulus (e.g., CD40 Abs versus trimerized ligand (CD40LT)), and the availability of potential cytoplasmic CD40-binding proteins have significant effects on the functional outcome of signaling through CD40. Despite the myriad of conflicting data presented in the literature, some general themes regarding CD40 signaling mechanisms have gained acceptance. Accumulating evidence supports the theory that initiation of signaling through TNFR family members requires oligomerization of the receptors with trimeric ligands (reviewed in Ref. 46). This clustering of receptors triggers the recruitment of members of the TNF receptor-associated factor (TRAF) family to their cytoplasmic domains. It has also been proposed that TRAFs trimerize and that their self-association is required for high-affinity binding to TNFR members such as CD40 (47, 48). The TRAFs are a family of adapter molecules that were first discovered based on their interactions.
FIGURE 3. Putative signaling pathways for CD40 in a B lymphocyte.
with members of the TNFR family such as TNFRII (p75) (49) and CD40 (50, 51).

Presently, there are six members of the TRAF family (TRAF1-TRAF6), and all of them except TRAF4 has been implicated in CD40 signaling. TRAF4 was isolated from metastatic tumor cells (52) and is unusual in the fact that it is predominantly a nuclear protein and it has not been definitively shown to associate with any TNFR family member; however, a recent TRAF4 knockout mouse indicates that it is involved in the development of the lung (53).

TRAF protein structure can be generally divided into two basic domains: 1) a carboxy-terminal TRAF domain, and 2) an amino-terminal domain with zinc and RING finger motifs (49) (Fig. 4). The TRAF domain can be further divided into the TRAF-C (C-terminal portion) and TRAF-N (N-terminal portion) regions. The TRAF-C region is highly conserved and is critical for the interaction of TRAF molecules with cytoplasmic receptor tails as well as with signaling proteins such as NF-κB-inducing kinase (NIK) (54). The TRAF-N region contains predicted coiled-coiled structures and mediates TRAF self-association into homo- and hetero-oligomers (55, 56). With the exception of TRAF1, all TRAFs contain an amino-terminal RING finger domain and 5-7 zinc finger domains that appear to be required for downstream signaling (57). For now, the TRAFs are thought to lack any enzymatic activity of their own and, therefore, must bind to other cytoplasmic molecules that are capable of activating transcription factors downstream.

All of the TRAFs have been shown to associate with the cytoplasmic tail of CD40, except for TRAF4 (47, 58) (Fig. 5). TRAF2, TRAF5, and TRAF6 have been implicated in signaling through CD40, leading to activation of NF-κB (55, 58-60). Other signaling events that have been reported to occur upon CD40 engagement include the
FIGURE 4. Schematic representation of TRAF molecules. The TRAFs are highly conserved at their carboxyl terminus, and this region (TRAF-C) is important for binding to the cytoplasmic tails of their associated receptors as well as to downstream signaling molecules. The TRAF-N domain contains coiled-coiled structures and mediates the homo- and hetero-oligomerization between TRAFs. With the exception of TRAF1, all TRAFs contain an amino-terminal RING finger domain and 5-7 zinc finger domains that appear to be required for downstream signaling.
FIGURE 5. Predicted association of TRAFs 2, 3, 5, and 6 with the cytoplasmic tail of CD40.
activation of tyrosine kinases (Lyn), AP-1, NF-AT, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinases (MAPKs), and phosphatidylinositol 3-kinase (PI-3K) (61-66). The task of dissecting the CD40-induced signaling pathways is well underway, and it appears that regulation of CD40 signal transduction is highly cell-type specific. As a result, details regarding TRAF- and CD40-TRAF-mediated signaling events are conflicting. For example, studies using TRAF2 knockout mice and transgenic mice indicate that TRAF2 is essential for JNK activation, but is not required for NF-κB activation (67, 68). However, splenocytes from TRAF2-deficient mice showed defective CD40-induced proliferation and a complete loss of NF-κB activation in response to CD40 triggering (69). In another study, Leo et al. found that TRAF2 binding to CD40 is not essential for JNK or NF-κB activation in human embryonic kidney cells (HEK) 293 cells (70).

TRAF2 and TRAF3 bind to a shared consensus sequence on the cytoplasmic tail of CD40, and although competitive binding is suggested as a negative role for TRAF3 in CD40 signaling (71), its molecular function is unclear. Importantly, TRAF3 knockout mice die ten days after birth from wasting and are immune deficient (72). A recent study investigating the molecular interaction of TRAF3 with CD40 proposed that CD40 may undergo conformational changes when bound to different TRAF family members (73). Another important mediator of CD40 signaling is TRAF6, which binds a membrane-proximal region and demonstrates redundant as well as unique signaling mechanisms (74, 75). Using mutational analysis, Pullen and coworkers demonstrated in HEK 293 cells that optimal NF-κB and JNK activation by CD40 required the mutual binding site for TRAF2 and TRAF3 as well as the site for TRAF6 (76). Therefore, CD40 signaling could
be regulated by competitive binding of TRAFs, conformational changes in CD40, as well as integration of multiple signals from different pathways.

Aside from the TRAF adapter proteins, one other molecule has been shown to bind directly to CD40's cytoplasmic tail and it is a protein tyrosine kinase (PTK) called Janus kinase 3 (Jak3) (77). Hanissian and Geha initially reported that Jak3 is constitutively associated with CD40 in human B cell lines (BJAB, Daudi, Ramos) and that CD40 ligation with a monoclonal antibody induced tyrosine phosphorylation and activation of Jak3; in addition, these investigators reported that Jak3 phosphorylation led to the activation and translocation of a member of the family of signal transducers and activators of transcription (termed STATs), specifically STAT3 (77). STATs when properly phosphorylated will homo- and heterodimerize and move to the nucleus where they can bind to DNA regions such as the IFN-γ-activated sequence (GAS) element and the IFN-stimulated regulatory element (ISRE), thus activating transcription (reviewed in Ref. 78). However, in primary murine B cells, engagement of CD40 by sCD40L resulted in tyrosine phosphorylation of STAT6, but no Jak phosphorylation was identified (79).

In a follow-up study, members of the group that initially identified the activation of Jak3 by CD40 demonstrated that human B cells deficient in Jak3 did not have any functional defects and concluded that Jak3 was not involved in CD40 induction of proliferation, Ig class switching, or up-regulation of CD23, ICAM-1, CD80, and lymphotoxin-α (LT-α) expression (80). Most recently, Revy and colleagues have compared CD40-induced signal transduction in primary human monocytes and primary human resting tonsillar B cells and discovered that, while Jak3 is associated with the cytoplasmic tail of CD40 in both cell types, only CD40-activated monocytes induce Jak3 phosphorylation and
STAT5a nuclear translocation (81). Furthermore, these investigators noted that triggering of CD40 on monocytes and B cells led to NF-κB activation, but the individual components of the NF-κB complexes formed were different in the two cell types as shown by supershift assays. Specifically, p50 was involved in CD40 activation of NF-κB in both B cells and monocytes, but p65 was involved only in B cell complexes and not complexes formed in monocytes (81). Taken together, these studies are a good example of the complexity found in CD40-induced signal transduction.

Proinflammatory Cytokines

**TNF-α.** TNF-α is the prototypical member of the TNF superfamily of molecules, which includes CD40L, FASL, CD30L, and LT-α (Fig. 2) (reviewed in Ref. 82). These transmembrane (type II) glycoproteins show homology within their C-terminal extracellular domains and act as ligands for the TNF receptor family. Generally, members of the TNF family can be found as both a membrane-bound protein or as a proteolytically cleaved, soluble protein (reviewed in Ref. 83). TNF-α is produced as a 26-kDa precursor protein with a N-terminal sequence that can be proteolytically cleaved to release a 17k-Da TNF peptide. The precursor protein has some activity, such as tumor cell cytotoxicity, but mature TNF-α is thought to be responsible for the majority of known inflammatory activities. Upon release, TNF-α trimerizes to form a homotrimeric complex capable of aggregating its receptors (84).

TNF-α was first discovered as a proinflammatory soluble factor present in the supernatants of macrophages (85). Beutler and Cerami noted a wasting disease in rabbits and traced the activity to a serum factor which they named cachectin (86). Separately,
Coley had noted a necrotic effect on tumors in patients with streptococcal infections, and subsequent investigation resulted in the isolation of TNF-α as well as its primary exogenous stimulant, LPS (as cited in Ref. 86). It is now known that TNF-α is critical to the host response and inflammation; however, TNFα is a double-edged sword. If it is produced in low concentrations, it serves to protect the host from a variety of pathogens. Conversely, in high concentrations, TNF-α can cause septic shock, cachexia, and eventually contribute to the death of the host.

The main sources of TNF-α are activated macrophages and monocytes, but other sources include T cells, B cells, NK cells, mast cells, neutrophils as well as non-hematopoietic cells (87). LPS is one of the most potent inducers of TNF-α gene expression. TNF-α has a short half-life (20 min), and after a LPS-induced response will be cleared from the blood completely in 4-6 h. Other cytokines can induce the expression of TNF-α, including IL-1, IFNγ, GM-CSF, and TNF-α itself. Conversely, glucocorticoids can inhibit TNF-α gene transcription. Additional inhibitory molecules include TGF-β, IL-6, and IL-10.

The cells that express receptors for TNF-α and thus can respond to it include virtually all the cell types in the body. As described earlier, members of the TNFR family include TNFRI (p55), TNFRII (p75), CD40, CD30, and Fas (Fig. 1). As stated earlier, these receptors demonstrate homology in their extracellular domains that is hallmarked by multiple cysteine repeats (reviewed in Ref. 46). Specifically, TNFRI (p55) mediates most of the known biologic functions of TNFα. TNFRI knockout mice display severe immunodeficiencies and a resistance to LPS (reviewed in Ref. 82). TNFRII (p75) is also a receptor for TNFα, but does not show significant phenotypic
alterations when knocked out in mice. A form of regulatory control is provided by the secretion and shedding of TNFRs. Soluble TNFR is increased by the same stimuli as TNFα, thus helping to control its biological activity (88).

Although members of the TNFR family share homology in their cysteine-rich extracellular domains, they vary widely intracellularly. All of the members lack an intrinsic kinase or phosphatase activity, making other methods of signal transduction necessary. Using yeast two-hybrid systems, adapter molecules were isolated that provided the link between TNFRs and downstream signaling events. These novel proteins, named TRAFs, were found to associate with TNFRs and have been characterized (49). Presently, six TRAFs have been identified.

The functions of TNF-α are wide-ranging and include cell differentiation, activation, cytotoxicity, and proliferation. As stated above, in vivo effects of TNF-α include the ability to induce fever and acute-phase proteins, as well as cachexia and a syndrome resembling septic shock (87). Chronic TNF-α exposure eventually suppresses the responses of Th1 and Th2 cells and attenuates T cell receptor signaling (89). At the cellular level, TNF-α is known to activate leukocytes, monocytes, and fibroblasts. From activated cell sources, TNF-α synergizes strongly with IFN-γ to induce the expression of a secondary cascade of proinflammatory molecules such as chemokines and adhesion molecules. For example, TNF-α induces epithelial cells to express ICAM-1 and also synergizes with IL-4 and IFN-γ to increase VCAM-1 expression on endothelial cells (reviewed in Ref. 90). Neutrophils increase their binding to endothelia in vitro when stimulated by TNF-α and also display increased degranulation and respiratory bursts. TNF-α is capable of inducing its own production as well as IL-1, prostaglandins, IL-6,
and IFN-γ expression (91). Another activity of TNF-α is proliferation of fibroblasts
during repair after damage has occurred because of inflammation or injury. This fibrosis
may be the culprit of TNF-α-induced cachexia under chronic inflammatory conditions,
such as is seen in tuberculosis.

As a major player in inflammation and immunity, TNF-α is involved in airway
pathologies such as asthma and airway hyperreactivity resulting from injury (trauma).
TNF-α and TNFRs are considered to be the main antiviral activity, and viruses have
developed elaborate methods of escaping immune surveillance (89). It has been
hypothesized that TNF-α plays a role in the interstitial pneumonitis and the trapping of
neutrophils (lesions) as seen in acute-respiratory distress syndrome (ARDS) (92). High
levels of TNF-α have been detected in the BAL fluids of ARDS patients (93). TNF-α
exerts many of the effects seen in allergic asthma. For example, it can activate and
differentiate macrophages, prompt neutrophils to degranulate, stimulate the release of the
pleiotropic cytokines IL-1 and IL-6, stimulate the release of arachidonic acid, and also
promote extensive pulmonary fibrosis (tissue remodeling) (92).

IL-1β. IL-1 was first noted as a product made by macrophages that acted as a
comitogen for thymocytes and lymphocytes (94). There are two distinct forms of IL-1
encoded by different genes, IL-1α and IL-1β. Although there is not much sequence
homology between the two, they bind shared receptors and display very similar
functional properties (reviewed in Refs. 95, 96). The most significant difference between
them is that IL-1β is cleaved by a cysteine protease (IL-1 converting enzyme (ICE)) and
released while IL-1α is not typically secreted (97). There is another IL-1 family member
that serves as a specific receptor antagonist (IL-1ra), and it can bind to receptors for IL-1 but cannot trigger the signal required for cell activation (96). Another unique feature of IL-1 is employed by its receptors. Two receptors exist that can bind to all three of the IL-1 family members: Type I (IL-1RI) and Type II (IL-1RII). However, only IL-1RI transduces a signal, and that means that IL-1RII can serve as a "decoy receptor" for IL-1 (98). Signal transduction by the IL-1RI is mediated by TRAF adaptor proteins, particularly TRAF6 (74). TRAF6 interacts with IL-1 receptor-associated kinase (IRAK) which, true to its name, is recruited to the IL-1 receptor (99). The ultimate result of this signaling cascade is the activation of the transcription factor, NF-κB, a well described activator of inflammatory mediator genes (100).

