Role of Mitogen-activated Kinases in Cd40-mediated T Cell Activation of Monocyte/macrophage and Vascular Smooth Muscle Cell Cytokine/chemokine Production

Denise M. Milhorn
East Tennessee State University

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ROLE OF MITOGEN ACTIVATED KINASES IN CD40-MEDIATED T CELL
ACTIVATION OF MONOCYTE/MACROPHAGE AND VASCULAR SMOOTH
MUSCLE CELL CYTOKINE/CHEMOKINE PRODUCTION

A Dissertation
Presented to
the Faculty of the Department of Biochemistry and Molecular Biology
James H. Quillen College of Medicine
East Tennessee State University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Biomedical Sciences

by
Denise M. Milhorn
August 1999
APPROVAL

This is to certify that the Graduate Committee of

Denise Marie Milhorn

met on the

Twenty-fifth of June, 1999

The committee read and examined her dissertation, supervised her defense of it in an oral examination, and decided to recommend that her study be submitted to the Graduate Council in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences.

Signed on behalf of the Graduate Council

Chair, Graduate Committee

Dean, School of Graduate Studies
ABSTRACT

ROLE OF MITOGEN ACTIVATED KINASES IN CD40-MEDIATED T CELL ACTIVATION OF MONOCYTE/MACROPHAGE AND VASCULAR SMOOTH MUSCLE CELL CYTOKINE/CHEMOKINE PRODUCTION

by

Denise M. Milhorn

Atherogenesis is characterized by the infiltration of immune competent cells such as monocytes/macrophages and T cells to the site of lesion formation, resembling a chronic inflammatory response. Histological studies have shown the T cells present at these lesions are activated, CD154+, CD4+ T cells of the Th1 or inflammatory phenotype. The presence of these cells may be critical in regulating vascular inflammation due to their capacity to activate proinflammatory activities in CD40+ cells in the vasculature including monocytes/macrophages and vascular smooth muscle cells (SMC), through the CD40:CD154 receptor ligand interaction. This dissertation represents efforts to determine the functional consequences acquired by SMC in response to CD40 ligation by activated CD154+ T cells, and to elucidate components of the signaling pathway(s) activated in response to CD40 signaling in both monocytes and SMC.

To study the consequences of CD40 stimulation, primary human monocytes and aortic SMC were treated with plasma membranes purified from CD154+, CD4+ T cells. The ability of CD40 signaling to initiate cytokine/chemokine mRNA and protein synthesis was assayed by RNase protection assay, and enzyme linked immunosorbent assay (ELISA), respectively. Western blot analysis was used to determine the activation state of members of the mitogen activated protein kinase (MAPK) family following CD40 stimulation. In addition, the activation of the transcription factor, NFκB, which has been demonstrated to contribute the induction of cytokine/chemokine expression was analyzed by electromobility shift assay (EMSA). Analysis of SMC cell surface expression of receptors responsive to T cell ligands, and the analysis of CD40-mediated regulation of SMC adhesion and co-stimulatory molecules was analyzed by flow cytometry.

The results presented in this dissertation demonstrate that SMC, like monocytes/macrophages, are capable of interacting with T cells in a manner that results in reciprocal activation events. SMC were shown to present antigen to, and activate T
cells. In turn T cell stimulus resulted in the activation of proinflammatory function in SMC initiated through the CD154:CD40 interaction. CD40 stimulation of SMC resulted in the production of the chemokines interleukin 8 (IL-8) and macrophage chemotactic protein-1 (MCP-1), and the upregulation of intercellular adhesion molecule (ICAM). Examination of the intracellular signaling pathways activated through CD40 signaling revealed the involvement of MAPKs in the pathway leading to induction of proinflammatory activity. Evaluation of CD40 signaling in monocytes demonstrated the activation of the MAPK family members ERK1/2, but not the MAPK family members p38 or c-jun-N-terminal kinase (JNK). In contrast, CD40 signaling in SMC was shown to result in ERK1/2 and p38 activation, and both of these kinases were shown to play a critical role in the induction of chemokine synthesis. An examination of the ability of anti-inflammatory cytokines to modulate CD40 signaling in monocytes and SMC demonstrated that the anti-inflammatory cytokines IL-4 and IL-10 abrogate CD40-mediated induction of inflammatory cytokine production by monocytes. This inhibition was shown to be a result of a negative influence of IL-4 and IL-10 on CD40 mediated ERK1/2 activation in monocytes. However, IL-4 and IL-10 did not inhibit SMC proinflammatory responses indicating a difference in the intracellular responses to these cytokines by the two cell types.

These studies contribute to the understanding of the interaction between SMC and CD4 + T cells through the receptor:ligand pair CD40:CD154, and its role in the maintenance of vascular inflammation. The identification of the signaling components of the monocyte and SMC CD40 signaling pathways will identify control points that may serve as targets for anti-inflammatory therapies.
ACKNOWLEDGEMENTS

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Finally, I would like to acknowledge the professors that served on my graduate committee, Dr. Bob Stout, Dr. Mitch Robinson, Dr. Lou Ernest-Fonberg, and Dr. Guha Krishnaswamy. I greatly appreciate your support, and taking the time from your busy schedules to serve on my committee.
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<tr>
<td>SMC</td>
<td>Smooth Muscle Cell</td>
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<td>VSMC</td>
<td>Vascular Smooth Muscle Cell</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>ICAM</td>
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<td>RPA</td>
<td>RNAse Protection Assay</td>
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<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
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<td>Sos</td>
<td>Son of sevenless</td>
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<tr>
<td>PAK</td>
<td>p21 Activated Kinase</td>
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<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
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<td>IκB</td>
<td>I kappa B</td>
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<td>PMA</td>
<td>Phorbol Myristic Acetate</td>
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<td>HB-EGF</td>
<td>Heparin Binding Epidermal Growth Factor</td>
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<td>Basic Fibroblast Growth Factor</td>
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CHAPTER 1
INTRODUCTION

Cell Interactions and the Role of Cytokines and Chemokines in Atherosclerosis

Atherogenesis is characterized by the infiltration of immune competent cells, such as monocytes/macrophages and T lymphocytes at the site of a vascular lesion resembling a chronic inflammatory response. Tissue sections of atherosclerotic plaques have revealed that the T lymphocytes at the site of the vascular lesion are activated, CD154+, CD4+ T helper cells of the Th1 or inflammatory phenotype (Mach and others 1998). These activated T cells may interact with cells in the vasculature through the receptor-ligand interaction of CD154, expressed on activated T cells with its receptor CD40 expressed on a diverse group of cells including smooth muscle cells and monocytes/macrophages (Figure 1). This interaction has been shown to result in the up regulation of costimulatory and adhesion molecule expression, and the induction of inflammatory cytokine and chemokine synthesis in monocytes, and may result in similar functional consequences in SMC. We hypothesize that the interaction between activated T cells and monocytes, and activated T cells and SMC through the receptor-ligand pair CD40:CD154 results in inflammatory cytokine and chemokine synthesis contributing to the pathogenesis of atherosclerosis.

The presence of activated CD4+ T cells at atherosclerotic lesions has been well established (Mach and others 1998). However the role of T cells in the maintenance
Figure 1. Overview of Cell:Cell Interactions Occurring in Vascular Inflammation. A cross-sectional view of an artery illustrating cell:cell interactions which may be occurring through the receptor-ligand pair CD40:CD154 during atherogenesis.
and/or exacerbation of atherosclerosis has not been elucidated. Research thus far has indicated that T cells appear early in the onset of atherosclerosis and localize to the site of insult or injury to the artery. T cells transmigrate through the endothelium from the media to the intima of the aorta where they have the ability to interact with many cells in the vasculature through the receptor-ligand pair CD154:CD40. Recently there has been increasing amounts of evidence that support a critical role of T cells in regulating vascular inflammation or atherosclerosis (Mach and others 1998). Treatment with antibodies against murine CD154 limits atherosclerosis in susceptible mice, and the atheroma of mice treated with anti-CD154 antibodies contains significantly fewer immune competent cells suggesting the involvement of inflammatory pathways and a role for CD40 signaling in atherosclerosis (Mach and others 1998). Additionally, T cells have the capacity to produce growth factors such as basic fibroblast growth factor (bFGF) and heparin binding epidermal growth factor (HB-EGF) which are potent mitogens for SMC proliferation (Rolfe and others 1995), and several cytokines including IFNγ, a known co-stimulus for macrophage activation, which enhances macrophage effector function and induces or enhances MHC class II expression by many cells including macrophages, endothelial cells and SMC.

