December 1994

Role of the Cd40-cd40 Ligand Interaction in Cd4(+) T Cell Activation of Monocyte Interleukin-1 Synthesis

David H. Wagner
East Tennessee State University

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Role of the CD40-CD40 ligand interaction in CD4+ T cell activation of monocyte interleukin-1 synthesis

Wagner, David Hal, Jr., Ph.D.
East Tennessee State University, 1994
ROLE OF THE CD40-CD40 LIGAND INTERACTION IN CD4+ T CELL
ACTIVATION OF MONOCYTE INTERLEUKIN-1 SYNTHESIS

A Dissertation
presented to
the Faculty of the Department of Biochemistry
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 David H. Wagner, Jr.

December 1994
APPROVAL

This is to certify that the Graduate committee of

David H. Wagner, Jr.

met on the

4th day of November, 1994

The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council and the Associate Vice-President for Research and Dean of the Graduate School, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences.

Jill Suttles
Chair, Graduate Committee

David A. Johnson

W. Scott Champney

Carol Duk

Paul J. Monaco

Signed on behalf of the Graduate Council

Associate Vice-President for Research and Dean, School of Graduate Studies
ROLE OF THE CD40-CD40 LIGAND INTERACTION IN
CD4+ T CELL ACTIVATION OF MONOCYTE INTERLEUKIN-1 SYNTHESIS
ABSTRACT

Role of the CD40-CD40 ligand in CD4⁺ T cell activation of monocyte

Interleukin-1 synthesis

by

David H. Wagner, Jr.

Most studies of the induction of cytokine synthesis in monocytes have used an exogenous triggering agent such as Lipopolysaccharide (LPS). However, during nonseptic chronic inflammatory responses (e.g., rheumatoid arthritis) monocyte activation occurs as a result of T cell generated signals. This report demonstrated that plasma membranes from anti-CD3 activated peripheral CD4⁺ T cells (TmA) but not from resting CD4⁺ cells (TmR) induced monocytes to synthesize IL-1 in the absence of costimulatory cytokines. The expression kinetics of the molecule(s) unique to activated T cells which interact with monocyte receptors to induce IL-1 demonstrated that optimal expression occurred at 6h post activation. This matched Lederman's, et al., (1992) previously reported kinetics of expression of CD40 ligand (CD40L) on activated peripheral T cells, implicating the CD40-CD40L interaction as a candidate for the initiator of IL-1 induction in monocytes. In this work, it was demonstrated that the signal could be reduced up to 85% by addition of 5c8, a monoclonal anti-CD40L antibody. In addition, a monoclonal anti-CD40 IgM (BL-C4) induced resting monocytes to synthesize IL-1. Experiments demonstrated that crosslinking the CD40 molecules on monocytes was critical for IL-1 induction. TmA but not TmR also up-regulated cell surface expression of adhesion/costimulatory molecules on monocytes including CD40, ICAM-1, and LFA-3. Anti-CD40 signaling up-regulated expression of ICAM-1 and LFA-3. Experiments suggested that signaling through CD40 may utilize a protein tyrosine kinase (PTK) mediated pathway but not a protein kinase C mediated pathway and studies using THP-1, a premonocytic cell line, indicated that the transcription factor, NF-κB, was activated through anti-CD40 signaling. Since CD40 ligand-transfected cells alone did not induce IL-1 but TmA did, it was considered that an additional costimulatory cell surface molecule was required. Preliminary experiments suggested that CD69 may be required. In summary, these results indicate that contact-dependent T cell-monocyte interactions, alone, can activate inflammatory cytokine production by resting monocytes and that a critical component of this interaction is the CD40-CD40L signaling event.
FULL TITLE: T Cell Activation of Monocyte IL-1 Synthesis

PROJECT #94-013s

PROJECT DIRECTOR: Jill Suttles, Ph.D.

MULTI-INSTITUTIONAL PROJECTS:

Sponsoring Organizations:

Principal Investigator:

GENERAL DESCRIPTION OF RISKS AND BENEFITS: see attached informed consent

INFORMED CONSENT PROCEDURES: see attached informed consent

PROCEDURES FOR PROMPT REPORTING:

Any changes or adverse reactions will be reported to the Chairman of the Institutional Review Board utilizing standard reporting procedures. See policy statement on the next page.

The Board will review this project at least at (12) month intervals.

I, Anthony DeLuca, Ph.D., Chairman of the Institutional Review Board, endorse the above Certificate of Special Assurance and certify that this project (which includes the protocol and informed consent) has received full IRB approval and that ETSU has established the Institutional Review Board satisfying the requirements of the 45 C.F.R. Sec. 46.

Dr. Anthony DeLucia, Chairman

August 9, 1994
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“d. All project directors will be advised in writing to notify the IRB Chairperson immediately of any proposed changes in an activity and of unanticipated problems involving risk to subject or others. This information will be immediately passed directly by the Chairperson to all members of the IRB. No changes in a research activity may be initiated without prior IRB review and approval, except where necessary to eliminate apparent hazards to the subject.”

“e. Any such problems on HHS funded projects including adverse reaction to biologicals, drugs, radiolotope labelled drugs, or to medical devices, shall be reported directly by the project director to the HHS and to the IRB members.”
INFORMED CONSENT FORM

Principal Investigator: Jill Suttlcs, Ph.D.

Project Title: Analysis of T cell-Monocyte CD40 Signaling

I understand that this is a research experiment.

I understand that I am being asked to participate in an experiment which is designed to determine the mechanism by which immune cells are activated to produce immune system hormones.

If I decide to participate in this experiment I will be asked, at a prescheduled time that does not conflict with my working hours, to come to the Department of Biochemistry laboratories, where I will be asked to donate 50 ml (1.7 ounces or .12 of a pint) of blood which will be drawn from my arm vein using a sterile disposable needle and syringe.

I understand that I will not be asked to donate blood anymore than once a month. Each donation will be totally voluntary and informed consent will be obtained at the time of each donation. I understand that I may withdraw from the study at any time without penalty by calling the office of Dr. Jill Suttlcs, telephone number 929-5916.

I understand that the only expected side effects that I will experience because of this experiment is the transient local pain and slight bruising of the skin associated with having my blood drawn. There is an extraordinarily remote possibility of a local infection at the puncture site. There is also the potential risk of fainting in response to having blood drawn.

I understand that potential benefits to me as a participant include gaining some information about my own immune system including the number of peripheral blood mononuclear cells that I have per ml of blood and the ability of these cells to respond to activation signals. I understand that I will not be charged for any of these tests.

I understand that I will be immediately notified if any of the results of the study might affect my willingness to continue to participate in the study. I may contact Dr. Jill Suttlcs concerning the research or my rights or concerning any medical problem that I feel may relate to the study. I understand that I may be withdrawn from the study at any time by the above mentioned investigator, if, in her opinion, it would be inadvisable for me to participate or if the study is terminated.

I understand that a copy of the results of the study will be kept on file in the Department of Biochemistry at the Medical School for a period of at least 10 years. I understand that all medical information gained in this study will be treated confidentially, and any publications resulting from this study will not use my name or release information about my specific medical condition.
I also understand that while my rights and privacy will be maintained, the Secretary of the Department of Health and Human Services and the ETSU Institutional Review Board do have free access to any information obtained in this study should it become necessary and I freely and voluntarily choose to participate. I understand that I may withdraw at any time without prejudice to me. I also understand that while East Tennessee State University (ETSU) does not provide compensation for medical treatment other than emergency first aid for any physical injury which may occur as a result of my participation as a subject in this study, claims arising against ETSU or any of its agents or employees may be submitted to the Tennessee Claims Commission for disposition to the extent allowable as provided under TCA Section 9-8-307. Further information concerning this may be obtained from the Chair of the Institutional Review Board.

I agree that I will participate in the study.

Date ___________________________ Signature of Volunteer

Date ___________________________ Signature of Principal Investigator

Date ___________________________ Signature of Witness
ACKNOWLEDGMENTS

There are many people for whose assistance I am grateful. First, I would like to thank my mentor and advisor, Dr. Jill Suttles who introduced me to the exciting field of immunology and resurrected my scientific career, as well as helping me to improve all my running PRs. (Jill, I still think we can create a new journal "Running Immunology "). I would like to thank the members of my committee including Dr. Robert Stout, Dr. Paul Monaco, Dr. Scott Champney and Dr. David Johnson. I am especially grateful to Dr. Ellen Rasch for seeing that I was supported throughout this entire venture. I owe a debt of gratitude to Bob Miller for helpful discussions, technical assistance and equally important for being a basketball confidant (GO BUCS).

