June 1993

Mechanisms of T Cell-mediated Macrophage Activation: Role of Antigen Specific and Antigen Nonspecific Cognate Interactions

Xiang Tao
East Tennessee State University

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Mechanisms of T cell-mediated macrophage activation: Role of antigen specific and antigen nonspecific cognate interactions

Tao, Xiang, Ph.D.

East Tennessee State University, 1993
MECHANISMS OF T CELL-MEDIATED MACROPHAGE ACTIVATION
ROLE OF ANTIGEN SPECIFIC AND ANTIGEN NONSPECIFIC COGNATE INTERACTIONS

A Dissertation
presented to
the Faculty of the Department of Microbiology
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 Xiang Tao
June 1993
APPROVAL

This is to certify that the Graduate committee of

Xiang Tao

met on the

3rd day of June, 1993

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.

[Signatures]

Chair, Graduate Committee

[Signatures]

Signed on behalf of the Graduate Council

Associate Vice-President for Research and Dean, School of Graduate Studies
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ROLE OF ANTIGEN SPECIFIC AND ANTIGEN NONSPECIFIC
COGNATE INTERACTIONS
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Role of Antigen Specific and Antigen Nonspecific Cognate Interactions

by
Xiang Tao

Macrophages play an important role in host antimicrobial immunity and in non-septic inflammatory reactions. An interaction between T cells and macrophages is a critical step in initiating both specific and nonspecific immune responses to antigenic stimulation. The current study examines the role of cognate T cell-macrophage interaction in activation of macrophage effector functions and induction of macrophage early activation gene expression.

Viable resting TH2 clone cells can activate IFNγ-primed macrophages to produce reactive nitrogen intermediates or express cytostatic activity. The activating signal is mediated by cognate membrane contact between T cells and macrophages as evidenced by the ability of plasma membranes of the activated T cells to activate the IFNγ-primed macrophages. In contrast to the antigen-specific interaction of macrophages with viable resting TH2 cells, the activation of IFNγ-primed macrophages by membranes of activated TH2 cells does not display antigen specificity. Plasma membranes of the resting T cells cannot activate the IFNγ-primed macrophages. Monoclonal antibody against CD4, which blocks the interaction between CD4 and class II MHC molecules on macrophages, inhibits the activation of IFNγ-primed macrophages by viable resting TH2 cells but does not inhibit the ability of fixed activated TH2 cells to activate the macrophages. To examine the intracellular events in macrophages initiated by cognate signaling, the expression of a panel of macrophage early activation genes, c-myc, c-fos, JE, IP10, D3, TNFα and IL-α, are analyzed by dot blot hybridization. Plasma membranes of activated TH2 cells induce the expression of all these genes in macrophages stimulated for 1-4 hour. In contrast, the plasma membranes of resting TH2 cells are unable to induce the expression of most of the genes examined. These results suggest that the T cell-macrophage interaction involves reciprocal activation of both cells - an antigen specific activation of the T cells which results in the acquisition of T cell membrane components involved in antigen nonspecific activation of the macrophages.
DEDICATION

I dedicate this dissertation to my wife and my family for their love, encouragement, and support.
ACKNOWLEDGMENTS

I am most grateful to my Ph.D. Committee Chairman Dr. Bob Stout. It is his vision and wisdom that has made the accomplishment of this project possible. I am enormously indebted to him for his invaluable and indispensable guidance, advice and training during my graduate study in his laboratory. As both a mentor and friend, he taught me science, and also helped me in understanding and appreciating the culture and the way of life in this great nation. I am particularly thankful (as I will always be) for his kind decision of accepting me into his lab as a graduate student. I wish also thank Dr. Jill Suttles who has been consistently helping me in every stage of my dissertation research. Her timely advice and constructive criticism has been invaluable to the completion of this work. My heartfelt thanks also go to the other members of my committee, Dr. Bill Campbell, Dr. David S. Chi and Dr. Michael Gallagher. Without their input, advice, expertise and experience, the smooth accomplishment of this project would have been impossible. I am also grateful to the Departmental Chairman, Dr. Dwight Lambe for his supporting me with a graduate assistantship during all these years, and for many other helps he has kindly offered me and my wife, Yining. A special note of thanks goes to my good friend Dr. Chuanfu Li for his friendship and for his generous assistance in carrying out those dot blot hybridization experiments. I would also like to thank many other people in
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Ferguson, Dr. Laffan, Betty, Jerry, Tove, Barbara and Janette.
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have succeeded in my graduate study.
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ABBREVIATIONS

Act. Activated
Ag Antigen
APC Antigen-presenting cell
CD Cluster of differentiation (e.g., CD4)
ConA Concanavalin A
Conalb. Conalbumin
CTL Cytotoxic T lymphocyte
D10 D10.G4 (a T cell clone)
G-CSF Granulocyte-colony stimulating factor
GM-CSF Granulocyte-macrophage-colony stimulating factor
HLA Human histocompatibility leukocyte antigen
ICAM-1 Intercellular adhesion molecule-1
IFN Interferon-γ (e.g., IFNγ)
Ig Immunoglobulin
IL Interleukin (e.g., IL-1)
LFA-1 Lymphocyte function-associated antigen-1
LPS Lipopolysaccharide
M-CSF Macrophage-colony stimulating factor
MDP Muramyl dipeptide
MHC Major histocompatibility complex
MP1 Monocyte chemoattractant protein 1
Mφ Macrophage
NGMMA NG-monomethyl-L-arginine
NK cell Natural killer cell
PDGF Platelet-derived growth factor
<table>
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<th>Acronym</th>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PM$_r$</td>
<td>Plasma membranes isolated from resting T cells</td>
</tr>
<tr>
<td>PM$_a$</td>
<td>Plasma membranes isolated from activated T cells</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified protein derivative</td>
</tr>
<tr>
<td>RNI</td>
<td>Reactive nitrogen intermediates</td>
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<tr>
<td>ROI</td>
<td>Reactive oxygen intermediates</td>
</tr>
<tr>
<td>rTNF$_{\alpha}$</td>
<td>Recombinant tumor necrosis factor-$\alpha$</td>
</tr>
<tr>
<td>TcR</td>
<td>T cell antigen receptor</td>
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<tr>
<td>TGF$\beta$</td>
<td>Transforming growth factor-$\beta$</td>
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Overview of Macrophage Functions and Effector Mechanisms

Macrophages are mononuclear phagocytes constituting a major host regulatory and effector system. These mononuclear phagocytes are derived from bone marrow myeloid precursors which, after extensive development in the marrow, enter the blood as monocytes and migrate from the blood to the tissues where they mature into tissue macrophages (Furth et al. 1972, Furth 1989). Macrophages display a variety of important biological functions and play many essential roles in the host immune system. These functions include antimicrobial and antitumor activities, mediating and regulating autoimmune inflammatory reactions, and presenting antigen to T lymphocytes (Table 1). By presenting antigens to T lymphocytes, macrophages activate T cells and initiate antigen-specific cell-mediated immunity (Unanue 1984). The mechanisms by which macrophages exert these antimicrobial, antitumor activities and autoimmune inflammatory effector functions include the release of reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), cytokines, such as tumor necrosis factor-α (TNFα), interleukin (IL)-1, IL-6, interferon (IFN)-α, IFN-β, transforming growth factor-β (TGF-β), and the release of a variety of toxic and nontoxic inflammatory substances, such
as neutral protease, lysozyme, complement and prostaglandin (Table 1). These effector molecules constitute the basis of macrophage cytostatic, cytotoxic, and regulatory effects on nonspecific (natural) immunity (Adams and Hamilton 1984, Green et al. 1991, Kolb and Kolb-Bachofen 1992). Reactive oxygen intermediates (ROI), including superoxide anion, hydrogen peroxide and hydroxyl radicals, are produced by macrophages during oxidative burst (Babior 1984). Earlier studies have shown that the production and intracellular release of ROI are a major microbicidal mechanism employed by monocytes and macrophages (Adams and Hamilton 1984). Engagement of Fc receptors, complement receptors, receptors for mannose terminal glycoproteins on macrophages, or exposure of macrophages to phorbol 12-myristate 13-acetate (PMA) or lipopolysaccharide (LPS), can stimulate an oxidative burst resulting in release of ROI (Johnston 1981, Nathan and Rook 1977). However, some other studies have revealed that induction of ROI does not always correlate precisely with induction of macrophage microbicidal or tumoricidal function (Adams and Marubi 1983, Johnson et al. 1984, Cohen et al. 1982). In addition, cytocidal activity and phagocytosis are not necessarily accompanied by a respiratory burst, suggesting that other effector mechanisms also play important roles in macrophage antimicrobial and antitumor activities.

In recent years, an increasing number of studies have
Table 1 Roles of Macrophages in Immunity

<table>
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<th>Anti-microbial activity</th>
<th>Anti-tumor activity</th>
<th>Autoimmune inflammation</th>
<th>Antigen processing &amp; presentation</th>
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<tr>
<td>Effector Mechanisms</td>
<td>Reactive oxygen intermediates</td>
<td>Reactive nitrogen intermediates</td>
<td>Cytokines (TNFα, IL1, IL6, etc.)</td>
<td>Other inflammatory substances (complement, prostaglandins, etc.)</td>
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demonstrated that the release of RNI by macrophages is an essential mechanism by which macrophages exert cytostatic or cytotoxic effects against a wide array of pathogens and tumor cells (Green et al. 1991, Stuehr and Nathan 1989). The major component of RNI is nitric oxide, a simple and relatively unstable radical derived from L-arginine by oxidation of a terminal nitrogen in the guanidino group (Kolb and Kolb-Bachofen 1992). This molecule has been recognized as a potent mediator in many systems, such as a blood vessel relaxing factor in the cardiovascular system, as a neurotransmitter in the neural system and as a defense molecule in the immune system (Lowenstein and Snyder 1992). In the immune system, nitric oxide is produced mainly by activated macrophages and is toxic to many intracellular microorganisms, some extracellular parasites and a wide range of tumor cells (Duerksen et al. 1992, Summersgill et al. 1992, Green et al. 1991, Fischer et al. 1992, Bermudez 1993) (Table 2). The mechanism of nitric oxide cytotoxicity is not fully known, but the findings from many studies indicate that this molecule can covalently react with intracellular iron, and therefore inactivate the iron-sulfur-containing enzymes inside target cells (Green et al. 1991). The consequences of the enzyme inactivation by nitric oxide may involve inhibition of DNA replication, inhibition of the citric acid cycle, and inhibition of mitochondrial respiration in susceptible target cells (Green
<table>
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<td>Chlamydia trachomatis</td>
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<td>Schistosoma mansoni</td>
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<td>Plasmodium falciparum</td>
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<td>Entamoeba histolytica</td>
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<td>Francisella tularensis</td>
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<td>Naegleria fowleri</td>
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<td>Mycobacterium tuberculosis</td>
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<tr>
<td>Listeria monocytogene</td>
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<td>Legionella pneumophila</td>
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<tr>
<td>Cryptococcus neoformas</td>
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<tr>
<td>Tumor cells</td>
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<td>Normal tissue cells (e.g. islet cell)</td>
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et al. 1991). Besides being hostile to microbes and tumor cells, nitric oxide has also been found to be toxic to healthy tissues of the host, such as pancreatic islet cells, and thus may contribute to autoimmune inflammatory damage (Kolb and Kolb-Bachofen 1992).