Smooth muscle cells and monocytes/macrophages are thought to play a crucial role in the process of atherogenesis. Both monocytes/macrophages and SMC have the propensity to become lipid-laden foam cells which contribute to the formation of the atherosclerotic plaque. Additionally, monocytes/macrophages and SMC may amplify the inflammatory process resulting in atherosclerosis due to their ability to express
class II histocompatibility (MHC class II) (Warner and Libby 1989). Class II expression in atherosclerotic lesions in vivo has been well documented (Hansson and others 1986) suggesting that antigen presentation may be occurring at atherosclerotic lesions resulting in the activation of T cells. Antigens present in atherosclerotic plaques may include modified lipoproteins, neoantigens revealed through tissue destruction and determinants arising from infectious agents (Libby and Hansson 1991). Furthermore, monocytes/macrophages and SMC have been shown to express CD40 which can interact with CD154 present on activated T cells. Recent research has shown that ligation of CD40 on atheroma-associated cells (i.e. monocytes/macrophages and SMC) in vitro activates functional attributes such as the production of cytokines, chemokines, and adhesion molecules which are some of the critical components required for the directional migration of leukocytes during inflammatory processes. The inflammatory cytokines, tumor necrosis factor (TNF) α and IL-1 β are both know to be involved in inflammatory diseases and are produced by several cell types including monocytes/macrophages and SMC. Interleukin (IL)-8 and monocyte chemotactic protein-1 (MCP-1) are well characterized members of the C-X-C and C-C chemokine subfamilies, respectively (Beasley and others 1995), are pro-inflammatory in nature, and are produced by several cell types including vascular smooth muscle cells and monocytes/macrophages. Histological studies have demonstrated these inflammatory cytokines and chemokines to be present in atherosclerotic plaques, suggesting that they may play a crucial role in the development, maintenance, and pathogenesis of atherogenesis by promoting the directed migration of inflammatory cells (Warner and
Libby 1989; Wang and others 1998; Liu and others 1997; Terkeltaub and others 1998). MCP-1 mRNA has been detected in several cells including macrophages, endothelial cells and SMCs in atherosclerotic arteries of patients undergoing a bypass revascularization (Seino and others 1995). Recent reports have shown that mice deficient in MCP-1 that were fed a high cholesterol diet deposited less lipids and had fewer macrophages present within their aortic walls as compared to the wildtype control (Boring and others 1998). Similarly, mice deficient in the MCP-1 receptor, CCR-2, had a overall decrease in atherosclerotic lesion size, and had significantly fewer macrophages and monocytes present in the aortas as compared to the wildtype control (Gu and others 1998). IL-8 is chemotactic for several cell types including T cells and neutrophils at picomolar and nanomolar concentrations respectively (Banka and others 1991), and may be involved in the recruitment of T cells to the intima of a blood vessel contributing to the pathogenesis of atherosclerosis (Larsen and others 1989).

**Structure and Function of CD40**

CD40 is a transmembrane glycoprotein of approximately 45kDa, a member of the tumor necrosis receptor superfamily, which also includes the TNF receptor (TNFR1 and TNFR2), nerve growth factor receptor, and Fas (CD95). CD40 is expressed by a variety of cell types including B cells (Clark 1990), monocytes/macrophages (Alderson and others 1993), dendritic cells (Caux and others 1994), thymic epithelial cells (Galy and Spits 1992), endothelial cells (Yellin and others 1995), keratinocytes (Peguet-Navarro and others 1996), and smooth muscle cells (Mach and others 1998). CD40
interacts with its ligand, CD154, a type II membrane protein of approximately 39 kDa, and a member of the TNF superfamily. CD154 is preferentially expressed on activated, but not resting T cells (Lynch and Ceredig 1989; Noelle and Snow 1992). Engagement of CD40 by its ligand CD154 has been reported to induce functional changes on these cell types that may contribute to inflammatory responses, including the secretion of pro-inflammatory cytokines and chemokines, the up-regulation of costimulatory and adhesion molecules (Stout and Suttles 1996), and effects on cellular proliferation (Yellin and others 1995). CD40 and its receptor CD154 has been identified as a receptor:ligand pair which contributes to contact dependent signaling between monocytes and T cells. The importance of CD154 in vivo was revealed by studies of X-linked Hyper-IgM Syndrome patients. Hyper-IgM Syndrome is an immunodeficiency characterized by the absence of circulating IgA and IgG and the absence of germinal centers, which is a result of defects in the gene encoding CD154 (Klein and Miedema 1995). CD40 was initially identified on the surface of B cells (Banchereau and others 1994) where CD40 signaling provides critical costimulatory signals required for proliferation, isotype switching, germinal center formation, and the generation of memory B cells (Grabstein and others 1993). The signaling pathways activated through CD40 in B cells involve activation of the src family PTK's, PKC, Jak3, serine/threonine kinases, MAPK family members (JNK, p38 and ERK1/2) and PLCγ2 (Aagaard-Tillery and Jelinek 1996; Padmore and others 1997; Hanissian and Geha 1997).

In monocytes/macrophages, CD40 ligation has been shown to induce monocyte-
derived inflammatory cytokine synthesis (Wagner and others 1994), nitric oxide production (Stout and others 1996), and to rescue monocytes from undergoing apoptosis (programmed cell death) induced by the deprivation of serum factors (Suttles and others 1996). Additionally, our laboratory has demonstrated that CD40-mediated rescue from apoptosis is critically dependent on the activation of PTK but does not appear to involve the serine/threonine kinase PKC family (Suttles and others 1996). Our laboratory has also observed activation of the transcription factor Nuclear Factor-kappa B (NF-κB) in response to CD40 signaling in monocytes. NF-κB is a transcription factor involved in the transcription of several genes. It is present in the cytosol in an inactive state, complexed with the inhibitory I kappa B (IκB) proteins (Baeuerle and Baltimore 1988; Beg and others 1993; Finco and others 1994). Activation of NF-κB occurs through phosphorylation of IκB-α at serine 32 and 36, resulting in the release and nuclear translocation of active NF-κB where it can bind relevant gene promoters (Brown and others 1995; Brockman and others 1995; Chen and others 1996). The release of active NF-κB results from the phosphorylated IκB conjugating with ubiquitin followed by proteasome-mediated degradation of IκB (Chen and others 1996).

CD40 does not contain cytoplasmic sequences with catalytic activity and has been shown to signal through cytoplasmic adaptor proteins of the TNF receptor-associated factor family (TRAF), which activate intracellular signaling pathways (Dadgostar and Cheng 1998). All TRAF family members contain homologous C-terminal TRAF domains, TRAFs 2 through 6 contain an N-terminal ring finger and zinc finger-like
domains (Dadgostar and Cheng 1998), and TRAF 3 and TRAF 5 contain an isoleucine zipper domain (Cheng and others 1995; Ishida and others 1996; Nakano and others 1996). TRAFs 2, 5 and 6 interaction with CD40 has been associated with activation of the transcription factor NF-κB (Ishida and others 1996), however the disruption of CD40 cytoplasmic sequences involved in TRAF interaction does not eliminate NF-κB activation (Hsing and others 1997). Additionally, signaling through CD40 can initiate TRAF mediated activation of MAPK family members (Natoli and others 1997), however the role of the TRAF family members in CD40-mediated activation of monocytes/macrophages and SMCs has not been investigated.

Previous research in our laboratory has demonstrated modulation of CD40-mediated monocyte inflammatory cytokine synthesis by the Th2-derived anti-inflammatory cytokines IL-4 and IL-10 (Poe and others 1998). Several studies have shown inhibition of monocyte/macrophage inflammatory function in response to lipopolysaccharide (LPS) (Moore and others 1993). In vivo, it has been shown that these cytokines reduce autoimmune inflammatory diseases, and IL-10 is being used in clinical trials to treat autoimmune diseases (Horsfall and others 1997; Narula and others 1998). The mechanism by which IL-4 and IL-10 inhibit inflammatory cytokine synthesis has not been elucidated, however previous research in our laboratory suggested that the effectiveness of these cytokines may result from the inhibition of the signaling pathway(s) activated in response to CD40 ligation in monocytes. Our laboratory has recently shown that IL-4 and IL-10 act early in the CD40 signaling cascade by modulating the overall PTK induction in monocytes.
Both IL-4 and IL-10 signal through receptors which associate with and activate the Janus family (Jak) tyrosine kinases upon ligation (O'Shea 1997; Leonard and O'Shea 1998). The Jaks in turn, activate proteins called Signal Transducers and Activators of Transcription (Stats). The Stats translocate to the nucleus where they regulate the expression of various genes (Darnell 1997) (Figure 2). The Stats are inducers of a family of negative regulators of cytokine signaling, the suppressor of cytokine signals (SOCS) which work as a negative feedback system to inhibit the Jaks either directly or non-directly (Nicholson and Hilton 1998). CD40-mediated activation of the Jaks in monocytes has not been investigated, however in B cells studies have shown that CD40 may associate with Jak3 (Hanissian and Geha 1997). The data thus far indicate that IL-4 and IL-10 inhibit the early PTK activity initiated from CD40 ligation suggesting that IL-4 and IL-10 may induce protein tyrosine phosphatase (PTP) activity, resulting in the suppression of the CD40 signaling pathway in monocytes, and thus suppression of ERK1/2 activity.