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<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>APPROVAL</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>IRB ASSURANCE #M1194</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiv</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Biology and Biochemistry of IL-1</td>
<td>1</td>
</tr>
<tr>
<td>Monocyte/Macrophage Activation Leading to</td>
<td>4</td>
</tr>
<tr>
<td>Induction of IL-1</td>
<td></td>
</tr>
<tr>
<td>CD40</td>
<td>9</td>
</tr>
<tr>
<td>2. MATERIALS AND METHODS</td>
<td>13</td>
</tr>
<tr>
<td>Cell Preparation</td>
<td>13</td>
</tr>
<tr>
<td>Antibodies</td>
<td>14</td>
</tr>
<tr>
<td>T Cell Purification and Activation</td>
<td>15</td>
</tr>
<tr>
<td>T Cell Membrane Preparation</td>
<td>16</td>
</tr>
<tr>
<td>Metabolic Labeling of Monocyte Proteins,</td>
<td>17</td>
</tr>
<tr>
<td>Immunoprecipitation and SDS Electrophoresis of</td>
<td></td>
</tr>
<tr>
<td>IL-1</td>
<td></td>
</tr>
<tr>
<td>Flow Cytometric Analysis</td>
<td>18</td>
</tr>
<tr>
<td>Cell-ELISA</td>
<td>18</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>C</td>
<td>Celcius</td>
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<tr>
<td>Ca</td>
<td>Calcium</td>
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<tr>
<td>cAMP</td>
<td>cyclic Adenosine monophosphate</td>
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<td>CD4+</td>
<td>Helper T cells</td>
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<tr>
<td>CD8+</td>
<td>Cytotoxic T cells</td>
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<tr>
<td>cDNA</td>
<td>complementary Deoxyribonucleic acid</td>
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<tr>
<td>CRE</td>
<td>cAMP Responsive element</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate buffered saline</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene Glycol-bis(β-aminoethyl Ether)N,N,N',N'-Tetraacetic acid</td>
</tr>
<tr>
<td>ELAM</td>
<td>Endothelial-leukocyte adhesion molecule</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>Fab</td>
<td>Fraction antigen binding</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluoresence activated-cell sorter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>FcγR</td>
<td>Fragment, crystalline gamma receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>GM-CSF</td>
<td>Granulocyte/monocyte colony stimulating factor</td>
</tr>
<tr>
<td>gp</td>
<td>glycoprotein</td>
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<tr>
<td>h</td>
<td>hour</td>
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<tr>
<td>H-7</td>
<td>1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>I.O.D.</td>
<td>Integrated optical density</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
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<td>ICE</td>
<td>IL-1β convertase enzyme</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
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<tr>
<td>L</td>
<td>ligand</td>
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<tr>
<td>LFA</td>
<td>Lymphocyte function-associated antigen</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>M</td>
<td>Molar</td>
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<tr>
<td>Mg</td>
<td>Magnesium</td>
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<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
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</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
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<tr>
<td>mRNA</td>
<td>messenger Ribonucleic acid</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
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<tr>
<td>NF</td>
<td>Nuclear factor</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood leukocytes</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
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<tr>
<td>PKC</td>
<td>Protein kinase-C</td>
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<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
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<tr>
<td>pmoles</td>
<td>picomoles</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethlysulfonyl fluoride</td>
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<tr>
<td>PTK</td>
<td>Protein tyrosine'kinase</td>
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<td>SDS</td>
<td>Sodium dodecylsulfate</td>
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<tr>
<td>TBAM</td>
<td>T cell-B cell activation molecule</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper (CD4+ T cell)</td>
</tr>
<tr>
<td>Tm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Activated T cell membranes</td>
</tr>
<tr>
<td>Tm&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Resting T cell membranes</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>V</td>
<td>Voltage</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VLA</td>
<td>Very late activation antigen</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cell surface molecules on antigen presenting cells and the ligand counterparts on T cells</td>
</tr>
<tr>
<td>2</td>
<td>Activated T cell plasma membranes (Tm(^A)) but not resting T cell plasma membranes (Tm(^R)) induce human monocyte IL-1(\beta) synthesis</td>
</tr>
<tr>
<td>3</td>
<td>Kinetics of expression of T cell IL-1-inducing determinants</td>
</tr>
<tr>
<td>4</td>
<td>Expression of CD40 on plastic adherent human monocytes</td>
</tr>
<tr>
<td>5</td>
<td>Anti-CD40L (5c8) blockade of contact-dependent CD4(^+) induction of monocyte IL-1(\beta) synthesis</td>
</tr>
<tr>
<td>6</td>
<td>Stimulation of monocyte IL-1 synthesis with anti-CD40 antibodies</td>
</tr>
<tr>
<td>7</td>
<td>Requirement for crosslinking of monocyte CD40 for IL-1(\beta) induction</td>
</tr>
<tr>
<td>8</td>
<td>Blockade of BL-C4 induction of IL-1(\beta) with G28-5</td>
</tr>
<tr>
<td>9</td>
<td>Expression of CD69 on D1.1 cells and expression of CD40L on Hut-78 cells</td>
</tr>
<tr>
<td>10</td>
<td>Hut-78 plasma membranes in combination with D1.1 plasma membranes induce IL-1(\beta) synthesis in monocytes</td>
</tr>
<tr>
<td>11</td>
<td>Effects of protein kinase inhibitors on monocyte total protein synthesis</td>
</tr>
<tr>
<td>12</td>
<td>Effects of protein kinase inhibitors on IL-1(\beta) induction</td>
</tr>
<tr>
<td>13</td>
<td>Activation of NF-κB in THP-1 cells mediated through CD40 signaling</td>
</tr>
<tr>
<td>14</td>
<td>Responsive regions in the IL-1(\beta) gene</td>
</tr>
</tbody>
</table>
Chapter 1

INTRODUCTION

Biology and Biochemistry of IL-1

Interleukin (IL)-1 is a cytokine with numerous biological effects. When produced in limited concentrations during a normal immune response, IL-1 helps sustain the immune response by acting as a costimulus for T cell activation (Farrar et al., 1980). For instance, IL-1 induces the expression of IL-2, a T cell growth factor, in CD4+ T helper type 1 (Th1) cells. Each T cell subtype is designated by its ability to produce specific cytokines. Furthermore, IL-1 induces expression of the IL-2 receptor in all T cell subtypes, thus promoting T cell expansion. IL-1 also augments the ability of antigen presenting cells (APC) such as monocytes/macrophages, B cells, and dendritic cells, to activate T cell-dependent immune responses by up-regulating the expression of adhesion and costimulatory molecules on the APC cell surface (Dinarello, 1991). IL-1 is produced primarily by activated macrophages, however it has been shown to be synthesized by a variety of cell types including fibroblasts, keratinocytes, endothelial cells, smooth muscle cells, and synovial cells (Matsushima et al., 1985; and Dinarello, 1984).

Overproduction of IL-1, as seen during chronic inflammatory diseases as well as bacterial infection and septic shock, results in injurious effects such as tissue destruction and exacerbation of host inflammatory responses. Tissue destruction results from the induction type IV collagenase and elastase gene expression (Lomedico et al.,
IL-1 amplifies inflammatory responses through the induction of phospholipase A2 synthesis which leads to the production of lipid mediators of inflammation, leukotrienes, (Dinarello, 1991) and through induction of eicosanoid synthesis, prostaglandin E2, in synovial cells (Dayer et al., 1986). IL-1 increases the expression of adhesion molecules including intracellular adhesion molecule 1 (ICAM 1), vascular cell adhesion molecule (VCAM) and endothelial-leukocyte adhesion molecule (ELAM) on the surface of surrounding tissues including endothelial and vascular smooth muscle cells (Dinarello, 1991). These adhesion molecules bind circulating neutrophils, lymphocytes and monocytes resulting in expansion of the inflammatory response.

Other biological effects of IL-1 as reviewed by Dinarello (1991) include the induction of inflammatory cytokine genes such as Tumor necrosis factor-α (TNFα), IL-6 and IL-1 itself. IL-1 exhibits endocrine consequences including the induction of lipolysis in adipocytes, and glucocorticoids in adrenal glands, as well as increasing production of prostaglandins, and promoting effects such as somnolence, anorexia, hypoalgesia and fever in brain tissue. IL-1 also has been shown to induce kappa immunoglobulin light chain synthesis, up-regulation of the T cell growth factor (TGF)-β receptor and down-regulation of IL-1 receptor, tumor necrosis factor (TNF) receptor and epidermal growth factor receptor.

The IL-1 designation refers to two separate proteins, IL-1α and IL-1β, which are products of different genes but perform essentially the same biological functions (Lomedico et al., 1985; and Auron et al., 1984). Although IL-1α and IL-1β share
only 26% amino acid identity each binds to the same receptors (type I and type II) and with equal affinity (Dinarello, 1991). IL-1α and IL-1β are synthesized as 33kD precursors both of which are proteolytically processed to a 17kD mature form (Giri et al., 1985). Precursor IL-1α has biological activity but IL-1β requires processing to the mature form in order to bind to the IL-1 receptors (Black et al., 1988). Precursor IL-1α reportedly is processed by calcium dependent proteases such as calpain (Kobayashi et al., 1990). A specific enzyme for processing precursor IL-1β, IL-1β-converting enzyme (ICE) that's localization apparently is restricted to monocytes/macrophages, has been identified (Thornberry et al., 1992; and Kostura et al., 1989). Intracellular IL-1 is found exclusively as the precursor form (Giri et al., 1985; Suttles et al., 1990) while both forms have been detected extracellularly (Suttles et al., 1990; and personal observations). Processing therefore must occur extracellularly or at the plasma membrane. Other enzymes, though not accepted as the primary processing enzymes, also cleave both IL-1α and IL-1β to various biologically active sizes. It has been demonstrated that cathepsin-G, collagenase, and elastase are capable of processing IL-1α and IL-1β precursors (Hazuda et al., 1990), although these enzymes only may process IL-1 precursors after tissue damage.

Neither IL-1α nor IL-1β have an N-terminal or internal signal peptide sequence (Hazuda et al., 1990) which is considered necessary for secreted proteins. Furthermore, it has been reported that IL-1 is not associated with secretory vesicles or transported across the endoplasmic reticulum (Singer et al., 1988; and Suttles et al., 1990). Therefore, IL-1α and IL-1β must utilize some novel secretory mechanism. It
has been proposed that the principal mechanism of secretion is cell injury (Hogquist et al., 1991). While this is an accepted mechanism of IL-1 release, there is evidence that some type of secretory mechanism distinct from the typical signal-peptide mechanism of secretion does exist (Hazuda et al., 1988; Hazuda et al., 1990; Singer et al., 1988; Hogquist et al., 1991; and Koch et al., 1990). We have seen that ethylenediaminetetraacetic acid (EDTA) diminishes the ability of activated macrophages to secrete IL-1β, suggesting that extracellular divalent cations such as calcium and magnesium may play a role in the secretory mechanism of IL-1 (unpublished observations).

Monocyte/Macrophage Activation Leading to Induction of IL-1

Most studies of induction of IL-1 synthesis and secretion in monocytes/macrophages have used the bacterial endotoxin, lipopolysaccharide (LPS) a potent stimulator of cytokine synthesis in monocytes/macrophages. While such studies are certainly relevant to the induction of cytokines during septic conditions such as bacterial infection and septic shock, IL-1 production in rheumatoid arthritis and other chronic inflammatory autoimmune diseases is not mediated by LPS. It has been reported that there are activated CD4+ T cells in the synovial tissues of patients with rheumatoid arthritis (Jahn et al., 1987; Koch et al., 1990). T cells and monocytes/macrophages form conjugates during a normal immune response and this conjugate formation during abnormal chronic inflammatory responses may also result in the overproduction of IL-1.
During antigen presentation events, T cells interact with macrophages in an antigen dependent, major histocompatibility complex type II (MHC-II) restricted fashion with cell-contact mediated and cytokine mediated signals resulting in mutual activation of both T cells and macrophages. Studies of the interactions between T cells and macrophages have demonstrated that the cell contact-mediated contribution is critical for monocyte/macrophage activation (Stout, 1993; Stout and Suttles, 1992; and Suttles et al., 1994). It was demonstrated that the activation of macrophages to produce reactive nitrogen intermediates, nitric oxide, requires a combination of signals (Tao and Stout, 1993). These signals provided by LPS and interferon-γ (IFNγ), a cytokine produced by activated T helper type 1 (Th1) cells, lead to establishment of a TNFα autocrine loop (Stout, 1993; Stout and Suttles, 1992). It is well established that LPS induces the synthesis and secretion of TNFα, as well as other inflammatory cytokines (Sherry and Cerami, 1988). IFNγ induces upregulation of cell surface proteins including the TNFα receptor (Tsujimoto et al., 1986). Therefore, newly synthesized and secreted TNFα binds to its cell surface receptor and results in a signaling cascade that induces effector functions such as production of nitric oxide and interferon-β (IFNβ) in the macrophage (Stout and Suttles, 1992). The necessity of the TNFα autocrine loop was confirmed since macrophages activated by LPS and IFNγ in the presence of anti-TNFα antibodies did not produce nitric oxide [effector function] (Stout and Suttles, 1992; Oswald et al., 1992).