The cytokines produced by macrophages exert both regulatory and effector functions in the immune system (Durum and Oppenheim 1989). TNFα, IL-1 and IL-6, although not produced exclusively by macrophages, are the major cytokines produced by macrophages (Durum and Oppenheim 1989). In addition to killing directly many types of tumor cells, TNFα also plays essential roles in regulating the functions of the cells involved in inflammatory reaction (Beutler and Cerami 1989). For example, TNFα can 1) stimulate macrophages or other cell types to produce IL-1, IL-6, IL-8 and TNFα itself; 2) stimulate the expression of adhesion molecules on endothelial cells and leukocytes; 3) activate inflammatory leukocytes and neutrophils to kill microbes; and 4) function as a costimulator for T cell activation and stimulate antibody production by B cells. Many of the TNFα functions overlap with the functions of IL-1 (Dinarello 1985, Dinarello 1989). In addition, both TNFα and IL-1 are pyrogens causing a rise in body temperature (Dinarello 1989). IL-1 and IL-6 are more potent than TNFα in regulating T cell and B cell activation (Le et al. 1989, Matsuda et al. 1989).
Macrophages also produce TGFβ, IFNα and IFNβ. The biological roles of these cytokines are very diverse ranging from up-regulation of class II MHC expression (IFNα), increase in natural killer cell activity (IFNα), enhancement of wound healing (TGF-β) to antiviral activity (IFNα, INFβ), inhibition of lymphocyte proliferation (TGFβ, IFNα, IFNβ) and inhibition of IL-2 effects including IL-2 receptor expression (TGFβ) (Pestka et al. 1987, Assoian 1987, Chieftez 1987). Macrophages also secrete macrophage-colony stimulating factor (M-CSF), granulocyte-macrophage-colony stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF) which regulate the differentiation and growth of hematopoietic stem cells (Coleman et al. 1988, Sieff 1987, Rich 1988). In addition to the array of cytokines discussed above, macrophages can release, upon appropriate stimulation, a variety of toxic and nontoxic metabolites such as lysozyme, proteases, complement components and arachidonic products—prostaglandins (Gordon et al. 1974, Werb and Gordon 1975, Brade and Bentley 1980, Bonney and Davues 1984). These substances are basically toxic or inhibitory to microorganisms, neoplastic cells and normal tissues, contributing to the host inflammatory reactions (Auger and Ross 1992). In summary, the macrophage is the central component of host natural immune defense system with a wide array of functions and diverse effector mechanisms.
Macrophage Activation

In order to perform those important biological functions in the host immune system as discussed above, macrophages need to be activated (Adams and Hamilton 1984). Different levels of activation of different functions (e.g., phagocytosis, secretive capacity or cytolytic activity) can be achieved by providing appropriate stimuli to macrophages. Conversely, the response of macrophages to a stimulus may be quantitatively and qualitatively different depending on the stage of differentiation (in bone marrow, in the peripheral blood or within the tissues) that the macrophages are and where in the body the macrophages reside (splenic macrophages, alveolar macrophages or peritoneal macrophages (Adams and Hamilton 1984, Hurme et al. 1992). In vitro studies have indicated that macrophages are stimulated by a variety of events. These stimuli are varied, from engagement of Fc receptors (Kinet 1989), encounter with microbial components such as lipopolysaccharide (LPS) (Adams 1991), to interaction with cytokines (Durum and Oppenheim 1989), and physical contact with lymphocytes (Stout 1993). The contemporary concept of macrophage activation has been viewed by many investigators as acquisition of competence by macrophages for microbicidal or tumoricidal function (Adams and Hamilton 1992). Numerous studies have demonstrated that activation of macrophage microbicidal or tumoricidal
function is a result of a multistep cascade of events involving sequential stimulation of macrophages with at least two essential agents, IFNγ and LPS (Adams and Hamilton 1984). IFNγ is a cytokine produced by activated T lymphocytes. LPS is the cell wall component of Gram-negative bacteria, and is also known as endotoxin. Treatment of macrophages with IFNγ induces macrophages into a state termed "primed". Primed macrophages are not cytostatic or cytolytic but readily become so when triggered by traces of bacterial LPS (Adams and Hamilton 1984). Therefore both the priming signal delivered by IFNγ and triggering signal delivered by LPS are required for induction of macrophage microbicidal or tumoridical function. Although some other microbial components besides LPS such as mycobacterial purified protein derivative (PPD) and muramyl dipeptide (MDP) can also deliver the triggering signal (Green et al. 1991) to primed macrophages, IFNγ is still the only known cytokine to date to be capable of priming macrophages for cytostatic or cytotoxic activity. Stimulation of macrophages with IFNγ has been shown to cause many biological effects in macrophages including increased surface expression of class II MHC molecules (Basham and Merigan 1983, Becker 1984) lymphocyte function-associated adhesion molecule-1 (LFA-1) and other adhesion molecules (Dustin et al. 1986), elevated chemotaxis and phagocytosis, secretion of ROI and enhanced ability to present antigen
(Adams and Hamilton 1984, Nathan et al. 1983, Schreiber and Celada 1985). On the other hand, stimulation of non-primed macrophages with LPS can effectively induce macrophage secretory capacity resulting in production of cytokines and inflammatory substances such as lysozyme and prostglandins (Auger and Ross 1992). IFNγ can augment these responses of macrophages to LPS. For example, it has been demonstrated that production of TNFα by macrophages stimulated with LPS plus IFNγ is 2-fold higher than by the macrophages stimulated with LPS alone (Stout and Suttles 1992). Furthermore, IFNγ has been found to induce macrophages to express receptors for TNFα (Tsujimoto et al. 1986, Ruggiero et al. 1986), indicating that this cytokine plays an important role in the autocrine regulatory activities of TNFα.

TNFα is produced at a very early stage by LPS-stimulated monocytes or macrophages (the secretion of the protein starts as early as 30 minutes after stimulation, peaks 4-8 hours) (Anderson et al. 1992, Abrams et al. 1992, Stout and Suttles 1992). TNFα can mimic the ability of LPS to synergize with IFNγ in the activation of many functions of macrophages including cytostatic or cytotoxic activities and RNI generation (Stout and Suttles 1992, Drapier et al. 1988, Ding et al. 1988, Green et al. 1990). It is believed that many of the effects of LPS on macrophage activation are secondary to the endogenous induction of macrophage TNFα.
production which exerts autocrine effects on the macrophages (Beutler and Cerami 1989).

Although many of the functions of macrophages stimulated by LPS and/or IFNγ have been identified and extensively studied, the knowledge about the intracellular events initiated by these agents in macrophages is still sketchy. Several groups have reported that LPS can initiate both protein kinase C (PKC)-dependent and PKC-independent signal transduction pathways in the macrophages whereas IFNγ-mediated signaling have been most closely associated with the enhanced exchange of Na+/H+ across the macrophage cell membrane (Rosen et al. 1989, Prpic 1987, Uhing and Adams 1989). The link between the second messengers generated in response to LPS or IFNγ stimulation and the final initiation of gene transcription in macrophages is murky just as in the activation of many other cell types. Studies of macrophage gene expression in response to the stimulation of LPS and/or IFNγ have been focused mainly on the expression of pro-inflammatory cytokine genes such as TNFα, IL-1, JE, IP10; a number of proto-oncogenes such as c-myc, c-fos; and the LPS-responsive genes such as DJ1. Expression of mRNA for TNFα and IL-1 by macrophages is induced by LPS but not by IFNγ (Yu et al. 1990, Myers et al. 1989). IFNγ can enhance the LPS-stimulated expression of mRNA for TNFα. The appearance of transcripts for TNFα precedes the appearance of IL-1 mRNA (Yu et al. 1990, Myers
et al. 1989), correlating with the kinetics of the protein synthesis of these two cytokines. The effect of LPS on induction of macrophage TNF\(\alpha\) mRNA is due to the enhancement in the transcription of TNF\(\alpha\) gene whereas the effect of LPS on IL-1 gene expression appears to involve stabilization of IL-1 mRNA rather than increased transcriptional activity (Yu et al. 1990).

C-Myc and c-Fos are well characterized cellular proto-oncogenes expressed very early (several minutes) after mitogenic stimulation in many cell types such as fibroblasts, macrophages, T cells and B cells (Abbas et al. 1991). The products of c-myc and c-fos are nuclear factors which act within the nucleus to regulate cell growth and cell activation through transcriptional regulation of other genes, such as the IL-2 gene (Crabtree 1989). In macrophages, the mRNA for c-myc and c-fos can be induced as early as 2 minutes after stimulation of macrophages with LPS or the PKC activator-PMA (Introna et al. 1986), indicating that protein kinase C is involved in the expression of these proto-oncogenes. It was observed that the expression of these genes did not correlate with induction of cellular proliferation in the macrophages but rather was more closely related to macrophage activation (Introna et al. 1986). JE is a competence gene originally characterized in platelet-derived growth factor (PDGF)-stimulated fibroblast (Zullo et al. 1985, Cochran et al. 1983). Its product is now known to
be an inflammatory cytokine that directs chemotaxis of monocytes, and is a homologue of monocyte chemoattractant protein 1 (MCP1) (Rollins et al. 1990). The expression of JE in macrophages can be stimulated by LPS via a PKC-dependent transductional pathway (Introna et al. 1987). However, like IL-1 mRNA induction, the JE gene is regulated post-transcriptionally (Koerner et al. 1987). The expression of c-myc, c-fos and JE in macrophages cannot be induced by stimulation with IFNγ alone (Introna et al. 1986, Yu et al. 1990). IP10 and D3 are the genes transcribed in LPS-stimulated macrophages (Tannebaum et al. 1988, Yoshimura et al. 1987). The function of the protein encoded by D3 has yet to be determined whereas the IP10-encoded product has been shown to be a pro-inflammatory protein belonging to the MP-1 and IL-8 cytokine family (Yoshimura et al. 1987, Oppenheim et al. 1991). Besides LPS, IFNγ can also induce the expression of IP-10 and D3 genes in macrophages (Narumi and Hamilton 1991, Narumi et al. 1992). It should be pointed out that most of the studies of activation of macrophage gene expression or other macrophage functions were conducted using resident or sterilely elicited mouse peritoneal macrophages. These macrophages can be phenotypically and differentiatively different from macrophages obtained from other sources, such as lung, liver, spleen, bone, brain and bone marrow (Adams and Hamilton 1992, Paulnock 1992, Hurme et al. 1992).
Therefore, one should exercise caution in applying the data obtained from peritoneal macrophages to macrophages derived from other sources. Clearly, more studies are needed to examine and compare the mechanisms regulating activation of macrophages of different organ origins.

**T Cell-Macrophage Interaction**

A central role of macrophages in the immune system is antigen processing and presentation to T lymphocytes. This role allows macrophages to interact physically with T lymphocytes and to initiate antigen-specific T cell immune responses against invading microorganisms or antigens (Unanue and Allen 1987). When a foreign substance (antigen) invades the body, macrophages, as a major component of the host phagocytic system, can phagocytose the antigen and subsequently digest the antigen into small peptides inside the cells. The antigenic peptides are placed in a physical association with major histocompatibility complex (MHC) molecules and transported to the surface of macrophages as antigen(Ag)-MHC complexes (Unanue 1984, Babbit et al. 1985). These complexes are presented by macrophages to T lymphocytes that express the specific antigen receptors for the complexes (Marrack and Kapler 1986) (Fig. 1). The binding of the Ag-MHC complex to the T cell receptor (TcR) delivers a signal to T cell for its activation (Moller 1987, Schwartz 1985). Many studies have demonstrated that the
signal generated by the interaction between Ag-MHC complex and TcR is necessary but not sufficient for activation of T cells (Mueller et al. 1989). Some other signal termed "costimulatory signal" from macrophages as antigen-presenting cells is also required for T cell activation. A great deal of effort has been made by many workers in order to unravel the nature of the costimulatory signal. The data generated from these studies indicate that although some cytokines or some surface molecules of antigen-presenting macrophages are essential for costimulating T cell activation, there is no single cytokine or surface molecule that can account for all the costimulatory signals needed for activation of the different subsets of T cells, such as T\textsubscript{h}1, T\textsubscript{h}2 and CD8\textsuperscript{+} T cells. Those most important macrophage-derived proteins involved in costimulatory activities are secreted cytokines such as IL-1, IL-6 and membrane-bound molecules such as B7, ICAM-1 and LFA-1 (Fig. 1).

