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
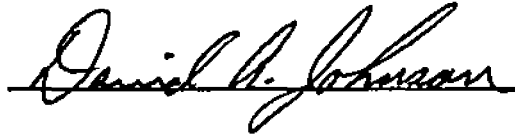
David H. Wagner, Jr.

met on the

_4th_ day of _November_, 1994
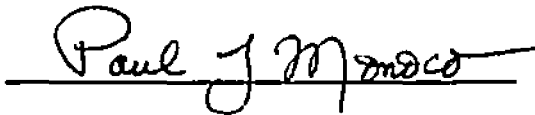
      The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council and the Associate Vice-President for Research and Dean of the Graduate School, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences.

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Chair, Graduate Committee

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Signed on behalf of
the Graduate Council

Associate Vice-President for
Research and Dean, School of
Graduate Studies

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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 Lipolpoysaccharide (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 (Tm^A) but not from resting CD4$^+$ cells (Tm^R) 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. Tm^A but not Tm^R 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 Tm^A 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.

EAST TENNESSEE STATE UNIVERSITY
IRB ASSURANCE #M1194

CERTIFICATE OF SPECIAL ASSURANCE

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 DeLucia, 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.

August 9, 1994
DATE

Dr. Anthony DeLucia, Chairman

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Principal Investigator: Jill Suttles, 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 Suttles, 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 Suttles 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

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LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
C	Celcius
Ca	Calcium
cAMP	cyclic Adenosine monophosphate
$CD4^+$	Helper T cells
$CD8^+$	Cytotoxic T cells
cDNA	complementary Deoxyribonucleic acid
CRE	cAMP Responsive element
DMSO	Dimethylsulfoxide
DPBS	Dulbecco's phosphate buffered saline
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene Glycol-bis(β-aminoethyl Ether)N,N,N',N'-Tetraacetic acid
ELAM	Endothelial-leukocyte adhesion molecule
ELISA	Enzyme linked immunosorbant assay
EMSA	Electrophoretic mobility shift assay
Fab	Fraction antigen binding
FACS	Fluoresence activated-cell sorter

FcγR	Fragment, crystalline gamma receptor
FCS	Fetal Calf Serum
FITC	Fluorescine isothiocyanate
GM-CSF	Granulocyte/monocyte colony stimulating factor
gp	glycoprotein
h	hour
H-7	1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine
HRP	Horse radish peroxidase
I.O.D.	Integrated optical density
ICAM-1	Intracellular adhesion molecule-1
ICE	IL-1β convertase enzyme
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
KCl	Potassium chloride
kDa	Kilodalton
L	ligand
LFA	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
M	Molar
Mg	Magnesium
MgCl₂	magnesium chloride

MHC	Major histocompatibility complex
μg	Microgram
ml	Milliliter
mRNA	messenger Ribonucleic acid
NaCl	sodium chloride
NF	Nuclear factor
ng	Nanogram
NK	Natural killer cells
nm	nanometer
PBL	Peripheral blood leukocytes
PHA	Phytohemagglutinin
PKC	Protein kinase-C
PMA	Phorbol 12-myristate 13-acetate
pmoles	picomoles
PMSF	Phenylmethylsulfonyl fluoride
PTK	Protein tyrosine kinase
SDS	Sodium dodecylsulfate
TBAM	T cell-B cell activation molecule
TGF	Transforming growth factor
Th	T helper (CD4$^+$ T cell)
Tm^A	Activated T cell membranes
Tm^R	Resting T cell membranes

TNF	Tumor necrosis factor
U	Units
V	Voltage
VCAM	Vascular cell adhesion molecule
VLA	Very late activation antigen

LIST OF FIGURES

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.,

1

1985; and Dinarello, 1991). IL-1 amplifies inflammatory responses through the induction of phospholipase A_2 synthesis which leads to the production of lipid mediators of inflammation, leukotrienes, (Dinarello, 1991) and through induction of eicosanoid synthesis, prostaglandin E_2, 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.