Many types of cells produce both forms of IL-1, including T and B cells, macrophages, dendritic cells, endothelia, neutrophils, smooth muscle cells, and fibroblasts. Monocytes and macrophages can be induced to increase production of IL-1 by stimulation with IL-1, TNF-α, GM-CSF, and endotoxin. Conversely, IL-1 production can be inhibited by prostaglandin-E2 and corticosteroids (reviewed in Ref. 101). IL-1 is a pluripotent cytokine that contributes to inflammation in many of the same ways as described earlier for TNF-α. As an endogenous pyrogen, it induces fever, neutrophilia, and induction of other proinflammatory mediators such as IL-6, chemokines, and adhesion molecules (101). IL-1β is an important growth factor for T cells and B cells and as a costimulatory molecule during antigen-dependent and antigen-independent immune responses. IL-1 induces the production of many other cytokines such as IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, RANTES, GM-CSF, IFN-γ, TNF-α, and platelet-derived growth factor (PDGF). The release of PDGF upon IL-1 stimulation may be the reason
that IL-1 induces fibroblasts to proliferate. IL-1β can synergize with TNF-α and IFN-γ to induce the production of ICAM-1 and VCAM-1 by vascular endothelia and airway epithelial cells (102). Importantly, IL-1β, along with TNF-α, is a major factor found in the bronchoalveolar lavage fluid (BAL) of asthmatics (reviewed in Ref. 103). Corticosteroid therapy can selectively inhibit IL-1β expression without affecting the receptor antagonist, IL-1ra (104).

**Leukocytic Chemotaxis and Transmigration**

During an inflammatory response, it is necessary for hematopoetic cells to traffic out of microvessels and into sites of injury and infection. This process requires specific signals that orchestrate cell adhesion and migration (reviewed in Ref. 105). The initial signal is provided by proinflammatory cytokines released at the site of tissue damage which, in turn, triggers chemoattractant cytokine (chemokine) secretion by resident cells. As a result, a chemogradient specific for certain subsets of leukocytes is established, and the local endothelium becomes activated. Next, adhesion molecules are expressed by activated endothelia, allowing leukocytes to bind the vessel wall, escape the peripheral circulation (diapedesis), and travel into compromised tissue. Both chemokines and adhesion molecules provide activation signals for trafficking immune cells, thus regulating this first leg of the inflammatory cascade. This general scheme of migration is referred to as the multistep paradigm of leukocyte recruitment (105) (Fig. 6). However, the lung is unique because all of the blood in the human body passes through it and because the architecture of the alveolar bed promotes pooling of polymorphonuclear leukocytes (PMNs) and alveolar macrophages, which are easily marginated if necessary.
rolling leukocyte to blood flow extravasation

activated endothelium

activation firm adhesion

interstitial cells

proinflammatory cytokine gradient

glycosaminoglycan integrin

VCAM, ICAM chemokine receptor
FIGURE 6. Model of leukocyte chemotaxis and migration. Cytokines and chemokines are released in local tissue upon insult and trigger the activation of the postcapillary venular endothelium. Activated endothelium expresses adhesion molecules that allow circulating leukocytes marginate and to slow down and "roll" along the surface of endothelial cells. Upon contact of leukocytes with endothelia, the repertoire of adhesion molecules changes to induce firm adhesion and diapedesis. Leukocytes then migrate between endothelial cells and traffic into the extracellular matrix.
Therefore, the sheer stresses that blood-borne immune cells encounter are absent, and instead, cells continually roll along the alveolar capillary membrane (106). Another important aspect of lung biology is the distribution of its resident lymphoid cells.

In contrast to other organs, the lung vascular bed contains large numbers of lymphocytes (reviewed in Ref. 107). These cells are distributed between the interstitium, the microvascular bed, the alveolar space, BALT, and throughout the epithelium. In the upper airways, there are approximately 18 intraepithelial lymphocytes (IEL) present per 100 epithelial cells, and these are predominantly CD8$^+$ cytotoxic T cells (108). In contrast, in the bronchial lamina propria, there are greater numbers of CD4$^+$ Th cells that are CD45RO$, a marker expressed by memory T cells (109). There are also large numbers of dendritic cells, which represent the principal APC in the lung (110). Although the alveolar macrophage was once thought to be the primary APC in the airway, it is now clear that they are poor APCs because they do not express B7, which is essential to T cell proliferation (clonal expansion) (111). Alveolar macrophages represent the bulk of cells found in BAL fluid (85%), and lymphocytes make up the majority of the remainder (112). Mucociliary clearance is not an active defense mechanism in the distal airway (alveolar space) and alveolar macrophages as well as PMNs are responsible for clearance of foreign particles and microbes (106). It is important to keep in mind that the chemokines and adhesion molecules that are involved in the margination process might be different in the lung because of the unusual anatomical differences in the microvasculature.
Chemokines: RANTES, IL-8, and MCP-1. Chemokines make up a large family of small cytokines (8-12 kDa) that selectively chemoattract subset populations of immune cells (reviewed in Ref. 113-115). Since the discovery of the first chemokines over a decade ago, 40 to 50 members have been reported (reviewed in Ref. 116). The four subfamilies are distinguished based on the spacing of highly conserved NH$_2$-terminal cysteines and are named accordingly: CXC, CC, C, and CX$_3$C (reviewed in Ref. 116, 117). The CXC chemokines can be further subdivided by the presence or absence of a motif containing three amino acids (glutamine-leucine-arginine; ELR) that precedes the first cysteine (118). The presence and placement of the ELR motif is thought to provide a potent neutrophilic signal, while ELR\textsuperscript{-} chemokines attract other cell types (119). Lymphotactin is the only member of the putative C family and preferentially attracts T lymphocytes but not monocytes (120). Neurotactin (also referred to as fractalkine) is the lone member of the CX$_3$C family and was identified in the brain (121). The relatively new C and CX$_3$C families are mentioned for completeness, but will not be discussed further.

Chemokine actions are mediated through G-protein-coupled, seven-transmembrane-spanning receptors that are mostly coupled to G$_{ai}$ proteins, rendering them inhibitable by pertussin toxin (reviewed in Ref. 113, 117). An interesting aspect of chemokine receptor biology is their promiscuity. Chemokine receptors can bind more than one chemokine with varying affinities. Therefore, the chemokine receptors are named based on the class of chemokines that they bind (see Table 1).

The first chemokine characterized was IL-8, which was purified by several groups as a monocyte-derived factor that attracts neutrophils (91, 122, 123). IL-8 is the
TABLE 1. Examples of chemokines and the cells that they attract.

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Chemokine</th>
<th>Target Cells</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXC</td>
<td>IL-8</td>
<td>neutrophils, immature T cells, basophils</td>
<td>71,103,</td>
</tr>
<tr>
<td></td>
<td>ENA-78</td>
<td>neutrophils</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>GRO-α</td>
<td>neutrophils</td>
<td>117</td>
</tr>
<tr>
<td>CC</td>
<td>RANTES</td>
<td>memory T cells, eosinophils, NK cells, basophils, dendritic cells</td>
<td>97,112, 113</td>
</tr>
<tr>
<td></td>
<td>MCP-1</td>
<td>monocytes, memory T cells, basophils, NK cells</td>
<td>108,109,110</td>
</tr>
<tr>
<td></td>
<td>Eotaxin</td>
<td>eosinophils</td>
<td>116</td>
</tr>
<tr>
<td>C</td>
<td>Lymphotactin</td>
<td>T cells</td>
<td>101</td>
</tr>
<tr>
<td>CX3C</td>
<td>Neurotactin/fractalkine</td>
<td>mast cells, monocytes, microglia</td>
<td>102</td>
</tr>
</tbody>
</table>

Abbreviations: IL-8 (interleukin-8), ENA-78 (epithelial neutrophil activating protein-78), GRO-α (growth related oncogene-alpha), RANTES (regulated on activation, normal T expressed and secreted), MCP-1 (monocyte chemotactic protein-1).

A prototypical CXC/ELR+ chemokine and can be made by many different cell types including monocytes, T lymphocytes, neutrophils, fibroblasts, endothelia, and epithelia. Cells produce IL-8 in response to exogenous stimuli such as mitogens as well as endogenous cytokines. IL-1 and TNF. IL-8 promotes the adhesion of neutrophils to endothelia by inducing neutrophils to express β2-integrins (124). IL-8 also stimulates histamine release by basophils (125). There are two receptors for IL-8, namely CXCRI and CXCRII, and both are expressed by neutrophils. However, CXCRII can bind not only IL-8 with high affinity, but also the other neutrophil-attracting chemokines. In contrast, CXCRI has only been shown to bind IL-8 with high affinity (126).
The CC chemokines represent the other major family of chemoattractant cytokines. The best characterized of these is monocyte chemoattractant protein-1 (MCP-1). In response to stimuli, MCP-1 is produced by monocytes, fibroblasts, endothelial cells, and epithelial cells. MCP-1 attracts monocytes with a potency comparable to that of IL-8 for neutrophils (127). In the presence of endothelial cells in vitro, MCP-1 attracts activated CD4+ and CD8+ memory T lymphocytes, but not B cells or NK cells (128). However, in the absence of endothelial cells, MCP-1 can attract NK cells as well as T lymphocytes (128). Another critical function of MCP-1 is the ability to activate basophils to release histamine (129). Early in chemokine receptor investigation, CCR2 was designated as a MCP-1 specific receptor, but other MCPs can bind CCR2, and MCP-1 is now thought to also bind to the chemokine receptor CCR4 (reviewed in Ref. 117).

Another CC chemokine, RANTES was first discovered as an inducible activity from T lymphocyte cell lines and circulating lymphocytes (130). RANTES attracts monocytes, CD4+, CD8+, and CD45RO+ T lymphocytes, as well as NK cells (reviewed in Ref. 116). RANTES is a strong chemoattractant for eosinophils and induces histamine release by basophils (131, 132). Similar to MCP-1, RANTES is produced by monocytes, fibroblasts, endothelium, and epithelium in response to a variety of stimuli. However, RANTES is also produced by lymphocytes, airway smooth muscle cells, and eosinophils (reviewed in Ref. 103). Several chemokine receptors demonstrate specificity for RANTES, including CCR1, CCR3, CCR4, and CCR5.

Each of the chemokines discussed above has been shown to be a mediator involved in airway inflammatory conditions (for details, see Refs. 103, 116). Briefly, anti-IL-8 antibodies can significantly reduce tissue damage during reperfusion injury.
In a murine pulmonary inflammation induced model, MCP-1 expression immediately precedes monocyte and macrophage infiltration of the lung. Soon after, T lymphocytes and eosinophils migrate into the challenged lung in response to RANTES expression (134).

**Adhesion Molecules: ICAM-1 and VCAM-1.** Adhesion molecules facilitate the interaction of circulating leukocytes with the endothelial cells lining the vessels and with the epithelium lining the airway (reviewed in Ref. 135). Leukocyte emigration from the bloodstream into extracellular matrices has been investigated extensively and divided into several discreet steps that include rolling, activation, and extravasation (diapedesis) (Fig. 6). These sequential events are regulated by several families of adhesion molecules such as selectins, cadherins, immunoglobulins, addressins, and integrins (reviewed in Refs. 105, 136, 137).

During an inflammatory response in the airway, it is necessary for immune cells to traverse the epithelial barrier that lines the lung. This process has not been studied as extensively as EC-leukocyte interactions, but some of the same molecules are thought to be involved. In particular, epithelia in the lung express the immunoglobulin supergene family members, ICAM-1 and VCAM-1 (reviewed in Refs. 135, 138). ICAM-1 and VCAM-1 interact with members of the integrin family which are expressed on trafficking leukocytes.

ICAM-1 is a 90-kDa transmembrane protein that is expressed in low levels on most peripheral blood leukocytes as well as on nonlymphoid cells such as vascular endothelial cells, mucosal epithelial cells, and dendritic cells (139). ICAM-1 is strongly
up-regulated by the proinflammatory mediators, TNF-α, IFN-γ, and IL-1β. Ligands for ICAM-1 include the β2-integrins, LFA-1 (CD11a/CD18), and Mac-1. LFA-1 is constitutively expressed on the majority of leukocytes and mediates firm cell adhesion on vascular endothelia in inflamed tissues (136). The importance of the interaction of β2-integrins and ICAM-1 in inflammation is highlighted by a congenital disease called leukocyte adhesion deficiency (LAD Type I) (140). This disease is the result of a defect in the CD18 gene of the β2-integrin family, and patients suffering from it are unable to mount an acute neutrophilic response. Corresponding evidence in CD18-deficient mice shows that these mice also lack the ability to recruit neutrophils to sites of inflammation. Additionally, epithelial cells infected by parainfluenza virus increased ICAM-1 expression on their surface and demonstrated increased neutrophil adhesion (141).

Despite the importance of ICAM-1/LFA-1 interactions, blocking antibodies to LFA-1 or ICAM-1 does not completely inhibit immune cell binding to endothelia and/or epithelia; therefore, additional adhesion molecules must be involved.

VCAM-1 was identified on endothelial cells as another immunoglobulin gene family member that was capable of binding leukocytes (142). VCAM-1 is expressed on lung epithelial cells as well as renal tubular cells and keratinocytes (138). VCAM-1 is rapidly induced by cytokines such as TNF-α and IL-1β. VCAM-1 recognizes the β1-integrin, very late antigen-4 (VLA-4), which is primarily expressed by lymphocytes, monocytes, and eosinophils, but not neutrophils. Selective recruitment of eosinophils into the airway in asthmatics may be mediated by the interactions between VCAM-1 and VLA-4 (143).
Th1/Th2 immunity and CD40

As discussed earlier, targeted disruption of CD40 and CD40L in mice results in defective cellular and humoral responses, which are mediated through the interaction of APCs and Th cells (25). CD4+ T cells are divided into Th1 and Th2 subsets based on their distinctive functions and are distinguishable by their production of a panel of cytokines (144). Thus, Th1 cells produce IL-2, IFN-γ, and TNF-β and are important for cell-mediated immunity. Th1 responses are critical to the clearance of intracellular organisms such as viruses and protozoans, but can also lead to delayed-type hypersensitivity reactions. In contrast, Th2 cells express IL-4, IL-5, IL-6, IL-10, and IL-13 and are essential to the generation of humoral responses, including vigorous IgE and IgG1 responses, as well as the induction of eosinophilia. Th2-dominated responses protect against extracellular microbes such as parasites and are thought to suppress Th1 responses, but may also promote immediate hypersensitivity (allergic) reactions (reviewed in Ref. 145).