**MAPK Signaling**

The mitogen-activated protein kinases (MAPK) family members are a group of protein serine/threonine kinases that are activated in response to a variety of extracellular stimuli and regulate a number of cellular responses. Along with other signaling pathways, the MAPK family members mediate signal transduction by differentially altering the phosphorylation status of transcription factors (Sivaraman and others 1997; Das and Vonderhaar 1996; Tong and other 1997) (Figure 3). There are
Figure 2. Jak/Stat Signaling Cascade
Figure 3. The ERK1/2, JNK, and p38 MAPK Signaling Cascades. The inhibitor PD98059 inhibits Raf-1 phosphorylation of MEK1/2, and therefore the phosphorylation of ERK1/2. The inhibitor SB203580 inhibits phosphorylation of p38 MAPK.
three members of the MAPK family: extracellular signal regulated kinase (ERK1/2), c-Jun-N-terminal kinase (JNK) and p38. The ERK1/2 signaling cascade involves the activation of receptor tyrosine kinases (which have src homology) which provide the binding site for the adaptor protein, growth receptor-bound protein 2 (GRB2), which localizes the guanine nucleotide exchange factor, son of sevenless (SOS), to the plasma membrane. SOS activates Ras by exchange of GTP for GDP (Winston and Hunter 1996; Sagata 1997; Takeuchi and others 1999; Hildt and Oess 1999). The Ras-GTP activated Raf phosphorylates a dual specificity kinase MAPK/ERK kinase1/2 (MEK1/2), which is phosphorylated on serine 218 and 222 (Avruch and others 1994). Active MEK1/2 dually phosphorylates ERK1/2 on theonine 183 and tyrosine 185 (Burack and Sturgill 1997; Cobb and others 1996). Active ERK translocates to the nucleus to phosphorylated a variety of transcription factors including Elk-1 (Gille and others 1992; Cheng and others 1996), C/EBPβ (NF-IL-6) (Nakajima and others 1993), ATF-2 (Abdel-Hafiz and others 1992), and NF-κB (Dadgostar and Cheng 1998).

The JNK signaling pathway is activated in several ways including exposure to UV radiation, heat shock, or inflammatory cytokines (Su and Karin 1996; Hibi and others 1993) and is mediated by Rac and cdc42, two small GTP binding proteins (Davis 1994). Activated cdc42 binds to PAK65 which activates MEK kinase (MEKK-1). MEKK-1 phosphorylates MEKK-4 at serine 219 and serine 223. The active MEKK-4 phosphorylates JNK which translocates to the nucleus and activates a variety of transcription factors including c-Jun (Kyriakis and others 1994; Derijard and others...
1995), ATF-2 (Gupta and others 1995) and Elk-1 (Cavigelli and others 1995).

The p38 MAPK family member is activated in response to several stimuli including inflammatory cytokines, endotoxins and osmotic stress (Tong and others 1997; Han and others 1994; Rouse and others 1994). P38 is activated by MEK kinase kinase-3 (MKK3) a dual specificity kinase. Following phosphorylation p38 translocates to the nucleus and phosphorylates and activates transcription factors including ATF-1, CREB (Tan and others 1996), Elk-1 (Price and others 1996), CHOP (Wang and Ron 1996), and ATF-2 (Raingeaud and others 1996).

The sites of phosphorylation are conserved between the MAPK family members, however, these sites are located within distinct dual specificity phosphorylation motifs: TEY for ERK, TPY for JNK, and TGY for p38 (Davis 1994; Derijard and others 1994; Raingeand and others 1995). Additionally, the MAPK family members share sequence homology ~45% sequence homology between JNK and ERK (Moriguchi and others 1996), and ~50% homology between p38 and ERK (Tong and others 1997).

This dissertation represents the results of studies designed to evaluate the role of CD40 signaling in two key cells found at atherosclerotic lesions, SMC and monocytes/macrophages, which contribute to the maintenance and/or exacerbation of vascular inflammation. We investigated the functional activities expressed by SMC in response to CD40 ligation, and critical components involved in CD40-mediated SMC chemokine synthesis. Additionally we examined the role of MAPK family members in CD40-mediated synthesis of inflammatory cytokines and chemokines in
monocytes/macrophages and SMC, and evaluate the role of anti-inflammatory cytokines on CD40-mediated activation of ERK1/2 MAPK family member(s), a critical component in CD40-mediated induction of monocyte IL-1β and TNFα synthesis in monocytes and IL-8 and MCP-1 synthesis in SMC. Gaining a better understanding of CD40-mediated activation of specific kinases, transcription factors and inflammatory cytokines and chemokines, and how these events can be modulated by natural mediators will help identify potential targets for drug intervention.
CHAPTER 2
MATERIALS AND METHODS

Control of endotoxin contamination

All cell culture reagents used were either certified as low endotoxin when purchased, or were ensured low endotoxin as determined by chromogenic limulus assay (BioWhittaker, Walkersville, MD). Stock solutions containing >1 ng/ml (10 endotoxin units/ml) were considered unacceptable. Stock solutions were diluted in assays such that endotoxin levels did not exceed 1 pg/ml.

Inhibitors/ Reagents

The MEK1/MEK2 inhibitor PD98050 was obtained from New England BioLabs, Inc. (Beverly, MA). The p38 MAPK inhibitor SB203580, the PTK inhibitor Herbimycin A and the PKC inhibitor R0-31-75499 were purchased from Calbiochem (La Jolla, CA). H7 was purchased from Sigma Chemical Co. (St, Louis, MO). IL-4 and IL-10 were purchased from R&D Systems (Minneapolis, MN). Sodium orthovanadate (Na$_3$VO$_4$) was acquired from Fisher Scientific, (Fair Lawn, NJ).

Antibodies

The following mAB's were prepared from culture supernatants of hybridomas purchased from American Type Culture Collection (ATCC, Rockville, MD): IgG
mouse anti-human CD3 (OKT-3), IgG mouse anti-human CD8 (OKT-8), IgG mouse anti-human monocyte (3C10), IgG mouse anti-human B cell (LYM-1), IgM mouse anti-human NK cell (hNK-1), and IgG mouse anti-human CD40 (G28-5). BioMag* goat anti-human IgG and IgM was obtained from PerSeptive Diagnostics, Inc. (Cambridge, MA). FITC-conjugated donkey anti-mouse IgG (H + L) was purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). IgG mouse anti-human CD154 mAb was obtained from Genzyme (Cambridge, MA). Rabbit antibodies recognizing the active, phosphorylated (Thr^{183}, Tyr^{185}) form of ERK1/2 were acquired from Promega (Madison, WI) and New England BioLabs. Rabbit antibodies recognizing phosphorylated p38 (Thr^{180}, Tyr^{182}) and phosphorylated JNK (Thr^{183}, Tyr^{185}) were purchased from New England BioLabs. Horseradish peroxidase-conjugated F(ab')\textsubscript{2} donkey anti-rabbit AB was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Monoclonal mouse anti-human CD154 was prepared from culture supernatant of the hybridoma 5C8 (American Type Culture Collection, Rockville, MD). The monoclonal mouse IgG anti-human CD40 used for flow cytometry was prepared from culture supernatant of the hybridoma G28-5 (American Type Culture Collection, Rockville, MD). The FITC-conjugated donkey anti-mouse IgG (H + L) was purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA).

**Cell Lines**

Cell lines used in these studies included 293 (human embryonic kidney, ATTC), stable transfectants of 293 which express high levels of CD154 (Yellin and others 1994), the human T cell leukemia line Jurkat (ATCC) and the CD154\textsuperscript{+} subclone of
Jurkat, D1.1 (Yellin and others 1991). Both D1.1 and 293-CD154 transfectants were gifts of Dr. Seth Lederman, Columbia University. Cell lines were maintained in RPMI 1640 (Hyclone, Logan, UT), containing 100mM HEPES, 50 μg/ml gentamicin, and 5% fetal bovine serum (FBS) (R-5). The 293-CD154 transfectants (created by co-transfection with pcDNA1-CD154 and pRSVneo) were periodically passaged in 200 μM G418 (Gibco/BRL). The human aortic smooth muscle cell line (SMC) was obtained from Clonetics Corporation (San Diego, CA). The SMC line designated AOSMC 2634-3 by Clonetics, was characterized phenotypically as SMC and found to be virus-free. The line was maintained in SmGM*-3 BulletKit* (Clonetics) containing 0.5μg/ml human recombinant Epidermal Growth Factor, 5mg/ml Insulin, 1μg/ml human recombinant Fibroblast Growth Factor, 50mg/ml Gentamicin, and 2% FBS (Clonetics Corporation). The SMC cell line was rested in Human Endothelial-SFM* Gibco/Life Technologies, Grand Island, NY). SMC were cultured in 25cm tissue culture flasks (Corning, Incorporated, Corning NY).