APC — LFA-3, ICAM-1, ICAM-2, HLA/A, B7, CD40

T cell — CD2, LFA-1, TcR, CD28/CTLA-4, CD40L
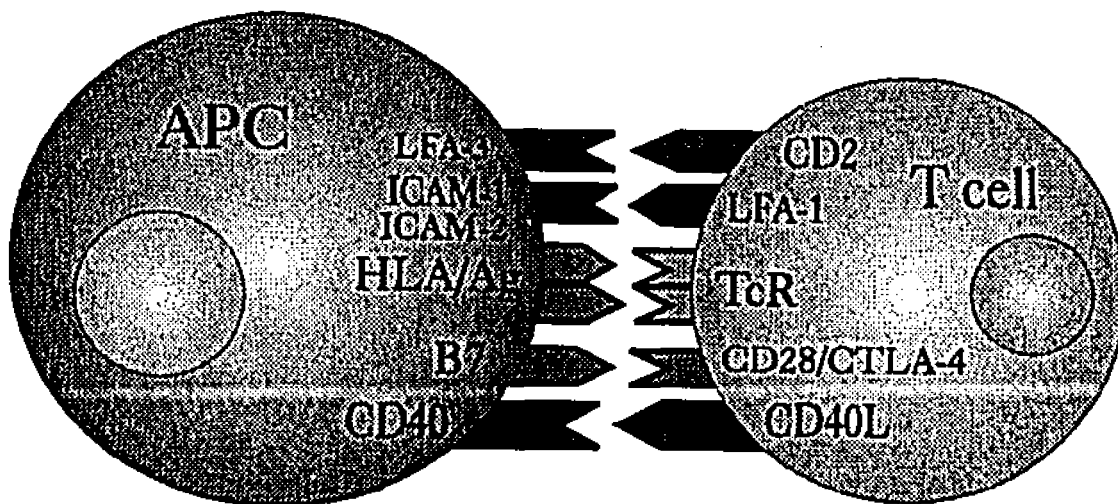
concentrations (Alderson et al., 1993) and recently CD40 has been reported on plastic adherent monocytes/macrophages (Wagner et al., 1994).

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-βA, 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-kB site and NFβA, an element reported to be specific for the IL-1ß gene, likewise have been reported (Shirakawa et al., 1993; Buras et al., 1994). It was further suggested that the NF-βA 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 (Tm^A) 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). Tm^A 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$^+$, Th0, Th1 and Th2, (Banchereau et al., 1994). CD40L has not been detected on CD8$^+$ cells or on resting CD4$^+$ cells but it has been reported that CD8$^+$ 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$^+$ 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 5×10^6 cells per well in

24-well tissue culture plates (Falcon Primaria, Lincoln Park, NJ) in RPMI 1640

(Hyclone, Logan, UT), containing $2 \times 10^{-5}M$ 2-mercaptoethanol, 100mM HEPES, and

gentamicin $50\mu g/ml$, 5% FCS (henceforth designated as R-5), $100\mu M$ 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, $100\mu M$ 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 Camfolio 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 10^6/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 MgCl$_2$, 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 ^{35}S-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

^{14}C-labeled molecular weight markers including myosin at 205 killodaltons (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/mm^2 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 $100\mu l$ 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, $10\mu g/ml$ 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 1mg/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 $MgCl_2$, 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 ^{32}P-gamma ATP (ICN Biomedicals Inc., Irvine, CA). Nuclear extracts

were incubated with gel shift binding buffer (4% glycerol, 1mM $MgCl_2$, 0.5mmM

EDTA, 0.5mM DTT, 50mM NaCl, 10mM Tris, pH 7.5, 0.05mg/ml salmon sperm

DNA), and ^{32}P-oligonucleotides at approximately $5x10^5$ 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 ^{32}P-

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.

Chapter 3

RESULTS

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 (Tm^A) were compared to plasma membranes from resting peripheral T cells (Tm^R) for their ability to induce IL-1β synthesis in resting, adherent monocytes. IL-1 synthesis was monitored by metabolic labeling using ^{35}S-labelled methionine and immunoprecipitation of monocyte cell lysates with anti-IL-1 antibodies. Tm^A successfully induced IL-1β synthesis (Figure 2), whereas Tm^R at the same concentration ($25\mu g/ml$) did not induce IL-1β synthesis above background. The ability of Tm^A to activate monocytes was titrated across a range of $10-25\mu g/ml$, 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 (Tm^A) but not resting T cell plasma membranes (Tm^R) induce human monocyte IL-1β synthesis.

Plastic adherent monocytes were incubated with either Tm^A or Tm^R for 5h in the presence of 50μCi ^{35}S-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, Tm^A at 25μg/ml; and Lane 4, Tm^R at 25μg/ml.
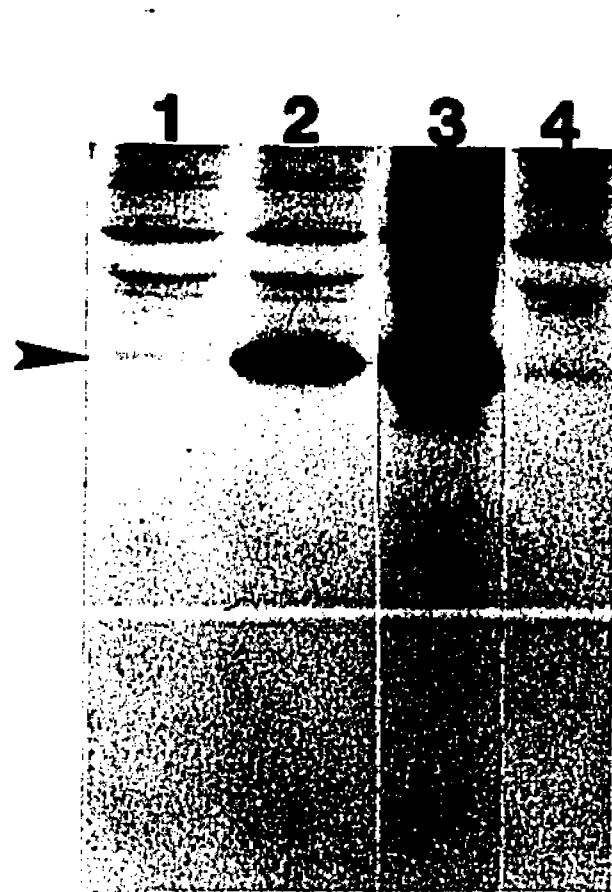
Kinetics of T cell expression of monocyte activating determinants