IL-1, originally known as lymphocyte activating factor, has long been implicated as a T cell subset costimulator (Weaver and Unanue 1990). It is now clear from studies in several laboratories that IL-1 is a requisite costimulator for the growth of selected CD4\textsuperscript{+} murine T-cell clones belonging to T\textsubscript{h}2 subset of T cells (Weaver and Unanue 1990, Kurt-Jones et al. 1987, Greenbaum et al. 1988, Mueller et al. 1989). IL-1 is not essential for growth of other types of T cell clones, such as T\textsubscript{h}1 or CD8\textsuperscript{+} cytotoxic T
Figure 1

Schematic Diagram of T Cell-Macrophage Interaction

MACROPHAGE

T CELL

T CELL ACTIVATING CYTOKINES

MACROPHAGE ACTIVATING CYTOKINES

LFA-3
CD40
MIIC-II
B7
ICAM
CD2
CD4
CD8
LFA-1
TcR
lymphocytes (CTL) (Kurt-Jones et al. 1987, Greenbaum et al. 1988, Weaver et al. 1988, Williams and Unanue 1990). Several studies have revealed that IL-6 can act as an important signal for T cell proliferation and differentiation (Luger et al. 1989, Le et al. 1989, Matsuda et al. 1989). This cytokine can greatly enhance the mitogen or anti-CD3-mediated activation of naïve CD4+ T cells, the differentiation of CD8+ CTL precursors and the proliferation of thymocytes. But in some other model systems, IL-6 could not replace the macrophage-derived costimulatory signal needed for T cell activation (Mueller et al. 1989, Williams and Unanue 1990).

Adhesion molecules such as LFA-1 and intercellular adhesion molecule-1 (ICAM-1) have been known to be important for antigen-presenting cell-mediated T cell activation (Bierer and Burakoff 1988). Whether their roles are restricted merely to augmenting adhesion between T cells and macrophages or are involved in triggering intracellular signaling in T cells is not completely clear. Recently, several studies have demonstrated that the B7/BB1 molecule expressed on antigen presenting cells (APC) such as macrophages is able to deliver a distinct and critical costimulatory signal to T cells, particularly to murine T(H)1 CD4+ T cells, upon interacting with its counterreceptor-CD28 (Razi-Wolf et al. 1992, Koulova et al. 1991, June et al. 1990). This costimulatory signal initiates or greatly
enhances IL-2 production by T cells by augmenting IL-2 gene transcription and/or stabilizing IL-2 mRNA (Lindsten et al. 1989, Fraser et al. 1991). Binding of CD28 with specific antibody or B7/BB1 transfectant could prevent the T cells from becoming tolerized by the stimulation of TcR alone (Razi-Wolf et al. 1992). These studies indicate that interaction between macrophage B7/BB1 and T cell CD28 represents an important costimulatory pathway for T cell activation. Upon activation, T cells produce an array of lymphokines which are required for regulation of growth, differentiation and activation of other cell types in the immune system, such as macrophages, B cells, CTL and natural killer (NK) cells. IFNγ is produced by activated T cells, and is the most potent macrophage activating lymphokine (Drysdale et al. 1988, Adams and Hamilton et al. 1984). Therefore, the interaction between T cells and antigen-presenting macrophages results in a reciprocal activation of T cells and macrophages and leads to an amplification of both specific and nonspecific immune responses (Fig. 1).

As most of the studies on the interaction between T cells and antigen-presenting macrophages have focused on the mechanisms of T cell activation, little attention has been given to the mechanisms of macrophage activation that could possibly result from this cognate interaction. It is conceivable that binding between TcR and Ag-MHC or between adhesion molecules and their ligands on T cells and
macrophages can somehow send an activating signal(s) to macrophages. Several reports have revealed that antibodies to HLA-DR, LFA-3, CD45 and CD44 can stimulate human monocytes to release IL-1 (Palacios 1985, Webb et al. 1990). Other studies have also shown that the physical contact between T cells and murine macrophages or between activated T cells and human monocytes can induce IL-1 protein synthesis or mRNA expression in the macrophages or monocytes (Weaver and Unanue 1986, Landis et al. 1991). The physical contact involved both antigen-specific, MHC-restricted interactions and antigen-nonspecific interactions between T cells and macrophages, suggesting that both class II MHC molecules and adhesion molecules may play signaling roles in these effects. Indeed, one study using B cell transfectants expressing truncated MHC molecules has indicated that MHC molecules play a critical role in signal transduction in B cells during B cell antigen presentation to T lymphocytes (St-Pierre et al. 1989). A few recent studies also demonstrated that crosslinking of class II MHC molecules with antibody or with T cell receptors could induce the expression of B7/BB1 molecules on B cells (Koulova et al. 1991, Nabavi et al. 1992, Watts et al. 1993). Since both IL-1 and B7/BB1 are important costimulatory molecules for T cell activation, these studies suggest that the physical interaction between T cells and antigen presenting cells can directly stimulate the costimulatory functions of APC,
leading to reciprocal activation of T cells. B7/BB1 is also expressed by monocytes (Freedman et al. 1991) and peritoneal macrophages (Razi-Wolf et al. 1992). Whether the induction of the expression of this molecule on macrophages can be mediated by class II MHC molecules awaits further study. A number of early studies have suggested that T cell-mediated activation of macrophage antimicrobial effector functions may also occur via interactions that do not involve lymphokine secretion (Simon and Sheagren 1971, Simon and Sheagren 1972). Later studies by Stout and Bottomly (1989) and by Panosian et al. (1985) have provided evidence supporting a role of cell-cell contact in T cell-mediated activation of macrophage effector functions. In summary, the interaction between T cells and macrophages is an essential step in generation of cell-mediated immune response. The physical interaction between T cells and macrophages is not only required for T cell activation but probably also essential for activation of macrophage costimulatory activity and effector functions.

Rationale and Goals

The roles of activating macrophage effector functions have been traditionally ascribed to two most potent macrophage activating factors, T cell-secreted IFNγ and bacterial endotoxin, LPS. These two agents work in concert to activate macrophage antimicrobial effects during
microbial infection (Adams and Hamilton 1987). Macrophages are also activated during a number of nonseptic inflammatory reactions, such as contact-mediated delayed hypersensitivities, autoimmune inflammation, anti-tumor responses, atherosclerosis and Graft versus host reactions (Duerksen et al. 1992, Kolb and Kolb-Bachofen 1992, Martin et al. 1992, Mantovani et al. 1992, Ross 1993, Hoffman et al. 1992). The mechanisms of macrophage activation in these situations have been less clear. As discussed above, two studies have indicated that T lymphocytes are able to provide a cell contact-mediated signal (cognate signal) for activation of macrophage effector functions (Stout and Bottomly 1989, Panosian et al. 1985). This cognate signal was observed to be antigen-specific and MHC-restricted. Recent studies have demonstrated that T cell-dependent B cell activation also involve both cognate (cell contact-mediated) and noncognate (e.g., IL4) signals (Noelle and Snow 1990). However, although the initial interaction resulting in T cell activation is antigen-specific, the cognate signal leading to B cell activation (proliferation and antibody production) is mediated by antigen-nonspecific receptor-ligand interactions, such as binding of p39 on activated T cells to CD40 on B cells (Noelle and Snow 1990, Noelle et al. 1992). Whether the cognate signaling of macrophage activation involves a similar series of antigen specific and antigen-nonspecific interactions has not yet
been determined. The current study is undertaken to investigate the role of cognate interaction in T cell-mediated macrophage activation. The activation of macrophages is determined by assaying macrophage cytostatic activity or macrophage production of reactive nitrogen intermediates which have been currently recognized as the major mediators of macrophage anti-microbial and antitumor activities (Green et al. 1991). To address the mechanics of cognate signaling of macrophage activation, the ability of viable T cells versus the ability of paraformaldehyde-fixed resting and activated T cells or the plasma membranes isolated from resting and activated T cell clones to activate macrophages is examined. Additionally, the intracellular events initiated by the cognate signaling in macrophages and the expression of a panel of early activation genes in macrophages stimulated by fixed T cells or by T cell plasma membranes also is investigated. The results of this study are discussed in the context of current theories regarding T cell-macrophage interaction.
Materials and Methods

Materials

Animals

Inbred female mice (C57BL/6J, CAF1, Balb/byJ and DBA/2J) were obtained from Jackson laboratories (Bar Harbor, ME). Only mice between the ages of 7 and 14 weeks were used for experimentation.

Media

The complete culture medium used for macrophage activation experiments was RPMI-1640 with L-glutamine (Hyclone Laboratories, Inc., Logan, UT), supplemented to 5% with fetal bovine serum (FBS, prescreened for endotoxin levels < 0.05 ng/ml, Hyclone), 10 mM HEPES (Gibco BRL Life Technologies, Inc., Grand Island, NY), 50 μg/ml gentamycin (Sigma Chemical Co., St. Louis, MO), 50 μM 2-mercaptoethanol (Sigma Chemical Co.), and 1 mM sodium pyruvate (Sigma Chemical Co.). The culture medium for maintaining T cell clones was the same as the macrophage activation medium described above with addition of 1-5% CASS (conditioned medium of ConA-stimulated rat spleen cells) and oxalacetic acid-insulin cocktail (Sigma Chemical Co., final concentrations were 132 μg/ml for oxalacetic acid and 0.2
u/ml for insulin). The culture medium for generation of macrophages from murine spleen cells was the same as the macrophage activation medium described above without addition of 2-mercaptoethanol. The medium for growing bacteria was LB containing 1% (w/v) sodium chloride, 1% (w/v) bacto-tryptone and 0.5% bacto-yeast (w/v) (Difco Laboratories, Detroit, MI).

Buffers and Solutions

For preparation of cells, phosphate buffered saline (PBS) was 0.15 M sodium chloride, 5.11 mM sodium phosphate (dibasic) and 1.56 mM potassium phosphate (monobasic). Dulbecco’s phosphate buffered saline (DPBS) was 0.90 mM calcium chloride (anhydrous), 2.68 mM potassium chloride, 1.47 mM potassium phosphate monobasic, 0.49 mM magnesium chloride, 136.9 mM sodium chloride and 8.06 mM sodium phosphate (dibasic).

For preparation of plasma membranes from T cells, homogenization buffer was a mixture of 20 mM Tris-chloride, 10 mM sodium chloride and 0.1 mM magnesium chloride. 0.1 mM phenylmethylsulfonyl fluoride (Fisher Scientific, Norcross, GA) and 0.5 μg/ml RQ1 DNase (Promega, Madison, WI) were added to the homogenization buffer prior to use. 41% sucrose (Fisher Scientific) solution (w/v) was prepared in the homogenization buffer.