**CD4⁺ T cell Purification and Activation**

CD4⁺ T cells were purified by negative magnetic panning from elutriation-enriched T cell populations. Cells were incubated in R-5 with mAB's against cell surface molecules generated from the hybridomas OKT-8 (anti-CD8⁺ T cell), 3C10 (anti-monocyte), LYM-1 (anti-B cell), and hNK-1 (anti-NK cell), used as culture supernatants at dilutions of 1:10, for 30 min at RT. Cells were then treated with BioMag® iron-conjugated antibodies to murine IgG and IgM (PerSeptives Diagnostics,
Cambridge, MA) for 30 min with gentle shaking at 4°C. Cells were diluted with DPBS in 75 cm² flasks (Fisher Scientific) and the CD4⁺ populations were removed via 27 megagauss Oerstead magnets (PerSeptives Diagnostics). A sample of the purified population was stained with an FITC conjugated anti-CD4 mAb and analyzed by flow cytometry on a FACSTAR® flow cytometer (Becton Dickinson, San Jose, CA). Resulting populations were typically found to be greater than 95% CD4⁺. CD4⁺ T cells were then rested in R-5 alone, or activated for 6 h in R-5 by incubation with 10ng/ml phorbol myristic acetate (PMA) (Sigma) and 0.5μM ionomycin (Calbiochem, San Diego, CA). Expression of CD154 on activated, but not resting, CD4⁺ T cells was then confirmed by flow cytometric analysis on a FACSTAR® plus flow cytometer (Becton Dickinson).

**Monocyte Isolation and Culture**

Blood was collected from normal, healthy human volunteers and PBMC's were isolated over a Ficoll density gradient (Fico-Lite-LymphoH, Atlanta Biologicals, Norcross, GA). PBMC's were plated at a density of 5 x 10⁶ cells/well in 24-well tissue culture plates (Falcon Primaria, Lincoln Park, NJ) in RPMI 1640 (Hyclone, Logan, UT), containing 100 mM HEPES, 50 μg/ml gentamicin, and 5% FBS (R-5). Monocytes were isolated by plastic adherence for 1 h at 37 °C after which nonadherent cells were removed by Pasteur pipetting during 2 washes with Dulbecco's PBS (DPBS). Cells were maintained in R-5 overnight prior to treatment.
**T-Cell Plasma Membrane Preparation**

For the preparation of purified plasma membranes, resting and activated purified CD4^+ T cells were resuspended in a hypotonic buffer containing 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM MgCl₂, and 50 μg/ml phenolmethylsulfonyl fluoride (PMSF) for 30 min on ice. The cells were then homogenized using a PowerGen 35 homogenizer (Fisher Scientific) until completely disrupted as determined microscopically. Disrupted cells were centrifuged at 500 x g for 5 min to remove nuclei, then centrifuged at 95,000 x g for 30 min using a Ti-50 rotor in a Beckman L5-65 Ultracentrifuge. Cell debris was resuspended in 35% (wt/vol) sucrose/hypotonic buffer then layered on 73% (wt/vol) sucrose/hypotonic buffer. Hypotonic buffer was layered on the 35% sucrose and the samples were centrifuged using a SW50.1 rotor at 130,000 x g for 1 h to separate plasma membranes. The plasma membrane layer (at the 73%-35% interface) was collected and diluted 1:5 with hypotonic buffer, then centrifuged again for 1 h at 130,000 x g to pellet purified plasma membranes. The membrane pellets were resuspended in PBS and total protein was determined by microtiter plate protocol of the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). The BCA protein assay was read on a Biotek Instruments microtiter plate reader at 561nm.

**Flow Cytometric Analysis**

Smooth muscle cells were harvested from tissue culture flasks with .025% Trypsin/EDTA (Clonetics Corporation). SMC were labeled with the appropriate
dilution of the primary antibody 30 min at room temperature, washed in DPBS, and 
stained with a FITC-conjugated donkey anti-mouse IgG (H + L) secondary antibody 
for 30 min at RT. Cells were washed in DPBS and analyzed for CD40, CD95 or 
ICAM surface expression on a FACSTAR® plus flow cytometer (Becton Dickinson, San 
Jose, CA).

**Electrophoretic Mobility Shift Assay (EMSA)**

After treatment or stimulation as required by the particular assay, cells were 
collected and lysed to isolate nuclei based on the method of Buras and others (1994). 
Briefly, cells were lysed in hypotonic buffer containing 10 mM KCl, 0.3 M sucrose, 10 
mM β-glycerol phosphate, 0.2 mM EDTA, 0.4% nonidet P-40, 1 mM PMSF and 1 
μg/ml each of leupeptin and pepstatin, on ice for 30min with gentle agitation. 
Following lysis, nuclei were collected by centrifugation at 11,500 x g for 5min. 
Nuclear proteins were extracted by incubating nuclei in a buffer containing 25% 
glycerol, 0.3 M KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM 
PMSF, and 1 μg/ml each of leupeptin and pepstatin for 30 min on ice. Extracts were 
centrifuged at 11,500 × g and supernatants were collected as nuclear extracts. Protein 
determinations were performed by the BCA micro protein assay. Extracts were 
aliquoted and stored at -80° C. Binding assays were performed using a gelshift assay 
kit consensus oligonucleotide containing the binding element for NF-κB (Genekea, 
Quebec, Canada) that was end-labeled using 32P-gamma ATP (Amersham Pharmacia 
Biotech, Inc., Piscataway, NJ). Nuclear extracts were incubated with gel shift binding
buffer and $^{32}$P-end-labeled oligonucleotide at approximately $5 \times 10^5$ cpm. Binding reactions were done per Geneka protocol. Following binding reactions, samples were analyzed on 5% polyacrylamide gels. Following electrophoresis, gels were dried onto Whatman filter paper and exposed to x-ray film from several hours to overnight at room temperature. X-ray films were analyzed by scanning densitometry using the UNSCAN-IT-gel automated digitizing system, Silk Scientific Corp., Orem, UT.

**Western Blot Analysis**

Prior to stimulation, monocytes were pretreated with 50 µM Na$_3$VO$_4$ for 20 min to negate PTP effects on tyrosine-phosphorylated cellular proteins during stimulation. After monocyte or smooth muscle cell treatment/stimulation in 24-well plates, cells were lysed in 50 µl boiling treatment buffer (125 mM Tris, pH 6.8, 2% SDS, 20% glycerol, 1% beta-mercaptoethanol, and 0.003% bromophenol blue) containing 1 mM each PMSF and sodium orthovanadate. Samples were boiled an additional 5 min prior to protein separation by SDS-PAGE on 15% minigels. Gels were equilibrated in transfer buffer (48 mM Tris, pH 9.2, 39 mM glycine, 1.3 mM SDS, and 20% methanol) for 15 min prior to transfer. Protein transfer to nitrocellulose membranes (Corning Costar Corp., Kennebunk, ME) was performed at 15V for 30 min using a Trans-Blot$^\text{TM}$ SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Richmond, CA). Dried membranes were blocked by gentle agitation in PBS containing 0.1% Tween 20 and 1% BSA (blocking buffer) for 30 min at 37° C. Membranes were incubated with
the relevant primary Ab in blocking buffer for 30 min at 37° C, and then washed for 10 min ×2 washes in PBS containing 0.1% Tween 20 (wash buffer), followed by incubation with the relevant (HRP-conjugated) secondary Ab for 30 min at 37° C (if primary antibody not HRP-conjugated), and then washed for 10 min ×3 in wash buffer. Ab-bound proteins were detected using an ECL™ Western blotting analysis system (Amersham Corp., Arlington Heights, IL) and the membranes were exposed to Kodak X-Omat LS X-ray film (Eastman Kodak, Rochester, NY). X-ray films were analyzed by scanning densitometry using the UN-SCAN-IT-gel automated digitizing system.