Certain cytokines promote the development of either a Th1 or Th2 immune response in vivo and in vitro (reviewed in Ref. 146). Briefly, IFN-γ and IL-12 are thought to be the major cytokines involved in the development of Th1 responses, and IL-4 and IL-13 are the main cytokines that drive Th2 differentiation (147). Since CD40-CD40L interactions mediate the ability of APCs to upregulate costimulation molecules and to produce cytokines, it is likely that CD40-CD40L interactions play some role in Th cell polarization (3). Infection by *Lieshmania major*, an intracellular pathogen, in CD40L knockout mice revealed that these animals did not mount an effective Th1-type immune response (148). The impairment of the CD40L knockout mice to establish a
Th1 response was the result of macrophages not being able to produce IL-12, and administration of recombinant IL-12 was enough to restore effective immunity in these mice. Similarly, in a murine collagen type II-induced arthritis model, Stüber et al. showed that treatment with anti-CD40L antibodies blocked IL-12 production and slightly enhanced IL-4 production, therefore preventing the priming of Th1 cells and severe disease (149). A follow-up study confirmed the ability of antibodies to CD40L to prevent IL-12 secretion by dendritic cells in vitro and the partial ability of IL-4 to disrupt CD40-induced IL-12 induction (150). Subauste et al. concluded that CD40-CD40L interaction is required for an effective Th1-mediated immune response during infections in humans by *Toxoplasma gondii* (151). Patients with HIGM are susceptible to this parasite as a result of impaired IFN-γ and IL-12 production induced by CD40; however, cytokine stimulation and clearance of the infection was corrected in vitro by treatment with CD40LT. Recently, Howland and coworkers compared the roles of CD28-B7 and CD40-CD40L interactions in Th cell skewing using TCR transgenic mice lacking either CD28 or CD40L (152). They discovered that in vitro and in vivo, T cells from CD28-deficient mice were less proliferative in the presence of antigen than CD40L-deficient T cells, but that CD40 is required for a sustained Th1-mediated response. Together, these studies indicate that CD40-CD40L engagement is important to the development of Th1 immune responses through the induction of IL-12 by APCs. Therefore, it is possible that in the absence of CD40 or CD40L that a Th2 response would be the default response type. However, a murine cardiac allograft rejection study suggests that blocking CD40-CD40L interaction not only inhibits Th1 cytokines but also stimulates the production of Th2 cytokines, IL-4 and IL-10 (153). A similar study reported that blocking CD40-
CD40L in the same mouse model did not affect cytokine production at all (154). Therefore, it is still unclear whether CD40-CD40L interactions have a direct effect on Th2-mediated immunity.

**Dissertation Objectives**

The work presented here addresses the role of CD40 expressed by airway epithelial cells. The binding of CD40 to CD40L plays a central role in the generation of immune responses and inflammation. Specifically, CD40-CD40L interactions have been implicated in the development and regulation of pulmonary inflammation. There are many types of cells in the airway that express CD40 and could potentially participate in the induction and amplification of inflammatory processes, but the role of epithelial CD40 has not been previously investigated in depth. Epithelial cells are the first line of defense in the lung and are the target cells for many respiratory pathogens. They also represent the most abundant cell type, and any change in their physiological state would make a significant contribution to the airway milieu. This dissertation examines functional roles for epithelial CD40 including regulation of chemokine and adhesion molecule expression. The possible regulation of chemokines (IL-8, RANTES, MCP-1) and adhesion molecules (ICAM-1, VCAM-1) by epithelial CD40 would be relevant to future studies and potential treatments of pulmonary inflammation. Another goal of this work was to investigate the underlying mechanism of CD40 activation in airway epithelial cells. The literature suggests that CD40 signaling mechanisms are highly cell type-specific, and here we investigate the differences that might exist in epithelial cells. Overall, the objective of this body of work is to specifically expand the knowledge.
regarding CD40 expression and function in an airway epithelial model system, and to
generally contribute valuable information regarding airway inflammation.
PROINFLAMMATORY AND TH2-DERIVED CYTOKINES MODULATE CD40-MEDIATED EXPRESSION OF INFLAMMATORY MEDIATORS IN AIRWAY EPITHELIA: IMPLICATIONS FOR THE ROLE OF EPITHELIAL CD40 IN AIRWAY INFLAMMATION

by

STACIE M. PROPST, RAQUIA DENSON, EMILY ROTHSTEIN, KIM ESTELL, AND LISA M. SCHWIEBERT

Journal of Immunology 165: 2214-2221, 2000

Copyright 2000 by The American Association of Immunologists

Used by permission

Format adapted for dissertation

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
ABSTRACT

Cytokines produced by activated macrophages and Th2 cells within the lung play a key role in asthma-associated airway inflammation. Additionally, recent studies suggest that the molecule CD40 modulates lung immune responses. Because airway epithelial cells can act as immune effector cells through the expression of inflammatory mediators, the epithelium is now considered important in the generation of asthma-associated inflammation. Therefore, the goal of the present study was to examine the effects of proinflammatory and Th2-derived cytokines on the function of CD40 in airway epithelia. The results show that airway epithelial cells express CD40 and that engagement of epithelial CD40 induces a significant increase in expression of the chemokines RANTES, monocyte chemoattractant protein (MCP-1), and IL-8 and the adhesion molecule ICAM-1. Cross-linking epithelial CD40 had no effect on expression of the adhesion molecule VCAM-1. The proinflammatory cytokines TNF-α and IL-1β and the Th2-derived cytokines IL-4 and IL-13 modulated the positive effects of CD40 engagement on inflammatory mediator expression in airway epithelial cells. Importantly, CD40 ligation enhanced the sensitivity of airway epithelial cells to the effects of TNF-α and/or IL-1β on expression of RANTES, MCP-1, IL-8, and VCAM-1. In contrast, neither IL-4 nor IL-13 modified the effects of CD40 engagement on the expression of RANTES, MCP-1, IL-8, or VCAM-1; however, both IL-4 and IL-13 attenuated the effects of CD40 cross-linking on ICAM-1 expression. Together, these findings suggest that interactions between CD40-responsive airway epithelial cells and CD40 ligand+ leukocytes, such as activated T cells, eosinophils, and mast cells, modulate asthma-associated airway inflammation.
INTRODUCTION

The molecule CD40 is a member of the TNFR family, which includes the TNF receptor, nerve growth factor receptor, and Fas. It is a 50-kDa integral membrane glycoprotein that was identified originally on B lymphocytes and demonstrated to play a central role in the regulation of humoral and cell-mediated immunity (reviewed in Ref. 1). For example, CD40 engagement of B cells has been shown to up-regulate the expression of B7 (CD80), ICAM-1, CD23, and lymphotoxin α (LTα) (2). Moreover, the importance of CD40 in the regulation of immune responses is underscored by the observation that interruption of the CD40-CD40 ligand (CD40L) interaction halts the development and progression of several autoimmune diseases, including experimental encephalomyelitis, collagen-induced arthritis, and lupus, as well as responses to transplantation Ags observed in graft-vs-host disease (reviewed in Ref. 2). CD40L is a type II transmembrane protein classified as a member of the TNF family. It is expressed on activated CD4+ T cells, activated CD8+ T cells, eosinophils, mast cells, basophils, NK cells, and activated dendritic cells (1).

Airway epithelial cells form a continuous pseudostratified layer in the lung, creating a tight barrier that protects underlying tissue from the external environment. As such, airway epithelial cells have been described classically as barrier cells that are involved in homeostasis; these cells respond to a variety of environmental stimuli, resulting in the alteration of their cellular functions such as ion transport and movement of airway secretions. Recent evidence, however, suggests that airway epithelial cells might also act as immune effector cells in response to noxious endogenous or exogenous stimuli. Several studies have shown that airway epithelial cells express and secrete...
various immune molecules, such as lipid mediators, oxygen radicals, adhesion molecules, and a wide variety of cytokines, including chemokines (reviewed in Ref. 3). Through the expression and production of these immune molecules, the epithelium is now thought to be important in the initiation and exacerbation of inflammatory responses within the airway.

T lymphocytes play a major role in the pathogenesis of allergic airway disease, including asthma-associated inflammation. Elevated numbers of activated T cells have been observed in the bronchoalveolar lavage (BAL) fluid and bronchial tissue of asthmatic patients; the majority of these T cells are CD4+ (reviewed in Ref. 4). CD4+ T cells are categorized into Th1 and Th2 subsets with respect to their lymphokine production. Specifically, Th1 cells produce IL-2, TNF-β, and IFN-γ and are important for the development of cell-mediated immunity. In contrast, Th2 cells express IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 and are essential in the generation of humoral immune responses, including IgE production, as well as in the induction of eosinophilia. Because IgE synthesis and eosinophilia are hallmarks of allergic airway disease, it has been postulated that asthma-associated inflammation is mediated by a Th2 response.

Many reports have indicated that interactions between CD40 and its ligand, CD40L, control the development of humoral and cell-mediated immune responses. In particular, studies utilizing CD40 or CD40L knockout mice have suggested that CD40 ligation promotes inflammatory responses within the airway (5-7). Despite this observation, it is unclear which resident cell type(s) of the airway is critical in facilitating CD40-mediated airway inflammation. Because epithelial cells have been implicated in airway inflammatory responses, it is likely that cell-cell interactions between CD40-
responsive airway epithelial cells and leukocytes that express CD40L, including T lymphocytes, eosinophils, and mast cells, can initiate and/or exacerbate lung inflammation. Therefore, the present study examined the expression of CD40 on airway epithelial cells and its role in amplifying inflammatory responses within the lung. Results described herein demonstrate that airway epithelial cells express CD40 and that cross-linking of CD40 up-regulates the expression of inflammatory mediators, including the chemokines, IL-8, RANTES, and monocyte chemoattractant protein (MCP-1) and the adhesion molecule ICAM-1. Moreover, these results demonstrate that the proinflammatory cytokines TNF-α and IL-1β and the Th2-derived cytokines IL-4 and IL-13 modulate the positive effects of CD40 engagement on chemokine and adhesion molecule expression in these cells.

MATERIALS AND METHODS

Cell culture

Experiments were performed with the human airway epithelial cell line 9HTEo' (tracheal; a gift from Dr. Dieter Gruenert, University of California (San Francisco, CA (8)). The 9HTEo' cell line was cultured in LHC-8 medium (Biofluids, Rockville, MD) containing 5% FCS, 1% penicillin-streptomycin, and 0.2% Fungizone. Cells were grown at 37°C in a 5% CO₂ environment and on Vitrogen 100 (Collagen, Palo Alto, CA)-coated flasks; Vitrogen 100 contains collagen types I and IV.
Immunohistochemical analysis of CD40 protein expression

Lungs from BALB/c mice were excised, quick-frozen in liquid nitrogen to preserve Ag immunoreactivity, and sectioned. Frozen sections of the upper airway were then fixed in 3% formaldehyde (TEM grade, Tousimis, Rockville, MD) and rinsed in PBS, and nonspecific sites were blocked in PBS containing 1% BSA. Samples were stained with a rat anti-mouse CD40 Ab (1C-10, 10 μg/ml, PharMingen, San Diego, CA) or rat IgG (10 μg/ml, Sigma, St. Louis, MO) as a negative control for 1 h at room temperature. Samples were then rinsed in PBS and incubated with a mouse anti-rat Ig secondary conjugated to rhodamine fluorochrome (Molecular Probes, Eugene, OR) for 1 h at room temperature. Samples were then rinsed with PBS, mounted in a solution containing 0.1% p-phenylenediamine (Sigma), and analyzed via confocal microscopy.

Analysis of CD40 surface protein expression

To examine CD40 surface expression, airway epithelial cells were cultured with TNF-α, IL-1β, IL-4, or IL-13 at the concentrations and time points indicated. After culture, cells were lifted with HBSS (Ca²⁺ and Mg²⁺ free) containing 10% FCS and 0.05 M EDTA, washed twice with 1X PBS containing 0.2% BSA, and then stained with a mouse anti-human anti-CD40 mAb (10 μg/ml; G28.5, a gift from Dr. Randolph Noelle, Dartmouth Medical School, Lebanon, NH) for 30 min on ice. Parallel samples were stained with mouse IgG1κ immunoglobulin (10 μg/ml, Sigma) as an isotype-matched Ab control. After incubation, cells were washed as above and then incubated with the secondary Ab, FITC-conjugated goat anti-mouse F(ab')₂ IgG1 (diluted 1:100, BioSource, Camarillo, CA) for 30 min on ice. Cells were again washed as above, resuspended in 1X
PBS containing 0.2% BSA, and analyzed via flow cytometry (Becton Dickinson, FACS-caliibur; University of Alabama at Birmingham Core Facility).

**Analysis of IL-8, MCP-1, and RANTES protein expression**

To analyze IL-8, MCP-1, and RANTES protein expression, cells were cultured in the presence and absence of soluble CD40L (sCD40L; mCD8hgp39, a gift from Dr. R.J. Noelle) with or without TNF-α, IL-1β, IL-4, or IL-13 (R&D Systems, Minneapolis, MN) at the concentrations indicated for 18 h at 37°C. After culture, supernatants were harvested and prepared for ELISA of IL-8, MCP-1, or RANTES protein content (BioSource); cells were harvested and counted to account for differences in cell number. ELISAs were performed according to the manufacturer’s protocol (limits of detection, <3 pg/mL).

**Analysis of ICAM-1 and VCAM-1 surface protein expression**

To analyze ICAM-1 and VCAM-1 surface protein expression, cells were cultured in the presence and absence of sCD40L, with or without TNF-α, IL-1β, IL-4, or IL-13 (R&D Systems) at the concentrations indicated for 18 h at 37°C. After culture, cells were harvested and analyzed via flow cytometry as described above for CD40 detection with the exception that mAbs directed against ICAM-1 and VCAM-1 were utilized (R&D Systems).
Statistical analysis

Data are expressed as the mean ± SD for replicate determinations as indicated. Statistical significance was determined by ANOVA. \( p \leq 0.05 \) was considered significant.

RESULTS

CD40 surface protein expression in airway epithelial cells

To examine CD40 expression in primary epithelial cells, immunohistochemical analysis of BALB/c mouse lung tissue was performed. Mouse lung tissue was utilized for this analysis due to: 1) the difficulty in obtaining normal human lung tissue; and 2) the potential for phenotypic changes of primary cells cultured in vitro. Therefore, lungs from BALB/c mice were excised, quick-frozen in liquid nitrogen to preserve Ag immunoreactivity, and sectioned. Frozen sections of the upper airway were then stained with either a mAb directed against murine CD40 or an appropriate negative control. As shown in Fig. 1, epithelial cells lining the bronchioles of the upper airway stained brightly for CD40 (Fig. 1A), whereas negative control samples displayed little cross-reactivity (Fig. 1B).