For mini-prep of plasmids, glucose buffer consisting
of 25 mM Tris (pH 8.0), 50 mM glucose and 10 mM EDTA (Sigma Chemical Co.). Alkaline lysis buffer was 0.2 M NaOH with 1% SDS prepared fresh each time by mixing 5 ml of 10% SDS (Sigma Chemical Co.) and 1 ml of 10 M NaOH in 44 ml of H₂O. Sodium acetate was 3 M with pH of 4.8 adjusted with glacial acetic acid. TE buffer was 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA. Lysozyme solution was prepared by dissolving lysozyme (Sigma Chemical Co.) in glucose buffer (8 mg/ml) prior to use.

For purification of DNA inserts from the plasmids, gel loading buffer contained 50% (v/v) glycerol, 0.2 M EDTA (pH 8.3) and 0.05% (w/v) bromphenol blue. Tris-Acetate-EDTA (TAE) electrophoresis buffer was prepared as a 10x stock solution containing 0.4 M Tris, 0.2 M sodium acetate and 10 mM EDTA. Low salt buffer contained 0.15 M sodium chloride, 20 mM Tris (pH 8.0) and 0.5 mM EDTA. High salt buffer contained 1 M sodium chloride, 20 mM Tris and 0.5 mM EDTA.

For dot blotting and hybridization, sodium chloride-sodium citrate (SSC) was prepared as 20x stock solution containing 3 M sodium chloride and 0.3 M sodium citrate (pH 7.0, adjusted with 1 M HCl). SSPE buffer was prepared as 2x stock solution containing 0.36 M sodium chloride, 20 mM sodium phosphate (monobasic) and 20 mM EDTA. Prehybridization buffer was 0.5 M Na₂HPO₄/NaH₂PO₄ (pH 7.0), 7% SDS and 100 µg/ml denatured Herring sperm DNA (Promega). Hybridization buffer was the same as the prehybridization
buffer with addition of radio-labeled DNA probes. Washing buffer 1 consisted of 0.1 M Na₂HPO₄/NaH₂PO₄ and 1% SDS. The buffer was diluted by a 1:10 dilution with distilled water to generate washing buffer 2.

Reagents

Recombinant interferon-γ (rIFNγ) was obtained from R&D Systems (Minneapolis, MN) and from Amgen Biologics (Thousand Oaks, CA). Recombinant tumor necrosis factor-α (rTNFα) was obtained from Genzyme Corporation (Cambridge, MA). LPS (Escherichia coli, 011:B3W) was obtained from Sigma Chemical Co. (St. Louis, IL). Conalbumin, egg albumin (ovalbumin), concanavalin A (con A), mitomycin-c, NG-monomethyl-L-arginine (NGMMA), ampicillin and tetracycline were also obtained from Sigma Chemical Co. Paraformaldehyde was purchased from Fischer Scientific (Norcross, GA). ³H-thymidine was obtained from Amersham (Arlington heights, IL). ³²P-dCTP was obtained from New England Nuclear Inc. (Boston, MA). Random primed DNA labeling Kit (Prime-a-Gene Labeling System) and restriction endonucleases including EcoR I, Bam HI, Xba I, Pst I and Bgl II were purchased from Promega (Madison, WI).

Nylon Wool

Nylon wool (LP-1 Leuko-Pak Leukocyte Filters) was obtained from Femwall Laboratories.
Monoclonal Antibodies

Monoclonal hamster anti-murine CD3 antibody (Leo, O., et al., 1987) was purified from the culture supernatant of 145-2C11 hybridoma (provided by Dr. Jeffrey Bluestone of the University of Chicago) by 50% saturated ammonium sulfate precipitation and protein G-Sepharose 4B (Pharmacia LKB Biotech, Inc. Piscataway, NJ) affinity column chromatography followed by dialysis and filtration through a 0.22-m filter. Hybridoma GK1.5 (Wilde et al. 1983) producing rat anti-murine CD4 (IgG₂b) was obtained from the American Type Culture Collection (Rockville, MD). The culture supernate of GK1.5 was titrated by flowcytometric analysis for binding to CD4 on the murine spleen cells and was used directly in macrophage activation inhibition study.

Plasmids

Plasmid containing partial murine TNFα cDNA (Caput et al. 1986) was obtained from Dr. Bruce Beutler (Southwestern Medical Center, Dallas, TX). Plasmid containing partial murine IL-1α cDNA was obtained from Dr. Jill Suttles (East Tennessee State University, Johnson City, TN). Plasmids containing partial cDNAs for murine IP-10 and D3 genes (Tannenbaum et al. 1988) were obtained from Dr. Thomas Hamilton (The Cleveland Clinic Foundation, Cleveland, Ohio). Plasmid containing cDNA for murine JE gene (Rollins et al. 1988) was obtained from Dr. Charles Stiles (Dana Farber
Cancer Institute, Boston, MA). Plasmids containing genomic DNAs for murine c-myc (Grace et al. 1982) and c-fos (Curran et al. 1983) were purchased from American Type Culture Collection. Plasmid containing cDNA for chicken β-actin gene was obtained from Dr. Philip Musich (East Tennessee State University, Johnson City, TN).

**Autoradiography Materials**

Kodak X-OMAT AR (XAR 5) X-ray films (8 x 10 inches) were purchased from Sigma Chemical Co. Kodak GBX developer and GBX fixer were also obtained from Sigma Chemical Co.

**T Cell Clones**

The I-A<sup>k</sup>-restricted, conalbumin-specific T<sub>H</sub>2 clones, D10.G4 and AK8 (Kim et al. 1985, Janeway et al. 1982), were obtained from Dr. Kim Bottomly (Yale University). The I-A<sup>k</sup>-restricted, ovalbumin-specific T<sub>H</sub>1 clone, J6-19 (Wilde and Fitch 1984), was obtained from Dr. Frank Fitch (The University of Chicago).

**Methods**

**Macrophage Preparation**

Splenic macrophages were generated as described previously in detail (Stout 1985, Stout and Suttles 1992).
Briefly, spleen cells were resuspended to $4 \times 10^6$/ml in macrophage generation medium and were added to 96-microtiter culture plate in 0.2 ml aliquots. For extraction of total RNA from the macrophages, the $4 \times 10^6$/ml spleen cells were added to 24-clusterwell culture plates in 2 ml aliquots of macrophage generation medium. The cells were then cultured for 5 days after which the nonadherent cells were removed by washing the wells of the culture plates with warm DPBS (0.15 M, pH 7.3) for three times.

**T Cell Preparation**

For preparing fresh splenic T cells, murine fresh spleen cells prepared as described previously (Stout and Fisher 1983) were resuspended to $5 \times 10^7$/ml in DPBS supplemented with 2% of FBS and were incubated with nylon wool packed in a 12-ml syringe (0.6 g nylon wool per $1 \times 10^8$ cells) for 45 minutes at 37°C. The nylon wool was then washed with warm DPBS. Nonadherent cells were collected (Mishell and Shiigi 1980) and used as fresh T cells in this study.

For preparing $T_n^2$ or $T_n^1$ cells, D10.G4, AK8 and J6-19 were maintained as previously described (Stout and Bottomly 1989), using CAF1 spleen cells as antigen (conalbumin for D10.G4 and AK8, ovalbumin for J6-19) presenters and feeding every 4-5 day with clone medium containing 1-5% rat CASS between restimulation cycles. For the described
experimentation and assays, the clones were harvested 14-18 days after antigen stimulation, centrifuged over lympholyte-M (Accurate Scientific and Chemical Corp., Westbury, NY) to eliminate dead cells and debris, and washed twice with DPBS supplemented with 2% FBS. These cells were used as resting T cells.

To obtain activated T cells, the affinity column-purified monoclonal anti-CD3 was immobilized to 24-clusterwell culture plates (Costar, Cambridge, MA) by incubating the plates at 4°C overnight with 2 μg/ml anti-CD3 (1 ml/well). After incubation, the plates were rinsed three times with PBS. $1\times10^6$/ml $T_H^2$ clone cells or fresh splenic T cells were added to the plates and cultured at 37°C for 6 hours and 24 hours, respectively, and were harvested from the plates as activated T cells. A 6 hour stimulation period was found to be optimal for generating T cells from the $T_H^2$ clones capable of cognate signaling of B cells (Tohma and Lipsky 1991) and macrophages (Stout and Suttles 1993) after being paraformaldehyde fixed. A 24 hour stimulation period was found to be optimal for activating fresh splenic T cells to deliver cognate signal for B cell activation (Tohma and Lipsky 1991).

**Preparation of $T_H^2$ Supernatant**

Purified D10.G4 or AK8 $T_H^2$ cells were cultured at a concentration of $1\times10^6$/ml in the medium containing 2 μg/ml
concanavalin A (con A) for 24 hours. The supernatant was subsequently harvested and treated with α-monomethyl-D-mannoside (20 mg/ml) to neutralize residual con A. The supernatant was sterilized by filtration through a 0.22 μm filter and stored at -20°C.

Fixation of T Cells with Paraformaldehyde

Paraformaldehyde fixation of T cells was performed as described previously (Noelle et al. 1989). Briefly, resting or activated T cells were resuspended to 1x10^7/ml in PBS followed by addition of an equal volume of 0.8% paraformaldehyde prepared in PBS. After 5 minutes incubation at room temperature in dark, an equal volume of 0.2 M lysine (Sigma Chemical Co.) prepared in sterile distilled water was added. Cells were washed three times in DPBS supplemented with 2% FBS and centrifuged through a cushion of FBS. The fixed cells were normally incubated in complete culture medium for 3-4 hours at 37°C before use to leach any possible remaining paraformaldehyde or T cell-derived soluble factors.

T Cell Plasma Membrane Preparation

The membrane isolation procedure is based on that of Maeda et al (Maeda et al. 1983). Endotoxin-free conditions were maintained throughout the procedure, using endotoxin free water and glassware, and monitoring all buffers by the
chromogenic endotoxin assay (BioWhittaker, Inc., Walkersville, MD). Resting or activated T cells (5x10⁷-1x10⁸) were resuspended in 15 ml cold homogenization buffer (20 mM Tris·Cl/10 mM NaCl/0.1 mM MgCl₂/0.1 mM phenylmethylsulfonyl fluoroide/0.5 µg of DNase I per ml). Cells were disrupted in a dounce homogenizer. The homogenate was layered over 41% (w/v) cold sucrose in homogenization buffer and the membranes were separated from soluble proteins and nuclei by centrifugation at 95,000 x g for 1 hour. The white interfacial band consisting of plasma membranes was collected and washed twice in serum-free DPBS. The membrane pellet was resuspended to 1-2 µg/ul in DPBS. The yield was normally 100-200 µg plasma membrane protein from an input of 5x10⁷-1x10⁸ T cells. The plasma membrane preparations were assayed for endotoxin contamination and were found to contain less than 0.1 pg LPS/µg membrane protein.

Cytostatic Activity Assay

The assay for macrophage cytostatic activity was described previously (Stout 1985). Briefly, the macrophages were cultured for 24 hours in 96-well microtiter plates with the stimulators as indicated in the individual experiments. The cultured macrophages were then washed three times with DPBS supplemented with 2% FBS to remove the stimulators. When the stimulators were viable T cells, the macrophages
were further treated with 20 μg/ml mitomycin-c (50 μl per well) for 30 minutes at 37°C and were subsequently rinsed with DPBS for three times. Afterward, P815 tumor cells (derived from a DBA/2 mastocytoma) were added to the macrophages at 200 μl of 2.5x10⁶/ml cells per well. The cells were incubated overnight and subsequently pulsed for 4 hours with 1 μCi/ml ³H-thymidine, harvested and counted on a Beckman LS7000 Scintillation Spectrometer. The reported percent inhibition or percent cytostatic activity is calculated by the following formula:

\[ \% \text{ inhibition} = (1 - \frac{X}{Y}) \times 100 \]

where \% inhibition is the cytostatic activity, \(X\) is the cpm ³H-thymidine incorporation of P815 in the presence of macrophages, and \(Y\) is the cpm ³H-thymidine incorporation of P815 in the absence of macrophages.