Having demonstrated that Tm^A are capable of inducing monocytes to synthesize IL-1β through contact-dependent interactions alone, it was of interest to determine the expression kinetics of the T cell surface molecules which induced IL-1β 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-1β production. Image analysis of the 33 kDa IL-1β 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 5×10^5, 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 4×10^5 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.
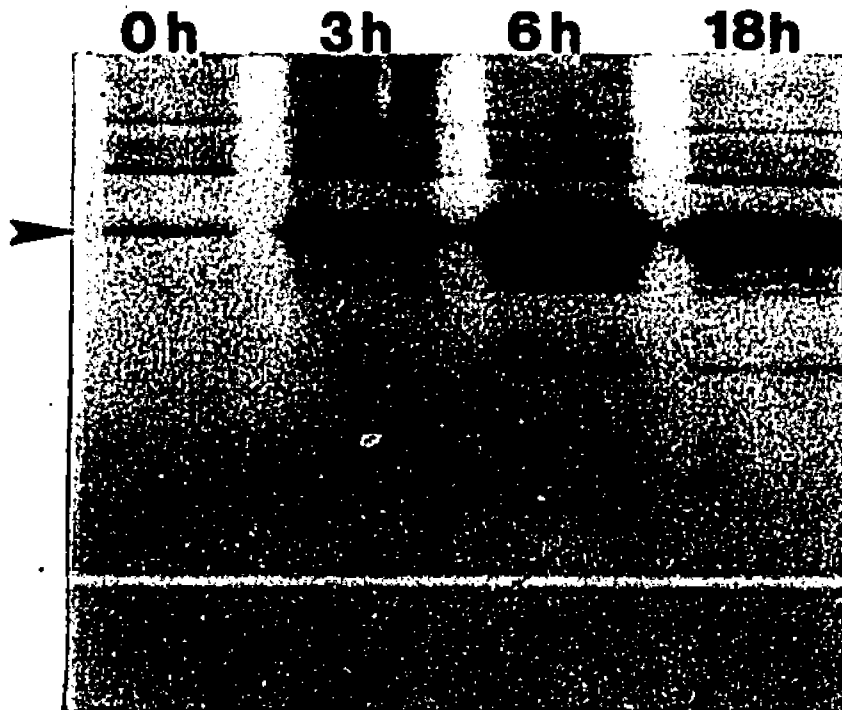
feasibility of a potential CD40-CD40L interaction in IL-1 induction. Adherent monocytes were removed from tissue culture plates and labeled with the monoclonal anti-CD40 antibody, G28-5, followed by FITC-conjugated rabbit anti-mouse IgG F(ab')$_2$. Flow cytometry revealed that resting plastic adherent 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 Tm^A, but not Tm^R 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 1×10^6/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 5×10^5 were incubated in the presence or absence of LPS+IFNγ,

Tm^R or Tm^A labeled using cell-ELISA with anti-CD40 (G28.5) followed by horse

radish peroxidase conjugated goat anti-F(ab')$_2$.
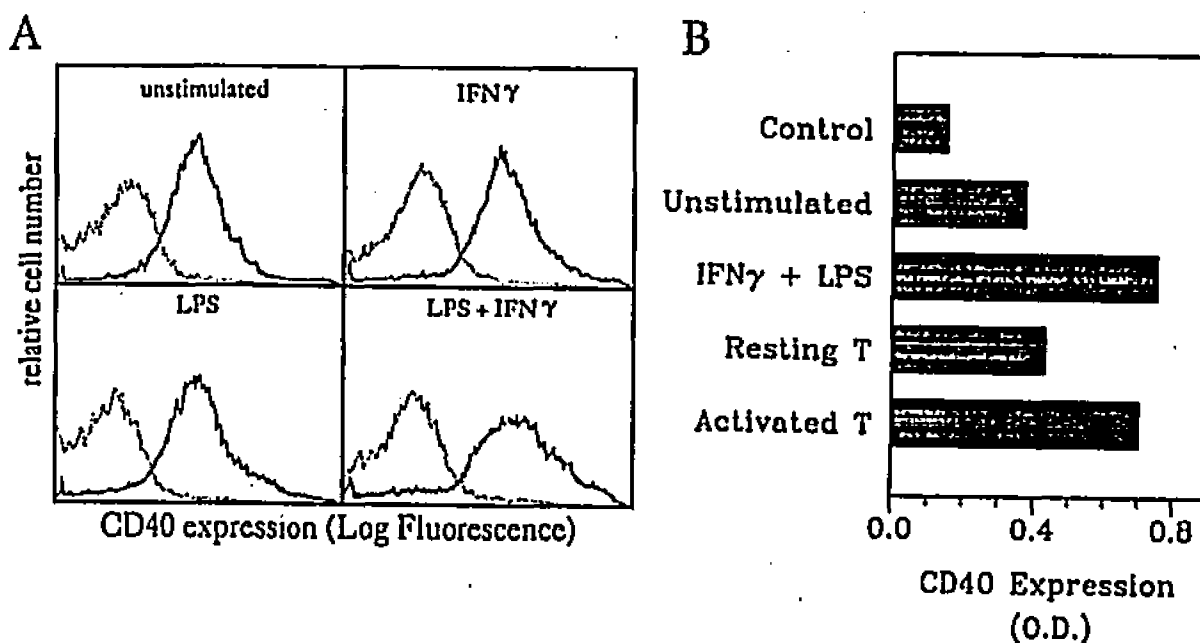
and CD40 on monocytes induces IL-1, the ability of the monoclonal anti-CD40L (T-BAM) antibody, 5c8 (Lederman et al., 1992), to interfere with the ability of Tm^A or activated, fixed CD4$^+$ cells to induce monocyte IL-1 synthesis was tested. Activated peripheral CD4$^+$ cells were fixed with paraformaldehyde 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 25μg/ml. The cells were