To determine whether human airway epithelial cells can express CD40 in vitro, CD40 surface protein expression was examined on the 9HTE0 human airway epithelial cell line directly via flow cytometry. There are limitations to using in vitro cultures of airway epithelial cells. The phenotype of an airway epithelial cell, whether immortalized or primary, may not reflect its phenotype in vivo. However, the 9HTE0 cell line utilized for the studies described herein has been clearly described as "epithelial" (8). Thus, the use of this cell line provides unambiguous results for airway epithelial expression of
FIGURE 1. Airway epithelial cells express CD40 in vivo. Lungs from BALB/c mice were excised, quick-frozen in liquid nitrogen, and sectioned. Sections of mouse upper airway were analyzed via immunohistochemical analysis utilizing an anti-CD40 Ab followed by the appropriate secondary Ab conjugated to rhodamine. Representative results of three separate experiments are shown.
CD40. Flow cytometric analysis revealed that ~100% of 9HTEo' cells stained positively for CD40 expression (Fig. 2A). The negative control HT-29, a human intestinal epithelial cell line that does not express CD40 (9), did not stain for CD40 (Fig. 2B). The change in mean fluorescence intensity for 9HTEo' cells staining positively for CD40 expression was ~4-fold greater than controls.

Experiments were performed to determine whether the cytokines TNF-α, IL-1β, IL-4, and/or IL-13 modulated CD40 expression on airway epithelial cells in vitro. For these experiments, 9HTEo' cells were cultured separately with these various cytokines at increasing concentrations, ranging from 0.1 to 100 ng/ml, and increasing time points, ranging from 18 to 72 h. As presented in Fig. 2A, neither TNF-α nor IL-1β altered airway epithelial CD40 expression at the concentrations and time points examined. IL-4 and IL-13 also did not affect CD40 expression on airway epithelial cells (data not shown).

**CD40-mediated expression of chemokines**

CD40 is known to play a role in cell-cell interactions that result in the modulation of immune responses (2). To determine whether cross-linking CD40 on airway epithelial cells induced the expression of chemokines, 9HTEo- cells were cultured in the presence and absence of sCD40L and then analyzed for protein expression of the chemokines RANTES, MCP-1, and IL-8 via ELISAs. The 9HTEo- cell line was utilized as a model airway epithelial cell for these studies, given that 9HTEo- cells express CD40 (Fig. 2) and can be induced to express a variety of immune molecules, including chemokines and adhesion molecules (10). The chemokines IL-8, RANTES, and MCP-1 were examined.
FIGURE 2. Airway epithelial cells express CD40 surface protein. The airway epithelial cell line 9HTEo* was cultured in the presence and absence of TNF-α or IL-1β (each at 1 ng/ml) for 18 h at 37°C. After culture, 9HTEo* cells were lifted and stained with an isotype-matched control or anti-CD40 Ab (A) as described in the text; HT-29 cells (B) were included as a negative control. In the right-hand corner of each histogram is the percent positive for each cell sample tested. Representative results of three or more independent experiments are shown. Similar results were observed in 9HTEo* cells stimulated with TNF-α or IL-1β at 0.1 or 100 ng/ml for 48 or 72 h at 37°C (data not shown).
in these experiments because each has been implicated in facilitating leukocyte migration into the airway lumen during an inflammatory response (reviewed in Ref. 11). In particular, MCP-1 mediates monocyte and basophil chemotaxis and activation whereas IL-8 primarily induces the migration of neutrophils. RANTES induces the chemotaxis of eosinophils, monocytes, and CD45 RO⁺ memory T lymphocytes. The data in Fig. 3 demonstrate that CD40 ligation induces chemokine expression in 9HTEo⁻ cells. Specifically, sCD40L up-regulated RANTES, IL-8, and MCP-1 expression in 9HTEo⁻ cells from undetectable levels to 0.6 ng/10⁶ cells, 1.3 ng/10⁶ cells, and 2.2 ng /10⁶ cells, respectively (Fig. 3A). Importantly, sCD40L induced the expression of RANTES, IL-8, and MCP-1 in a dose-dependent manner; sCD40L-induced IL-8 expression is shown in Fig. 3B as a representative example. The effects of sCD40L on chemokine expression in airway epithelial cells were blocked by a mAb directed against CD40L (TRAP1, data not shown).

**CD40-mediated expression of adhesion molecules**

The adhesion molecules ICAM-1 and VCAM-1 facilitate leukocyte migration within the lung. Specifically, ICAM-1 binds β₂ integrins expressed on a variety of cell types, including lymphocytes and neutrophils, whereas VCAM-1 binds VLA-4 found on eosinophils (reviewed in Ref. 12). To determine whether CD40 engagement modulated expression of ICAM-1 and/or VCAM-1 on airway epithelia, 9HTEo⁻ cells were cultured with or without sCD40L. Cells were then collected and analyzed for the surface expression of ICAM-1 and VCAM-1 via flow cytometry. As observed in Fig. 4A, ~20% of 9HTEo⁻ cells stained positively for ICAM-1 expression; however, sCD40L enhanced
FIGURE 3. CD40 engagement induces chemokine expression in airway epithelial cells. 9HTEo' cells were cultured with or without sCD40L at a single concentration (A, 400 ng/ml) or increasing concentrations as indicated (B) for 18 h at 37°C. After culture, supernatants were collected, and the cells were counted to control for variances in cell number between samples. ELISAs specific for RANTES, IL-8, or MCP-1 were performed on the supernatants to analyze protein expression. Results are reported as nanograms protein/10^6 cells (n = 3; *, p ≤ 0.05 relative to carrier control).
FIGURE 4. CD40 engagement induces adhesion molecule expression in airway epithelial cells. 9HTEo' cells were cultured with or without sCD40L at a single concentration (A, 400 ng/ml) or increasing concentrations as indicated (B) for 18 h at 37°C. After culture, cells were harvested and analyzed for ICAM-1 and VCAM-1 surface expression via flow cytometry. Results are reported as percent positive (n = 3; *, p ≤ 0.01 relative to carrier control).
basal ICAM-1 expression ~3-fold. The dose response for sCD40L-mediated induction of ICAM-1 expression is shown in Fig. 4B. The change in mean fluorescence intensity for CD40-mediated modulation of ICAM-1 expression in 9HTEo' cells was ~2-fold greater than the respective isotype matched controls (data not shown). The effect of sCD40L on ICAM-1 expression in airway epithelial cells was blocked by a mAb directed against CD40L (TRAP-1, data not shown). Fig. 4A also demonstrates that 9HTEo' cells express little or no detectable VCAM-1 on the cell surface. Importantly, CD40 engagement on 9HTEo' cells did not induce the expression of VCAM-1 above basal levels (Fig. 4A). Because VCAM-1 could be shed from the cell surface, supernatants from these cultures were analyzed for the presence of soluble VCAM-1 via ELISA; however, these analyses did not detect the presence of soluble VCAM-1 (data not shown), suggesting that sCD40L does not induce VCAM-1 surface expression on 9HTEo' cells.

*Effects of proinflammatory cytokines and Th2 cytokines on CD40-mediated expression of chemokines*

In the asthmatic lung, airway epithelial cells are exposed to a variety of soluble mediators, including proinflammatory and Th2-derived cytokines, that alter their cellular activity and function. To examine the effects of proinflammatory and Th2-derived cytokines on CD40-mediated expression of the chemokines RANTES, IL-8, and MCP-1, 9HTEo' cells were cultured in the presence and absence of sCD40L with and without the proinflammatory cytokines TNF-α and IL-1β or the Th2-derived cytokines IL-4 and IL-13. Chemokine expression was then monitored via ELISA. TNF-α and IL-1β were utilized in these experiments because previous studies have demonstrated that these cytokines are elevated during an inflammatory response of the airway (13) and can
modulate airway epithelial expression of immune molecules (reviewed in Ref. 3). IL-4 and IL-13 were included in these experiments because they are Th2-derived cytokines that have been reported to alter the activity of airway epithelial cells (14, 15).

As demonstrated in Fig. 5, TNF-α and/or IL-1β enhanced the effects of CD40 engagement on RANTES, IL-8, and MCP-1 expression. Specifically, cells cultured with TNF-α alone expressed RANTES at ~0.4 ng/10⁶ cells; however, TNF-α synergized with sCD40L to increase RANTES expression to 2.0 ng/10⁶ cells or ~3- to 5-fold over that amount observed with either stimulus alone (Fig. 5A). Similarly, cells stimulated with IL-1β alone expressed approximately 1.0 ng/10⁶ cells; yet, IL-1β synergized with sCD40L to up-regulate MCP-1 expression to nearly 7.0 ng/10⁶ cells or between 3.5- and 7-fold greater than that observed with either stimulus alone (Fig. 5B). Interestingly, TNF-α and IL-1β each combined with sCD40L to up-regulate IL-8 expression in airway epithelial cells (Fig. 5C). In detail, TNF-α and IL-1β alone induced IL-8 expression from undetectable levels to ~1.0 ng/10⁶ cells and 1.0 ng/10⁶ cells, respectively (Fig. 5C). Importantly, both TNF-α and IL-1β combined with sCD40L in a synergistic fashion to enhance CD40-mediated IL-8 expression to ~3.7 and 3.0 ng/10⁶ cells, respectively, or between 2- and 3-fold greater than that observed with either stimulus alone. In contrast, neither IL-4 nor IL-13 induced RANTES, MCP-1, or IL-8 expression and, moreover, did not modulate the effects of CD40 cross-linking on chemokine expression in these cells (data not shown).

To further examine the effect of CD40 engagement on the sensitivity of airway epithelial cells to proinflammatory cytokines, additional experiments were performed. Specifically, 9HTEo⁺ cells were cultured in the presence and absence of sCD40L in
FIGURE 5. TNF-α and/or IL-1β enhance CD40-mediated effects on chemokine expression. 9HTEo' cells were cultured with or without sCD40L (400 ng/ml) in the presence and absence of TNF-α or IL-1β (each at 1 ng/ml) for 18 h at 37°C. After culture, supernatants were collected, and the cells were counted to control for variances in cell number between samples. ELISAs specific for RANTES (A), MCP-1 (B), and IL-8 (C) were performed on the supernatants to analyze protein expression. Results are reported as nanograms protein/10^6 cells (n = 3; *, p ≤ 0.05 relative to carrier control; †, p ≤ 0.05 relative to sCD40L alone and TNF-α or IL-1β).
combination with TNF-α or IL-1β at increasing concentrations. Alternatively, cells were preincubated with sCD40L for varying time periods and then exposed to TNF-α or IL-1β at a single concentration. After culture, supernatants from these cultures were harvested and examined for IL-8, RANTES, or MCP-1 protein expression via ELISA. As demonstrated in Fig. 6, ligation of CD40 increased the sensitivity of 9HTEo’ cells to the effects of TNF-α and IL-1β in a dose-dependent manner with regard to IL-8 expression; similar results were observed for the effects of TNF-α on RANTES expression and IL-1β on MCP-1 expression (data not shown). In contrast, priming the cells with sCD40L for 2 or 6 h before exposure with TNF-α or IL-1β did not alter the effect of either cytokine on IL-8 expression (Fig. 7); similar results were observed for RANTES and MCP-1 expression (data not shown).

Effect of proinflammatory and Th2 cytokines on CD40-mediated expression of adhesion molecules

The effects of TNF-α and IL-1β as well as IL-4 and IL-13 on CD40-mediated expression of the adhesion molecules ICAM-1 and VCAM-1 in airway epithelial cells were examined. Although TNF-α and IL-1β each induced the expression of ICAM-1 between 2- and 3-fold above basal levels, neither of these stimuli modulated CD40-mediated expression of ICAM-1 (Fig. 8A). Interestingly, IL-4 and IL-13 increased ICAM-1 expression modestly in these cells; however, both of these cytokines decreased CD40-mediated ICAM-1 expression significantly (Fig. 8A). In contrast, TNF-α alone, but not IL-1β, IL-4, or IL-13, induced expression of VCAM-1 in airway epithelial cells.
FIGURE 6. CD40 ligation enhances the sensitivity of airway epithelial cells to the effects of TNF-α and IL-1β with regard to IL-8 expression. 9HTEo cells were cultured with or without sCD40L (400 ng/ml) in the presence and absence of TNF-α (A) or IL-1β (B) at increasing concentrations as indicated for 18 h at 37°C. After culture, supernatants were collected, and the cells were counted to control for variances in cell number between samples. An ELISA specific for IL-8 was performed on the supernatants to analyze protein expression. Results are reported as nanograms protein/10⁶ cells (n = 3; *, p < 0.05 relative to carrier control; †, p < 0.05 relative to sCD40L alone and TNF-α or IL-1β alone).
FIGURE 7. Ligation of CD40 does not prime airway epithelial cells to respond to TNF-α or IL-1β. 9HTEo− cells were preincubated with sCD40L (400 ng/ml) for 0, 2, or 6 h at 37°C before exposure to TNF-α (A) or IL-1β (B) (each at 1 ng/ml) for an additional 18 h at 37°C. After culture, supernatants were collected, and the cells were counted to control for variances in cell number between samples. An ELISA specific for IL-8 was performed on the supernatants to analyze protein expression. Results are reported as nanograms protein/10⁶ cells (n = 3; *, p ≤ 0.05 relative to carrier control; †, p ≤ 0.05 relative to sCD40L alone and TNF-α or IL-1β alone).
FIGURE 8. TNF-α, IL-1β, IL-4, and IL-13 have no effect on CD40-mediated adhesion molecule expression. 9HTEo* cells were cultured with or without sCD40L (400 ng/ml) in the presence and absence of TNF-α, IL-1β, (each at 1 ng/mL), IL-4 (10 ng/ml), or IL-13 (5 ng/ml) for 18 h at 37°C. After culture, cells were harvested and analyzed for ICAM-1 (A) or VCAM-1 (B) surface expression via flow cytometry. Results are reported as nanograms protein/10⁶ cells (n = 3; *, p ≤ 0.05 relative to carrier control; †, p ≤ 0.05 relative to sCD40L alone and TNF-α or IL-1β alone).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
(Fig. 8B). Importantly, TNF-α synergized with sCD40L to increase VCAM-1 expression between 3- and 4-fold over that observed with either stimulus alone (Fig. 8B).