**Nitrite Assay**

Nitrite production by macrophages was detected by mixing 50-μl aliquots of culture supernatants from macrophages cultured with stimulators for 48 hours or as indicated otherwise in the individual experiments with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2.5% \(\text{H}_3\text{PO}_4\)) and incubating at room temperature for 10 minutes. The absorbance at 570 nm was measured in an automated plate reader (VMAX, Molecular Devices Corporation, Menlo Park,
CA). Nitrite concentration (expressed as nmol per microwell) was calculated from a NaN02 standard curve.

**Activation of Macrophages for mRNA Assay**

Macrophages generated in 24-well plates were incubated in complete culture medium (1 ml per well) with stimulators including plasma membranes isolated from resting T cells (40 µg per well), plasma membranes isolated from activated T cells (40 µg per well) and LPS (100 ng per well) for a period of 1-4 hours, after which the macrophages were washed twice with DPBS to remove the stimulators and were subjected to the isolation of total RNA.

**Cell Incubation**

All the cell incubations were carried out at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

**Preparation of DNA Probes**

The plasmids containing DNA for the genes examined in this study were prepared from bacterial cells according to a mini-prep procedure described by Davis et al. (Davis et al. 1988). Briefly, the bacterial cells containing plasmids were grown overnight in 50 ml of LB medium containing 50 µg/ml of appropriate antibiotics (ampicillin for all of the plasmids except the plasmid containing c-fos which is tetracycline-resistant). Cells were resuspended in 0.9 ml
of glucose buffer with addition of 0.3 ml of 8 mg/ml lysozyme solution and were incubated for 5 minutes at room temperature. Alkaline lysis buffer (2.4 ml of 0.2 M NaOH with 1% SDS) was added to the cells, which were subsequently placed on ice for 5 minutes. Ice-cold sodium acetate (1.8 ml of 3 M) was added to the cells. The cells were centrifuged at 12,000 x g at 4°C for 10 minutes. The supernate containing plasmid DNA was collected and extracted with an equal volume of mixture of phenol:chloroform:isoamylalcohol (25:24:1) (Fisher Scientific). The plasmid DNA was precipitated by 100% ice-cold ethanol (Fisher Scientific) and resuspended in 50 µl of TE buffer for further use.

The DNA inserts for the genes examined in this study were purified from the plasmids by digesting the plasmid DNA with appropriate restriction endonucleases (4-10 u of enzymes per 1 µg plasmid DNA) for 1 hour at 37°C (Davis et al. 1988). The total digestion reaction volume was 50 µl. The reaction buffers, provided by Promega, contained Tris-HCl, NaCl, MgCl₂ and DTT. The concentration of each component in the buffers slightly varied for each restriction endonuclease according to manufacturer's specifications. Murine TNFα cDNA probe was a 0.5 Kb fragment from the plasmid pUC-9 digested with EcoR I (Caput et al. 1986). Murine IL-1α cDNA probe was a 0.5 Kb fragment from the plasmid PRSV-mIL-1 digested with Bgl II. IP-10 and
D3 fragments were 0.5 Kb and 0.6 Kb, respectively and both were in the EcoR I site of the pBS plasmid (Tannenbaum et al. 1988). The JE cDNA insert with size of 0.6 kb was obtained from the plasmid pcJE-1 cleaved with EcoR I (Rollins et al. 1988). A 2.0 Kb fragment of c-Fos DNA was released from the vector pBR 322 digested with both EcoR I and Bam HI (Curran et al. 1983). A 4.8 Kb insert for c-Myc was released from the vector pSV2 digested with both Xba I and Bam HI (Grace et al. 1982). Digesting pGEM-4 plasmid containing chicken β-actin cDNA with restriction endonuclease Pst I yielded a 1.7 Kb fragment of the insert. As described by Sambrook et al (Sambrook et al. 1989), the digests were resolved by electrophoresis in a mini-gel made of 1.2% agarose (Difco Laboratories) containing 0.5 µg/ml ethidium bromide (Sigma Chemical Co.) in 1 x TAE buffer for 30-60 minutes at 8-10 Volts/CM. The DNA fragment was recovered by inserting a piece of DEAE-81 paper (Schleicher and Schuell Inc., Keene, NH) into the gel in front of the fragment desired. After the DNA of that fragment has completely migrated onto the paper, the paper was removed from the gel and was washed 2-3 times with low salt buffer (this buffer was discarded) followed with treatment of the paper with high salt buffer at 65°C for 45 minutes. The buffer was saved and was extracted 2 times with an equal volume of butanol (Fisher Scientific) to remove the ethidium bromide. DNA was subsequently precipitated with ice-cold
ethanol from the buffer, and was dried and redissolved in 20 μl dH₂O.

**Radiolabeling of DNA Probes**

The DNA inserts purified as described above were radiolabeled by random primer labeling method using the Prime-a-Gene Labeling System kit following the instructions by the supplier (Promega). Briefly, 25-50 ng of the denatured DNA to be labeled was mixed with 10 μl of 5x labeling buffer, 2 μl of a mixture of the nonlabeled dTTP, dGTP and dATP (20 μm each), 2 μl of nuclease-free BSA, 5 μl of (α-³²P)dCTP (50 μCi, 3000Ci/mmole) and 5 units klenow enzyme. The final volume was 50 μl. The mixture was incubated at room temperature for 60 minutes. The DNA probes were routinely labeled to a specific activity of >10⁹ cpm/μg.

**RNA Preparation from Macrophages**

Total RNA from macrophages was isolated by using a commercial reagent Trisolv purchased from Biotecx Laboratories, INC. (Houston, TX) according to manufacturer's instructions. Briefly, adherent macrophages generated in 24-clusterwell plates were lysed directly in the wells by adding the TRISOLV (1 ml per well, 4 wells sequentially) and were homogenized. Chloroform (0.2 ml per 1 ml of the TRISOLV) was added to the homogenate and the mixture was
vigorously shaken for 15 seconds followed by a
centrifugation at 12,000 g for 15 minutes at 4°C. The top
aqueous phase was transferred to a fresh tube and was mixed
with isopropanol (0.5 ml per 1 ml of TRISOLV used for
homogenization). After 5-10 minutes incubation of the
mixture at room temperature, total RNA was pelleted by
centrifugation at 12,000 g for 10 minutes at 4°C. The RNA
pellet was washed once with 75% ethanol before being
dissolved in diethylpyrocarbonate (DEPC) (Sigma Chemical
Co.)-treated water. Concentration of the RNA and its purity
were determined by obtaining the A260 and A280 readings
(260/280 ratio ranged from 1.6 to 2.0). The yield was
normally 5-10 μg per 1x10^6 macrophages.

**Dot Blot Hybridization**

The dot blotting method is based on that described by
Sambrook et al (Sambrook et al. 1989) with some
modification. RNA samples were denatured by mixing 50 μl of
RNA with 30 μl of 37% formaldehyde (Fisher Scientific) and
20 μl of 20 x SSC at 65°C for 15 minutes followed by
chilling the mixture on ice. Denatured RNA samples were
blotted (2 μg RNA per blot) onto the nylon membranes (Zeta-
Probe Blotting Membranes purchased from Bio-Rad
Laboratories, Richmond, CA) using a 96-well manifold (Gibco
BRL Life Technologies) applied with vacuum. The membranes
were heated in an oven at 80°C for 2 hours and were
subsequently prehybridized and hybridized according to the method described by Church and Gilbert (Church and Gilbert 1984) with some modification. Briefly, the membranes were prehybridized for 3 hours at 65°C in 0.5 M Na₂HPO₄/NaH₂PO₄, 7% SDS and 100 μg/ml denatured Herring sperm DNA. The membranes were then hybridized with denatured (α-³²P)-dCTP-labeled probes at 65°C in the same solution for 15 hours. At the end of hybridization, the membranes were washed twice for 30 minutes at room temperature in washing buffer 1. Subsequently the membranes were washed twice for 30 minutes in washing buffer 2 at 65°C. Afterward, the nylon membranes were exposed to Kodak X-OMAT AR X-ray films within the intensifying screens (Fischer Scientific) at -80°C for 2-7 days after which the films were developed and fixed in Kodak GBX developer and fixer solutions. To ensure that equivalent amounts of RNA were blotted to each well, the blots were washed in 2 x SSPE with 50 % formamide (Fisher Scientific) to strip the probes off the membranes and rehybridized with the probe for β-actin.
CHAPTER 3
Results

Viable T\(_{\text{h}}\)2 Cell-Mediated Induction of Macrophage Nitrite Production

D10.G4 is a T\(_{\text{h}}\)2 clone which does not secrete IFN\(\gamma\). This clone recognizes conalbumin presented on I\(-\)A\(^k\) and is also alloreactive with I\(-\)A\(^b\) (Janeway et al. 1982). To assess the ability of D10.G4 to induce macrophage nitrite production, viable resting D10.G4 (4x10\(^4\)/well) were cultured with I\(-\)A\(^k\)-positive macrophages in the presence or absence of conalbumin. Nitrite production was induced only in those cultures to which both conalbumin and IFN\(\gamma\) (50 u/ml) were added (Fig. 2). The amount of nitrite produced increased from 0.635 ± 0.03 to 4.988 ± 0.84 nmol/well as the concentration of conalbumin was increased from 1-1000 \(\mu\)g/ml.

To examine the MHC specificity of D10.G4-mediated induction of macrophage nitrite production, the ability of D10.G4 and another T\(_{\text{h}}\)2 clone, AK8, to activate I\(-\)A\(^b\) macrophages was tested. Unlike D10.G4, AK8 is not alloreactive with I\(-\)A\(^b\). Nitrite production was induced in I\(-\)A\(^b\) macrophages cultured with IFN\(\gamma\) and D10.G4 T cells but not in I\(-\)A\(^b\) macrophages cultured with IFN\(\gamma\) and AK8 T cells (Fig. 3). The amount of nitrite produced increased from 3.99 ± 0.54 to 6.23 ± 0.88 nmol/well as the number of D10.G4 cells added per microwell to the cultures increased from 1 to 8x10\(^4\).
To determine if Th2 cells could stimulate nitrite production with kinetics similar to that stimulated by LPS, nitrite accumulation was monitored during a 48 hour period in cultures of C57BL/6J macrophages stimulated with either LPS (100 ng/ml) or viable resting D10.G4 (4x10⁴/well) in the presence or absence of 50 u/ml IFNγ. Nitrite was detected in culture supernates of macrophages incubated for 48 hours in the presence of both LPS and IFNγ (4.15 ± 0.68 nmol/well) or in the presence of both D10.G4 and IFNγ (4.44 ± 0.42 nmol/well) (Fig. 4). Nitrite production was about 4 fold lower in macrophage cultures which did not contain IFNγ or which contained IFNγ alone (in the absence of LPS or D10.G4). Nitrite accumulation after 24 hours incubation was approximately 5 fold lower than accumulation after 48 hours incubation of macrophages cultured with IFNγ plus either LPS or D10.G4 (Fig. 4). In addition, it is known that induction of macrophage nitrite production by LPS is mediated through a L-arginine-dependent pathway which could be blocked by NG-nitromonomethyl-L-arginine (NGMMA) (Green et al. 1991). To determine if T cell-mediated nitrite production shares the same pathway, L-arginine analog, NGMMA, was added to the cultures containing viable D10.G4, IFNγ and C57BL/6J macrophages. NGMMA, at the concentration of 5 mM, completely blocked the nitrite production induced by D10.G4 or by LPS (Fig. 5).
Induction of Macrophage Nitrite Production by Supernatant From Activated T\(_{\text{H}2}\) Cells

It has been previously demonstrated that the cytokines produced by T\(_{\text{H}2}\) cells could not activate macrophages effector functions even in the presence of exogenous IFN\(\gamma\) (Stout and Bottomly 1989, Mosmann and Coffman 1989). To determine if the T\(_{\text{H}2}\) cytokines can induce macrophage nitrite production, the supernatant from D10.G4 or AK8 stimulated with concanavalin (conA) for 24 hours was cultured with C57BL/6J macrophages for 48 hours in the presence of IFN\(\gamma\). Nitrite production was induced only in the macrophages cultured with undiluted supernatant (Table 4). Supernatant from D10.G4 was more effective than supernatant from AK8 in inducing the nitrite production. However, the amount of nitrite induced by supernatant from either D10.G4 or AK8 was about 3 times less and 6 times less, respectively, than that induced by LPS. It was also observed that the ability of the supernatant to induce macrophage nitrite production was completely diminished by a serial 2-fold dilution of the supernatant ranging from 1:2 to 1:16 (Table 4).