Analysis of Chemokine Production

Analysis of IL-8 and MCP-1 production by SMC stimulated through CD40, was examined by ELISA. SMC were plated in 96 well microtiter plates and rested for 10 days in serum free medium. The cells were then treated with IFNγ for 5 days to up-regulate CD40 expression. SMC were co-incubated with activated T cell membranes (TmA), resting T cell membranes (TmR), 293 parent cell line, 293-CD154 transfectants, Jurkat cell line, or D1.1 cells. Supernatants were harvested after an 18-h incubation and assayed by enzyme-linked immunosorbant assay (ELISA) using R&D system's Quantikine™ human enzyme linked immunosorbant assay system or OptEIA™ sets for human IL-8 and MCP-1, PharMingen (San Diego, CA).
RNAse Protection Assay

Following stimulation of SMC, cells were lysed and RNA was isolated using TRIzol® Reagent (Life Technologies/Gibco) per TRIzol® protocol. The Riboquant® Multi-Probe (PharMingen) RNAse protection assay system was used to detect mRNA species. The multi-probe was labeled with α-32P-UTP (Amersham), and hybridized to the RNA. RNAse treatment and purification of protected probes was performed per Riboquant® protocol. Samples were analyzed on QuickPoint™ precast gels (NOVEX, San Diego, CA), and exposed to Kodak X-Omat LS X-ray film.

Alloreactive Proliferation Assay

A total of 4x10^5 SMC per well were plated in 96-well tissue culture plated in SMGM®-3, and 100U/ml recombinant human IFNγ was added after overnight culture. On day 3, cultured cells were washed with PBS, and treated with 50 μg/ml mitomycin C (Sigma Chemical Co.) to ensure that they were unable to contribute to the measurement of [3H]-thymidine incorporation used to assay T cell proliferative responses. In some experiments, the SMC were fixed, with freshly prepared 0.8% paraformaldehyde at room temperature (RT) for 10 minutes, rather than mitomycin C-treated. The cells were washed with PBS prior to addition of 2x10^4 human CD4+ T cells per well with or without IL-1 (1% P388D1 supernatant) as a source of costimulus. Three days after addition of the T cells, the cells were pulsed with 0.5 μCi/well [3H]thymidine for 3 h and harvested on glass fiber filters for scintillation counting.
CHAPTER 3

RESULTS

CD40 Signaling in Monocytes

CD40 Activation of ERK1/2 in Monocytes

Previous research done in our laboratory demonstrated that CD40-mediated monocyte inflammatory cytokine synthesis was dependent on activation of PTKs (Poe and others 1998). To further investigate the activation of PTKs, the role of MAPK family members in T cell activation of monocytes through CD40 signaling was evaluated. Stimulus was provided by purified plasma membranes from CD4+ T cells which had been activated for 6 hours (a time point at which CD154 expression was optimal) designated TmA. Purified plasma membranes from resting (CD154+) T cells, designated TmR, were used as a control. Plasma membrane preparations were titrated for activity based on membrane protein concentrations. In the experiments presented in this dissertation, membranes were used at 10μg of protein/ml. The ability of TmA to induce MAPK phosphorylation was evaluated over a 2 hour time period postactivation. ERK1/2 phosphorylation was evident at 10 minutes, and declined at 2 hours (Figure 4A, top panel). The blot was stripped and reprobed with antibody recognizing both phosphorylated and nonphosphorylated forms of ERK present demonstrating that the differences observed were not due to differences in the level of ERK1/2 protein, or artifacts of gel loading (Figure 4A, bottom panel).

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Figure 4. CD40-Mediated Activation of ERK1/2 in Monocytes. A, analysis of ERK1/2 phosphorylation. Monocytes were left unstimulated or stimulated with LPS for 30 minutes, or TmA for 10, 30, 60 or 120 minutes. After stimulation lysates were harvested and analyzed by western blot using an antibody specific for the phosphorylated form of ERK1/2 (top panel) or antibodies recognizing total ERK1/2 (bottom panel). B, analysis of p38 and JNK phosphorylation. Monocytes were stimulated as in A, lysates were harvested and analyzed by western blot using antibodies specific for the phosphorylated forms of p38 and JNK.
Although LPS stimulation resulted in the activation of the MAPK family members p38 and JNK, treatment of the monocytes over the same time period examined in 3A did not induce or enhance p38 or JNK phosphorylation (Figure 4B). These data suggested that CD40 signaling in monocytes resulted in the phosphorylation of the MAPK family member ERK1/2, but not in the phosphorylation of p38 or JNK over the time period examined (Suttles and others 1999).

**IL-4 and IL-10 Inhibit CD40-Mediated Monocyte ERK1/2 Phosphorylation**

In previous work, IL-4 and IL-10 were found to inhibit CD40-induced activation of PTK activity and the subsequent synthesis of inflammatory cytokines (Poe and others 1997). Additionally our previous work indicated a role of MEK and ERK activation in this pathway, therefore we hypothesized that IL-4 and IL-10 acted by interference of the signaling cascade leading to ERK1/2 activation. To test this hypothesis, monocytes were pretreated for an 18-h period with IL-4 or IL-10 prior to TmA stimulation and the cell lysates were assayed for phosphorylation of ERK1/2. ERK1/2 activation was decreased in a dose-dependent manner with both IL-4 and IL-10 (Figure 5). Quantification by scanning densitometry indicated that a dose of 50 ng/ml IL-4 resulted in 79% decrease in the level of phosphorylation observed over background in response to TmA. A 90% decrease was observed with treatment of IL-10 at 50 ng/ml. Neither of these cytokines affected the level of expression of ERK’s during this time period as shown by Western blots of the same samples, reprobed with an antibody reactive with
Figure 5. Effects of IL-4 and IL-10 on CD40-Mediated ERK1/2 Activation in Monocytes. Monocytes were pretreated with IL-4 or IL-10 at 5, 10, and 50 ng/ml, as indicated, then left unstimulated (control) or stimulated with TmA for 30 minutes. Cell lysates were harvested and analyzed by western blot for level of ERK1/2 phosphorylation using anti-phospho-ERK1/2 antibodies (top panel). The film was scanned and the digitized image analyzed for band density. The histogram represents density (total pixels minus background x 10^3) of the ERK2 bands (middle panel). The membrane was stripped and reprobed using antibodies recognizing total ERK (bottom panel).
total ERK1/2, which show equivalent levels of ERK1/2 regardless of treatment (Fig 5, bottom panel).

**Evidence of T Cell-SMC Interactions**

**SMC Express CD40**

Monocytes can interact with activated CD4+ T cell through several receptor:ligand pairs including the interaction of CD40 on monocytes with its natural ligand CD154 present on activated CD4+ T-cells. This interaction results in the up-regulation of pro-inflammatory cytokine synthesis. Both monocytes and SMC appear to play a role in vascular inflammation by interacting with T cells. We next examined SMC for CD40 and other receptors which have the ability to interact with T cells similar to monocyte:T cell interactions. Using the monocyte:T cell model we hypothesized that vascular smooth muscle cells may interact in a similar way contributing to the development of vascular inflammation. To address the hypothesis we phenotyped the SMC to determine the expression of the cell surface molecule CD40. SMC were treated over a period of 0 to 5 days with Th1 cytokine IFN-γ, which is a costimulus for macrophage activation and has been shown to up-regulate MHC-II expression in other cell lines. Cells were harvested and stained with anti-CD40 antibodies and analyzed by flow cytometry for CD40 expression (Figure 6). Untreated cells were negative for CD40 expression. However, IFNγ treatment induced CD40 expression which increased over the five day period. The experiment was carried out through day 7.
Figure 6. IFNγ Up-regulates CD40 Expression on SMC. A, CD40 surface expression on SMC. SMCs were left untreated or treated with 100U/ml of IFNγ for 3 days. Cells were harvested and stained for CD40 surface expression. Cells were analyzed by flow cytometric analysis. B, Kinetics of IFNγ treated on CD40 surface expression on SMC. SMC were left untreated or treated with IFNγ over time period of 1 to 5 days. Cells were harvested and analyzed by flow cytometric analysis.
(data not shown), however, expression declined consistently after day 5.

**SMC Express CD95**

Monocytes can interact with activated T cells through several receptor:ligand pairs in addition to the CD40:CD154 interaction. Monocytes express CD95 (Fas), a member of the TNF receptor superfamily. CD95 binds to its ligand CD95L (Fas ligand) expressed on T cells. This interaction can lead to programmed cell death or apoptosis. Apoptosis of SMC has been demonstrated *in vivo* and *in vitro* (Bennett and others 1995). Apoptosis of SMC observed in atheroma suggest that this process contributes to vascular remodeling that results in intimal thickening and fibrous cap formation. We hypothesized that SMC express CD95, which could interact with T cells through the CD95:CD95L receptor:ligand interaction similar to the monocyte-T cell model, resulting in apoptosis, and vascular remodeling. To address this hypothesis, SMC were analyzed for CD95 expression by flow cytometry (Figure 7).