To further examine the effect of CD40 engagement on the sensitivity of airway epithelial cells to the effects of TNF-α with regard to VCAM-1 expression, 9HTEO− cells were cultured in the presence and absence of sCD40L in combination with TNF-α at increasing concentrations. In addition, cells were preincubated with sCD40L for varying time periods and then exposed to TNF-α at a single dose. After culture, cells were harvested and examined for VCAM-1 surface expression via flow cytometry. ICAM-1 expression was not monitored in these experiments because neither TNF-α nor IL-1β had a significant effect on sCD40L-induced ICAM-1 expression in 9HTEO− cells (Fig. 8). As demonstrated in Fig. 9A, ligation of CD40 increased the sensitivity of 9HTEO− cells to the effects of TNF-α in a dose-dependent manner with regard to VCAM-1 expression. In contrast, priming the cells with sCD40L for 2 or 6 h before exposure with TNF-α did not alter the effect of this cytokine on VCAM-1 expression (Fig. 9B).

DISCUSSION

The data presented herein demonstrate that airway epithelial cells, in vitro and in vivo, express CD40. Moreover, these data show that ligation of CD40 expressed on the airway epithelial cells up-regulates the expression of inflammatory mediators, including the chemokines, IL-8, RANTES, and MCP-1 and the adhesion molecule ICAM-1. Engagement of epithelial CD40 had no effect on VCAM-1 expression. Importantly, CD40 ligation enhanced the sensitivity of airway epithelial cells to the effects of TNF-α and/or IL-1β on expression of RANTES, MCP-1, IL-8, and VCAM-1. In contrast,
FIGURE 9. CD40 ligation enhances the sensitivity of, but does not prime, airway epithelial cells to the effects of TNF-α with regard to VCAM-1 expression. A, 9HTEo' cells were cultured with or without sCD40L (400 ng/ml) in the presence and absence of TNF-α at increasing concentrations as indicated for 18 h at 37°C. B, 9HTEo' cells were preincubated with sCD40L (400 ng/ml) for 0, 2, or 6 h at 37°C before exposure to TNF-α (1 ng/ml) for an additional 18 h at 37°C. After culture, cells were harvested and analyzed for VCAM-1 surface expression via flow cytometry. Results are reported as nanograms protein/10^6 cells (n = 3; *, p ≤ 0.05 relative to carrier control; †, p ≤ 0.05 relative to sCD40L alone and TNF-α alone).
neither IL-4 nor IL-13 modified the effects CD40 engagement on the expression of RANTES, MCP-1, IL-8, or VCAM-1; however, both IL-4 and IL-13 attenuated the effects of CD40 cross-linking on ICAM-1 expression. These findings suggest that epithelial CD40 plays a role in airway inflammatory responses.

The epithelial barrier in the airway has two distinct surfaces, the apical (luminal) and the basolateral (serosal) surfaces. The apical surface is exposed to the environment directly whereas the basolateral surface is protected from the environment through the existence of tight junctions. Tight junctions facilitate selective transport of materials across the epithelial barrier and dictate sequestration of proteins made by epithelia to either the apical or the basolateral compartment. To date, there is no conclusive evidence that CD40 demonstrates a polarized pattern expression on airway epithelial cells. Our observations suggest that CD40 is expressed primarily on the apical surface; however, we can detect CD40, albeit to a lesser degree, on the basolateral surface (Fig. 1). A more definitive answer to this question is being pursued via colocalization studies and laser confocal microscopy. As stated in the Introduction, T lymphocytes play a major role in the pathogenesis of allergic airway disease. In fact, elevated numbers of activated T cells have been observed in the BAL fluid and bronchial tissue of asthmatic patients (reviewed in Ref. 4). In the light of the data presented herein, we anticipate that T lymphocytes, which express CD40L on activation (1), will encounter and interact with CD40 expressed on the basolateral and/or apical surface of the airway epithelium as these cells migrate from the circulation and into the airway lumen. The consequences of such cell-cell interactions, be it at the apical and/or basolateral surface, will trigger the epithelium to
express increased amounts of chemokines and adhesion molecules and thereby contribute to the airway inflammatory response.

Previous reports indicate that bronchial epithelial cells express CD40 (16, 17). In particular, Gormand et al. (17) have reported that the cytokines TNF-α and IFN-γ increased the basal expression of CD40 on bronchial epithelial cell lines. Moreover, their data suggest that ligation of CD40 expressed on bronchial epithelial cell lines enhanced the expression of IL-6 and GM-CSF from these cells; however, CD40 engagement did not alter the sensitivity of these cells to the effects of TNF-α with regard to IL-6 and GM-CSF expression.

The data presented herein contrast with those of Gormand et al. First, although our data also demonstrate CD40 expression in both lung tissue and airway epithelial cell lines, such expression was not modulated by the cytokines TNF-α, IL-1β, IL-4, or IL-13. Second, as was similarly observed by Gormand et al., our data demonstrate that the expression of inflammatory molecules, including RANTES, IL-8, MCP-1, and ICAM-1, was increased on ligation of CD40 on airway epithelial cells. Third, as stated above, our results indicate that CD40 engagement enhances the sensitivity of airway epithelial cells to the effects of proinflammatory cytokines TNF-α and/or IL-1β. Specifically, CD40 ligation enhanced the response of the cells to TNF-α with regard to the expression of IL-8, RANTES, and VCAM-1, as well as IL-1β, with regard to MCP-1 and IL-8 expression. Interestingly, CD40 ligation before cytokine exposure did not prime airway epithelial cells to respond to either TNF-α or IL-1β. Together, our results suggest that, as CD40L+ cells migrate into the lung and cross the epithelial barrier, ligation of epithelial CD40 will
render the epithelium more sensitive and responsive to the effects of proinflammatory cytokines present in the local microenvironment.

In addition to airway epithelial cells, other CD40-responsive cell types in the lung have been identified. Lazaar et al. (18) reported that cross-linking CD40 on airway smooth muscle cells with CD40L up-regulates the expression of the pleiotropic cytokine IL-6. Similarly, Sempowski et al. (19) demonstrated that cross-linking CD40 on lung fibroblasts with CD40L induces the expression of IL-6 and the chemokine IL-8; expression of both of these molecules was enhanced further in the presence of IFN-γ. Moreover, Zhang et al. (20) reported that engagement of CD40 on lung fibroblasts increased PGE2 synthesis via the induction of cyclooxygenase-2.

Recent reports suggest that CD40 plays a role in airway inflammatory responses in vivo. For example, Adawi et al. have reported that disruption of CD40-CD40L interactions blunts hyperoxic lung injury (21) and protects against radiation-induced pulmonary toxicity (22). Specifically, these authors demonstrated that mice pretreated with an Ab against CD40L protected against oxygen-induced lung injury as well as radiation-induced pneumonitis and fibrosis. Studies utilizing CD40 or CD40L knockout mice have also implicated CD40 in airway inflammatory responses in vivo. Wiley et al. (5) have shown that treatment of wild-type CD40 mice with sCD40L increased polymorphonuclear cell infiltration of the alveolar space and an accumulation of alveolar macrophages with increased Ia expression; such an increase in cell infiltration of the lungs was not observed in CD40L-treated CD40 knockout mice. Similarly, Lei et al. (7) have reported altered airway immune responses in CD40L knockout mice. These authors observed that CD40L knockout mice sensitized with OVA followed by an OVA aerosol
challenge, as a model of allergic airway inflammation, displayed a reduced airway inflammatory response when compared with similarly sensitized and challenged wild-type controls. Specifically, significantly less numbers of monocytes, lymphocytes, neutrophils, and eosinophils were detected in the BAL fluid of CD40L knockout mice at 72 h postchallenge as compared with controls. Moreover, decreased serum levels of OVA-specific IgE and IgG1 and IL-4 and decreased BAL levels of IL-4 and TNF-α were detected in the CD40L knockout mice as compared with wild-type controls; however, similar levels of IL-5 were detected in the serum and BAL fluid of both control and knockout mice. In addition, lung endothelial cell expression of VCAM-1 in OVA-sensitized and challenged CD40L knockout mice was reduced as compared with controls.

It is evident that CD40 plays a role in lung inflammation in vivo. Identifying the CD40-responsive cells within the airway that promote inflammation is critical in understanding the mechanisms that underlie airway inflammation and, moreover, in generating novel therapies that ameliorate inflammatory diseases such as asthma.

ACKNOWLEDGEMENTS

We thank Dr. Randolph Noelle, Dr. Etty Benveniste, and Albert Tousson for their assistance, and Dr. Dale Benos, the Department of Physiology and Biophysics, and the Department of Cell Biology for their continued support.

REFERENCES


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


TRAF3 ENHANCES CD40-MEDIATED SIGNALING IN AIRWAY EPITHELIAL CELLS

by

STACIE M. PROPST, KIM ESTELL, AND LISA M. SCHWIEBERT

Submitted to the Journal of Biological Chemistry

Format adapted for dissertation
ABSTRACT

CD40 is expressed by airway epithelial cells, and its activation regulates the expression of chemokines (RANTES, IL-8, MCP-1) and adhesion molecules (ICAM-1, VCAM-1). CD40 signaling is cell-type specific, but at this time, nothing is known about the mechanisms underlying CD40 signaling in airway epithelial cells. In this study, we investigated the signaling pathways involved in CD40-induced activation of a RANTES-luciferase (R1.4) promoter construct expressed in a tracheal epithelial cell line, 9HTEo'.

Tumor necrosis factor (TNF) receptor-associated factors (TRAFs) bind to the cytoplasmic tail of CD40 and act as adapter proteins that trigger downstream signaling events. Therefore, we utilized transient transfection assays to study the effects of overexpressing TRAF2 and TRAF3 in 9HTEo' cells. TRAF3 up-regulated RANTES promoter activity, while TRAF2 inhibited the activation of the RANTES promoter. The RANTES promoter contains binding sites for the transcription factor nuclear factor-κB (NF-κB), and CD40 has been shown to activate NF-κB in B cells and a human embryonic kidney cell line (HEK293). Inactive NF-κB is retained in the cytoplasm by inhibitors of NF-κB (IκBs) which mask its nuclear translocation signal. IκBs are inducibly phosphorylated by a multisubunit IκB kinase (IKK) which is composed of two catalytic subunits, IKK-α and IKK-β, both of which can properly phosphorylate IκB, thus targeting it for ubiquitination by the 26S proteasome and releasing NF-κB to translocate to the nucleus where it activates gene transcription. Therefore, we examined the involvement of IKKs in CD40-induced RANTES promoter activation. Specifically, overexpression of IKK-α and IKK-β activated the RANTES promoter, and overexpression of dominant negative forms of either of these kinases completely

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
inhibited CD40-induced RANTES promoter activation. In addition, we show here that cross-linking of CD40 with soluble CD40 ligand (sCD40L) leads to phosphorylation of IκB-α on Ser32 which is required for degradation of IκB. Taken together, these findings suggest that epithelial CD40, when bound by its ligand, activates NF-κB through TRAF3 binding and IκB phosphorylation. Importantly, these results also support the emerging role of epithelial cells as immune effector cells in the lung.

INTRODUCTION

CD40 is a member of the TNFR family which includes TNFRI (p55), TNFRII (p75), CD30, Fas, and low-affinity nerve growth factor receptor (LNFGFR) (reviewed in Refs. 1, 2). CD40 and its natural ligand, CD40L, play a central role in the regulation of humoral and cell-mediated immunity (3). Depending on the cell type and the local environment, interactions between CD40 and CD40L can modulate cell proliferation, differentiation, apoptosis, isotype switching, and inflammatory mediator production (4).

Members of the TNFR family display homology in their extracellular ligand-binding domains, which are composed of tandemly repeated cysteine-rich modules. The interactions of these modules create a three-dimensional structure that provides ligand specificity (reviewed in Ref. 5). Members of the TNF ligand family are trimerized, and their cognate receptors aggregate upon binding. In turn, receptor aggregation activates signal transduction. However, TNFR family members lack an intrinsic cytoplasmic enzymatic activity capable of transducing a signal to the nucleus (reviewed in Ref. 6). In 1994 Rothe and colleagues (7) identified a family of putative signal transducing proteins that associated with TNFRII (p75). These TNFR-associated factors (TRAFs) have
proven to act as unique signaling adapter proteins for the TNFR superfamily as well as for some members of other receptor families (7). To date, six TRAF molecules have been identified (TRAF1-TRAF6). Growing evidence supports the idea that the diverse array of biological functions displayed by members of the TNFR family is at least partially due to the nature of TNFR-TRAF interactions. Hence, the context in which transmembrane CD40 binds to its ligand (CD40L) will determine the functional result of that interaction. Differences in signaling could arise from the cell type expressing CD40 or CD40L, differentiation state of the cells involved, local environment (tissue specificities), the presence of modulatory factors, and the recruitment of individual TRAFs.

TRAF protein structure can be generally divided into two basic domains: 1) a carboxy-terminal TRAF domain, and 2) an amino-terminal domain with RING and zinc finger motifs (7). The TRAF domain can be further divided into the TRAF-C (C-terminal portion) and TRAF-N (N-terminal portion) regions. The TRAF-C region is highly conserved and is critical for the interaction of TRAF molecules with cytoplasmic receptor tails as well as with signaling proteins such as NF-κB-inducing kinase (NIK) (8). The TRAF-N region contains predicted coiled-coiled structures and mediates TRAF self-association into homo- and hetero-oligomers (9, 10). With the exception of TRAF1, all TRAFs contain an amino-terminal RING finger domain and 5-7 zinc finger domains that appear to be required for downstream signaling (11).

A number of studies have established that CD40 multimerization induces the transcription factor NFκB. This activation has been connected to TRAF2, TRAF5, and TRAF6 association with CD40 (9, 12). In contrast, TRAF3 has been shown to have an
inhibitory effect on TRAF2 positive CD40 signaling in B cells; however, the precise role of TRAF3 remains poorly defined (13). Presently, information regarding CD40 signaling is based primarily on studies performed with B cells and a human embryonic kidney cell line (HEK 293). A few studies have been performed in other cell types, and those indicate that signaling through CD40 is highly cell-type specific. To our knowledge, no one has examined the signaling mechanisms of CD40 in an airway epithelial cell system.