Induction of Macrophage Nitrite Production By Paraformaldehyde-Fixed T\(_{\text{H}2}\) Cells

The interaction between viable T\(_{\text{H}2}\) cells and the antigen-presenting macrophages results in both T cell
activation (Stout and Bottomly 1989) and macrophage activation as demonstrated above. It is known that activation of T cells is an antigen-specific, MHC-restricted event (Stout and Bottomly 1989, Unanue 1984). It is possible that the antigen-dependent activation of macrophages by T cells reflects that T cells need to be activated before they can activate macrophages. To test this hypothesis, resting T\textsubscript{H}2 cells and T\textsubscript{H}2 cells activated by immobilized anti-CD3 were fixed by 0.4% paraformaldehyde. The fixed T cells were cultured with I-A\textsuperscript{k} macrophages in the presence or absence of conalbumin or with I-A\textsuperscript{b} macrophages. Nitrite production was induced in the I-A\textsuperscript{k} macrophages cultured with fixed activated D10.G4 cells (4x10^5/well) in the presence of IFN\textgamma regardless of the presence or absence of antigen-conalbumin (Fig. 6). The amount of nitrite detected in the culture supernates of macrophages stimulated by fixed activated D10.G4 and IFN\textgamma in the presence of conalbumin (100 \mu g/ml) was about the same as in the supernates of macrophages stimulated by fixed activated D10.G4 and IFN\textgamma in the absence of conalbumin (2.901 ± 0.780 versus 2.948 ± 0.540 nmol/well). Nitrite production was not induced in the IFN\textgamma-primed macrophages stimulated by fixed resting D10.G4 cells (Fig. 6). Addition of conalbumin to the culture containing fixed resting D10.G4 and IFN\textgamma-primed macrophages did not increase the nitrite production by the macrophages. I-A\textsuperscript{k} macrophages were also incubated with an
increasing number of fixed activated D10.G4 cells per well in the presence or absence of conalbumin (Fig. 7). The amount of nitrite induced increased from 1.964 ± 0.07 to 4.112 ± 0.16 nmol/well in the supernates of macrophages cultured with fixed activated D10.G4 and IFNγ in the absence of conalbumin. As the T cell number per well increased from 1 to 8x10⁴, nitrite concentration increased from 1.324 ± 0.24 to 3.766 ± 0.34 nmol/well in the supernates of macrophages cultured with fixed activated D10.G4 and IFNγ in the presence of conalbumin (Fig. 7). The presence of conalbumin (100 µg/ml) in the cultures had no effect on the ability of fixed activated D10.G4 to activate the macrophages in the presence of IFNγ (50 u/ml). To examine the activation of macrophages by fixed resting Th2 versus fixed activated Th2 in the alloreactive system, IFNγ-primed I-Aᵇ macrophages were cultured with fixed resting or activated D10.G4 and AK8 cells. In contrast to the results obtained with viable resting D10.G4 cells (Fig. 3), fixed resting D10.G4 cells did not induce nitrite production in the IFNγ-primed I-Aᵇ macrophages, but fixed activated D10.G4 cells stimulated nitrite production (from 3.393 ± 0.45 to 5.008 ± 0.07 nmol/well as cell number increased from 2 to 8x10⁴/well (Fig. 8). In contrast to the results obtained with viable resting AK8 cells (Fig. 3), fixed activated AK8 effectively induced nitrite production in the IFNγ-primed I-Aᵇ macrophages (from 3.383 ± 0.18 to 5.415 ± 0.51 nmol/well
as cell number increased from 2 to 8x10^4/well) (Fig. 8). The amounts of nitrite induced in the IFNγ-primed macrophages stimulated by fixed activated D10.G4 or fixed activated AK8 were comparable (Fig. 8). The amount of nitrite detected in the supernate of the IFNγ-primed macrophages cultured with medium alone was 2.37 ± 0.17 nmol/well. Nitrite was not detected in the culture supernates of macrophages cultured with fixed resting or activated T cells in the absence of IFNγ.

Fresh T Cell-Mediated Induction of Macrophage Nitrite Production.

To determine if fresh T cells, as D10.G4 and AK8 T cell clones, can also induce macrophage nitrite production, C57BL/6J (I-A^b positive) macrophages were cultured with viable or fixed fresh splenic T cells purified from C57BL/6J and Balb/C mice. 4-8x10^5 viable Balb/C T cells/well (I-A^d positive) induced the macrophages to produce 3.531 ± 0.283 to 5.520 ± 1.493 nmol nitrite per well (Fig. 9), whereas the syngeneic C57BL/6J T cells did not induce any detectable amount of nitrite in C57BL/6J macrophages. To determine if the activated fresh T cells, as activated T_h^2 clones, do not require the presence of alloantigen in activation of macrophages, C57BL/6J and Balb/C T cells were activated by immobilized anti-CD3 and were subsequently fixed by 0.4% paraformaldehyde. Fixed activated syngeneic C57BL/6J T
cells and fixed activated allogeneic Balb/C T cells (4×10^5/well) induced equivalent levels of nitrite (1.986 ± 0.091 nmol/well and 2.121 ± 0.605 nmol/well, respectively) in C57BL/6J macrophages in the presence of IFNγ (Fig. 9). At 8×10^5/well, fixed activated C57BL/6J T cells induced more nitrite than fixed activated Balb/C T cells in the C57BL/6J macrophages (3.864 ± 0.304 versus 2.9 ± 0.161 nmol/well) in the presence of IFNγ. It is noted that the viable Balb/C T cells stimulated macrophage nitrite production in the absence of IFNγ, while fixed activated T cells induced nitrite production only in the presence of IFNγ (Fig. 9). Fixed resting C57BL/6J or Balb/C T cells did not activate IFNγ-primed macrophages to produce nitrite (data not shown).

**Induction of Macrophage Nitrite Production by Plasma Membranes From T_H2 Cells.**

To exclude the possibility of cytokines leaching from the fixed cells, the ability of isolated plasma membranes from T_H2 cells to activate macrophages was determined. Plasma membranes were isolated either from resting or from activated D10.G4 T cells and then added to I-A^b macrophage cultures in the presence or absence of IFNγ. Plasma membranes from activated D10.G4 T cells induced nitrite production in the macrophages in the presence of 50 u/ml IFNγ. The amount of nitrite produced increased from 1.097 ± 0.024 to 3.385 ± 0.181 nmol/well as the amount of plasma
membrane protein added per well was increased from 1-4 μg (Fig. 10). In contrast, membranes from resting D10.G4 T cells did not induce detectable nitrite production in the IFNγ-primed macrophages, agreeing with the results obtained with fixed resting D10.G4 cells. Nitrite production was not detected in macrophages cultured with either membrane preparation in the absence of IFNγ (Fig. 10). To analyze the antigen specificity and MHC restriction of the interaction between the macrophages and the T cell membrane preparations, I-A<sup>b</sup> macrophages were cultured with plasma membranes from either resting or from activated AK8 T cells. In agreement with the results obtained with fixed activated AK8 T cells, the plasma membranes of activated AK8 did induce significant nitrite production in IFNγ-primed macrophages in a dose-dependent fashion (1.45 ± 0.507 to 6.353 ± 0.309 nmol/well for 1 to 8 μg per well of membrane protein) (Fig. 11). As with membranes isolated from resting D10.G4 T cells, the membranes from resting AK8 T cells did not induce nitrite production in IFNγ-primed macrophages. Neither membrane preparation induced nitrite production in the absence of IFNγ (Fig. 11). To corroborate the apparent lack of antigen specificity displayed by membranes from activated AK8 cells, membranes isolated from activated D10.G4 cells were cultured with I-A<sup>k</sup>-positive macrophages in the presence or absence of conalbumin. As was observed with AK8 cells, the membranes of activated D10.G4 cells could
induce nitrite production in the absence of antigen (Fig. 12).

**Induction of Macrophage Cytostatic Activity by Fixed T Cells and Plasma Membranes**

Reactive nitrogen intermediates contribute to the macrophage cytostatic and cytotoxic activities which are the basis of macrophage anti-microbial and anti-tumor functions (Green et al. 1991, Kolb and Kolb-Bachofen 1992). To determine if macrophage cytostatic activity could be induced by T cells in a manner similar to the macrophage nitrite production induced by T cells, paraformaldehyde-fixed resting D10.G4 and activated D10.G4 were incubated with C57BL/6J macrophages for 24 hours. The macrophage cytostatic activity against the proliferation of tumor cell line P815 was measured. As demonstrated in Table 5, the proliferation of P815 was greatly inhibited by macrophages cultured with fixed activated D10.G4 T cells plus IFNγ (87% cytostatic activity) or LPS plus IFNγ (88% cytostatic activity). In contrast, the proliferation of P815 was only slightly inhibited by the macrophage cultured with fixed resting D10.G4 T cells plus IFNγ (26% cytostatic activity). Macrophages cultured with fixed resting or activated T cells alone did not exhibit cytostatic activity against P815. Macrophages cultured with LPS alone or IFNγ alone showed only low levels of cytostatic activity (16% for LPS and 17%
for IFNγ). To analyze the antigen specificity and examine the activation of macrophage cytostasis by fresh T cells, splenic T cells were obtained from C57BL/6J and DBA/2J inbred strains of mice. These T cells were activated by immobilized anti-CD3 and fixed by 0.4% paraformaldehyde. Both fixed activated C57BL/6J T cells (I-Ab positive) and fixed activated DBA/2J (I-Ad positive) T cells activated C57BL/6J macrophages (I-Ab positive) to express cytostatic activity in the presence of IFNγ (60% and 66%, respectively) regardless of the alloreactivity between the T cells and macrophages (Table G). Again, the fixed resting C57BL/6J or DBA/2J T cells did not induce significant cytostatic activity from the macrophages (< 10%). LPS or IFNγ alone did not induce detectable cytostatic activity. The combination of LPS and IFNγ elicited 61% cytostatic activity from the macrophages. The macrophages cultured with fixed activated C57BL/6J or DBA/2J T cells in the absence of IFNγ displayed low level of cytostatic activity (25% and 21%, respectively) (Table 6). To demonstrate conclusively that the physical contact (cognate interaction) between T cells and macrophages is involved in activation of macrophage cytostasis, plasma membranes isolated from anti-CD3-activated D10.G4 T cells were used to stimulate macrophages in the presence or absence of IFNγ. As was expected, the plasma membranes from activated D10.G4 T cells effectively induced macrophage cytostatic activity in the presence of
IFNγ (Table 7).