**SMC Present Antigen to CD4⁺ T cells**

Monocytes have the ability to display peptide-MHC complexes in a form that can be recognized by T cells, and are known as classical antigen presenting cells (APC). The interaction of T cells with monocytes through antigen presentation results in reciprocal activation involving both contact-dependent and cytokine-mediated signals. Since the possible SMC-T cell interactions seem to resemble the monocyte-T cell model, we were interested in exploring the possibility of antigen presentation by SMC...
Figure 7. SMC Express CD95. Analysis of cell surface expression of CD95(Fas) on SMC. SMC were left untreated or treated with IFNγ for 5 days. After 5 days of treatment the cells were harvested, stained for surface CD95, and analyzed by flow cytometric analysis.
to T cells. Previous work done in the murine system has shown that pure populations of vascular smooth muscle cells (VSMC) clones are capable of antigen processing and presentation via the exogenous pathway to syngeneic or histocompatibility matched CD4+ T cells. We have hypothesized that human SMC are also capable of antigen presentation to allogeneic CD4+ T cells. To determine this human SMC were treated with 100U/ml IFN-γ for 3 days, which had been shown in previous experiments to optimally induce expression of class II MHC in monocytes. SMC were IFN-γ primed for three days, washed with DPBS and treated with mitomycin C to ensure that the cells were unable to contribute to the measurement of [3H]-thymidine incorporation or fixed with 0.8% paraformaldehyde. The SMC were co-cultured with human allogeneic CD4+ T cells in the presence or absence of IL-1β as a source of co-stimulus. On day 3 of culture, wells were pulsed with [3H]-thymidine, and SMC were evaluated for their ability to activate the T cells by allo-antigen presentation. CD4+ T cells incubated in the presence of fixed allogeneic SMC proliferated excessively as compared to the mitomycin C treated group (Figure 8). These data indicate that the SMC are capable of allo-antigen presentation to CD4+ T cells, and therefore reciprocal activation.

Influence of CD40 Signaling on SMC Chemokine Production

Our laboratory has demonstrated that CD40 signaling in monocytes results in the activation of inflammatory cytokine synthesis and rescue from apoptosis. We have also demonstrated that both of these events are critically dependent on the activation of PTK and but does not exhibit dependence on the activity of the PKC family (Poe and others
Figure 8. Allo-Antigen Presentation to CD4\(^+\) T Cells by SMC. SMC were plated in 96-well culture plates, IFN\(\gamma\) treated, and either paraformaldehyde fixed or treated with mitomycin C. 10\(^5\) allogeneic CD4\(^+\) T were added to the SMC cultures in the presence or absence of IL-1\(\beta\) (1%P388D1 supernatant). On the third day T cell proliferation was assayed by [\(^3\)H]thymidine incorporation. The average cpm [\(^3\)H]thymidine incorporation ± standard deviation of triplicate samples.
1997) However, the activity of PTK and the activity of the serine/threonine PKC family has been demonstrated in CD40 signaling in B cells. We were interested in determining the nature of kinase involvement in CD40 signaling in SMC. CD40-mediated IL-8 and MCP-1 production were evaluated in SMC pretreated with inhibitors of PTK and PKC activity. SMC were treated either with herbimycin A, a potent, broad PTK inhibitor, or H-7, an inhibitor of PKC, prior to CD40 stimulation. The inhibitors were used at concentrations based on previous experiments (Suttles and others 1996), and caution was exercised to ensure that the concentrations used did not affect cell viability and total protein synthesis. Stimulus was provided by plasma membranes purified from CD4+ T cells which had been activated for 6 hours (a time point at which CD154 expression was optimal) as well as by co-culture of SMC with CD154 transfectants, or the CD154+ Jurkat T cell variant, D1.1 (Yellin and others 1991; Yellin and others 1994). SMC were pretreated with or without 0.5 μg/ml HerbA and 2 μg/ml H-7 for ~18 hours and then stimulated through CD40 with plasma membranes from either activated (CD154+) CD4+ T cells designated TmA or CD154-transfected 293 cells. Supernatants were collected for analysis at 18 hours. Controls consisted of supernatants from SMC stimulated with resting (CD154-) CD4+ T cells designated TmR or the 293 parent cell line. SMC IL-8 and MCP-1 synthesis was induced by co-culture with TmA and the CD154 transfectants, but not by co-culture with TmR or the control 293 population. Furthermore both IL-8 and MCP-1 production was suppressed by both HerbA and H-7 which suggests that both PTK and PKC activity is required for CD40-mediated activation of SMC IL-8 and MCP-1.
production (Figure 9). Additionally, we demonstrated that activation of SMC induced by co-incubation with CD154 transfectants is inhibited by the addition of anti-CD154 antibodies confirming that the primary activating component of this interaction is the CD40 ligand, CD154 (Figure 10). These data suggest that CD40-mediated IL-8 and MCP-1 synthesis in SMC is a CD40-mediated event and dependent on both PTK and PKC activity.

**CD40 Signaling in SMC results in Cytokine and Chemokine mRNA Synthesis**

SMC have the capacity to play an active role in vascular inflammation by their ability to produce a variety of cytokines including IL-1, TNF-α, IL-6, GM-CSF, IL-8, MCP-1, and monocyte chemotatic and activation factor (MCAF) (Ross 1993). The production of these cytokines by SMC may contribute to the recruitment and activation of leukocyte populations, resulting in vascular inflammation and plaque formation. Since we observed that CD40 signaling in SMC resulted in the induction of the inflammatory chemokine synthesis, we next addressed the question how specific is CD40-mediated chemokine and cytokine synthesis in SMC. To address this question SMC were stimulated with TmA for various time points, and the mRNA was extracted and evaluated for a panel of inflammatory cytokines and chemokines by an RNAse protection assay as described in materials and methods. We previously observed that PTK, PKC, MEK/ERK and p38 were involved in CD40-mediated induction of IL-8 and MCP-1 in SMC, therefore we hypothesized that activation of these kinases had an effect on CD40-mediated cytokine/chemokine mRNA synthesis. To determine the
Figure 9. Requirement for PTK and PKC Activity in CD40-Mediated SMC IL-8 and MCP-1 Synthesis. SMC were pretreated with the PTK inhibitor, HerbA (0.5µg/ml) or the PKC inhibitor H-7 (2µg/ml) for 18 hours. SMC were either stimulated with 293-CD154 cells or the control parent 293 cell line. After 18 hours stimulation, supernatants were removed and analyzed by ELISA.
Figure 10. IL-8 Production by SMC is a CD40-Mediated Event. SMC were left unstimulated (control) or stimulated for 18 hours with 293 parent cell, CD154 transfectants, or CD154 transfectants + antibody, as indicated. Supernatants were assayed for IL-8 production by ELISA.
involvement of PTK, PKC, ERK1/2 and p38 activity in CD40-mediated induction of cytokine/chemokine mRNA synthesis, SMC were pretreated with or without the PTK inhibitor, Herb A, the PKC inhibitor H-7, the MEK/ERK inhibitor PD98059, and the p38 inhibitor SB203580, and stimulated at several time points with TmA (figure 11). Synthesis of TGFβ-1, IL-8 and IL-1RA was observed with SMC stimulated with TmA. Inhibition of the cytokines/chemokines was seen with PD98059, SB203580 and Herb A treatment, but not with H-7. These data indicate that CD40 signaling in SMC results in the induction of cytokine/chemokine mRNA synthesis, and that activation of the MEK/ERK, p38 and possibly other pathways are involved.