Recently, we described CD40 expression on airway epithelial cells and found that engagement of CD40 on these cells stimulates the production of chemokines, IL-8, RANTES, and MCP-1, and adhesion molecules, ICAM-1 and VCAM-1 (14). The data presented here examine the underlying signaling mechanisms that may be involved in CD40-induced inflammatory mediator expression. To that end, we verified expression of TRAF2 and TRAF3 in airway epithelial cells and proceeded to examine their involvement in CD40 signaling. We restricted these initial studies to TRAF2 and TRAF3 because they are clearly the best characterized and because other investigators have shown that TRAF2 and TRAF3 have the highest binding affinities for CD40 (15). Specifically, we performed transient transfection assays to study RANTES promoter activity after cross-linking CD40 on airway epithelial cells. Our studies suggest that TRAF3 is involved in positive CD40 regulation of the RANTES promoter but that TRAF2 blocks CD40-induced RANTES promoter activation. Furthermore, our data suggest that CD40 signaling is dependent on the transcription factor NF-κB.
MATERIALS AND METHODS

Cell Culture

Experiments employed the human airway epithelial cell line 9HTEo- (tracheal; a gift from Dr. Dieter Gruenert, University of California, San Francisco, CA (16)). In addition, a bronchial cell line (also a gift from Dr. Dieter Gruenert, University of California, San Francisco, CA), 16HBE14o− was employed for TRAF detection experiments. Airway epithelial cells were cultured in LHC-8 media (Biofluids, Rockville, MD) containing 5% FCS, 1% penicillin/streptomycin, an 0.2% Fungizone (Gibco/BRL, Gaithersburg, MD). All cells were grown at 37°C in a 5% CO₂ environment and on Vitrogen 100 (Cohesion, Palo Alto, CA) -coated flasks (Vitrogen 100 contains collagen types I and IV).

Detection of TRAF molecule expression

Cells were cultured as described above and then lysed in lysis buffer (10 mM Tris, 0.15 mM NaCl, 0.5% Triton-X, and the protease inhibitors aprotonin, leupeptin, and pepstatin A (100 ug/ml each), and 10 uM PMSF); lysates were then spun to remove nuclei and cellular debris. Whole cell lysates (5 x 10⁶ cells/sample) were pretreated with glutathione-agarose (Sigma Chemical) for 2 h at 4°C and then immunoprecipitated with GST or GST-CD40cyt (a GST-CD40 fusion protein containing the cytoplasmic tail of CD40) each at 20 ug/ml and glutathione agarose for 18 h at 4°C. Precipitated proteins were washed three times with cold lysis buffer, eluted, electrophoresed, and immunoblotted with rabbit polyclonal antibodies specific for TRAF2 or TRAF3 (each at 1 ug/ml; Santa Cruz, Santa Clarita, CA) followed by a goat anti-rabbit-HRP secondary.
Ab (1:2000 dilution in lysis buffer, Sigma Chemical Co.) and developed via ECL chemiluminescence (Amersham, Piscataway, NJ).

**Transient transfection of TRAF molecules and RANTES plasmid constructs**

Cells were seeded on Vitrogen 100-coated wells of a 6-well plate. At approximately 75% confluence, cells were incubated with low-serum Opti-MEM I media (Gibco/BRL) containing Lipofectamine Plus (6 ug/well; Gibco/BRL) and TRAF constructs (0.5 ug/well). RANTES promoter-luciferase reporter constructs (2.0 ug/well), and a β-galactosidase construct (0.5 ug/well) for 6 h at 37°C. Control experiments were performed by replacing the TRAF constructs with an appropriate mock control. Full-length and mutant forms of TRAF molecules were utilized in these studies (Fig. 1A) and were generous gifts from Dr. Randolph Noelle, Dartmouth Medical School, Lebanon, NH; each construct contains a peptide tag, c-Myc or FLAG. The RANTES promoter-luciferase reporter constructs (Fig. 1B) were generated and provided by Dr. Hiroyuki Moriuchi, NIH (17). After incubation, the cells were washed with buffer, and media containing 5% FCS was added to each well. The cells were cultured for an additional 24 h at 37°C. Following transfection, cells were either stimulated with sCD40L (400 ng/ml) or left untreated for 18 h at 37°C. Cells were harvested, lysed, and then analyzed for luciferase activity via the Dual Luciferase Reporter assay system (Promega, Madison, WI) and the relative expression of the epitope-tagged TRAF molecules via immunoblotting with antibodies against their respective tags. Relative transfection efficiency was assessed through measurement of β-galactosidase activity (Promega).
A. TRAF constructs

<table>
<thead>
<tr>
<th>N-terminus</th>
<th>C-terminus</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAF Domain</td>
<td></td>
</tr>
<tr>
<td>Ring</td>
<td>Zinc Finger</td>
</tr>
</tbody>
</table>

wild-type TRAF →

mutant TRAF →

B. RANTES-luciferase promoter

1.4kB CD28RE NF-AT NF-κB (κB1, κB2) TATA

LUC

FIGURE 1. Maps of the TRAF and RANTES-luciferase promoter constructs.
Transient transfection of IκB kinase-α and IκB kinase-β constructs and RANTES plasmid constructs

Experiments were designed using the same procedures as described above except that cotransfections included IκB kinase-α (IKK-α) and IκB kinase-β (IKK-β) wild-type and dominant-negative constructs (gifts from Dr. Randolph Noelle, Dartmouth Medical School, Lebanon, NH), RANTES-luciferase promoter construct, and appropriate mock controls. After the 24-h recovery period (as described above), cells were stimulated or not with sCD40L (400 ng/ml) for 18 h at 37°C. Cells were then harvested and analyzed for luciferase and β-galactosidase activity as described above.

Analysis of IκBα phosphorylation

Cells were stimulated with or without sCD40L (400 ng/ml) for 0, 5, 10, 15, and 30 min. Following stimulation, cells were lysed with lysis buffer as described above and then examined for the presence of phosphorylated IκBα via immunoblotting. Specifically, equivalent amounts of protein (25 μg/lane; as determined via BioRad DC Protein Assay) for each sample were electrophoresed and transferred to a PVDF membrane. Nonspecific sites were blocked with Tris-buffered saline (TBS; 20 mM Tris-HCl, 140 mM NaCl, pH 7.6) containing 0.1% Tween and 5% nonfat dry milk; filters were then immunoblotted with a polyclonal rabbit Ab that recognizes IκBα-Ser32 (diluted 1:1000 in TBS containing 0.1% Tween and 5% BSA; New England Biolabs, Inc., Beverly, MA) followed by a goat anti-rabbit IgG antibody conjugated to HRP (diluted 1:2000 in TBS containing 0.1% Tween and 5% BSA). Immunoblots were developed using chemiluminescence. Blots were then stripped (0.2 N NaOH for 5 min at room temperature) and reprobed with a polyclonal rabbit Ab against IκBα (diluted...
1:1000 in TBS containing 0.1% Tween and 5% BSA; New England Biolabs, Beverly, MA) in order to verify equivalent IκBα protein expression in each sample.

RESULTS

**TRAf molecule expression in airway epithelial cells**

As described previously, CD40 receptor engagement triggers association with a family of signaling adapter molecules known as TRAFs (7). To demonstrate whether airway epithelial cells express TRAFs and if CD40 can potentially recruit these adapter proteins to its cytoplasmic domain upon receptor activation, we performed immunoprecipitation experiments using cell lysates derived from airway epithelial cells (9HTEo' and 16HBE14o'). Specifically, a fusion protein composed of GST and a portion of the cytoplasmic tail of CD40 was used as bait to detect the presence of TRAF2 and TRAF3 in whole cell lysates. Using Abs specific to the individual TRAFs, our results indicate that 9HTEo' cells and 16HBE14o' cells express TRAF2 and TRAF3 (Fig. 2) as well as TRAF5 and TRAF6 (data not shown). The presence of these adapter proteins in airway epithelial cells confirms their expression and availability for participation in CD40 signaling events.

**TRAf molecule involvement in CD40-triggered RANTES promoter activity**

TRAF2 and TRAF3 have recently been shown by in vitro binding studies and by plasmon resonance studies to have significantly higher binding affinities to the cytoplasmic tail of CD40 than do TRAF1, TRAF5, or TRAF6 (15, 18, 19). Additionally, TRAF2 and TRAF3 are thought to differentially regulate CD40 in B cells (13).
**FIGURE 2.** Airway epithelial cells express TRAF2 and TRAF3. 9HTEo- and 16HBE14o- cells were analyzed for TRAF2 (upper panel) and TRAF3 (lower panel) protein expression via immunoprecipitation with GST or GST-CD40cyt as described in the text. Representative results of three separate experiments are shown.
Presumably, this difference in regulation is due to a common binding motif in the cytoplasmic tail of CD40, but the exact mechanism is unknown. Therefore, we focused on the participation of TRAF2 and TRAF3 in CD40-induced RANTES promoter activity in the airway epithelial cell line, 9HTEo⁺. This cell line was utilized because it constitutively expresses CD40 on its surface and is responsive to stimulation by sCD40L. Maps of the TRAF constructs and the RANTES-luc promoter employed are shown in Figure 1. The mutant forms of the TRAF constructs lack the coding region for the ring domain and portions of the zinc-finger domain (Fig. 1A). These truncated forms of the TRAF proteins can bind to the cytoplasmic tail of CD40 but cannot signal downstream.

To verify the expression of TRAF constructs in these experiments, we probed equivalent amounts of cell lysates with epitope-specific antibodies (see inset, Fig. 3). The wild-type RANTES-luciferase reporter gene is composed of a 1.4 kB 5′-noncoding sequence of the RANTES gene fused to a luciferase reporter gene (R1.4) (Fig. 1B).

Cells treated with sCD40L and transiently transfected with a mock control for the TRAF constructs and the R1.4 construct showed a 2-fold increase of relative luciferase activity over that of untreated cells (Fig. 3). Clearly, CD40 engagement on these cells induced the activation of the RANTES promoter. However, when transfection assays included either TRAF2 or the TRAF2 mutant construct, luciferase activity returned to control levels. The result was the same in the presence or absence of stimulation by sCD40L. Hence, the promoter activation seen in the sCD40L stimulated controls was lost when either TRAF2 or mutant TRAF2 was overexpressed in 9HTEo⁺ cells.

Conversely, inclusion of TRAF3 and mutant TRAF3 overexpression enhanced promoter activation. In cells not treated with sCD40L, reporter luciferase activity
FIGURE 3. TRAF molecules modulate CD40-mediated RANTES promoter activation in airway epithelial cells. 9HTEo⁺ cells were transiently transfected with mock or TRAF and RANTES (R1.4) constructs as described in the text. Results are expressed as relative reporter activity (luciferase light units normalized by β-galactosidase activity). Samples were examined for full-length TRAF and mutant TRAF expression by immunoblotting with epitope-tagged specific antibodies (see inset). Results from three independent experiments are shown (*, p ≤ 0.05 with respect to unstimulated mock controls).
increased 4-fold above similarly untreated controls in the presence of TRAF3. When treated with sCD40L, reporter activity increased even further (Fig. 3). However, mutant TRAF3 overexpression diminished this CD40-induced activity, indicating that CD40 activation of the RANTES promoter is regulated through a TRAF3-dependent mechanism.

**Effects of mutated NF-κB binding sites in the RANTES promoter**

The transcription factor NF-κB is known to play a role in RANTES promoter activity (17). In addition, a number studies have described activation of NF-κB upon CD40 engagement and TRAF binding (12, 18-26). Therefore, we cotransfected 9HTEo- cells with either a control mock construct or the TRAF3 construct in combination with either a full-length RANTES-luc promoter construct or a RANTES-luc promoter construct containing mutated NF-κB sites (17). The loss of NF-κB binding sites eliminated CD40-induced RANTES activation (Fig. 4), indicating that epithelial CD40-signaling events involve the activation of NF-κB. Next, we examined whether RANTES promoter activation induced by TRAF3 overexpression was affected by the loss of functional NF-κB sites and found that RANTES promoter activation was significantly reduced. These results suggest that TRAF3 is acting through an NF-κB-dependent pathway. To further test this finding, we examined the involvement of possible intermediate signaling components, IKK-α and IKK-β, which are well described activators of NF-κB.
FIGURE 4. Ligation of CD40 activates the RANTES promoter via an NF-κB-dependent mechanism. 9HTEo' cells were transfected with a mock control or the full-length TRAF3 construct together with RANTES promoter constructs containing either wild-type (R1.4) or mutated (ΔκB) sites. Cells were stimulated with sCD40L and analyzed as described in the text. Samples were also examined for relative amounts of epitope-tagged TRAF3 expression via immunoblotting (see inset). Results from three separate experiments are shown (*, p < 0.05 relative to unstimulated mock controls).
IKK-α and IKK-β play a role in epithelial CD40 signaling

NF-κB is sequestered in the cytoplasm by members of the inhibitory IκB family (27). Phosphorylation of IκB proteins by kinases such as the serine/threonine kinases IKK-α and IKK-β triggers degradation of IκBs, and NF-κB is released as a consequence. Once released, NF-κB can migrate to the nucleus as an active transcription factor. Therefore, we investigated the participation of both kinases in CD40 receptor-mediated signaling. As described for earlier studies, transient transfection assays were used to examine the ability of CD40 to activate the RANTES promoter. The constructs used encode either a mock control, wild-type, or dominant-negative forms of IKK-α or IKK-β. The dominant-negative forms of IKK-α and IKK-β each contain alanine substitutions of conserved lysine residues in the kinase domain, thereby rendering the kinases catalytically inactive (11, 28).

IKK-α overexpression increased RANTES promoter activity 2-fold above untreated mock control (Fig. 5). When 9HTEo' cells were simultaneously treated with sCD40L, however, RANTES promoter activity increased ~4-6-fold above untreated mock control. IKK-α dominant-negative overexpression returned RANTES promoter activity to control levels whether in the presence or absence of stimulation by sCD40L. The results of these experiments indicate that functional IKK-α is required for CD40-induced RANTES promoter activation. Next, we studied the overexpression of IKK-β and IKK-β dominant-negative constructs (Fig. 6) and found that the results were similar to those observed for IKK-α and IKK-α dominant-negative overexpression. These results suggest that two IκB kinases, IKK-α and IKK-β, are required for the activation of the RANTES promoter through CD40 engagement on 9HTEo' airway epithelial cells.
FIGURE 5. IKK-α is necessary for CD40 activation of the RANTES promoter. 9HTEo cells were transfected with either a mock construct, IKK-α wild-type construct (WT), or IKK-α dominant-negative (DN) construct in conjunction with the wild-type RANTES promoter construct. Cells were stimulated with sCD40L and analyzed for reporter activity as described in the text. Results from three separate experiments are shown (*, $p < 0.05$ relative to unstimulated mock controls).
FIGURE 6. IKK-β is necessary for CD40 activation of the RANTES promoter. 9HTEo<sup>−</sup> cells were transfected with either a mock construct, IKK-β wild-type (WT) construct, or IKK-β dominant-negative (DN) construct in conjunction with the wild-type RANTES promoter construct. Cells were stimulated with sCD40L and analyzed for reporter activity as described in the text. Results from three separate experiments are shown (*, p < 0.05 relative to unstimulated mock controls).
These kinases are thought to form active heterodimers upon stimulation and to be responsible for the phosphorylation of specific serines on IκB-α and IκB-β (28).