metabolically labeled for a 4h 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

25μg/ml; Lane 4, monocytes incubated with activated CD4$^+$ cells plus 5c8 at 10μg/ml;

Lane 5, monocytes incubated with activated CD4$^+$ cells plus 5c8 at 1μg/ml. Data is

representative of three experiments. Arrow indicates 33kDa intracellular form of IL-

1β.
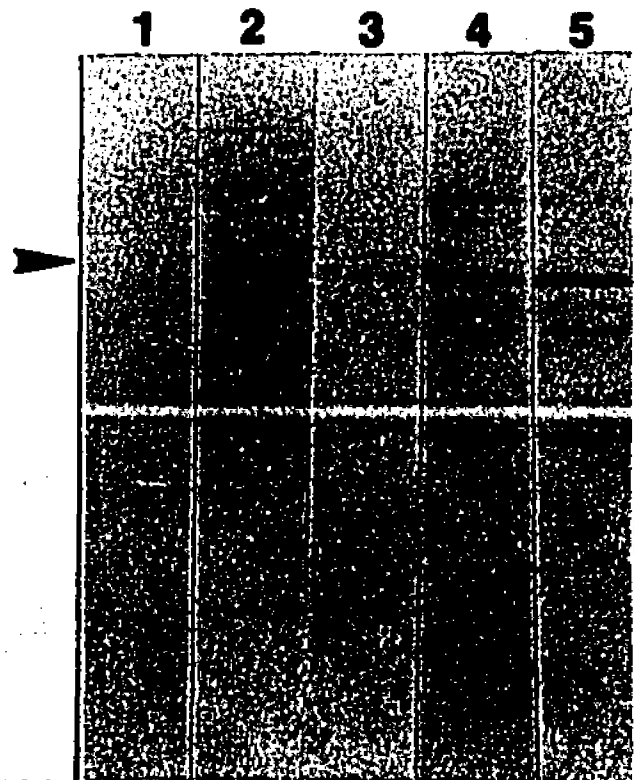
Anti-CD40 Stimulation of Monocyte IL-1 Synthesis

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 $1\mu g/ml$ to $25\mu g/ml$.

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 $0.1\mu g/ml$ to $10\mu g/ml$ 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).

1 2 3 4   1 2 3 4

A   B
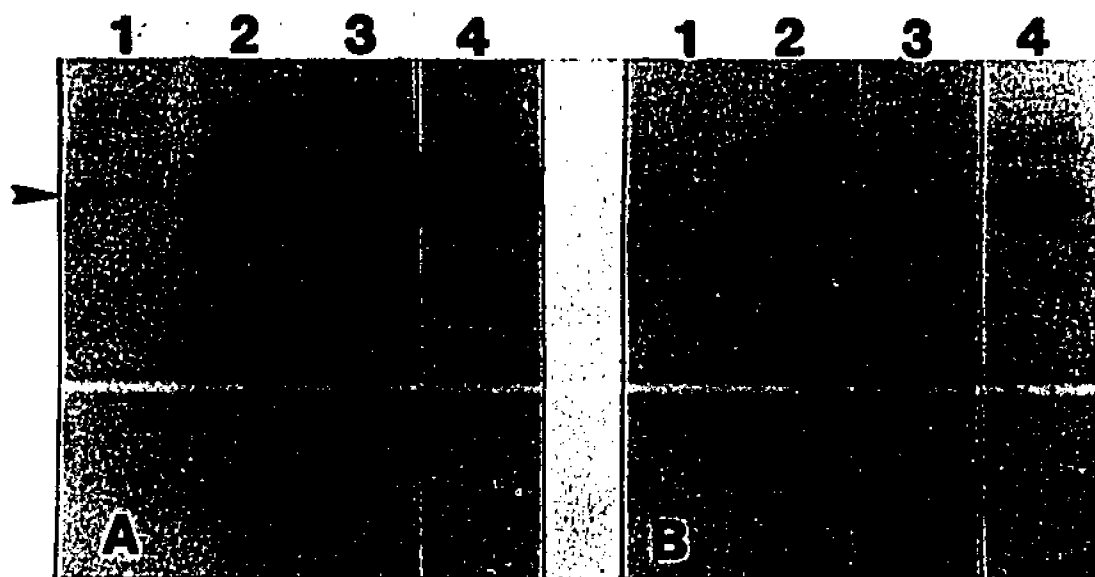
Figure 7. Requirement for crosslinking of monocyte CD40 for IL-1β induction.