Later studies have demonstrated that activated T cells could replace the LPS signal resulting in macrophage effector function. Stout and Bottomly (1989) have
shown that macrophages are activated to cytostatic activity by cell-contact mediated interactions with Th1 cells alone and by Th2 coupled with recombinant interferon-gamma (IFNγ). Activated Th1 cells produce IFNγ whereas activated Th2 cells do not. Stout and Suttles (1993) later showed that the signals necessary to induce cytostatic activity in IFNγ primed macrophage could be delivered by cell-contact mediated interactions with murine Th2 cells. The activation signal could not be delivered by supernatants of activated T cells (cytokines) or if cell contact was prevented. However, paraformaldehyde fixed, activated T cells, either Th1 or Th2, were able to deliver the signal (Stout and Suttles, 1993). These reports indicate that a critical part of the signal for macrophage activation is transduced through cell-contact mediated events.

These studies in murine systems have established that activated T cells through cell-contact mediated events, induce early gene activation events in macrophages (TNFα production) and in the presence of recombinant cytokines such as IFNγ late gene events are induced (cytostatic activity). Several molecules have been identified on monocyte/macrophage cell surfaces which form receptor-ligand pairs with molecules on activated T cells during conjugate formation (Figure 1). These molecules include: Intracellular adhesion molecule [ICAM-1 also known as CD54] (Springer, 1990), Lymphocyte function-associated antigen [LFA-3 or CD58] (Webb et al., 1990), and Very late activation antigen [VLA-4 or CDw49d] (Springer, 1990). B7.1, B7.2/CD80 [BB1] has been detected in very low concentrations on monocytes/macrophages, but reportedly it can be up-regulated by IFNγ (Freedman et al., 1991). It has also been demonstrated that CD40 is expressed on peripheral monocytes in extremely low
Figure 1. Cell surface molecules on antigen presenting cells [APC] such as monocytes/macrophages and the ligand counterparts on T cells.

Several cell surface receptor - ligand molecules have been identified during T cell:monocyte/macrophage conjugate formation. Molecules on monocyte/macrophage cell surfaces include LFA-3, ICAM-1/ICAM-2, B7.0, B7.1, and B7.2 (though B7 is expressed in low concentrations on monocytes (APC) but upregulated by cytokines such as IFNγ), and recently CD40. The corresponding ligands on T cells include CD2, LFA-1, CD28/CTLA-4, and CD40 ligand. Potentially, any of these receptor-ligand pairs could participate in signaling events which lead to reciprocal activation of both cell types.
concentrations (Alderson et al., 1993) and recently CD40 has been reported on plastic adherent monocytes/macrophages (Wagner et al., 1994).

Monocytes do not constitutively synthesize IL-1, but as previously mentioned, synthesis of IL-1 can be induced by exogenous triggering agents such as LPS. It has been demonstrated that following LPS stimulation, IL-1 mRNA is rapidly transcribed without further protein synthesis (Fenton et al., 1987). This suggests the presence of preexisting transcription factors which become activated through a second messenger system. It has been determined that there are two responsive regions in the IL-1β gene. The first identified region was the cap site-proximal region, just upstream of the start codon (Fenton et al., 1987) and the second region which is much further upstream is the LPS/PMA responsive region (Shirakawa et al., 1993). It was demonstrated that mutations or deletions within either of these regions will prevent IL-1β transcription/translation (Shirakawa et al., 1993). Fenton et al., (1987) reported the presence of elements including NF-κB, AP-1, and NF-IL6 in the cap site-proximal responsive region (-49 to -38). The presence of several enhancer elements located in the LPS/PMA responsive region (positions -3307 to -2666), including four cAMP responsive elements (CRE), three AP-1 sites, one NF-κB site and NFκA, an element reported to be specific for the IL-1β gene, likewise have been reported (Shirakawa et al., 1993; Buras et al., 1994). It was further suggested that the NF-κA element confers tissue specificity for IL-1β (Buras et al., 1994). Once the cell receives an appropriate signal, any of the preexisting cytoplasmic transcription factors may become activated, migrate to the nucleus and interact with the appropriate
enhancer elements resulting in IL-1β induction. The activation of a particular factor(s) would depend upon the specific signaling pathway employed by the cell contact-mediated interactions between a ligand on activated T helper cells and its corresponding receptor on macrophages. Many of the previously mentioned receptor-ligand interactions could result in activation of various transcription factors.

**CD40**

A recently identified cell-contact dependent interaction that has proven to be critical for Th induced B cell activation was CD40L:CD40 (Parker, 1993). When B cell CD40 was blocked by antibody, Th dependent B cell activation was inhibited (Noelle et al., 1992). Potentially this interaction could occur between activated T cells and other APCs such as monocytes/macrophages. CD40 is a cell surface receptor which is a member of the TNFα receptor superfamily. Other members of this superfamily include, NGFR (nerve growth factor receptor), CD27 (expressed on T cells and B cells), CD30 (a T cell activation molecule), OX40 (an activated T cell-antigen found only in rats), and Fas/Apo 1 (a T cell antigen that induces apoptosis) [reviewed by Banchereau et al., 1994a]. The CD40 protein is a phosphorylated glycoprotein which has been reported thus far to be expressed on B cells, thymic epithelial cells, dendritic cells (Banchereau et al., 1994a) and very recently on human monocytes (Alderson et al., 1993; Wagner et al., 1994). In B cell studies, crosslinking of CD40 by anti-CD40 monoclonal antibodies induced proliferation of resting B cells, and antibodies in combination with costimulating agents induced full activation of B
cells (Clark and Ledbetter, 1986). Also, it has been shown that latex beads which contain covalently attached anti-CD40 monoclonal antibody (Luxembourg and Cooper, 1994), or L-cells (a fibroblast cell line), transfected with the human Fcγ receptor, type II, (FcγRII), then treated with monoclonal anti-CD40 antibodies (Larson and LeBien, 1994), induced B cells to a substantial proliferation response, further demonstrating the effect of CD40 crosslinking on B cells. The importance of the CD40 signal in B cell activation was reiterated since it was demonstrated that CD40 signals are required for effective class switching. Cytokines including IL-4, IL-5 and IL-10, in combination with an anti-CD40 monoclonal antibody or CD40L-fusion protein induced B cell antibody production and class switching (Splawski et al., 1993; Grabstein et al., 1993; and Banchereau et al., 1994b). Anti-CD40 antibodies or cytokines alone were ineffective.

Sequence analysis of the cDNA encoding human CD40 determined that the primary structure is composed of 277 amino acids with a 193 amino acid extracellular domain, a 22 amino acid transmembrane segment and a 62 amino acid intracellular tail (Stamenkovic et al., 1989). The intracellular tail reportedly has no enzymatic activity (Ren et al., 1994), therefore CD40 must associate with another protein such as a protein kinase for signal transduction. It has been demonstrated that signal transduction following CD40L or CD40 monoclonal antibody engagement of the CD40 molecule induced phosphorylation of at least four distinct intracellular substrates in nonresting B cells (Uckun et al., 1991). These authors showed that protein tyrosine kinase (PTK) inhibitors, including herbimycin A and genistein, prevented phosphorylation of
intracellular substrates in B cells following stimulation through CD40. PTK inhibitors have been shown to block B cell aggregation (Kansas and Tedder, 1991). The protein kinase-C (PKC) inhibitor, H-7, was shown to abrogate the stimulation of five serine/threonine specific protein kinases, although the role of PKC during CD40 signal transduction has not been defined (Uckun et al., 1991). Other recent studies have shown that blockade of interaction between activated T cell plasma membranes (TmA) and B cells via a CD40L monoclonal antibody prevented the phosphorylation of various PTKs (Marshall et al., 1994). Contrary to earlier reports, it was demonstrated that PKC was not involved in CD40 signaling in B cells (Marshall et al., 1994). TmA interactions with B cells did not induce rises in intracellular Ca^{2+} levels, cAMP production, or PKC activity, effects which are mediated through the PKC pathway.

The ligand for CD40, CD40L (Armitage et al., 1992) also known as gp39 (Noelle, et al., 1992), and T cell - B cell activation molecule [TBAM] (Lederman et al., 1992), has been reported to be expressed on the cell surface of activated CD4{\textsuperscript{+}}, Th0, Th1 and Th2, (Banchereau et al., 1994). CD40L has not been detected on CD8{\textsuperscript{+}} cells or on resting CD4{\textsuperscript{+}} cells but it has been reported that CD8{\textsuperscript{+}} cells activated by lectins such as phytohemagglutinin (PHA) will express some CD40L (Roy et al., 1993). The expression of CD40L is transient on activated CD4{\textsuperscript{+}} cells with maximal expression reported to be at 6-8h post anti-CD3 activation and no detectable expression was seen at 24h post anti-CD3 activation (Roy et al., 1993; Lederman et al., 1992).

This project was designed to demonstrate that the signals which induce IL-1 synthesis in monocytes/macrophages are communicated solely through cell-contact
mediated interactions of activated Th cells with resting monocytes and that
CD40:CD40L interactions are critical for the induction of IL-1. Furthermore, we
examined the signaling mechanisms involved. Potential cell surface interactions
between macrophages and T cells which were examined included LFA-3:CD2, ICAM-
1:LFA-1, and B7/BB1:CD28/CTLA-4, and CD40:CD40L. Signal transduction
mechanisms involved in IL-1 induction were explored by looking at activation of
known transcription factors, specifically Nf-κB, which are specific for elements in the
IL-1 gene, and by use of drugs which block protein tyrosine kinases and protein kinase-
C. Determination of the T cell-contact mediated interactions which are involved in the
induction of IL-1 synthesis and the subsequent signal transduction mechanisms could
lead to therapies which will better control inflammation.
Chapter 2

MATERIALS AND METHODS

Cell Preparation

Blood was collected from normal human volunteers and peripheral blood leukocytes (PBL) were isolated over diatrizoate/Ficoll gradients (Ficoll-lite, Atlanta Biologicals, Atlanta, GA). PBL's were plated at a density of 5 x 10⁶ cells per well in 24-well tissue culture plates (Falcon Primaria, Lincoln Park, NJ) in RPMI 1640 (Hyclone, Logan, UT), containing 2 x 10⁻⁵ M 2-mercaptoethanol, 100mM HEPES, and gentamicin 50μg/ml, 5% FCS (henceforth designated as R-5), 100μM EDTA. Monocytes adhered to the plastic culture plates within 45 minutes and were isolated by removal of nonadherent cells. Adherent monocytes were rinsed twice with saline and maintained overnight in R-5, 100μM EDTA. Since monocytes are so responsive to endotoxins, strict precautions were taken to ensure an endotoxin-free environment at all times. All reagents used during cell culture and monocyte labeling, including antibodies, were tested by chromogenic limulus assay (BioWhittaker, Walkersville, MD) to determine endotoxin levels. Endotoxin levels of greater than 1 ng/ml were considered unacceptable. When necessary, endotoxin was removed from reagents per manufacturer's instructions with Acticlean-Etox (Sterogene Bioseparations Inc., Arcadia, CA).
Antibodies

Anti-CD3 antibody was purified from culture supernatants of the hybridoma, OKT-3, (Van Voorhis et al., 1983) from American Type Culture Collection, ATCC, (Rockville, MD). The murine monoclonal IgM anti-human CD40, clone BL-C4, produced by Monosan, (The Netherlands), was purchased from Caltag, (San Francisco, CA). The monoclonal IgG1 anti-human CD40, G28-5, was a gift of Dr. E.A. Clark (Dept. of Microbiology, University of Washington, Seattle WA). Blockade experiments utilized the monoclonal anti-CD40L, 5c8, a gift of Dr. Seth Lederman (Columbia University, New York, NY) (Lederman et al., 1992). Anti-CD69, IgG1, monoclonal antibody was purchased from Becton Dickinson (San Jose, CA). IgM isotype controls and goat anti-mouse IgG F(ab’)_2 were obtained from Southern Biotechnology Associates, Inc., (Birmingham, AL). Horse radish peroxidase (HRP) conjugated, goat F(ab’)_2 anti-mouse IgM was purchased from Caltag Laboratories (San Francisco, CA) and HRP-conjugated rabbit F(ab’)_2 anti-mouse IgG was from Southern Biotechnology Associates, Inc. Anti-BB-1 (CD80), an IgG1 monoclonal antibody, anti-LFA-3 IgG2a monoclonal antibody, and anti-ICAM-1 IgG2b monoclonal antibody were purchased from Camflio division of Becton Dickinson (San Jose, CA).