Inhibition of T\textsubscript{H2} Cell-Mediated Induction of Macrophage Nitrite Production by Anti-CD4

CD4 is the T cell receptor co-receptor which binds to the nonpolymorphic regions of class II MHC molecules (Janeway et al. 1989). Both TcR and CD4 are believed to interact with the same MHC molecules during the activation of T cells by antigen-presenting macrophages (Janeway et al. 1989). The data presented in this study have suggested that interaction between TcR and the antigen-presenting class II MHC molecules are required for T cell activation but are not essential for macrophage activation. To examine this phenomenon further, monoclonal antibody against CD4 was added to the cultures containing macrophages and the T\textsubscript{H2} cells. As shown in Figure 13, anti-CD4 significantly inhibited the induction of macrophage nitrite production by viable D10.G4 T cells (39% inhibition by a 1:40 dilution of anti-CD4 and 53% by a 1:20 dilution of anti-CD4). In contrast, anti-CD4 did not inhibit the fixed activated D10.G4 T cell-mediated induction of macrophage nitrite production (Fig. 13).

Comparison of T\textsubscript{H1} and T\textsubscript{H2} in Induction of Macrophage Nitrite Production

The data presented above have clearly demonstrated a
role of cognate interaction in T cell-mediated macrophage activation. It is unknown which surface components on activated T cells contribute to the cognate signaling of macrophages. Several studies have suggested that membrane TNFα is involved in activation of macrophage effector functions. Membrane TNFα is known to exist on Th1 cells (Liu et al. 1989, Birkland et al. 1992), but has not been reported to be present on Th2 cells. To determine if membrane TNFα plays an important role in cognate activation of macrophages, the ability of fixed activated Th1 and Th2 cells in inducing macrophage nitrite production was examined. It is noted that equal numbers of fixed activated Th1 (J6-19) and Th2 (D10.G4 and AK8) induced approximately the same magnitude nitrite production in macrophages primed by IFNγ (Fig. 14).

Induction of Nitrite Production by TNFα

Although the results from the current study appear to exclude membrane TNFα as an effector of T cell-mediated macrophage activation, numerous studies have demonstrated an important role of endogenous TNFα produced by macrophages in activation of macrophage effector functions (Green et al. 1991, Stout and Suttles 1992). A role of TNFα in inducing splenic macrophage nitrite production was demonstrated in the current study. Nitrite production increased as the recombinant TNFα increased from 30 u/ml to 1000 u/ml (Table
8). The induction was dependent on the presence of IFNγ in the macrophage cultures since TNFα (from 30 u/ml to 1000 u/ml) alone did not induce detectable nitrite (data not shown). However, the amount of nitrite induced by TNFα was considerably less than that induced by LPS (Table 8).

**Titration of LPS in Induction of Macrophage Nitrite Production**

It has been realized that a trace amount of endotoxin (also known as LPS) can trigger some biological activities from macrophages. In order to assess the role of the possible contaminating endotoxin in the culture medium and the plasma membrane preparations in the T cell-mediated induction of macrophage nitrite production, LPS was titrated from 1 pg/ml to 10 μg/ml to determine the lowest concentration of LPS that could synergize with IFNγ to activate macrophages. As shown in Figure 14, optimal induction of nitrite production occurred only when both IFNγ and LPS were added to the macrophages. A synergistic effect was observed in the macrophages cultured with IFNγ (50 u/ml) and LPS ranging from > 100 pg/ml to 10 μg/ml (Fig. 14). The chromogenic limulus assay for endotoxin revealed that the level of contaminating LPS in the medium or in the plasma membrane preparations was far below the lowest concentration of LPS necessary to synergize with IFNγ in induction of macrophage nitrite production (data not shown).
Finally, to investigate the intracellular events initiated in macrophages by cognate T cell-macrophage interaction, plasma membranes isolated from D10.G4 cells were cultured with macrophages for 1 to 4 hours. The expression of mRNA for a panel of early activation genes by macrophages were examined by dot blot hybridization. These genes are proto-oncogenes c-Myc and c-Fos, cytokine genes TNFα and IL-1α, macrophage inflammatory protein genes or LPS-responsive genes JE, IP-10 and D3. The results of two repeated experiments are shown in Fig. 16. One hour after treatment of the macrophages with the plasma membranes (40 µg/ml) or LPS (100 ng/ml), expression of the mRNA for all genes examined was induced in macrophages treated with LPS or with plasma membranes from activated D10.G4 T cells (Fig. 16 A). The expression of mRNA for c-myc, c-fos and D3 was much weaker but still detectable in the macrophages treated with plasma membranes from resting D10.G4 T cells. In macrophages cultured in medium alone, the mRNA for c-myc and D3 was weakly expressed. It is noted that the intensity of the blot representing the mRNA for each gene examined varies from one probe to another in the macrophages treated with LPS or with the plasma membranes from activated D10.G4 T cells. For example, the intensity of the blots for TNFα, c-myc and c-fos is much stronger than the blots for D3, IL-1α,
JE and IP10. This variance is probably due to some experimental variations in this study such as the different radio-labeling efficiencies of each DNA probe rather than the differential regulation of the expression of mRNA among these genes. The expression of mRNA for TNFα, IL-1α, c-myc, JE and IP10 continued after 4 hours of treatment of macrophages with plasma membranes from activated D10.G4 T cells and LPS (Fig. 16 B). Particularly, the accumulation of mRNA for IL-1α and JE increased in both plasma membrane-treated and LPS-treated macrophages. With the exception of mRNA for IL-1α, the mRNA for all other genes examined was not detected in macrophages treated with plasma membranes from resting D10.G4 T cells or in macrophages cultured with medium alone. Surprisingly, the mRNA for c-myc disappeared in the macrophages treated with LPS for 4 hours. The expression of mRNA for c-myc appeared to decrease in macrophages treated with plasma membranes from activated D10.G4 T cells for 4 hours (Fig. 16 B). The expression of mRNA for c-fos and D3 in the macrophages cultured for 4 hours was not examined in this experiment.
Table 3  Antigen Specificity of $T_{H2}$ Clones

<table>
<thead>
<tr>
<th>$T_{H2}$ Clones</th>
<th>Antigen Specificity</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Conalb-Ia$^k$</td>
</tr>
<tr>
<td>D10.G4</td>
<td>+</td>
</tr>
<tr>
<td>AK8</td>
<td>+</td>
</tr>
</tbody>
</table>
Antigen-specific induction of macrophage nitrite production by T^2 cells. CAF1/J MΦ (I-A^k +) were cultured for 48 hours with \(4 \times 10^4\)/well viable resting D10.G4 T cells in the presence or absence of 50 u/ml IFNγ and graded doses of antigen-conalbumin. The supernates of the cultures were subsequently assayed for nitrite production. Each point represents the average of triplicate cultures ± S.D.
Figure 3

Induction of nitrite production by allogeneic interactions between TH2 cells and macrophages. C57BL/6J Mφ (I-Ab +) were cultured for 48 hours with the indicated numbers of viable resting D10.G4 or AK8 TH2 cells in the presence or absence of 50 u/ml IFNγ. The supernates of the cultures were assayed for nitrite production. Each point represents the average of triplicate cultures ± S.D.
Comparison of T<sub>h</sub>2 cells and LPS in induction of macrophage nitrite production. C57BL/6J Mϕ were cultured for 24 or 48 hours with medium alone, IFNγ (50 u/ml) alone, LPS (100 ng/ml) alone, viable resting D10.G4 T cells (4x10<sup>4</sup>/well) alone, or a combination of IFNγ plus either LPS or T cells. The supernates of the cultures were subsequently assayed for nitrite production. Each point represents the average of triplicate cultures ± S.D.
Blocking of T cell- or LPS-mediated nitrite production by NG-monomethyl-L-arginine. C57BL/6J Mφ were cultured for 48 hours with viable resting D10.G4 T cells (4x10^4/well) plus IFNγ (50 u/ml) or with LPS (100 ng/ml) plus IFNγ in the presence or absence 5 mM NG-monomethyl-L-arginine (NGMMA). The supernates of the cultures were assayed for nitrite production. Each point represents the average of triplicate cultures ± S.D.
Table 4  Effect of TH2 Supernatant On Macrophage Nitrite Production

<table>
<thead>
<tr>
<th>Dilutions</th>
<th>D10.G4 Supernatant</th>
<th>AK8 Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Undiluted)</td>
<td>1.803 ± 0.123</td>
<td>0.992 ± 0.076</td>
</tr>
<tr>
<td>1:2</td>
<td>0.590 ± 0.066</td>
<td>0.359 ± 0.051</td>
</tr>
<tr>
<td>1:4</td>
<td>0.405 ± 0.144</td>
<td>0.164 ± 0.066</td>
</tr>
<tr>
<td>1:8</td>
<td>0.195 ± 0.037</td>
<td>0.303 ± 0</td>
</tr>
<tr>
<td>1:16</td>
<td>0.108 ± 0.062</td>
<td>0.098 ± 0</td>
</tr>
</tbody>
</table>

Supernatants from 24 hour concanavalin A (conA)-stimulated D10.G4 and AK8 T cells were cultured for 48 hours with Mφ in the presence of 50 u/ml recombinant IFNγ. The supernates of the cultures were subsequently assayed for nitrite production. The results from a representative of more than three experiments are presented above. Nitrite production was not detected in the supernate of Mφ cultured with medium alone. Mφ treated with IFNγ (IFNγ-primed Mφ) produced nitrite of 0.536 ± 0.342 nmol/well. IFNγ-primed Mφ treated with LPS (50 ng/ml) produced nitrite of 5.872 ± 0.534 nmol/well. Each value represents the average of triplicate cultures ± S.D.
Induction of macrophage nitrite production by fixed activated Th2 cells but not by fixed resting Th2 cells. CAF1/J Mφ were cultured for 48 hours with paraformaldehyde-fixed resting or activated (Act.) D10.04 T cells (4x10⁶/well) in the presence or absence of 100 μg/ml antigen (Ag) and/or IFNγ (50 u/ml). The supernates of the cultures were subsequently assayed for nitrite production. Each point represents the average of triplicate cultures ± S.D.
Antigen-nonspecific induction of macrophage nitrite production by fixed activated T\textsubscript{H}2 cells. CAF1/J M\textsuperscript{Φ} were cultured for 48 hours with IFN\textgamma and the indicated numbers of paraformaldehyde-fixed activated D10.G4 T cells in the presence or absence of antigen (100 \mu g/ml). The supernates of the cultures were subsequently assayed for nitrite production. Each point represents the average of triplicate cultures \pm S.D.
MHC non-specific induction of macrophage nitrite production by fixed activated T\(_h\)2 cells. IFN\(\gamma\)-primed C57BL/6J (I-\(A^b\) +) M\(\phi\) were cultured for 48 hours with the indicated numbers of fixed resting or activated D10.G4 T cells (alloreactive with I-\(A^b\) MHC molecules and AK8 (not alloreactive with I-\(A^b\)). The supernates of the cultures were subsequently assayed for nitrite production. Each point represents the average of triplicate cultures ± S.D.
Induction of macrophage nitrite production by splenic T cells. C57BL/6J Mφ were cultured for 48 hours with viable splenic C57BL/6J T cells or viable splenic Balb/C (I-A^d +) T cells. The Mφ were also cultured with paraformaldehyde-fixed activated C57BL/6J or Balb/C splenic T cells in the presence of 100 u/ml IFNγ. The supernates of the cultures were subsequently assayed for nitrite production. Each point represents the average of triplicate cultures ± S.D.
Activation of macrophages by plasma membranes from activated D10.G4 T cells. C57BL/6J Mφ were cultured for 48 hours with the indicated amount of plasma membranes (PM) isolated from activated or resting D10.G4 T cells in the presence or absence of 50 u/ml IFNγ. The culture supernates were assayed for nitrite production. Each point represents the average of triplicate cultures ± S.D.
Activation of I-A$^b$ macrophages by plasma membranes from activated AK8 T cells. C57BL/6J Mφ were cultured for 48 hours with the indicated amount of plasma membranes isolated from activated or resting AK8 T cells in the presence or absence of 50 u/ml IFNγ. The culture supernates were assayed for nitrite production. Each point represents the average of triplicate cultures ± S.D.
Antigen-independence of macrophage activation by plasma membranes from activated D10.G4 T cells. CAF1/J MΦ were cultured for 48 hours with medium alone, plasma membranes (4 μg/well) from activated D10.G4 T cells alone, IFNγ (50 u/ml) alone or plasma membranes in the presence of IFNγ with or without antigen-conalbumin (100 μg/ml). The supernates of the cultures were assayed for nitrite production. Each point represents the average of triplicate cultures ± S.D.
Table 5 Activation of Mφ Cytostatic Activity by Fixed T<sub>H</sub>2-D10.G4 Cells