Monocytes were incubated with G28-5 at 25μg/ml for 20 min. An F(ab')₂ anti-

murine 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.
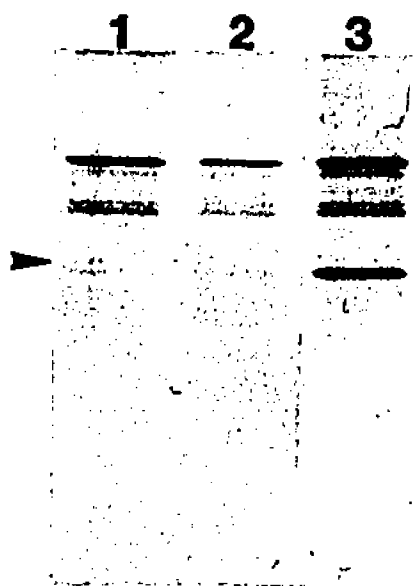
signaling in monocytes, as has been demonstrated in studies of B cell CD40 signaling.

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 Tm[A] 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 Tm[A] 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 Tm^A'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 Tm^R

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$^+$, CD40L^{lo}) 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.
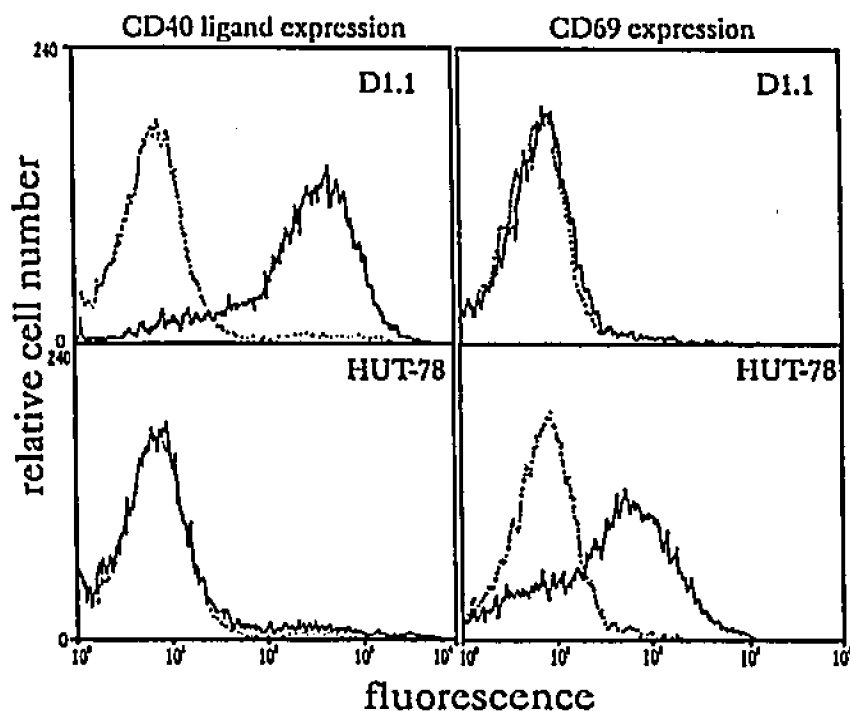
Figure 10. HUT-78 plasma membranes in combination with D1.1 plasma membranes induce IL-1β synthesis in monocytes.