Purification of CD4^+ cell populations and immunoprecipitation experiments employed monoclonal antibodies generated from hybridomas including anti-monocyte, 3C10 (IgG2b) (Hoffman et al., 1980), anti-B cell, LYM-1 (IgG2a), anti-NK, hNK-1 (IgM) (Abo and Balch, 1981) and anti-CD8, OKT-8 (IgG2a) (Van Voorhis et al., 1983) and anti-IL-1β (clone H6-A) (Kenney et al., 1987) all purchased from ATCC.
T Cell Purification and Activation

T cells were purified from the plastic-nonadherent cells on prewarmed (37°C) nylon-wool columns by incubating at 37°C for 45 min. T lymphocytes were eluted off the column with 25ml Dulbecco's phosphate buffered saline (DPBS) containing 2% fetal bovine serum (FBS). The nylon wool purified T cells were either incubated in R-5 (resting) or activated by plating on anti-CD3 coated plates. T cells were activated for 3-18h at 37°C during kinetic studies and for 6h for other experiments. Following incubation, activated T cells were washed from the plates with DPBS, centrifuged and resuspended in an appropriate medium, for either membrane preparation or paraformaldehyde fixation. Cells were fixed by incubation in 1.0% paraformaldehyde for 15 min at 4°C. Fixed cells were then rinsed 3 times with PBS, resuspended in PBS and incubated at 37°C for 18h prior to use.

The CD4+ population was purified by magnetic panning. Plastic nonadherent cells were incubated with monoclonal antibodies against cell surface molecules which had been generated from hybridomas (described above) including 3C10 (monocytes), LYM1 (B cells), anti-hNK1 (natural killer cells), and anti-CD8 (CD8+ lymphocytes), for 30 min on ice. The monoclonal antibodies were used as culture supernatants. Cells were then treated with iron conjugated antibodies to murine IgG and IgM from PerSeptives Diagnostics (Cambridge, MA) for 30 min with shaking at 4°C. Cells were diluted to 1 x 10⁶/ml with sterile DPBS and the CD4+ populations were removed via 27 megagauss Oerstead magnets (PerSeptives Diagnostics). A sample of the purified
population was stained with FITC conjugated anti-CD4 and analyzed by flow
cytometry. This population was typically found to be greater than 95% CD4⁺.

T Cell Membrane Preparation

Plasma membranes were prepared using a modification of the method of Noelle,
et al., (1991). Briefly, resting and activated T cells, or transformed T cell lines
including D1.1(CD40L expressing T cell line), HUT-78, or Jurkat (T cell leukemia cell
line, CD40L⁻) were resuspended in a hypotonic buffer containing 50mM Tris-HCl,
pH 7.4, 25mM KCl, 5mM MgCl₂, and proteinase inhibitors, phenyl methane sulfonyl
fluoride (PMSF) at 50μg/ml, leupeptin and pepstatin at 1μg/ml for 30 min on ice. The
cells were then Dounce-homogenized until cells were completely disrupted as
determined microscopically. Disrupted cells were then centrifuged at 500xg for 5 min
to remove nuclei, then centrifuged at 95,000xg for 30 min in a Beckman L5-65
Ultracentrifuge. Cell debris was homogenized 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
130,000xg, for 2h 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 2h at 180,000xg to pellet membranes. The membrane pellet was
resuspended in PBS and total protein was determined by a microtiter plate protocol
based on the method of Lowry, et al., (1953) or by a microtiter plate protocol of the
bicinchoninic acid (BCA) protein assay from Pierce (Rockford, IL). The BCA protein
assay was read on a Biotek Instruments microtiter plate reader at 561nm. The micro-
Lowry assay was read on a Molecular Devices VMAX plate reader at 693nm.

**Metabolic Labeling of Monocyte Proteins, Immunoprecipitation, and SDS**

**Electrophoresis of IL-1**

The induction of IL-1 in monocytes by T cells was measured by metabolically
labeling plastic adherent monocytes with 50µCi 35S-methionine in methionine deficient
RPMI 1640 from Hyclone (Logan, UT) supplemented with 5µg/ml insulin and
transferrin. After labeling, the culture medium was removed, cells were rinsed with
saline and lysed with an immunoprecipitation buffer containing 25mM Tris-HCl pH
7.4, 1% Triton X-100, 1% Deoxycholate, 0.35M NaCl, 10mM EDTA, and 50µg/ml
PMSF. Lysed cells were immediately snap-frozen on dry ice and ethanol. Thawed
samples were immunoprecipitated with rabbit anti-human interleukin-1β (hIL-1β) or
anti-human IL-1α from R&D Systems (Minneapolis, MN) as described previously
(Suttles et al., 1990). Monoclonal antibody against IL-1β, generated from the
hybridoma H-6A (Kenney et al., 1987), was used as culture supernatant fluid. The
precipitates were electrophoresed through 15% polyacrylamide gels. All gels included
14C-labeled molecular weight markers including myosin at 205 kilodaltons (kDa), β-
galactosidase at 116kDa, bovine serum albumin at 66kDa, carbonic anhydrase at 29kDa
and lysozyme at 14.3kDa, obtained from Amersham International (Amersham, UK).
The gels were prepared for fluorography by soaking in autofluor from National
Diagnostics (Atlanta, GA) for 1h. Gels were then dried, and exposed to Kodak X-omat
LS X-ray film from Eastman Kodak Co (Rochester, NY). Autoradiographs were quantified by image analysis using a Millipore BioImage image analysis system. Relative levels of IL-1 synthesis were evaluated based on the integrated optical density (IOD), reported as OD/mm² of labeled bands.

**Flow Cytometric Analysis**

For flow cytometric analysis of CD40 expression, adherent monocytes were removed from tissue culture plates by incubation in PBS, 0.02% EDTA followed by repeated pipetting. The cells were washed in PBS and resuspended in 100µl of a 1:100 dilution of the monoclonal anti-CD40, G28-5 (IgG1) and incubated 30 min at 4°C. The cells were washed, resuspended in an FITC-conjugated rabbit anti-mouse IgG F(ab')₂ and incubated for 30 min at 4°C. After a final wash in PBS the cells were analyzed on a FACS* 420 with a Spectra-Physics 164-05 argon laser lasing at 488nm, as described previously (Suttles, et al., 1986).

**Cell-ELISA**

Monocytes, selected by plastic adherence on 96 well plates, were fixed with 0.05% glutaraldehyde for 30 min. The cells were washed twice with saline, then incubated in 10% horse serum, 10µg/ml human IgG in DPBS for 10 min for blockade of nonspecific binding. The cells were again washed in saline, then incubated with primary antibody suspended in the blocking solution. After washing, the cells were incubated 30 min in a secondary antibody, horse radish peroxidase conjugated goat anti-F(ab')₂ from Southern Biotech (Birmingham, AL). Antibody binding was
visualized by addition of peroxidase substrate tetramethyl benzidine dihydrochloride from Sigma Chemical Co. (St. Louis, MO) Plates were analyzed on a Vmax model microtiter plate reader from Molecular Devices (Menlo Park, CA) at 450nm.

**Protein Kinase Inhibitors**

Inhibitors of signal transduction included herbimycin A and genistein, potent PTK inhibitors, staurosporine, an inhibitor of phospholipid/calcium dependent protein kinase activity and H-7, a protein kinase-c inhibitor. Herbimycin A was obtained from Calbiochem (La Jolla, CA), resuspended in DMSO and used at concentrations ranging from 0.01μg/ml to 1.0μg/ml. Staurosporine, Sigma Chemical Co. (St. Louis, MO), was resuspended in DMSO at 1μg/ml, and used at concentrations ranging from 1ng/ml - 100ng/ml. H-7 and an H-7 analog, 1-(5-isoquinolinylsulfonyl-3-methylpiperazine, (Sigma Chemical Co.), were resuspended in water and used at concentrations ranging from 1.0μg/ml - 10μg/ml. Genistein, (Sigma Chemical Co.), was resuspended in 100mM DMSO and used over the range of 0.1μg/ml - 10μg/ml.

**Electrophoretic Mobility Shift Assays**

THP-1, a human monocytic cell line, was cultured in RPMI, supplemented with 5% FBS and for experiments, cells were transferred to 6-well multiwell plates at a concentration of 5x10^7/ml. THP-1 or peripheral monocytes at 2-5x10^7 were left as unstimulated or stimulated with LPS at 1μg/ml, or BC-L4, anti-CD40 IgM, at 1μg/ml. Cells were collected and lysed to isolate nuclei based on the method of Buras et al., (1994). Briefly, cells were lysed in hypotonic buffer containing 10mM KCl, 0.3M
sucrose, 10mM β-glycerol phosphate, 0.2mM EGTA, 0.4% nonidet P-40, 1mM PMSF and 1μg/ml each of leupeptin and pepstatin, on ice for 30 min with intermittent gentle agitation. Following lysis, nuclei were collected by centrifugation at 11,500g for 5 min. Nuclear proteins were extracted by incubating nuclei in a buffer containing 25% glycerol, 1.5mM MgCl₂, 0.2mM EDTA, 0.5mM dithiothreitol, 1mM PMSF and 1μg/ml each of leupeptin and pepstatin, and 0.4M KCl for 30 min on ice. Extracts were centrifuged at 11,500g and supernatants were collected as nuclear extracts.

Extracts were concentrated and reconstituted to 0.1M KCl using Microcon microconcentrators from Amicon (Beverly, MA). Protein determinations were done by the BCA micro protein assay. Extracts were aliquoted and frozen at -80°C.