*Ligation of epithelial CD40 triggers phosphorylation of IκB-α*

As stated earlier, NF-κB is retained in the cytoplasm of unactivated cells through interaction with the members of the IκB inhibitor family, including IκB-α (27). Phosphorylation at specific serines (Ser 32 and 36) immediately precedes degradation of IκB-α, releases NF-κB, and allows NF-κB to translocate to the nucleus where it can activate transcription (27); therefore, phosphorylation of IκB-α is a reliable marker of NF-κB activation. To further understand the role of NF-κB in CD40 signaling, we examined the effect of CD40 signaling on phosphorylation at serine 32 (Ser32). Because phosphorylation occurs rapidly in activated cells, we chose a time course of 0-30 min to examine the effects of CD40 ligation. As shown by Western analysis (Fig. 7A), CD40-sCD40L ligation induces an increase in the amount of phosphorylated IκB-α at Ser32 present in 9HTEo' cells. Densitometry (Fig. 7B) reveals that there is a 4-fold increase in this phosphorylated form of IκBα at 5 min and that levels decrease with time. These results provide additional evidence that engagement of CD40 by sCD40L on airway epithelial cells activates the RANTES gene promoter at least partly through an NFκB-dependent signaling pathway.

**DISCUSSION**

In our laboratory, we are interested in how epithelial cells in the airway participate in inflammation. Airway epithelial cells express many classically immune
FIGURE 7. CD40 ligation on airway epithelial cells enhances phosphorylation of \( I\kappa B\alpha \). 9HTEo' cells were stimulated in the presence and absence of sCD40L for the time points indicated at 37°C. After stimulation, lysates were analyzed for phospho-\( I\kappa B\alpha\) serine 32 content via immunoblotting; a positive control lysate (C; generated from TNF-\( \alpha \) stimulated HeLa cells) was included. Blots were stripped and reprobed for \( I\kappa B\alpha\) protein expression (A). Densitometric results were normalized to total \( I\kappa B\alpha\) protein content (B). Representative results of three independent experiments are shown.
molecules including CD40. CD40 activation in these cells up-regulates chemokines and adhesion molecules (14). Therefore, we initiated studies that examined how engagement of CD40 by sCD40L regulates the production of the chemokine, RANTES, in airway epithelial cells. Chemokines and adhesion molecules are considered to be proinflammatory mediators because they facilitate the migration of immune cells to sites of infection or injury. The transcription factor, NF-κB, is involved in the regulation of a large number of proinflammatory genes that are modulated in response to inflammation and infection, including chemokines and adhesion molecules.

The pathways that have been implicated in CD40 signal transduction are as varied as the functional outcomes. As a member of the TNFR family, CD40 lacks an intrinsic enzymatic activity that is capable of initiating a signaling cascade (6). Instead, adapter molecules called TNFR-associated factors (TRAFs) interact with the cytoplasmic domain of CD40 and trigger downstream molecules such as NFκB-inducing kinase (NIK) (8) and c-Jun NH₂-terminal kinase (JNK) (29). As stated earlier, six TRAFs have been identified (TRAF1-TRAF6) and all of them, excluding TRAF 4, have been implicated in the regulation of CD40 signaling. Studies have demonstrated that the intracellular domain of CD40 can associate with TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6 (7, 10, 21, 30). As yet, there is no evidence that TRAF4 can bind to CD40 (10, 31). TRAF5 interaction with CD40 appears to be indirect and it has been suggested that its role in CD40-mediated activation results from hetero-oligomerization between the isoleucine zipper motifs of TRAF3 and TRAF5 (10).

Our studies focused on the roles of TRAF2 and TRAF3 in epithelial CD40 signaling. The importance of TRAF2 and TRAF3 as ubiquitous signaling molecules is
highlighted by knockout and transgenic model research. TRAF2-deficient mice suffer from atrophy of the thymus and the spleen as a result of increased sensitivity to TNF-induced apoptosis and are defective in TNF-mediated stress-activated protein kinase (SAPK/JNK) activation (32, 33). Interestingly, effects on NF-κB activation were minimal, indicating that TRAF2 was nonessential for NF-κB activation. A similar phenomena was seen in lymphocytes from a mouse expressing a TRAF2 dominant-negative transgene (34). Loss of the TRAF3 gene in mice causes impaired T cell-dependent immunity and results in early postnatal lethality (35). Currently, it seems that TRAF5 can associate with receptors only through hetero-oligomerization with TRAF3, which could mean that the loss of TRAF3 may also result in the loss of TRAF5-mediated signaling.

Ongoing research is attempting to establish the connection between ligand binding to CD40, CD40 oligomerization, association of the TRAF adapter molecules to the CD40 cytoplasmic tail, and downstream transcriptional activation. There is some debate whether TRAFs associate with CD40 constitutively or associate upon CD40 engagement by CD40L. In the embryonic kidney epithelial cell line, HEK293, overexpression of TRAFs 2, 3, 5, and 6 results in constitutive association of TRAFs with CD40 and triggers NF-κB activation (9, 20). However, in B cells, association of TRAF2 and TRAF3 with CD40 requires recruitment by ligation with CD40L, but this association also results in NF-κB activation (30). TRAF2 is considered to be the molecule responsible for CD40 activation of NF-κB. In HEK293 cells, it was observed that overexpression of TRAF3 in relation to TRAF2 blocked NF-κB activation via CD40 (9).
Despite the evidence that TRAF3 associates with CD40, the role of TRAF3 in CD40-mediated signaling is still unclear.

Recently, a number of hypotheses have been suggested regarding TRAF3. Dadgostar and Cheng have examined the inability of TRAF3 to activate c-Jun terminal kinase (JNK) in comparison to TRAFs 2, 5, and 6 (36). They were able to convert TRAF3 to a potent activator of JNK by exchanging portions in its zinc finger region with that of TRAF5 or by forcing TRAF3 to the membrane by giving it a myristoylation signal. These effects suggest that TRAF3 may be somehow unavailable endogenously. Interestingly, a recent study by Ling and Goeddel describes a novel protein, MIP-T3 (microtubule-interacting protein that associates with TRAF3), that selectively binds to TRAF3 and microtubules, thus possibly sequestering TRAF3 (37). The stimulation of CD40 in HEK293 cells released TRAF3 from MIP-T3, and TRAF3 was subsequently recruited to the CD40 receptor. Lastly, van Eyndhoven and coworkers have cloned isoforms of TRAF3 resulting from splice-deletion variants capable of activating NF-κB in HEK293 cells (38).

We show here that airway epithelial cells express TRAF2 and TRAF3 endogenously (Fig. 1) in addition to TRAF5 and TRAF6 (data not shown). As compared to previous studies, the differences in TRAF2/TRAF3 involvement in CD40 signaling in these cells is striking. To date, TRAF3 has not been shown to activate NF-κB signaling, but in the tracheal epithelial cell line, 9HTEo⁺, we found that TRAF3 positively regulates CD40-induced activation of the RANTES promoter in an NF-κB-dependent fashion. CD40 was discovered as a B cell receptor, and the bulk of research done on CD40 has focused on traditional hematopoietic cell-specific functions such as humoral and cell-
mediated immunity (39, 40). Nonhematopoetic cell types are known to express CD40, but very little is known regarding the signaling pathways activated by CD40 in these cell types.

Our results suggest that TRAF association in airway epithelia is regulated differently than in B cells and HEK 293 cells when CD40 multimerization occurs. Specifically, we demonstrate that CD40 stimulation by sCD40L significantly up-regulates the activation of a RANTES promoter construct fused to a luciferase reporter gene (Fig. 3). Surprisingly, the addition of exogenous TRAF2 blocked this CD40-inducible activation, while adding TRAF3 stimulated RANTES promoter activity at least 4-fold in the absence of sCD40L and 7-fold in the presence of sCD40L as compared to the mock control. The expression of mutant TRAF3 in the absence of sCD40L was still capable of up-regulating RANTES activity 4-fold, suggesting that TRAF3 might be indirectly stimulating RANTES activation. However, in the presence of sCD40L, RANTES promoter activity dropped from a 7-fold induction to a 4-fold induction, suggesting that TRAF3 is directly involved in CD40-induced RANTES activation. It appears that the negative regulation by TRAF2 is not mediated through an alternate pathway because similar results were obtained when we substituted a dominant-negative construct that lacks the region thought to bind to downstream signaling proteins. However, it cannot be ruled out that mutant TRAF2 can still bind to another cytoplasmic protein that can transmit a negative signal, such as TRAF1. Alternatively, TRAF2 might bind to a site on the cytoplasmic tail of CD40 that blocks the binding of TRAF3. An appealing hypothesis for positive TRAF3 regulation of RANTES is that in resting cells, TRAF3 is sequestered and not available for receptor association. Engagement of CD40
could release TRAF3 as described by Ling and Goeddel (37) and make TRAF3 available to bind to CD40. This would also explain why addition of exogenous TRAF3 results in activation of the RANTES promoter. However, mutant TRAF3 had the same effect, and that suggests the possibility that TRAF3 is acting through TRAF5, which is capable of hetero-oligomerizing with TRAF3 (10). It is also possible that epithelial cell-specific adapter proteins exist that could be mediating CD40 signaling through binding to TRAF3 or that TRAF3 competes with another protein for a shared binding site on the cytoplasmic tail of CD40 and "pushes" it off to initiate a signaling cascade.

Once we determined that CD40-induction of the RANTES promoter involved TRAF3, we substituted a RANTES-luciferase promoter reporter construct that is incapable of binding to NF-κB because of site-directed mutations (Fig. 2) (17). Our results clearly indicate that CD40-TRAF3 induction of RANTES activity was dependent on NF-κB (Fig. 4). NF-κB in resting cells is retained in the cytoplasm by a family of inhibitory proteins called IκBs, which must be phosphorylated and degraded for NF-κB to migrate to the nucleus and activate gene transcription (reviewed in Ref. 27). Targeted phosphorylation of IκBs is mediated by the multi-subunit IκB kinase (IKK) which contains two catalytic subunits, IKK-α and IKK-β, each of which can properly phosphorylate IκBs (reviewed in Ref. 41). This multi-subunit kinase complex serves as the point of convergence for NF-κB-inducible signaling pathways, so we examined the effect of IKK-α and IKK-β in epithelial CD40 signaling. Expression of wild-type IKK-α in 9HTEo- cells stimulated the RANTES promoter as expected (Fig. 5). In the presence of sCD40L and IKK-α overexpression, induction of the promoter increased 2-fold above that of unstimulated samples. Expression of dominant-negative IKK-α in 9HTEo- cells,
whether treated with sCD40L or not, blocked activation of the promoter. The results of expressing IKK-β in 9HTEo' cells were remarkably similar (Fig. 6). These results are interesting because IKK-α and IKK-β have been shown to be regulated differently in some cases, but the proinflammatory mediator, LPS, has been described as activating both kinase subunits simultaneously (42). This set of experiments strongly supports the idea that CD40 signaling is mediated through NF-κB.

As mentioned before, IκB-α must be phosphorylated on specific serines (Ser 32 and 36) to be targeted for degradation by the 26S proteosome and release NF-κB. We treated 9HTEo' cells with sCD40L and looked for the presence of Ser 32-phosphorylated IκB-α because it is a reliable marker for NF-κB activation. The results of this experiment show a rapid phosphorylation of IκB-α upon CD40 cross-linking with sCD40L (Fig. 7) and further supports our suggestion that CD40 signaling in airway epithelial cells activates NF-κB.

Taken together, these results are intriguing because they are the first studies performed in airway epithelial cells and because they suggest that TRAF3 is capable of initiating a signaling pathway that activates NF-κB. Here we present data that overexpression of TRAF3, but not TRAF2, up-regulates RANTES promoter activity through an NF-κB-dependent pathway. Future studies in our laboratory will examine the proteins that bind to CD40 endogenously in order to address these possibilities. It is important to further analyze the specificity of TRAF signaling because the pleiotropic nature of receptors such as CD40 is probably dictated by finely regulated differences in adapter protein associations.
ACKNOWLEDGEMENTS

We thank Dr. Randolph Noelle and Dr. Hiroyuki Moriuchi for the research tools they generously shared with us. We thank Dr. Erik Schwiebert and Dr. Jim Collawn for their assistance in editing this manuscript.

REFERENCES


32. Yeh, W.-C., A. Shahinian, D. Speiser, J. Kraunus, F. Billia, A. Wakeham, J. L. de la Pompa, D. Ferrick, B. Hum, N. Iscove, P. Ohashi, M. Rothe, D. V. Goeddel, and T.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
SUMMARY

The object of this dissertation was to describe CD40 expression on airway epithelial cells, to examine the functional roles of that expression, and to study the underlying signaling mechanisms that are responsible for the outcomes of CD40 engagement on airway epithelial cells. These studies began with the hypothesis that epithelial cells in the airway can act as immune effector cells in the lung and that CD40 expression on airway epithelium is involved in the regulation of inflammatory mediators such as chemokines and adhesion molecules (Fig. 1).

Summary of Results

After confirming that airway epithelial cells do express CD40 in vitro and in vivo, we investigated whether CD40 expressed by a tracheal epithelial cell line (9HTEo') ligated by soluble CD40L (sCD40L) could up-regulate the production of the chemokines IL-8, RANTES, and MCP-1. We chose the 9HTEo' cell line as a model system because these cells display a high level of CD40 expression and they are responsive to the stimuli that we were interested in analyzing. As predicted, ligation of CD40 on 9HTEo' cells did significantly induce the production of IL-8, RANTES, and MCP-1 in a concentration-dependent manner. In addition, we looked at the combined effects of stimulation by proinflammatory cytokines, TNF-α or IL-1β, and sCD40L. The results displayed differential chemokine expression patterns dependent on the combination of stimuli. Specifically, sCD40L and TNF-α acted synergistically on the expression of IL-8 and...
FIGURE 1. Potential role of epithelial CD40 in airway inflammatory responses. This model depicts the potential role of CD40 expressed on airway epithelial cells in amplifying airway inflammation as suggested by the data presented in this dissertation. Engagement of epithelial CD40 by CD40L expressed on leukocytes that traffic into the airway during an inflammatory response results in the increased expression of chemokines and adhesion molecules.
RANTES, but there was no greater production of MCP-1 when cells were treated with sCD40L and TNF-α together. In contrast, sCD40L in combination with IL-1β had a synergistic effect on MCP-1 and IL-8 expression, but not on RANTES expression. These results suggest that CD40 on airway epithelium can contribute to the relative concentrations of individual chemokines expressed in the lung and influence the types of cells that are recruited into the airway. In addition to proinflammatory cytokines, the combinatorial effects of the Th2 cytokines, IL-4 and IL-13, were investigated because they have been reported to alter the activity of airway epithelial cells (155, 156). However, neither IL-4 nor IL-13 modified the expression levels of chemokines alone or in combination with sCD40L. These results confirm that airway epithelial cells expressing CD40 can respond to CD40L-bearing cells by modulating the relative levels of different chemokines present in the lung.