Monocytes were plastic adhered and incubated with plasma membranes from HUT-78 (CD40L^{lo} , CD69$^+$), D1.1 (CD40L$^+$, CD69$^-$) or a combination of the two membranes. Following metabolic protein labeling with ^{35}S-Methonine, 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 at15μg/ml . Arrow indicates 33kDa precursor form of IL-1β.
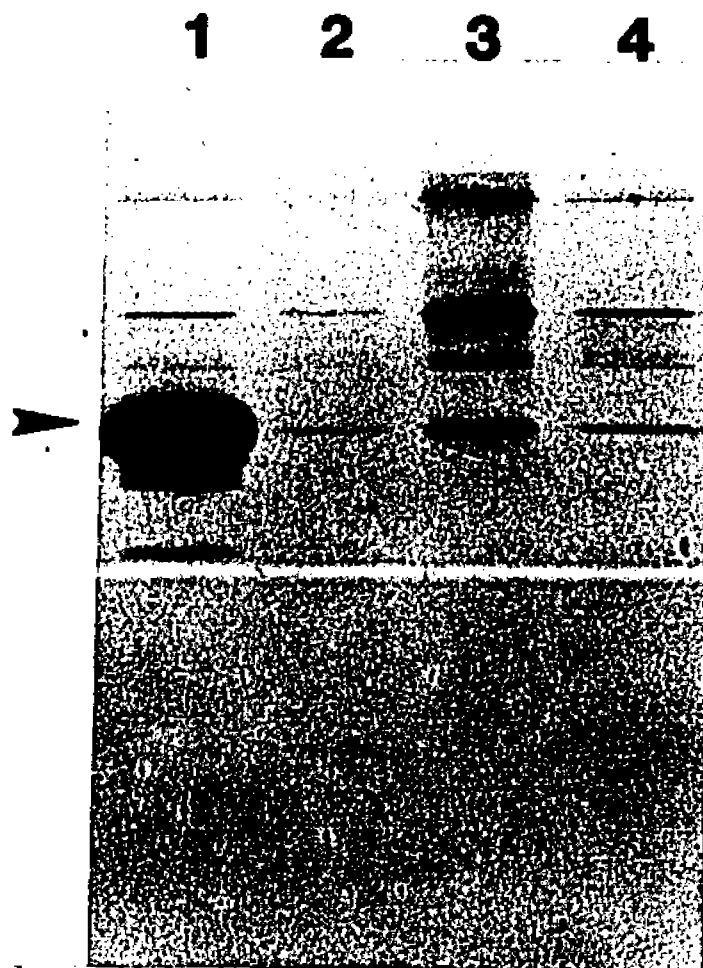
membranes alone and 100 times more effective than D1.1 (I.O.D. = 0.065) alone.

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

Treatment	BCA Protein in μg
untreated	7.86
LPS	7.58
anti-CD40	8.08

PKC Inhibitors:

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

PTK Inhibitor: 7.64

Herbimycin-A 1μg/ml	
Herbimycin-A 0.1μg/ml	8.02
Herbimycin-A 0.01μg/ml	7.74
	8.13

Mean = 7.664

s.d. = 0.324

Table 1. Cells were plated at 5×10^6 /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 $1\mu g/ml$ during ^{35}S-methionine metabolic labeling experiments for 4h. Samples of total cell protein were electrophoresed through SDS-polyacrylamide gels and visulaized by autoradiography. Lane 1, untreated; lane 2, LPS at $10\mu g/ml$; lane 3, anti-CD40 ($1\mu g/ml$) alone; lane 4, staurosporine at $100ng/ml$; lane 5, staurosporine at $10ng/ml$; lane 6, staurosporine at $1ng/ml$; lane 7, H-7 at $10\mu g/ml$; lane 8, H-7 at $1\mu g/ml$; lane 9, H-7(3Me) at $10\mu g/ml$; lane 10, herbimycin-A at $1\mu g/ml$; lane 11, herbimycin-A at $0.1\mu g/ml$; lane 12, herbimycin-A at $0.01\mu g/ml$.
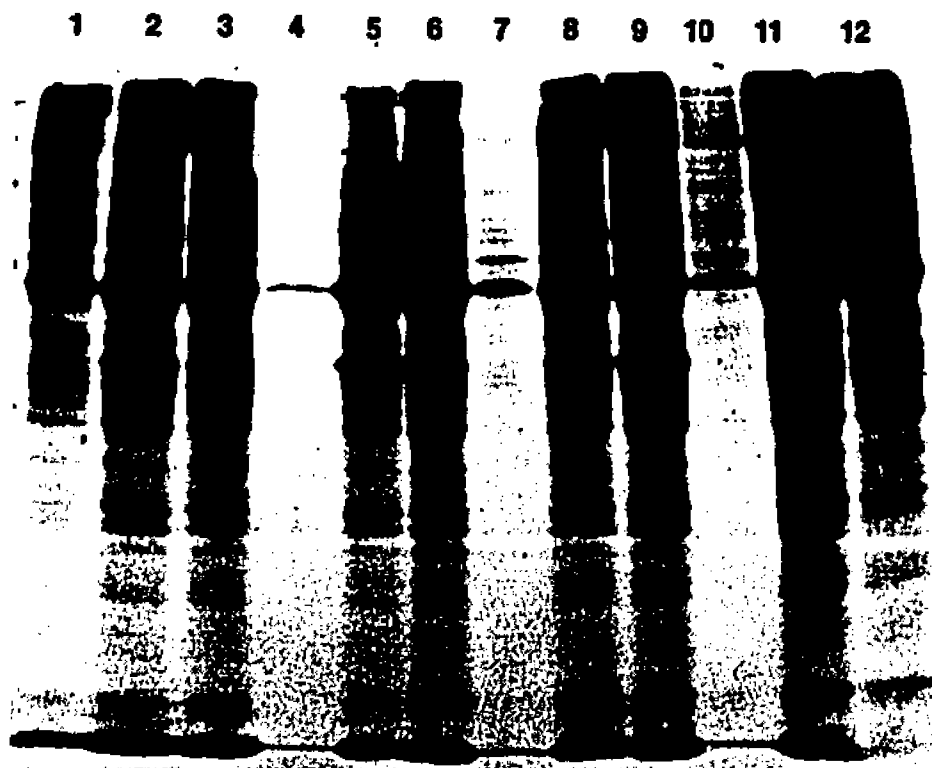
Figure 12. Effects of protein kinase inhibitors on IL-1β induction.