Binding assays were done using oligonucleotides, which included the binding elements for NF-κB from Promega Corp. (Madison, WI). NF-κB elements were end-labelled with ³²P-gamma ATP (ICN Biomedicals Inc., Irvine, CA). Nuclear extracts were incubated with gel shift binding buffer (4% glycerol, 1mM MgCl₂, 0.5mM EDTA, 0.5mM DTT, 50mM NaCl, 10mM Tris, pH 7.5, 0.05mg/ml salmon sperm DNA), and ³²P-oligonucleotides at approximately 5x10⁵ cpm. Binding reactions were done at room temperature for 30 min. Cold competition assays were done by first adding unlabelled oligonucleotides, 3.5pmoles, incubating for 20 min, then adding ³²P-labelled oligonucleotides. Following binding reactions, samples were prepared for electrophoresis by addition of 10x gel loading buffer (250mM Tris, pH 7.5, 0.2% bromphenol blue, 0.2% xylene cyanol and 40% glycerol). Samples were loaded onto 4% polyacrylamide gels which had been prerun for 1h at 100V constant voltage, and
electrophoresed at 100V constant voltage until the bromphenol blue dye front reached the bottom of the gel. Following electrophoresis, gels were dried onto Whatman filter paper and exposed to x-ray film overnight at room temperature.
Membrane Determinants on Activated, But Not on Resting T Cells Induce Resting Monocytes to Synthesize IL-1β

It has previously been demonstrated that contact-dependent signals induce macrophage effector function (Stout and Suttles, 1992; Stout and Suttles, 1993; Tao and Stout, 1993). Following this line of investigation, experiments were designed to test whether plasma membranes from activated peripheral T cells could induce IL-1 protein synthesis by monocytes obtained from the same individual, through contact-dependent interactions alone. Peripheral T cells were activated on anti-CD3 coated plates for 5 hours and plasma membranes prepared from the activated T cells (TmA) were compared to plasma membranes from resting peripheral T cells (TmR) for their ability to induce IL-1β synthesis in resting, adherent monocytes. IL-1 synthesis was monitored by metabolic labeling using 35S-labelled methionine and immunoprecipitation of monocyte cell lysates with anti-IL-1 antibodies. TmA successfully induced IL-1β synthesis (Figure 2), whereas TmR at the same concentration (25μg/ml) did not induce IL-1β synthesis above background. The ability of TmA to activate monocytes was titrated across a range of 10-25μg/ml, resulting in a dose-dependent response. These results suggest that activated T cells can provide all the necessary costimulatory molecules through cell surface interactions alone, to induce monocytes to synthesize IL-1β.
Figure 2. Activated T cell plasma membranes (TmA) but not resting T cell plasma membranes (TmR) induce human monocyte IL-1β synthesis.

Plastic adherent monocytes were incubated with either TmA or TmR for 5h in the presence of 50µCi 35S-methionine. Cell lysates were harvested, immunoprecipitated with anti-IL-1β monoclonal antibody, and electrophoresed on SDS-polyacrylamide gels. Proteins were visualized by autoradiography. The arrow indicates precursor IL-1β, 33kDa. Lane 1, unstimulated controls; Lane 2, LPS at 10µg/ml; Lane 3, TmA at 25µg/ml; and Lane 4, TmR at 25µg/ml.
Kinetics of T cell expression of monocyte activating determinants

Having demonstrated that Tm\(^A\) are capable of inducing monocytes to synthesize IL-1\(\beta\) through contact-dependent interactions alone, it was of interest to determine the expression kinetics of the T cell surface molecules which induced IL-1\(\beta\) synthesis. The kinetic studies were performed using purified CD4\(^+\) cells which were paraformaldehyde fixed post-activation. Plastic adherent monocytes were incubated with the same number of either fixed resting CD4\(^+\) cells, (0h), or CD4\(^+\) cells that had been activated on immobilized anti-CD3 for 3h, 6h, or 18h. As shown in Figure 3, CD4\(^+\) cells stimulated for 6h were most effective in the induction of monocyte IL-1\(\beta\) production. Image analysis of the 33 kDa IL-1\(\beta\) precursor bands reported an integrated optical density, (IOD) (density/mm\(^2\)) of 8.25 at 6h as compared to an IOD of 0.25 at 0h, an IOD of 3.46 at 3h, and an IOD of 2.41 at 18h. This suggested that maximal expression of the determinants on activated T cell surfaces which induce IL-1 synthesis in monocytes occurred at 6h post activation.

Expression and Upregulation of Monocyte CD40

Any of a number of activation-induced cell surface proteins could be considered as likely candidates for T cell-monocyte signaling. However, given the correlation of the expression kinetics of the IL-1 activating signal with those of expression of T cell CD40L (optimal at 6h) (Lederman et al., 1992), the possibility that CD40L may be contributing to the monocyte response was explored. First, the level of expression of CD40 on resting adherent monocytes was analyzed by flow cytometry to assess the
Figure 3. Kinetics of expression of T cell IL-1-inducing determinant(s).

Monocytes, at $5 \times 10^5$, were incubated with CD4$^+$ cells which had been left as resting (0h), or had been incubated on anti-CD3 coated plates for 3h, 6h, or 18h prior to immediate paraformaldehyde fixation. Fixed CD4$^+$ cells were added to monocytes at $4 \times 10^5$ cells/ml and incubated for 4h in the presence of $^{35}$S-methionine. Lysates were harvested, immunoprecipitated, and run on SDS polyacrylamide gels. The times designated above each lane indicate the length of time of anti-CD3 activation of the T cells. Data is representative of four experiments. The arrow indicates the 33kDa precursor, intracellular form of IL-1β.
feasibility of a potential CD40-CD40L interaction in IL-1 induction. Adherent monocytes were removed from tissue culture plates and labeled with the monoclonal anti-CD40 antibody, G28-5, followed by FITC-conjugated rabbit anti-mouse IgG F(ab')2. Flow cytometry revealed that resting plastic adherent monocytes exhibited significant expression of CD40 (mean fluorescence of 150) as compared to the IgG1 isotype control (mean fluorescence 15) (Figure 4a). Expression of CD40 could be upregulated slightly with either IFNγ or LPS, and to greater extent by treatment with the combination of both LPS and IFNγ (mean fluorescence 300). Analysis of expression using cell-ELISA demonstrated that TmA, but not TmR could also upregulate CD40 expression over 50% above the expression levels of unstimulated cells (Figure 4b). The cell-ELISA experiments confirmed that the combination of LPS and IFNγ substantially upregulated CD40 expression on adherent monocytes (Figure 4b), as initially demonstrated by flow cytometry. The analysis of CD40 expression suggested that resting adherent monocytes are capable of receiving signals from activated T cells through a CD40L-CD40 interaction, and that the contact-dependent interaction results in the upregulation of CD40 expression. Once it had been determined that interaction between CD40L on activated T cells and CD40 on monocytes was feasible, experiments were designed to block the interaction using antibodies to CD40L, and determine the result on IL-1β production in monocytes/macrophages.

**Blockade of T cell membrane signaling with anti-CD40L antibodies**

To directly address whether interaction between CD40L on activated T cells
Figure 4. Expression of CD40 on plastic adherent human monocytes.

(A). Analysis of CD40 expression by flow cytometry. Monocytes at 1x10^6/ml were left unstimulated or stimulated with IFNγ at 100U/ml, LPS at 10μg/ml, or IFNγ (100U/ml) plus LPS (10μg/ml) for 18h. Monocytes were incubated with G28-5, at 1:100 followed by an FITC-conjugated goat F(ab')2 anti-murine IgG and analyzed by flow cytometry. Isotype control labeling is seen as the dotted line. The profile of anti-CD40 labeled is represented by the solid line.

(B). Analysis of CD40 expression by cell-ELISA.
Adherent monocytes at 5x10^5 were incubated in the presence or absence of LPS + IFNγ, Tm^8 or Tm^4 labeled using cell-ELISA with anti-CD40 (G28.5) followed by horse radish peroxidase conjugated goat anti-F(ab')2.
and CD40 on monocytes induces IL-1, the ability of the monoclonal anti-CD40L (TBAM) antibody, 5c8 (Lederman et al., 1992), to interfere with the ability of Tm4 or activated, fixed CD4+ cells to induce monocyte IL-1 synthesis was tested. Activated peripheral CD4+ cells were fixed with paraformaldehyde and added to resting monocytes in the presence or absence of the 5c8 antibody. The antibody, 5c8, was titrated in these experiments over a concentration range of 1-25μg/ml. Control groups included fixed activated CD4+ cells alone, and fixed activated CD4+ cells incubated with an IgG2a isotype control. Throughout experiments, addition of 5c8 blocked signal transduction by activated CD4+ cells in a dose-dependent fashion as depicted in Figure 5. Data obtained through image analysis revealed an 85% reduction in IL-1β synthesis with the addition of 25μg/ml 5c8. A 50% reduction in signal was observed at an antibody concentration of 10μg/ml and a 30% reduction at 1μg/ml. Addition of an IgG2a isotype control at the same concentrations had no inhibitory effect on the ability of activated, fixed CD4+ cells to stimulate monocyte IL-1β synthesis. In initial studies to block the ability of activated T cells to induce monocyte IL-1 synthesis, other monoclonal antibodies were tested. Anti-ICAM-1, anti-CD80 (BB-1), and anti-LFA-3 were not effective at concentrations tested, in blocking the activated T cell signal for IL-1 synthesis. Also in separate experiments, none of these antibodies alone were agonistic towards IL-1 induction. The choice of these antibodies was based on previous demonstrations that the cell-surface molecules which these antibodies recognize are present on monocyte/macrophage cell surface, and that they may play some role in B cell signaling. These data suggest that ICAM-1, CD80 and LFA-3 interactions do not play a substantial role in the signaling of monocyte IL-1 synthesis.
Figure 5. Anti-CD40L (5c8) blockade of contact-dependent CD4+ induction of monocyte IL-1β synthesis.

Monocytes were incubated with 1 x 10^6 cells/ml anti-CD3 activated fixed CD4+ cells in the presence or absence of 5c8 at 1, 10 and 25 μg/ml. The cells were metabolically labeled for a 4 h incubation. Lane 1, monocytes incubated with fixed resting CD4+ cells alone; Lane 2, monocytes incubated with fixed activated CD4+ cells; Lane 3, monocytes incubated with fixed activated CD4+ cells plus 5c8 at 25 μg/ml; Lane 4, monocytes incubated with activated CD4+ cells plus 5c8 at 10 μg/ml; Lane 5, monocytes incubated with activated CD4+ cells plus 5c8 at 1 μg/ml. Data is representative of three experiments. Arrow indicates 33 kDa intracellular form of IL-1β.
Anti-CD40 Stimulation of Monocyte IL-1 Synthesis

In addition to the blockade studies, anti-CD40 antibodies were used to examine signals mediated through CD40 which may lead to IL-1 induction. Studies of B cell signaling have employed the IgG1 monoclonal anti-CD40 antibody, G28-5, to mimic the CD40L binding event. G28-5 has been found to stimulate B cell responses, selectively, when accompanied by costimulatory cytokines (Armitage et al., 1992, and Splawski, et al., 1993). G28-5 was not capable of stimulating monocyte IL-1 production over a broad range of concentrations, i.e. from 1μg/ml to 25μg/ml. However, the IgM monoclonal anti-CD40, BL-C4 proved to be a potent stimulus of both IL-1β and IL-1α production (Figure 6, a and b). BL-C4 was tested at concentrations ranging from 0.1μg/ml to 10μg/ml and was found to induce IL-1 synthesis in a dose dependent fashion. It was speculated that the ability of BL-C4, but not G28-5, to induce IL-1 may be due to the ability of pentameric IgM to crosslink CD40. The enhancement of B cell CD40 signaling through crosslinking has been observed previously (Clark and Ledbetter, 1986; Luxembourg and Cooper, 1994; Larson and LeBien, 1994). Experiments therefore were designed to examine the efficacy of crosslinking CD40 on monocytes. Plastic adherent monocytes were incubated with G28-5 followed by goat F(ab′)2 anti-murine IgG. Crosslinking G28-5 with goat F(ab′)2 anti-mouse IgG successfully induced IL-1β synthesis (Figure 7) whereas neither F(ab′)2 anti-mouse IgG nor G28-5, alone, had any effect. The effects of crosslinking on induction of IL-1α synthesis were not examined. These results suggest that crosslinking of CD40 receptors may be a requirement for efficient
Figure 6. Stimulation of monocyte IL-1 synthesis with anti-CD40 antibodies.