**CD40 Activation of ERK1/2 and p38 in SMC**

Previous studies from our laboratory demonstrated that CD40 signaling in monocytes is dependent on the activation of the ERK1/2 MAPK family members (Suttles and others 1999). In contrast, in B cells previous research has shown that CD40 signaling involves activation of the MAPK family members JNK, p38 and ERK1/2 (Swantek and others 1997; Sanghera and others 1996). We next addressed the question are the MAPK signaling pathway(s) activated in response to CD40 ligation in SMC. All three MAPK family members: ERK1/2, p38 and JNK were considered as candidates. To evaluate the ability of CD40 stimulation to activate the MAPK family members, SMC were treated with TmA or TmR over a 2 hour time period post-activation. After a 10 to 120 minute incubation period, cell lysates were harvested and analyzed for MAPK activation by Western blot using antibodies specific for the
Figure 11. CD40 Activation of Cytokine/Chemokine mRNA. SMC were left unstimulated or stimulated with TmA for 3, 6, or 19 hours in the absence or presence of 30mM PD98059, 30mM SB203580, 0.5mg/ml HerbA, or 2mg/ml H-7. Lane1, unstimulated; lane2, TmA 3hour; lane 3, TmA 6 hour; lane 4, TmA 19 hours; lane 5, 3 hour TmA + HerbA; lane 6, 6 hour TmA + HerbA; lane 7, 6 hour TmA + H-7; lane 8, 6 hour TmA + PD98059; lane 9, 6 hour TmA + SB203580.
phosphorylated (active) forms of ERK1/2, p38, and JNK. ERK1/2 and p38 phosphorylation was evident at 10 minutes and declined at 2 hours (Figure 12A and B). Treatment of SMC with TmA over the same 2 hour time period examined did not induce JNK phosphorylation (Figure 12C). Probing the blot with antibody recognizing the nonphosphorylated forms of JNK present revealed that the absence of phosphorylated JNK present was not due to artifacts of gel loading (data not shown). Lysates from UV-treated 293 cells (New England Biolabs) were used as a positive control for anti-phospho-JNK reactive proteins.

**CD40-Mediated IL-8 and MCP-1 Synthesis in SMC is Dependent Upon ERK1/2 and p38 MAPK Activity**

Since both ERK1/2 and p38 MAPK family members appear to be involved in CD40 signaling in SMC we next wanted to determine if the activation of ERK1/2 and p38 MAPKs have a functional significance in CD40-mediated induction of IL-8 and MCP-1 synthesis. The role of CD40-mediated activation of ERK1/2 and p38 in the induction of inflammatory chemokine synthesis was explored via upstream blockade of the ERK1/2 and p38 pathways. Activation of ERK1/2 is catalyzed by MEK1/2, which, itself, is activated through serine phosphorylation catalyzed by Raf family kinases (Davis 1993). Use of a specific MEK1/2 inhibitor, PD98059, which prevents Raf-mediated activation of MEK1/2 (Allessi and others 1995), (Figure 3), allowed us to evaluate the role of the MEK/ERK pathway in CD40-mediated induction of SMC IL-8 and MCP-1 synthesis. Additionally, use of a specific p38 inhibitor, SB203580, (Figure
Figure 12. CD40-Mediated Activation of ERK1/2 and p38 MAPK Family Members in SMC. SMC were either left unstimulated (control) or stimulated with LPS for 30 minutes, or TmA for 10, 30, 60 or 120 minutes as indicated. Cell lysates were then harvested and analyzed by Western Blot with antibodies recognizing the phosphorylated form of A, ERK1/2; B, p38; and C, JNK.
3), allowed us to evaluate the role of the p38 signaling pathway in CD40-mediated induction of SMC IL-8 and MCP-1. The influence of PD98059 and SB203580 on CD40-mediated SMC IL-8 and MCP-1 synthesis was analyzed by enzyme-linked immunosorbant assay of SMC supernatants. SMC were stimulated for 18 hours by CD154-transfected 293 cells (293-CD154) in the presence or absence of PD98059 and SB203580. Supernatants were then collected for analysis. Controls consisted of supernatants from SMC stimulated with the 293 parent cell line. SMC IL-8 and MCP-1 synthesis was induced in response to stimulation with the 293-CD154 cells, but not with the control 293 parent line. Additionally IL-8 and MCP-1 synthesis was suppressed by the addition of PD98059 and SB203580 (Figure 13A and B), and had no adverse effects on cell viability in a concentration range of 1-100μM tested. These data suggest that MEK activation of ERK1/2 and the activation of p38 are critical elements in the signaling pathways of CD40-mediated induction of SMC IL-8 and MCP-1 synthesis.

**CD40 Signaling in SMC Results in Activation of the Transcription Factor NF-κB**

The transcription factor NF-κB is in part critical for gene expression of many cellular products involved in inflammatory responses, such as TNF-α, IL-1β, IL-6, IL-8 and MCP-1 (Sharma and others 1998). Previous observations have shown that CD40-mediated IL-8 and MCP-1 require activation of ERK1/2 and p38, we hypothesized that CD40 signaling in SMC results in activation of NF-κB and that activation of both ERK1/2 and p38 is required. To determine the role of CD40
Figure 13. Requirement for ERK1/2 and p38 Activity in CD40-Mediated SMC IL-8 and MCP-1 Synthesis. SMC were left untreated (control) or stimulated with TmA in the presence or absence of the MEK/ERK inhibitor PD98059 or the p38 inhibitor SB203580. After 18 hours supernatants were harvested and analyzed by ELISA for: A, IL-8 production or B, MCP-1 production.
signaling in the activation of transcription factors contributing to inflammatory cytokine and chemokine synthesis in SMC, nuclear localization of NF-κB was evaluated by electrophoretic mobility shift assay (EMSA) as described in materials and methods. Additionally, the involvement of the MAPK family members ERK1/2 and p38 in CD40-mediated activation of NF-κB was examined. SMC were pretreated with or without the MEK/ERK inhibitor PD98059 and the p38 inhibitor, SB203580, and stimulated with TmA for 30 minutes. NF-κB was activated in SMC cells stimulated with TmA, and the amount of activation was decreased in cells pretreated with PD98059 and SB203580 (figure 14). These data suggest that CD40 signaling in SMC results in the activation and/or nuclear localization of the transcription factor NF-κB, and that the activation of the MEK/ERK and p38 pathways are critical.
Figure 1. CD40-Mediated Activation of NF-κB in SMC. SMC were left unstimulated (control) or stimulated with 1mg/ml TmA for 30 minutes in the absence or presence of 30mM SB203580 or PD98059. Cells were then lysed and nuclear proteins extracted and an EMSA was performed for NF-κB activation. Band densities were determined by scanning densitometry. Lane 1, unstimulated; lane 2, TmA; lane 3, TmA + SB203580; lane 4, TmA + PD98059; lane 5, cold competition assay of 30 minute TmA stimulation; lane 6, positive control; lane 7, mutant oligo.
The interaction of CD40 with its natural ligand CD154 contributes to normal cell-mediated responses as well as to chronic inflammatory diseases (Stout and Suttles 1996). Atherosclerosis, or vascular inflammation is characterized by the migration of immune cells (i.e. monocytes, T cells) to the site of injury, which transmigrate through the endothelium from the media to the intima of an artery. There are many cells which contribute to the development of the disease, however the presence of activated, CD154+ T cells at these atherosclerotic lesions may play a crucial role in the maintenance and/or exacerbation of inflammation. There are several known functional attributes acquired in response to CD40 ligation in monocytes/macrophages including the up-regulation of inflammatory mediators, and the increase of monocyte/macrophage longevity, both events contributing to the maintenance and/or exacerbation of inflammatory disease. Studies of tissue sections of atherosclerotic plaques have found the accumulation of activated (CD154+), CD4+ T lymphocytes (Mach and others 1997) which have the capacity to interact with CD40 expressing cells in the vasculature including endothelial, monocytes/macrophages, and smooth muscle cells. Previous research has shown that both monocytes/macrophages and SMC have the ability to contribute to vascular inflammation by the production of inflammatory cytokines/chemokines, tissue factor, the up-regulation of adhesion molecules, and both
have the propensity to become lipid-laden foam cells (Poe and others 1997; Mach and others 1997). In vascular inflammation the role of CD40, and the interactions that occur between major cell types contributing the formation of atherosclerotic plaques is currently under investigation. Experiments described in this dissertation were performed to determine the role of CD40 signaling in monocytes and SMC in the development of atherosclerosis. We examined the functional attributes acquired by SMC in response to CD40 ligation, and the role of MAPK signaling pathways activated in response to CD40 signaling in both monocytes and SMC.

Previous research done in our laboratory investigated the signaling pathways activated in response to CD40 ligation in monocytes resulting in the production of inflammatory cytokine synthesis. These studies revealed that the activation of PTK is a critical component in CD40-mediated activation of monocyte inflammatory cytokine synthesis, as well as rescue from apoptosis (Poe and others 1998). We evaluated the role of the MAPK family members as potential downstream mediators of CD40 signaling in monocytes and SMC. MAPK family members ERK1/2, p38 and JNK were all considered as likely candidates since previous reports have demonstrated that all three members are activated in response to CD40 signaling in B cells, and in LPS activation of monocytes (Li and others 1996; Sakata and others 1995; Sanghera and others 1996). The experiments presented in this dissertation employed primary human monocytes and primary aortic smooth muscle cells (of less than passage 6). Stimulus used in these studies included plasma membranes purified from activated (CD154⁺) CD4⁺ T cells and CD154 transfectants.
We observed CD40 signaling in monocytes resulted in the rapid phosphorylation and activation of the MAPK family member, ERK1/2, however over the same time period of stimulation, phosphorylation of p38 and JNK was not increased above background (Figure 4). Additionally, CD40-mediated activation of ERK1/2 in monocytes was accompanied by enhanced kinase activity and is a critical event in CD40-mediated monocyte IL-1β and TNFα synthesis (Suttles and others 1999). How ERK1/2 contributes to the transcriptional control of CD40-mediated induction of inflammatory cytokine synthesis is currently being investigated. Both IL-1β and TNFα genes are, in part, regulated by several transcription factors including NF-κB and NF-IL-6. NF-IL-6 is a direct substrate of ERK1/2 catalytic activity.