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 ^{35}S-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.
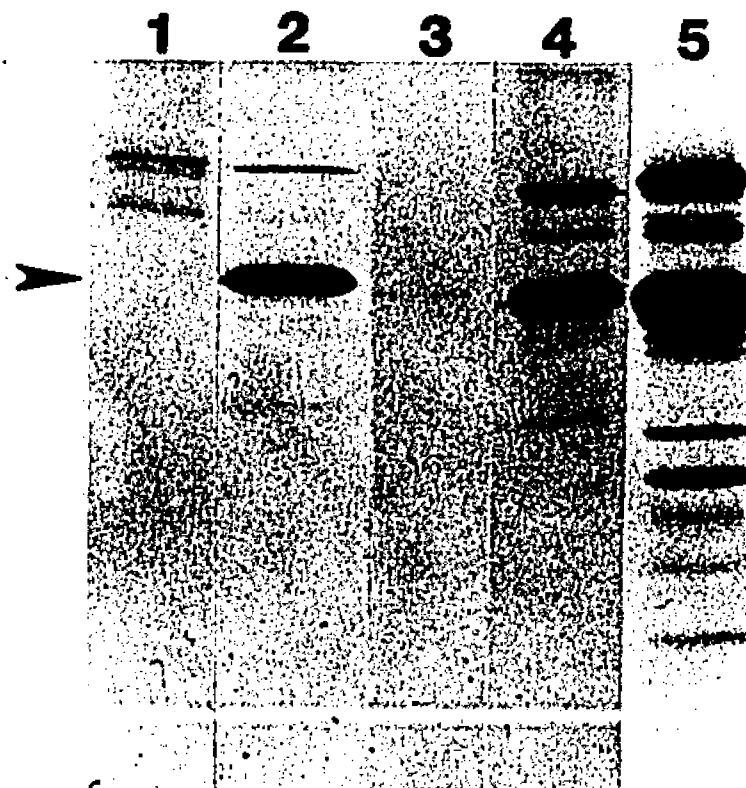
prevented total *de novo* protein synthesis but at 0.1μg/ml, herbimycin-A had no

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 p65subunit 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 ^{32}P-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.
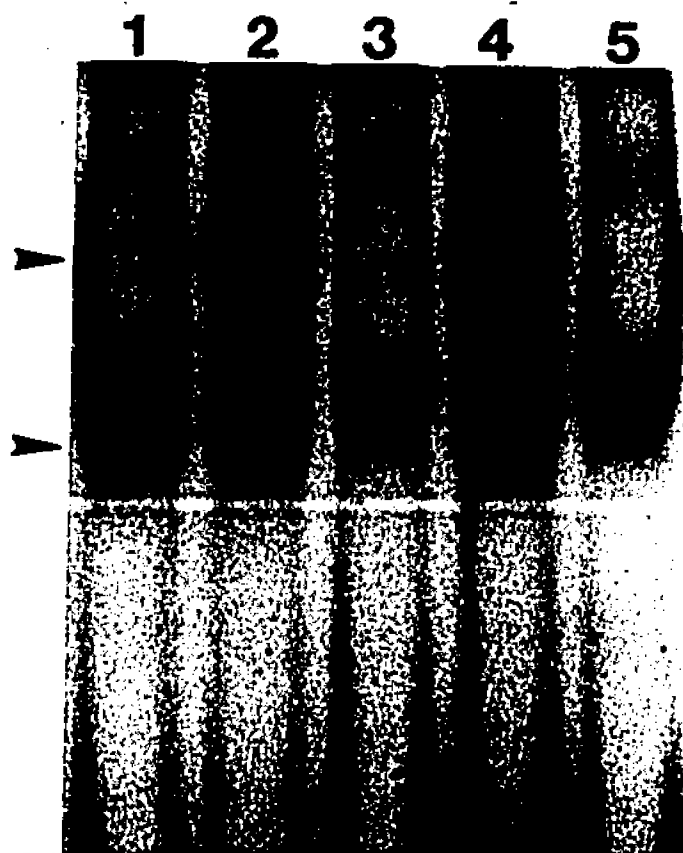
1990), LFA-3 (Webb et al., 1990), B-7 (Freedman et al., 1991) and recently CD40