Monocytes were incubated with BL-C4 during a 4h metabolic protein labeling period. Cell lysates were harvested and immunoprecipitated with anti-IL-1β (panel A) or anti-IL-1α (panel B). The arrows identify the IL-1β and IL-1α 33kDa precursor proteins. Panel A, induction of IL-1β synthesis: Lane 1, unstimulated monocytes; Lane 2, LPS at 10μg/ml; Lane 3, IgM isotype control at 1μg/ml; and Lane 4, BL-C4 at 1μg/ml. Panel B, induction of IL-1α: Lane 1, unstimulated monocytes; Lane 2, LPS at 10μg/ml; Lane 3, IgM isotype control at 1μg/ml; and Lane 4, BL-C4 at 1μg/ml. Bands seen below the arrow are 25kDa processing intermediates of IL-1β which has been reported previously (Suttles et al., 1990).
Figure 7. Requirement for crosslinking of monocyte CD40 for IL-1β induction.

Monocytes were incubated with G28-5 at 25μg/ml for 20 min. An F(ab')₂ antимurine IgG was added at 15μg/ml during a 4h metabolic protein labeling incubation. Cell lysates were harvested and immunoprecipitated with anti-IL-1β. Lane 1, G28-5, alone, at 25μg/ml; Lane 2 F(ab')₂, alone, at 15μg/ml; Lane 3, G28-5, 25μg/ml followed by F(ab')₂ 15μg/ml. Arrow indicates the 33kDa precursor form of IL-1β.
signaling in monocytes, as has been demonstrated in studies of B cell CD40 signaling. The specificity of effect of BL-C4 was evaluated by blockade experiments using G28-5. Since the G28-5 antibody bound to CD40 on monocytes, but did not initiate IL-1β synthesis, the ability of G28-5 to inhibit BL-C4 activation was examined. Plastic adherent monocytes were pre-incubated with G28-5 prior to BL-C4 stimulation. Pre-incubation of the monocytes with 10μg/ml G28-5 inhibited BL-C4 stimulus of IL-1β production by 77% as determined by image analysis of the autoradiograph (Figure 8).

Requirement of Costimulatory Signals In Addition to CD40

Experiments using TmA to activate resting, plastic adherent monocytes clearly demonstrated that all the signaling requirements for the induction of monocyte IL-1 synthesis are found on the activated T cell surface as demonstrated in Figure 1. As another means of examining the contribution of CD40L towards the induction of IL-1 in monocytes/macrophages, experiments were designed to test whether a human T cell leukemia line, D1.1, which constitutively expresses CD40L (Yellin et al., 1991), or a cell line transfected with a CD40L cDNA, 293/CD40L+, could induce IL-1 synthesis through cognitive signals. Plasma membranes were generated from D1.1, 293/CD40L+ and from a control parent line, Jurkat B2.7, which does not express CD40L (Yellin et al., 1991). D1.1 or 293/CD40L+, or Jurkatt plasma membranes were incubated with monocytes in metabolic labeling experiments at amounts equivalent to TmA that induced substantial IL-1 synthesis (25μg/ml), as demonstrated in Figure 1. However, de novo synthesis of IL-1 was not detected. This result was
Figure 8. Blockade of BL-C4 induction of IL-1β with G28-5.

Monocytes were stimulated with BL-C4 either alone, or after a 20 min pretreatment with G28-5, for a 4h labeling incubation. Cell lysates were immunoprecipitated with anti-IL-1β. Lane 1, G28-5, alone, at 25μg/ml; Lane 2, BL-C4, alone at 1μg/ml; Lane 3, G28-5 at 25μg/ml (pre-incubated 20 min) plus BL-C4 at 1μg/ml. Arrow indicates the 33kDa precursor form of IL-1β.
surprising since experiments demonstrated that direct blockade of CD40L:CD40 strongly diminished TmA's ability to induce IL-1β in monocytes, and experiments utilizing monoclonal IgM anti-CD40 antibodies were successful at inducing IL-1 synthesis. Combinations of membranes including D1.1 with B2.7, and D1.1 with TmR also did not induce IL-1 synthesis. These findings suggest that a costimulatory molecule in addition to CD40 on monocytes is required for cell-contact mediated induction of IL-1 and that the required costimulatory molecule is unique to the activated T cell surface.

It has been demonstrated that a T cell line, HUT-78, once activated, can induce IL-1 synthesis in THP-1, a monocytic cell line, through cell-contact dependent signals (Dayer et al., 1993). These authors demonstrated that an anti-CD69 monoclonal antibody was able to partially block the signal. It is possible therefore, that CD69 may provide the costimulus required for induction of IL-1 synthesis in monocytes. Experiments were designed to test this hypothesis. It was determined by flow cytometry analysis that D1.1 cells do not express CD69 (Figure 9), and that unactivated HUT-78 express only low levels of CD40L (Figure 9). Therefore monocytes were metabolically labeled in the presence of D1.1(CD40L+, CD69+) and HUT-78(CD69+, CD40Lb) plasma membranes. As controls, monocytes were incubated with either D1.1 or HUT-78 membranes alone. The combination of plasma membranes was synergistic towards induction of IL-1β synthesis in monocytes (Figure 10). Image analysis revealed that the combination of membranes (I.O.D. = 6.52) was 20 times more effective at inducing IL-1β synthesis than the HUT-78 (I.O.D. = 0.322)
Figure 9. Expression of CD69 on D1.1 cells and expression of CD40L on HUT-78 cells.

D1.1 cells, 1x10⁶/ml, were treated with an IgG anti-CD69 monoclonal antibody. Cells were then stained with a FITC-conjugated goat F(ab')₂ anti-murine IgG. Flow cytometry was performed as described in Materials and Methods. A negative control, second antibody alone, was included (dotted lines). The profile of anti-CD69 stained cells is indicated by solid lines.

HUT-78 cells, 1x10⁶, were treated with the anti-CD40L monoclonal antibody, 5c8, and stained with a FITC-conjugated goat F(ab')₂ anti-murine IgG. Flow cytometry was performed as described in Materials and Methods. Negative controls (dotted lines) were second antibody alone. The profile of CD40L expression is seen as solid lines.
Figure 10. HUT-78 plasma membranes in combination with D1.1 plasma membranes induce IL-1β synthesis in monocytes.

Monocytes were plastic adhered and incubated with plasma membranes from HUT-78 (CD40L⁻, CD69⁺), D1.1 (CD40L⁺, CD69⁻) or a combination of the two membranes. Following metabolic protein labeling with ³⁵S-Methionine, cell lysates were immunoprecipitated as described in Materials and Methods. Lane 1, anti-CD40 at 1µg/ml; lane 2, D1.1 plasma membranes at 15µg/ml; lane 3, D1.1 (15µg/ml) + HUT-78 (15µg/ml) membranes; lane 4, HUT-78 plasma membranes at 15µg/ml. Arrow indicates 33kDa precursor form of IL-1β.
membranes alone and 100 times more effective than D1.1 (I.O.D. = 0.065) alone.

Low level induction by the HUT-78 may be due to the low levels of CD40L on the cell surface. This data suggests that CD69 may indeed play a contributory role in the T cell contact-mediated induction of monocyte IL-1β synthesis.

Effects of Protein Tyrosine Kinase Inhibitors and Protein Kinase-C Inhibitors on Monocyte IL-1β Production

To evaluate the signal transduction events induced in monocytes through CD40 signaling, monocytes were pretreated with a variety of known signal transduction inhibitors prior to stimulation with the BL-C4, the IgM anti-CD40 antibody. Inhibitors of protein tyrosine kinase (PTK) regulated pathways and protein-kinase C (PKC) regulated pathways were tested at a variety of concentrations. Herbimycin-A has been described as a potent inhibitor of PTKs (Uckun et al., 1991; Weinstein et al., 1992; and Deans et al., 1993). While several PTKs have been identified from a variety of cell types, the specificity of herbimycin-A beyond that of a general PTK inhibitor has not been elucidated. The effects of H-7 and staurosporine, PKC pathway inhibitors (Uckun et al., 1991), were also examined. In initial experiments, the effects of various concentrations of the inhibitors on total cellular protein concentration and the effects of the inhibitors on preventing de novo protein synthesis were tested. At all concentrations tested there was no difference between total cellular protein levels as determined by BCA protein analysis (Table 1). There were, however, substantial effects on de novo protein synthesis (Figure 11). For example herbimycin-A at 1μg/ml
TABLE 1

The Effects of Protein Kinase Inhibitors on Monocyte Total Cellular Protein

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BCA Protein in µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>7.86</td>
</tr>
<tr>
<td>LPS</td>
<td>7.58</td>
</tr>
<tr>
<td>anti-CD40</td>
<td>8.08</td>
</tr>
</tbody>
</table>

**PKC Inhibitors:**

- Staurosporine 100ng/ml 7.36
- Staurosporine 10ng/ml 7.47
- Staurosporine 1ng/ml 7.64
- H-7 10µg/ml 7.03
- H-7 (3Me) 10µg/ml 7.42

**PTK Inhibitor:**

- Herbimycin-A 1µg/ml 7.64
- Herbimycin-A 0.1µg/ml 8.02
- Herbimycin-A 0.01µg/ml 8.13

Mean = 7.664
s.d. = 0.324

Table 1. Cells were plated at 5x10⁶ /ml and pretreated with herbimycin-A, staurosporine, H-7, or the H-7 analog, H-7(3Me) for 18h. Protein analysis was done on total cell lysates using a BCA-protein assay kit as described in Materials and Methods.
Figure 11. Effects of protein kinase inhibitors on monocyte total de novo protein synthesis.