Once chemokines are made and a chemogradient is established, certain subsets of lymphoid cells will respond and migrate along that gradient. Adhesion molecules make it possible for migrating cells to traverse cell barriers such as vascular endothelium and pulmonary epithelium. Intensive research has revealed a great deal of information about the mechanisms that permit immune cells to interact with and subsequently cross endothelium, but much less is known about immune cell trafficking across epithelial surfaces. Therefore, we investigated expression levels of the adhesion molecules ICAM-1 and VCAM-1 on 9HTEo⁺ cells stimulated by sCD40L. The results of these studies demonstrated a strong induction of ICAM-1 expression in the presence of sCD40L, but no significant change in VCAM-1 expression. The effects sCD40L stimulation of 9HTEo⁺ cells in the presence of proinflammatory cytokines (TNF-α, IL-1β) and Th2
cytokines (IL-4, IL-13) were also studied. The results showed that TNF-α and IL-1β were each capable of up-regulating ICAM-1 expression to the same extent as sCD40L; however, there were no additive or synergistic effects between them. Both of the Th2 cytokines, IL-4 and IL-13, did slightly enhance ICAM-1 expression independently and in combination with sCD40L as compared to similarly untreated cells. Interestingly, VCAM-1 expression was not induced by any of the stimuli alone, but sCD40L in combination with TNF-α significantly up-regulated the amount of VCAM-1 expression on the surface of 9HTEo⁺ cells. The results of these experiments show that epithelial CD40 engagement leads to increased expression of ICAM-1 and, in conjunction with TNF-α, leads to an increase in VCAM-1 expression as well. Therefore, CD40-induced expression of adhesion molecules could play a role in the movement of leukocytes and granulocytes across the pulmonary epithelium.

In addition to expression and function studies, we examined the signaling mechanisms of epithelial CD40 in the lung. CD40 is a member of the TNFR superfamily of receptors, and, like the cytokine receptor family, the members of the TNFR family lack any identifiable enzymatic activity that is capable of activating downstream kinases. Therefore, signaling through CD40 requires the recruitment of adapter proteins called TRAFs. Six TRAFs (TRAF 1-6) have been identified, but the studies in this dissertation focused on the role of TRAF2 and TRAF3 because they are the most thoroughly characterized and have the highest binding affinity for the cytoplasmic tail of CD40 (47). Initially, the presence of endogenous TRAF expression was examined, and it was found that 9HTEo⁺ cells do express TRAF2, TRAF3, TRAF5, and TRAF6. Next, we examined the effects that overexpressing TRAF2 and TRAF3 wild-type and dominant-negative
constructs had on a RANTES promoter construct fused to a luciferase reporter gene that was transiently transfected into 9HTEo' cells. We found that TRAF2 and TRAF3 differentially regulate CD40-mediated signaling in airway epithelial cells. Specifically, results indicate that CD40-induced activation of the RANTES promoter involved TRAF3, while TRAF2 had an inhibitory effect on RANTES promoter activity. This was unexpected because TRAF2 is considered to be a positive regulator of CD40 signaling in B cells and HEK 293 cells, and, in these cell types, TRAF3 has been shown to inhibit the positive actions of TRAF2. However, it is generally accepted that CD40 signaling is highly cell-type specific, and our findings are the first to suggest a positive role for TRAF3 in CD40-mediated signaling. The altered regulation of CD40 signaling in airway epithelial cells may explain some of the differences seen in CD40 functions on different cell types. Next, we investigated whether TRAF3 signaling was dependent on the transcription factor, NF-κB, by replacing the wild-type RANTES promoter with a RANTES promoter that has site-directed mutations in its NF-κB binding sites. NF-κB has been demonstrated to regulate the expression of genes that encode proinflammatory mediators, including chemokines and adhesion molecules. The results indicate that CD40-TRAF3 induction of the RANTES promoter is mediated through NF-κB. TRAF3 modulates CD40 signaling through its actions at the cell membrane, and NF-κB acts by translocating to the nucleus and activating gene transcription; however, the kinase cascade that activates NF-κB was still undetermined. Therefore, we attempted to define the intermediate players involved in CD40-mediated activation of NF-κB.

NF-κB is sequestered in the nucleus of resting cells by a family of inhibitors known as IκBs, which includes IκB-α and IκB-β (reviewed in Ref. 157). The IκBs
maintain NF-κB in the cytoplasm by masking its nuclear localization signal and can be phosphorylated on two amino-terminal serine residues (Ser 32 and Ser 36 for IκB-α, Ser19 and Ser23 for IκB-β) by the kinases IKK-α and IKK-β (reviewed in Ref. 158). This exposes IκB to ubiquitination and proteolytic degradation by the 26S proteosome, thus unmasking the nuclear localization signal of NF-κB and freeing it for nuclear translocation. Experiments were designed to discover whether the IκB kinases, IKK-α and/or IKK-β, are involved in CD40-mediated signaling. Overexpressing dominant-negative forms of IKK-α or IKK-β in 9HTEo` cells completely inhibited CD40-induced activation of the RANTES promoter, indicating that both of these kinases are needed for CD40 induction of NF-κB. These results are important because IKK-α and IKK-β are catalytic subunits of the IκB kinase (IKK) and the phosphorylation of IκB represents the point of convergence for most NF-κB-activating stimuli (158). To further confirm that CD40 signaling was capable of activating NF-κB, we asked if CD40 ligation led to phosphorylation of Ser32 on IκB-α and discovered that, indeed, phosphorylated IκB-α was rapidly induced in 9HTEo` cells after treatment with sCD40L. According to densitometric analysis, phosphorylated IκB-α levels were maximal at 5 min after CD40 ligation and diminished over time (30 min). Altogether, the signaling studies presented in this dissertation suggest that epithelial CD40 activation of NF-κB is mediated by TRAF3, inhibited by TRAF2, and that IKK-α and IKK-β are necessary for proper phosphorylation of IκB leading to release of NF-κB, which can then translocate to the nucleus and bind to promoters responsible for activating gene transcription.
Future Studies

There are some unanswered questions regarding epithelial CD40 expression and function that our laboratory will address in the future. Of course, the bulk of the data presented in this dissertation was performed in vitro and employed an epithelial cell line that was immortalized by viral transformation. However, it is important to compare these in vitro results with data collected from experiments done with primary airway epithelial cells and in vivo with an animal model. Presently, our unpublished observations suggest that CD40 is expressed less densely (~40%) on normal bronchial epithelial cells than on 9HTEo⁺ cells. This makes sense because most cells that express CD40 have constitutively low levels of surface protein, but can be induced to express higher levels of CD40 by stimuli such as IFN-γ, TNF-α, IL-1β, and possibly IL-4. Despite exhaustive efforts, we were not able to alter the expression of CD40 on airway epithelial cell lines or on normal bronchial epithelial cells in culture, but that does not mean that there is not regulation of CD40 expression in the normal lung. Gormand et al. (17) recently reported inducibility of CD40 on two human bronchial cell lines with TNF-α and IFN-γ, but the monoclonal antibody that was used to detect CD40 was different than the one we used (18). Another important question regarding airway epithelium in vivo is whether or not CD40 expression is polarized. As discussed in the Introduction, the epithelia of the airway form a tight barrier and display two distinct surfaces, apical (lumenal) and basolateral. Some proteins, such as the cystic fibrosis transmembrane regulator, are only expressed on one surface or the other, in the case of the cystic fibrosis transmembrane regulator, on the apical surface. Even though there is not conclusive data concerning polarized expression of CD40, our unpublished observations suggest that CD40 can be
expressed on both surfaces, and that scenario seems appropriate based its functional roles described here. Functionally, it is highly likely that epithelial CD40 is capable of regulating more than the expression of chemokines and adhesion molecules. Specifically, Gormand et al. reported that cross-linking CD40 on human bronchial cells up-regulates IL-6 and GM-CSF (17). We can confirm that CD40 regulates the expression of IL-6 (unpublished observation), and other investigators have reported that CD40 engagement up-regulates the expression of GM-CSF (reviewed in Ref. 5). Another possible role for CD40 on epithelial cells could be the regulation of free radicals, specifically, nitric oxide (NO) production. Importantly, CD40-CD40L interaction is required for the production of NO by macrophages (reviewed in Ref. 3). Epithelial cells in the airway constitutively produce the inducible isoform of nitric oxide synthase (iNOS), and a murine model of cystic fibrosis displays reduced levels of iNOS (159). Expression of iNOS is higher in asthmatic patients than in control subjects, but it is unclear whether it has protective or deleterious effects (160). At low levels, NO has a protective effect as an antioxidant; however, in concentrated amounts, NO can amplify inflammation (161). NO has also been reported to have direct effects on signaling molecules such as NF-κB (reviewed in Ref. 161).

The signaling mechanisms for the TNFRs are being studied intensively as the deluge of publications indicate; however, as more levels of control are revealed, the overall picture is increasingly confusing. The data regarding epithelial CD40-mediated signaling presented herein only scratches the surface of possible mechanisms involved, but they represent a solid beginning for more detailed study. For example, it is essential to investigate the involvement of TRAF5 and TRAF6 in our model system. Their
contribution to NF-κB activation through CD40 is well documented, and CD40 signaling in airway epithelia will not be complete without understanding the involvement of each of the TRAFs. TRAF5 forms hetero-oligomers with TRAF3 and might be responsible for the NF-κB activation triggered by CD40 ligation in 9HTE0' cells. TRAF2 and TRAF3 bind to overlapping consensus sequences on the cytoplasmic tail of CD40, but TRAF6 binds to a proximal region of the CD40 tail and has also been shown to activate NF-κB (59, 74). Although the TRAFs in 9HTE0' and 16HBE14o' cells were identified by immunoprecipitation using a GST-CD40 cytoplasmic tail fusion protein, it is important to demonstrate TRAF association with endogenous multimerized CD40. Another important question is whether the TRAFs are constitutively associated with epithelial CD40 or if they are recruited when ligation of CD40 occurs. It has been suggested that TRAF3 is sequestered by a novel protein, MIP-T3, in HEK293 cells until CD40 is ligated by CD40L (162). This model would make endogenous TRAF3 unavailable to bind receptors until receptor binding occurs. This could explain why expressing TRAF3 exogenously would subvert the normal regulation of CD40-mediated signaling.

Although NF-κB is clearly involved in CD40 signaling, it is important to analyze the involvement of other transcriptional factors in CD40-induced signaling in airway epithelial cells. Specifically, we have preliminary data that suggests AP-1 involvement in the activation of the RANTES promoter through CD40. Clearly, additional study is needed to delineate the mechanisms underlying epithelial CD40 signaling.

Transmembrane receptors act as sensors for cells and signal changes in the extracellular environment so that the cell can adjust appropriately. Receptors do this by transmitting signals into the cytoplasm and altering cellular functions such as protein
transport, translation, and transcription. This dissertation examined one receptor on one cell-type, but CD40 signaling in epithelial cells of the airway does not occur in a vacuum. As indicated by the functional studies, TNF-α, IL-1β, IL-4, and IL-13, through their respective receptors, alter the outcome of CD40 induction of chemokine and adhesion molecule expression. All of these receptors share common signaling adapter proteins (TRAFs) and activate multiple signaling pathways (Fig. 2). Cross-talk between these receptors is bound to occur on several levels and the resulting competition or synergy would dictate the final cellular outcome. Studying receptor cross-talk would help to unravel the complicated, but physiologically relevant, signaling networks in which CD40 participates.

Overall, the research presented in this dissertation implicates CD40 expression on airway epithelial cells as an important physiological trigger for immunity and inflammation in the lung. Almost all of the cells in the airway are capable of expressing CD40, and their individual roles are critical to the development of efficacious therapies for chronic inflammatory diseases. The epithelium is the first point of contact for many pathogens and cannot be overlooked as a cell type that can initiate and exacerbate inflammation. Pulmonary epithelial cells can be induced to express a vast array of inflammatory mediators, and the data presented here implicate CD40 signaling as a major pathway for regulation of proinflammatory mediator gene expression.
FIGURE 2. Model of CD40-mediated signaling pathways and potential cross-talk with cytokine receptors. The signaling pathways triggered via CD40, TNFR, IL-R, and IL-4R each involve TRAF molecules. These pathways diverge downstream of TRAF molecule involvement but may converge at other levels such as JNK and NIK activation. The activation of NIK and JNK leads to the activation of the transcription factors NF-κB and AP-1.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
GENERAL REFERENCES


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL
MEMORANDUM

DATE: April 3, 2000

TO: Lisa M. Schwiebert, PhD
MCLM 955
FAX: 975-9028

FROM: Clinton J. Grubbs, PhD, Chairman
Institutional Animal Care and Use Committee

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on March 29, 2000.

Title of Application: Role of CD40 in Airway Inflammation

Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office for Protection from Research Risks (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

CJG/miw
Name of Candidate    Stacie M. Propst

Graduate Program    Physiology and Biophysics

Title of Dissertation    Role of Epithelial CD40 in Airway Inflammation

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lisa M. Schwiebert</td>
<td></td>
</tr>
<tr>
<td>E.N. Benveniste</td>
<td></td>
</tr>
<tr>
<td>Daniel Bullard</td>
<td></td>
</tr>
<tr>
<td>Robert Jackson</td>
<td></td>
</tr>
<tr>
<td>Erik M. Schwiebert</td>
<td></td>
</tr>
<tr>
<td>Robert Carter</td>
<td></td>
</tr>
</tbody>
</table>

Director of Graduate Program

Dean, UAB Graduate School

Date 11/15/08