(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

Cell Surface Molecule	unstimulated	LPS	IFN γ	T^R	T^A	α-CD40	Isotype
CD80	7.2±1.5*	7.2±0.6	7.2±0.5	7.8±0.7	16.0±1.9	9.7±0.9	9.1±1.0
ICAM-1	17.3±2.3	n.d.	n.d.	23.0±0.2	57.3±2.2	44.1±4.0	9.8±1.0
LFA-3	9.5±1.6	11.1±0.3	12.4±1.3	10.1±0.7	17.4±2.6	10.9±0.7	9.1±1.0

*Values reported are arithmetic means±standard errors x10^{-2} of optical density units and representative of three experiments

Table 2. Peripheral blood mononuclear cells were plated at 5x10^5 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')$_2$]. T^A 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). It

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 Tm^A, 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^{lo}, 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-βA (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-βA. 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)

Figure 14. Responsive regions in the IL-1β gene.

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.

IL-1β Regulatory Sequences
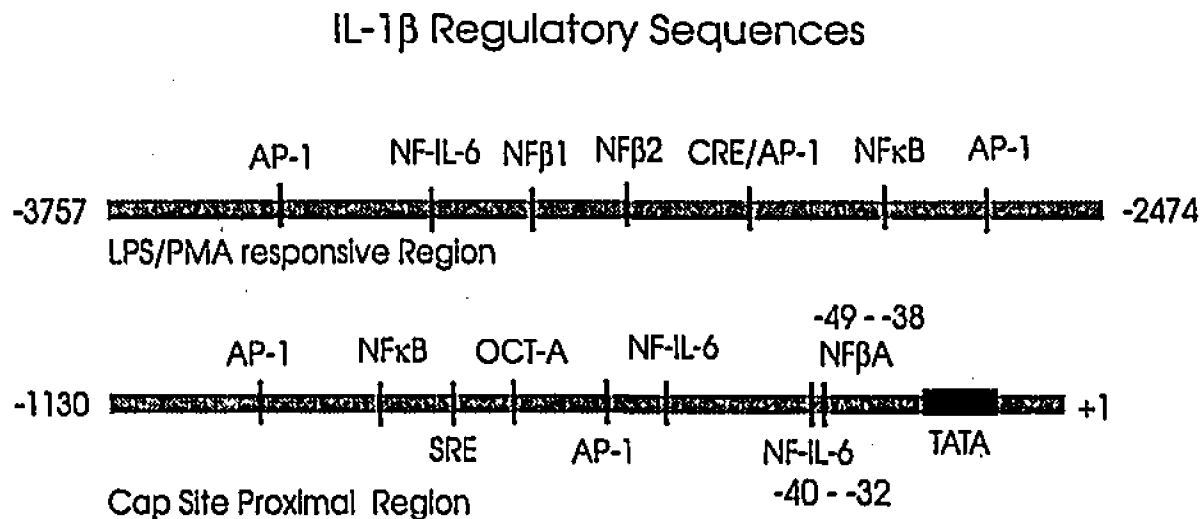
and CD40. ICAM-1 is thought to participate primarily as an adhesion molecule with little if any costimulatory activity (Springer, 1990). The effects of anti-CD40 and Tm^ to increase cell surface expression of ICAM-1 may be relevant to maintenance of cell-cell interactions. Upregulation of ICAM-1 would 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. Tm^ uregulation 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 interpretatoin of Tm^ 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.

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VITA

David H. Wagner, Jr.

Personal Data:	Date of Birth: August 9, 1962 Citizenship: USA
Education:	King College, Bristol, TN **B. S., Chemistry, 1984** *Magna Cum Laude*
	East Tennessee State University Johnson City, TN **M. S., Biology , 1987** "Indirect study of signal peptidase activity in the endoplasmic reticulum of cystic fibrosis fibroblasts"
Research Training:	Graduate Assistant, Department of Biochemistry, 1992-1994 Graduate Assistant, Department of Biophysics, 1987-1992 Graduate Assistant, Department of Pediatrics, 1985-1987
Society Memberships:	The American Society for Cell Biology Sigma Xi, Scientific Honor Society, Associate Member Gamma Beta Phi, National Honor Society
Awards:	College of Medicine Research Forum: Graduate Student Division- First Place, 1987. Presentation Title: "Signal peptidase activity in the endoplasmic reticulum of cystic fibrosis fibroblasts."

College of Medicine Research Forum: Graduate Student
Natural Sciences Division - First Place, 1990.
Presentation Title: "Tissue localization of the calcium-binding
protein, parvalbumin, and its mRNA in rainbow   trout."

College of Medicine Research Forum: Senior Graduate
Student Division-First Place, 1994. Presentation Title:
"Activation of monocyte IL-1 synthesis through CD40
signaling."

Academic Activities: President - Biomedical Graduate Student Assn., 1988-1989
                         Liaison Committee for Medical Education Review
                         Research Forum Task Force, 1988

Publications: Herd, J.K., D.H. Wagner, I.O. LeClair. 1990. " Abnormal
                         protein translocation as the elusive cause of cystic fibrosis:
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