Monocytes were preincubated with herbimycin-A, staurosporine, H-7 or an H-7 analog, H-7(3-Me), for 18h. Cells were then stimulated with anti-CD40 IgM at 1μg/ml during 35S-methionine metabolic labeling experiments for 4h. Samples of total cell protein were electrophoresed through SDS-polyacrylamide gels and visualized by autoradiography. Lane 1, untreated; lane 2, LPS at 10μg/ml; lane 3, anti-CD40 (1μg/ml) alone; lane 4, staurosporine at 100ng/ml; lane 5, staurosporine at 10ng/ml; lane 6, staurosporine at 1ng/ml; lane 7, H-7 at 10μg/ml; lane 8, H-7 at 1μg/ml; lane 9, H-7(3Me) at 10μg/ml; lane 10, herbimycin-A at 1μg/ml; lane 11, herbimycin-A at 0.1μg/ml; lane 12, herbimycin-A at 0.01μg/ml.
Figure 12. Effects of protein kinase inhibitors on IL-1β induction.

Monocytes were preincubated with herbimycin-A, staurosporine, H-7 or an H-7 analog, H-7[3-Me], for 18h. Cells were then stimulated with anti-CD40 IgM at 1µg/ml during 35S-methionine metabolic labeling experiments for 4h. Cell lysates were immunoprecipitated with the monoclonal anti-IL-1β antibody. Lane 1, untreated; lane 2, anti-CD40 at 1µg/ml; lane 3, herbimycin-A at 0.1µg/ml; lane 4, H-7 at 1µg/ml; lane 5, staurosporine at 10ng/ml. Arrow indicates 33kDa precursor form of IL-1β.
prevented total *de novo* protein synthesis but at 0.1μg/ml, herbimycin-A had no appreciable effect on synthesis (Figure 11). But at 0.1μg/ml, herbimycin-A substantially reduced IL-1β production (Figure 12). At concentrations which did not abolish total *de novo* protein synthesis, H-7 had very little effect on IL-1β synthesis (Figure 12). Staurosporine, likewise did not effect IL-1 synthesis at concentrations which did not abolish total protein synthesis. In fact, both H-7 and staurosporine apparently had activating effects on anti-CD40 IgM induced IL-1β production (Figure 12). It has been reported that PKC inhibitors enhance PTK activity (Mufson et al., 1992; and Shapira et al., 1993). These data suggest that signaling through CD40 may primarily utilize a protein tyrosine kinase regulated pathway and that PKC pathways apparently are not involved in signaling through CD40. It has been reported in B cell systems that plasma membranes from activated T cells induced PTK activities but did not increase cAMP, calcium, or PKC activity (Marshall et al., 1994). These authors also reported that an anti-CD40L monoclonal antibody, 5c8, could reduce PTK induction in B cells. The data presented here correlate with the finding that B cell signaling through CD40 induces PTKs but not PKC activity.

**Transcription Factors Activated Through CD40 Signaling**

In addition to the signal transduction information gained by using inhibitors to intracellular pathways, experiments were done to determine if the transcription factor, NF-κB would be activated through CD40 signaling. It has been demonstrated that NF-κB is activated in monocytes through LPS signaling (Drouet et al., 1991). For initial experiments, THP-1, a premonocytic cell line was used. It was determined by flow
cytometry that THP-1 cells constitutively express CD40, and can be induced to synthesize IL-1β by treatment with IgM anti-CD40 (BL-C4). Therefore, THP-1 were stimulated with BL-C4 for 45 min and nuclear extracts were prepared. Electrophoretic mobility shift assays (EMSA) demonstrated that CD40 signaling induced activation of NF-κB or an NF-κB like protein (Figure 13). Competition assays using unlabeled DNA oligonucleotides which bind NF-κB, were performed to verify that the bands indicated were NF-κB. Two band shifts were identified (Figure 13). NF-κB is composed of two protein subunits, p65 and p50, which form both heterodimers and homodimers (Moore et al., 1993). Moore et al (1993) reported that the heterodimer is the predominant form of NF-κB and the p65 subunit constitutes the primary homodimeric form. The two band shifts observed in Figure 13 may correspond to the homodimer and heterodimer forms but supershift assays using antibodies specific for p65 and p50 would be required to make such a determination. It was apparent that THP-1 constitutively activate 1 form of NF-κB (Lane 1, Figure 13), which is consistent with reports in B cell studies (Lalmanach-Girard et al., 1994). It has been reported that NF-κB binding elements are present in the IL-1β gene enhancer region (Fenton et al., 1987; Zhang and Rom, 1993; Hunninghake et al., 1992). These data suggest that signaling through CD40 activates NF-κB in the premonocytic cell line, THP-1, which appears to be a suitable model for studying activation of transcription factors through CD40 signaling.

**Effects of CD40 Signaling on The Expression of Monocyte Cell Surface Molecules**

Several costimulatory/adhesion molecules including ICAM-1/ICAM-2 (Springer,
Figure 13. Activation of NF-κB in THP-1 cells through CD40 signaling.

THP-1, a premonocytic cell line, were plated at 2.5x10^7 cells and treated with anti-CD40 IgM at 1μg/ml. Nuclear extracts were prepared and incubated with ^32P-labeled NF-κB binding oligonucleotides. Samples were electrophoresed through 4% polyacrylamide gels to generate electrophoretic mobility shift assays. Lane 1, untreated cells; lane 2, LPS at 10μg/ml; lane 3, LPS + unlabelled NF-κB in molar excess, 1.75pmoles (cold competition for LPS activation); lane 4, IgM anti-CD40; lane 5, IgM anti-CD40 + unlabelled NF-κB molar excess (cold competition for anti-CD40 activation). Arrows indicate band shifts.
1990), LFA-3 (Webb et al., 1990), B-7 (Freedman et al., 1991) and recently CD40 (Alderson et al., 1993, and Wagner et al., 1994) have been demonstrated on the cell surface of monocytes/macrophages. Experiments were designed to determine how T cell contact-dependent signals can regulate the expression of these cell surface molecules. Peripheral monocytes were plated on 96-well plates and stimulated with paraformaldehyde fixed activated T cells or paraformaldehyde fixed resting T cells, LPS, IFNγ, or with anti-CD40 IgM. Fixed, activated T cells and anti-CD40 IgM substantially upregulated the expression of ICAM-1, whereas resting T cells had a slight upregulatory effect as demonstrated by cell-ELISA assays (Table 2). This suggests that anti-CD40 signals, and signals provided through contact-dependent interactions with activated T cells upregulates ICAM-1. LFA-3 expression was not affected by anti-CD40, compared to isotype controls, but was upregulated by fixed, activated T cells (Table 2), while fixed resting T cells had no appreciable effect (Table 2). It has recently been reported that stimulation through CD40 upregulates the expression of CD80 (BB-1) on B cells (Yellin et al., 1994). The expression of CD80 (BB-1) was not altered by anti-CD40 stimulation or by fixed resting T cells compared to isotype controls. CD80 was upregulated two-fold by fixed, activated T cells.

These data suggest that contact-dependent interactions between adherent monocytes and activated T cells lead to up regulation of monocytic cell surface molecules including ICAM-1, LFA-3 and CD80 (BB-1). Additionally, it appears that CD40 signaling substantially upregulated the expression of ICAM-1. Unlike reports in B cells (Yellin et al., 1994), CD40 signaling did not upregulate the expression of CD80 (BB-1) in these experiments.
### TABLE 2

Regulation of Expression of Cell Surface Molecules on Adherent Monocytes

<table>
<thead>
<tr>
<th>Cell Surface Molecule</th>
<th>unstimulated</th>
<th>LPS</th>
<th>IFNγ</th>
<th>TR</th>
<th>TA</th>
<th>α-CD40</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD80</td>
<td>7.2±1.5*</td>
<td>7.2±0.6</td>
<td>7.2±0.5</td>
<td>7.8±0.7</td>
<td>16.0±1.9</td>
<td>9.7±0.9</td>
<td>9.1±1.0</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>17.3±2.3</td>
<td>n.d.</td>
<td>n.d.</td>
<td>23.0±0.2</td>
<td>57.3±2.2</td>
<td>44.1±4.0</td>
<td>9.8±1.0</td>
</tr>
<tr>
<td>LFA-3</td>
<td>9.5±1.6</td>
<td>11.1±0.3</td>
<td>12.4±1.3</td>
<td>10.1±0.7</td>
<td>17.4±2.6</td>
<td>10.9±0.7</td>
<td>9.1±1.0</td>
</tr>
</tbody>
</table>

*Values reported are arithmetic means ± standard errors x 10⁻² of optical density units and representative of three experiments.

Table 2. Peripheral blood mononuclear cells were plated at 5 x 10⁶ cells/well. Adherent cells were stained with the appropriate antibody at 1:100 (10μg/ml). Cells were rinsed to remove unbound antibody, and incubated with an HRP-conjugated second antibody [HRP-(Fab')₃]. TR were activated for 6h. Cell-ELISAs were performed as described in Materials and Methods. Optical densities were determined using a Vmax plate reader. n.d. = not done.
Chapter 4
DISCUSSION

The induction of a successful immune response is dependent upon the formation of physical conjugates between T helper (Th) cells and antigen presenting cells (APCs). The intent of this study was to define interactions during physical conjugate formation between monocytes and T cells, which result in activation of monocyte/macrophage IL-1 production. The initial interaction between T cells and monocytes/macrophages is antigen-dependent, and MHC-II restricted. Signals, both cell-contact mediated and cytokine mediated lead to reciprocal activation. Previous studies have demonstrated that paraformaldehyde fixed, activated T cells in the presence of IFNγ can induce nitric oxide and IFNβ production in monocytes/macrophages (Tao and Stout, 1993). These effector functions of monocytes/macrophages require a TNFα autocrine loop, and it has been demonstrated that activated T cells will induce transcription of TNFα message as well as protein synthesis and secretion (Stout, 1993; Stout and Suttles, 1992; and Suttles et al., 1994). Since fixed, activated T cells transduced the appropriate signals, cell-contact mediated signals alone were sufficient to induce TNFα message/protein production in monocytes/macrophages. This study has extended these earlier studies by identifying molecules involved in the contact-dependent T helper cell:monocyte/macrophage signal. Purified plasma membranes from activated, but not resting CD4+ T cells, or fixed activated cells, were capable of inducing monocyte IL-1 synthesis. The activating determinant(s) on the T cells are expressed optimally at 6h
post anti-CD3 activation, a kinetic profile which matches that of the expression of the CD40 ligand (Lederman et al., 1992). These data, and the observed expression of CD40 on adherent resting monocytes provided circumstantial evidence implicating CD40 as a candidate for the T cell-mediated IL-1 signaling event. The demonstration that 5c8, a monoclonal anti-CD40L (T-BAM) can effectively block the ability of fixed activated CD4+ cells to induce monocyte IL-1 synthesis (Figure 5) provided definitive evidence of the role of CD40-CD40L signaling in T cell-monocyte interactions.