The intent of our research is not only to determine how CD40 signaling in monocytes results in the activation of inflammatory cytokine synthesis, thus leading to inflammatory diseases such as vascular inflammation, rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease, but also in how to suppress the inflammatory process. Previous studies suggested that the anti-inflammatory cytokines IL-4 and IL-10 inhibited CD40-induced monocyte PTK activity, and CD40-mediated monocyte IL-1β production and together worked in a synergistic manner. Additionally, inhibition of CD40-mediated IL-1β synthesis, and activation of ERK1/2 in monocytes was observed in response to pretreatment with an PTK inhibitor suggesting that PTKs were a critical element in the monocyte CD40 signaling pathway (Poe and others 1997). IL-4 and IL-10 were evaluated for their ability to suppress CD40-mediated activation of ERK1/2 in monocytes. Both IL-4 and IL-10 modulated CD40-mediated
ERK1/2 phosphorylation in monocytes, suggesting that the MAPK, ERK1/2 may be their target of inhibition (Figure 5). Both IL-4 and IL-10 signal through receptors which associate with and activate Jaks upon ligation (O'Shea 1997; Leonard and O'Shea 1998). The Jaks in turn, activate the Stats which induce negative regulators of cytokine signaling SOCS, which work as a negative feedback system to inhibit the Jaks either directly or non-directly (Darnell 1997; Nicholson and Hilton 1998). IL-4 and IL-10 appear to act early in the signaling cascade since they inhibit PTK activity in CD40-mediated activation of monocyte inflammatory cytokine synthesis (Poe and others 1998). These data suggest that IL-4 and IL-10 may induce tyrosine phosphatase activity, resulting in the suppression of the CD40 signaling pathway in monocytes, and thus suppression of ERK1/2 activity.

Recent reports have shown that vascular inflammation involves the participation of immune competent cells and the activation of inflammatory pathways and mediators. Both monocytes and SMC are present at atherosclerotic plaques and may be the major progenitors of vascular inflammation (Mach and others 1997). We observed that SMC express CD40 (Figure 6) and other receptors that interact with T cells, similar to the monocytes including cell surface expression of CD95 (Figure 7), intercellular adhesion molecule (ICAM), the costimulatory molecules B7-1(CD80) and B7-2 (CD86) (data not shown), and both have the ability to present antigen to CD4 T cells (Suttles and others 1994). These data suggest that CD40 signaling in SMC results in the activation of signaling pathways and induction of inflammatory mediators in a manner similar to what occurs in monocytes.
The induction of chemokine synthesis may play an crucial role in the pathogenesis of atherosclerosis by promoting directed migration of immune competent cells to the site of injury. The observation that CD40 ligation in SMC induced the inflammatory chemokines MCP-1 and IL-8 suggested that SMC may play a critical role in the pathogenesis of atherosclerosis by enhancing the migration of immune competent cells to the atherosclerotic plaque. We further confirmed that the production MCP-1 and IL-8 was a CD40-mediated event by employing anti-CD154 antibodies and blocking the synthesis of the chemokines as compared to treatment with an isotype control antibody (Figure 10). Furthermore, we observed that CD40-mediated induction of MCP-1 and IL-8 synthesis was partially dependent on both PTK and PKC activity (Figure 9), suggesting that the CD40 signaling pathway in SMC may also have similarities to the signaling pathway(s) activated in B cells in response to CD40 ligation. MCP-1 induces tissue factor (TF), a transmembrane glycoprotein that initiates coagulation, mRNA synthesis in SMC (Schecter and others 1997). The exposure of TF during plaque rupture may induce acute thrombosis resulting in myocardial infarction. Further investigation of MCP-1 production and its effects on SMC may lead to a greater understanding of the pathways involved and provide important targets for inhibition of vascular inflammation and plaque thrombosis. The observation that CD40-mediated IL-8 and MCP-1 synthesis was, in part, dependent on PKC activity suggests that the PKC signaling pathway may play an important role in contributing to vascular inflammation and may be a potential target for modulation.

We observed that CD40 signaling in SMC resulted in the phosphorylation of the
MAPK family members ERK1/2 and p38, however we did not see activation of JNK over the time course examined (Figure 12). The critical dependence of both ERK1/2 and p38 activation on CD40-mediated IL-8 and MCP-1 were also important observations (Figure 13), suggesting that both pathways may contribute to the regulation of transcription factor activation resulting in the synthesis of these chemokines. These data suggest that CD40-mediated activation of SMC chemokine synthesis implements a distinctly different signaling pathway from that in monocytes and B cells. The ability of the MEK1/2 inhibitor PD98059 and the p38 inhibitor SB203580 to inhibit CD40-mediated SMC chemokine synthesis suggests that both MAPK signaling pathways are involved in the downstream activation of transcription factors resulting in chemokine production. Both IL-8 and MCP-1 are in regulated by several transcription factors including NF-κB (Roebuck and others 1999). SMC stimulated with TmA resulted in the activation of the transcription factor NF-κB (Figure 14). Additionally we observed that inhibitors of the MEK/ERK and the p38 signaling pathways suppressed activation of NF-κB suggesting that both of the MAPKs are involved in CD40-mediated activation of this transcription factor. Activation of NF-κB in collaboration with the transcription factors NF-IL-6 (C/EBP) and AP-1 regulate both IL-8 and MCP-1 transcription in most cells (Sharma and others 1998). These data suggest that the CD40 signaling in SMC results in the activation of the MEK/ERK and p38 MAPK signaling pathways which in turn activate transcription factors leading to the synthesis of the chemotactic factors IL-8 and MCP-1 at atherosclerotic lesions. We have demonstrated that CD40-mediated induction of VSMC
chemokine synthesis was in part regulated by PTK and PKC (Figure 9), this suggests that both of these pathways may be involved in activation of the MAPK pathways. Activation of PTK results in activation of ERK1/2, and activation of PKC may result in the activation of the MAPK p38.

Finally, these data suggest that monocytes/macrophages and SMC present at atherosclerotic lesions may be critical regulators of vascular inflammation. Both of these cells are capable of interacting with activated T cells through CD40:CD154 receptor-ligand pair resulting in the synthesis of inflammatory mediators. Accumulation of activated T cells and monocytes/macrophages at plaque formation sites and in the neointima, and the activation and proliferation of SMC contribute to the inflammatory reaction that takes place inside the plaques. The production of inflammatory cytokines including IL-1β and TNFα, and the secretion of chemotactic factors including IL-8 and MCP-1 facilitate aberrant migration of immune competent cells to that site. The delineation of the signaling pathways activated in response to CD40 signaling in monocytes and SMC will help us to gain a better understanding of the pathways activated resulting in the onset of inflammation, and will help us define targets of therapeutic intervention.

The ability of the Th2 cytokines IL-4 and IL-10 to abrogate CD40-mediated monocyte cytokine synthesis, and ERK1/2 phosphorylation suggests that there may be direct interaction between the Jak/Stat and ERK1/2 signaling pathways. However, significantly different to the observations in the monocyte system, neither IL-4 nor IL-10 suppress CD40-mediated SMC chemokine production or MAPK phosphorylation.
Monocytes and SMC exhibit many similar characteristics, but are distinctly unique in signaling pathways implemented in response to CD40 ligation (Figure 15). CD40 signaling in monocytes results in the activation of the inflammatory cytokines IL-1β and TNFα, which are both critically dependent on the activation of the MEK/ERK pathway, and can be suppressed by the anti-inflammatory cytokines IL-4 and IL-10. CD40 signaling in SMC results in the synthesis of the chemotactic factors IL-8 and MCP-1, which are both dependent on the activation of the MEK/ERK and p38 pathways suggesting divergence in the signaling pathway. However, CD40 signaling in both monocytes and SMC may result in the activation of divergent signaling pathways but with the downstream outcome being the activation of transcription factors which contribute to the production of inflammatory cytokines and chemokines.
Figure 15. Summary of CD40-Mediated Signaling Events in Monocytes and SMC
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