The CD40-CD40L interaction has been studied extensively in the context of T cell-B cell interactions (Noelle et al., 1992; Splawski et al., 1993; and Grabstein et al., 1993). As mentioned previously, B cell stimulation has been achieved using anti-CD40 monoclonal antibodies, or a CD40 ligand-Fc fusion protein, in the presence of costimulus (i.e., cytokines, anti-CD20 or anti-Ig) (Splawski et al., 1993; and Grabstein et al., 1993). B cell proliferation can be promoted by crosslinking of CD40 using the G28-5 monoclonal antibody (Clark and Ledbetter, 1986) or by crosslinking with anti-CD40 covalently attached to latex beads (Luxembourg and Cooper, 1994). Crosslinking appears to be a requirement for CD40 signaling of monocyte IL-1 synthesis, as well. IgG1 anti-CD40 (G28-5) was ineffective, whereas an IgM anti-CD40 (BL-C4), was proficient in the induction of IL-1. Crosslinking of G28-5 with the addition of an F(ab')2 allowed for the IL-1 signaling event to occur (Figure 5). The success of the BL-C4 IgM anti-CD40 in signaling may be due to the crosslinking ability of pentameric IgM or due to differences in the epitope to which BL-C4 binds. It has recently been suggested that two CD40 epitopes may exist in murine systems (Heath et al., 1994).
was shown that two different monoclonal antibodies generated against murine B cell CD40 differed in their ability to activate the B cells and that the two antibodies could not cross block one another. Human monocytes and B cells, may also have two CD40 epitopes. It is possible that the differences between G28-5 and BC-L4 in their ability to activate monocyte IL-1 synthesis may be due to their epitope specificity. The specificity of the BL-C4 generated signal was assured by the ability of the nonstimulatory G28-5 to block activation initiated by BL-C4. The ability of G28-5 to block BL-C4 activity does not preclude the existence of two CD40 epitopes. If the epitopes are in close association, G28-5 may result in stearic hinderance, partially blocking BL-C4's ability to bind to its epitope.

In a previous report, CD40 ligand-transfected cells were found to induce monocyte IL-6 and TNFα production, but only in the presence of costimulatory cytokines (GM-CSF, IL-3, or IFNγ) (Alderson et al., 1993). When membranes prepared from CD40L (T-BAM) transfectants, or from the Jurkat variant, D1.1, which express high levels of CD40L (T-BAM) (Lederman et al., 1992) were used in monocyte metabolic labeling experiments, they were not effective by themselves in inducing IL-1 (data not shown). Since TmA, alone, induced substantial amounts of IL-1, it is likely that T cell specific determinants in addition to CD40L are required for activation, and that these additional signals are mimicked by crosslinking CD40 molecules on monocyte cell surfaces as demonstrated by crosslinking of the IgG anti-CD40 and suggested by the activity of BL-C4. Other studies using adherent antibodies have implicated costimulatory molecules such as LFA-3:CD2. In the current study,
blockade of CD2:LFA-3, ICAM-1:LFA-1, and CD80:CD28 interactions did not affect activated T cells' ability to induce IL-1 synthesis in adherent monocytes. Although studies using antibody activation of monocyte receptors are enlightening, specific antibody blockade of signals generated by fixed T cells and T cell plasma membranes may best reveal the extent to which potential receptor-ligand pairs are contributing to functional intercellular signaling.

Preliminary experiments presented here (Figure 10), as well as a report from another laboratory (Dayer et al., 1993), suggest that the additional costimulatory molecule on activated T cells required for induction of monocyte IL-1 synthesis may be CD69. Experiments herein demonstrated that plasma membranes from D1.1 (CD40L+, CD69+) incubated with plasma membranes from HUT-78 (CD40L−, CD69+) induced IL-1 synthesis. The effects of the combined membranes were synergistic. The ligand for CD69 has not been identified so its presence on monocyte cell surfaces has yet to be determined.

Ultimately, the cell surface contact-mediated interactions result in activation of signaling pathways which induce IL-1 synthesis. It has been suggested that the CD40 signaling pathway in B cells employs a PTK pathway including member kinases such as p56 lyn (Ren et al., 1994). Results in the current study suggested that anti-CD40 signaling in monocytes also utilizes a PTK mediated pathway. Experiments demonstrated that herbimycin A, a potent PTK inhibitor, blocked BL-C4's ability to induce IL-1β in monocytes. H-7 and staurosporine, inhibitors of PKC pathways, did not block anti-CD40 mediated IL-1 induction suggesting that PKC mediated pathways
are not employed by anti-CD40 signaling. All inhibitors were used at concentrations that did not abrogate total protein synthesis as shown in Figure 11. These findings are similar to those reported in B cell studies which suggest that PTK but not PKC pathways are activated in T cell:B cell CD40 interactions (Marshall et al., 1994).

CD40 has been described as a member of the TNFα receptor superfamily (Banchereau et al., 1994a). Members of this superfamily are so designated by homologies within the extracellular domains. However, the cytoplasmic domains are quite varied. This suggests that a variety of signaling pathways may be employed by the various receptors. One such signaling pathway which has been implicated in signal transduction through the TNFα receptor is the sphingomyelin pathway (Kolesnick and Golde, 1994; and Yang et al., 1993). Sphingomyelin is cleaved by sphingomyelinase to generate ceramide and phosphocholine. Ceramide in turn activates a PTK cascade which results in activation of Raf leading to activation of NF-κB, a cytoplasmic nuclear transcription factor (Yang et al., 1993). While it has not been demonstrated directly, this pathway would be consistent with the results reported here showing that anti-CD40 signaling in monocytes leads to NF-κB activation (Figure 12) and that anti-CD40 signaling activates a PTK regulated pathway (Figure 13). It has been demonstrated that antibody blockade of the CD40:CD40L interaction between T cells and B cells prevents activation of NF-κB (Calmanach-Girard et al., 1994). As stated earlier, an NF-κB enhancer element is located in the human IL-1β gene, however the contribution of NF-κB binding within the IL-1β gene has not been determined.

It has been reported for LPS induction of monocyte IL-1 transcription/translation
that at least two transcription factors are required, NF-κA (Shirakawa et al., 1993; )
and NF-IL6 (Buras et al., 1994). It is not known yet if both/either of these
transcription factors are required for Tm\(^A\) induction of IL-1. Furthermore, the entire
complement of transcription factors necessary and sufficient for IL-1 induction is not
known. Since CD40L:CD40 interaction alone is not sufficient for IL-1 induction, as
demonstrated by the failure of D1.1 (CD40L\(^+\)) plasma membranes to induce IL-1, it is
likely that the combination of signals provided by both CD40 signaling and the other
required cell contact-mediated signal (potentially provided by CD69) results in
activation of the IL-1 transcription factors.

Two regulatory sequences have been identified in the IL-1β gene which are
reported to be required for IL-1 induction, and mutations or deletions within either of
these regions prevented IL-1 transcription (Shirakawa et al., 1993; and Buras et al.,
1994). The cap site-proximal region, lies just upstream of the start codon and includes
the TATA box as well as several transcription factor elements including binding sites
for NF-κB, NF-IL6 and NF-κA. The LPS/PMA responsive region, is much further
upstream and includes many of the same transcription factor elements (Figure 14).
Future work includes determining the transcription factors required for IL-1 induction
that are activated through anti-CD40 signaling. In addition, it will be necessary to
determine which transcription factors are activated through the secondary cell contact-
mediated costimulus (likely CD69L) signaling. Additional aspects of this study
determined the effects of either Tm\(^A\) or anti-CD40 on the expression of
monocyte/macrophage cell surface molecules such as LFA-3, ICAM-1, CD80 (BB-1)
Responsive regions have been identified in the IL-1β gene which have been demonstrated to be essential for IL-1 induction (Fenton et al., 1987; and Shirakawa et al., 1993). These regions include the cap site-proximal region which is just upstream of the start codon, and the LPS/PMA responsive region which is further upstream.

Several enhancer binding elements, including NF-κB, NF-IL-6, and NF-βA, have been mapped in these regions. This cartoon is a representation of the map of the IL-1β gene enhancer regions.
and CD40. ICAM-1 is thought to participate primarily as an adhesion molecule with little if any costimulatory activity (Springer, 1990). The effects of anti-CD40 and TmA to increase cell surface expression of ICAM-1 may be relevant to maintenance of cell-cell interactions. Upregulation of ICAM-1 would provide a cellular "glue" to allow receptor - ligand pairs that lead to signaling events to maintain contact for longer periods of time. For instance, CD40L is transiently expressed on activated T cells (Lederman et al., 1992) and ICAM-1 binding to its ligand, LFA-1, could facilitate interactions of CD40L with CD40 on monocytes by maintaining conjugate formation between the two cell types. TmA upregulation of LFA-3 and CD40 expression on monocytes may be indicative of interactions which facilitate expression of signal transducing molecules. Increased cell surface expression of these signaling molecules would lead to an increased likelihood of receptor - ligand encounters and therefore increased likelihood of a successful signaling event. The interpretation of TmA upregulation of CD80 is unclear since a functional role of CD80 in monocyte (APC) signaling has not been determined. Interestingly, LFA-3 expression was not affected by anti-CD40, suggesting that cell surface molecules other than CD40L on activated T cells are required to upregulate LFA-3 expression. It was determined in this study however that LFA-3 - CD2 interactions had little effect on activated T cells ability to induce IL-1 synthesis in monocytes.

In summary we have demonstrated that CD40L on activated T cells interacting with CD40 on adherent monocytes is critical for the induction of IL-1 synthesis in monocytes. The finding by Durie et al. (1993) that point mutations in the CD40L result
in X-linked hyper IgM syndrome, a disease that diminishes humoral immunity since B cells cannot switch from the IgM antibody class, emphasizes the crucial contribution of CD40-CD40L signaling in T cell activation of B cell function. Since patients with this deficiency do not appear to have overt defects in cellular immunity, it has been suggested that a functional CD40L is essential only to antibody-mediated immunity. However, the ability of 5c8 to block the T cell contact-dependent signal suggests that the CD40-CD40L interactions may make a significant contribution to the induction of inflammatory cytokines. The kinetics of expression of CD40L with activation suggests that after an antigen-specific activation event, CD4+ T cells have a time-limited capacity to activate IL-1 production in resting monocytes through cell contact, hence perpetuating inflammatory responses in the absence of further antigenic stimulus.

The effects of blocking the CD40L:CD40 interaction after the induction of arthritis in mice has been demonstrated. Durie et al., (1993) showed if mice are treated with an anti-CD40L antibody during the induction of arthritis, the arthritic symptoms are ablated. They reported that joints did not become inflamed, there was no demonstration of infiltration of inflammatory cells into subsynovial tissue, and no evidence of bone and cartilage destruction. These authors assert that the prevention of these typical arthritic effects arises from prevention of antibody production via T cell:B cell interactions. However, all of these deleterious effects in the arthritis model are also mediated through IL-1 (Banchereau et al., 1994a). The current study has demonstrated that through CD40L:CD40 interactions between T cells and macrophages, IL-1 is induced in monocytes. The prevention of the tissue damage seen
in anti-CD40L treated arthritic mice may result from blockade of activated T cell's ability to induce IL-1 in monocytes.
BIBLIOGRAPHY


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