<table>
<thead>
<tr>
<th>Activators</th>
<th>- IFNγ</th>
<th>+ IFNγ</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>40565 ± 726</td>
<td>33801 ± 4412 (17%)</td>
</tr>
<tr>
<td>Fixed resting D10.G4</td>
<td>46905 ± 1152 (0%)</td>
<td>30138 ± 5114 (26%)</td>
</tr>
<tr>
<td>Fixed activated D10.G4</td>
<td>42230 ± 1070 (0%)</td>
<td>5214 ± 259 (87%)</td>
</tr>
<tr>
<td>LPS</td>
<td>34238 ± 5068 (16%)</td>
<td>4815 ± 483 (88%)</td>
</tr>
</tbody>
</table>

Paraformaldehyde-fixed resting or activated D10.G4 T cells (4x10⁴/well) were cultured with C57BL/6J Mφ in the presence or absence of 50 u/ml IFNγ for 24 hours. Macrophages were subsequently assayed for cytostatic activity against the growth of P815. LPS as a positive control was added to Mφ at 100 ng/ml. Percentage cytostasis indicated within the parenthesis is based on the CPM of P815 on non-stimulated Mφ. Each value represents the average of triplicate cultures ± S.D.
Table 6  Activation of Mφ Cytostatic Activity by Fixed Splenic T Cells

<table>
<thead>
<tr>
<th>Activators</th>
<th>- IFNγ</th>
<th>+ IFNγ</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>105910 ± 5254</td>
<td>122799 ± 6306</td>
</tr>
<tr>
<td>Fixed Resting T Cells From C57BL/6J Mice</td>
<td>98397 ± 3429 (7%)</td>
<td>108525 ± 5521 (0%)</td>
</tr>
<tr>
<td>Fixed Activated T Cells From C57BL/6J Mice</td>
<td>80699 ± 2964 (24%)</td>
<td>42499 ± 3295 (60%)</td>
</tr>
<tr>
<td>Fixed Resting T Cells From DBA/2J Mice</td>
<td>79695 ± 1098 (25%)</td>
<td>96998 ± 6667 (8%)</td>
</tr>
<tr>
<td>Fixed Activated T Cells From DBA/2J Mice</td>
<td>83361 ± 2158 (21%)</td>
<td>35877 ± 4668 (66%)</td>
</tr>
<tr>
<td>LPS</td>
<td>115570 ± 7429 (0%)</td>
<td>41474 ± 3256 (61%)</td>
</tr>
</tbody>
</table>

Paraformaldehyde-fixed splenic T cells (5x10^5/well) were cultured with C57BL/6J Mφ in the presence and absence of 100 u/ml IFNγ for 24 hours. Macrophages were subsequently assayed for cytostatic activity against the growth of P815. LPS as a positive control was added to Mφ at 100 ng/ml. Percentage cytostasis indicated within the parenthesis is based on the CPM of P815 on non-stimulated Mφ. Each value represents the average of triplicate cultures ± S.D.
### Table 7: Activation of Macrophage Cytostatic Activity by Plasma Membranes from Activated D10.G4

<table>
<thead>
<tr>
<th>Activators</th>
<th>Concentration</th>
<th>CPM of P815 Cultured with Mφ</th>
<th>- IFNγ</th>
<th>+ IFNγ</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>33028 ± 830</td>
<td>26534 ± 2780 (20%)</td>
</tr>
<tr>
<td>PM</td>
<td>1 μg/well</td>
<td></td>
<td>25816 ± 886 (22%)</td>
<td>8199 ± 334 (75%)</td>
</tr>
<tr>
<td>PM</td>
<td>2 μg/well</td>
<td></td>
<td>26460 ± 627 (20%)</td>
<td>1662 ± 150 (95%)</td>
</tr>
<tr>
<td>LPS</td>
<td>100 ng/ml</td>
<td></td>
<td>30108 ± 2734 (9%)</td>
<td>1330 ± 176 (96%)</td>
</tr>
</tbody>
</table>

Plasma membranes (PM) isolated from activated D10.G4 T cells were cultured with Mφ in the presence or absence of 50 u/ml IFNγ for 24 hours. Macrophages were subsequently assayed for cytostatic activity against the growth of P815. Percentage cytostasis indicated within the parenthesis is based on the CPM of P815 on non-stimulated Mφ. Each value represents average of triplicate cultures ± S.D.
Inhibition of viable D10.G4 T cell-mediated macrophage activation by anti-CD4. C57BL/6J Mφ were cultured with or without anti-CD4 antibody for 48 hours with viable resting D10.G4 (4x10^4/well) or fixed activated D10.G4 T cells (4x10^4/well) in the presence of IFNγ (50 u/ml). The supernates of the cultures were assayed for nitrite production. Each point represents the average of triplicate cultures ± S.D.
Comparison of fixed activated T\(_h\),1 and T\(_h\),2 cells in activation of macrophages. C57BL/6J M\(\phi\) were cultured for 48 hours with IFN\(\gamma\) (50 u/ml) alone, fixed activated T\(_h\),2 D10.G4 or AK8 (4x10^4/well) plus IFN\(\gamma\) and fixed activated T\(_h\),1 J6-19 (4x10^4/well) plus IFN\(\gamma\). The supernates of the cultured were subsequently assayed for nitrite production. Each point represents the average of triplicate cultures ± S.D.
Table 8  Induction of Nitrite Production From IFN\(\gamma\)-Primed \(\Phi\) by TNF\(\alpha\)

<table>
<thead>
<tr>
<th>Stimulators</th>
<th>Concentration</th>
<th>Nitrite Produced (nmol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0.289 (\pm) 0.057</td>
</tr>
<tr>
<td>TNF(\alpha)</td>
<td>1000 u/ml</td>
<td>3.709 (\pm) 0.189</td>
</tr>
<tr>
<td></td>
<td>300 u/ml</td>
<td>2.660 (\pm) 0.670</td>
</tr>
<tr>
<td></td>
<td>100 u/ml</td>
<td>1.569 (\pm) 0.093</td>
</tr>
<tr>
<td></td>
<td>30 u/ml</td>
<td>1.421 (\pm) 0.289</td>
</tr>
<tr>
<td>LPS</td>
<td>50 ng/ml</td>
<td>8.852 (\pm) 0.936</td>
</tr>
</tbody>
</table>

Recombinant TNF\(\alpha\) of different concentrations was cultured with IFN\(\gamma\)-primed \(\Phi\) for 48 hours. The supernates of the cultures were subsequently assayed for nitrite production. Nitrite was not detected in the supernatant of \(\Phi\) cultured with TNF\(\alpha\) alone. Each point represents the average of triplicate cultures \(\pm\) S.D.
Figure 15

Titration of LPS in induction of macrophage nitrite production. C57BL/6J Mφ were cultured for 48 hours with the graded concentrations of LPS in the presence or absence of 50 μg/ml IFNγ. The supernates of the cultures were assayed for nitrite production. Each point represents the average of triplicate cultures ± S.D.
Figure 16

Induction of macrophage "early gene" expression by plasma membranes from D10.G4 T cells. Results from two similar experiments are presented. C57BL/6J Mφ were cultured for 1 hour (Figure 16 A) or 4 hours (Figure 16 B) with medium alone, plasma membrane from resting D10.G4 T cells (PMr, 40 μg/cluster well), plasma membranes from activated D10.G4 T cells (PMa, 40 μg/cluster well), and LPS (100 ng/ml). Total RNA were isolated from the macrophages and dot blotted to nylon membranes which were subsequently hybridized with the indicated 32p-labeled DNA probes and exposed to X-ray films.
<table>
<thead>
<tr>
<th>A.</th>
<th>PM₁</th>
<th>PM₀</th>
<th>LPS</th>
<th>Media</th>
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<tbody>
<tr>
<td>TNFα</td>
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<td>IL-1α</td>
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<td>c-Myc</td>
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<td>c-Fos</td>
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<tr>
<td>β-Actin</td>
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<tr>
<td>A.</td>
<td>PM₁</td>
<td>PM₀</td>
<td>LPS</td>
<td>Media</td>
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<tr>
<td>Protein</td>
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<td>PM₂</td>
<td>LPS</td>
<td>Media</td>
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<tr>
<td>TNFα</td>
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<tr>
<td>β-Actin</td>
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CHAPTER 4
DISCUSSION

Macrophages are major participants in host septic and nonseptic inflammatory reactions (Lewis and McGee 1992). In these reactions, macrophages are activated to become cytostatic/cytotoxic against microbial pathogens and normal tissue cells. Recently, reactive nitrogen intermediates have been shown to be the major mediators of macrophage cytostatic/cytotoxic activities (Green et al. 1991). Numerous studies have demonstrated that induction of macrophage cytostatic/cytotoxic effects and RNI production results from the exposure of macrophages to at least two stimuli sequentially (Adams and Hamilton 1984). IFNγ, a T cell-derived cytokine, provides a priming signal to macrophages. Microbial products such as LPS provide the triggering signal. These studies have clearly established the involvement of LPS in macrophage activation during septic inflammatory reactions. On the other hand, in nonseptic situations such as autoimmune inflammation, cutaneous hypersensitivity and antitumor immune responses, the microbial products may not be present. The second signal that is normally required to synergize with IFNγ in macrophage activation is not defined. Therefore the mechanisms by which macrophage effector functions are induced in the nonseptic inflammation are not fully
understood.

It has been realized for some time that T cells play a key role in the induction of macrophage cytostatic/cytotoxic activity and RNI production. With the demonstration of existence of two types of CD4+ T helper cells (Table 9), it was further revealed that it was predominantly the IFNγ-producing T\textsubscript{h1} cells that mediate the induction of cell-mediated reactions and macrophage activation (Mosmann and Coffman 1989, Stout and Bottomly 1989). T\textsubscript{h2} cells, which do not secrete IFNγ, cannot activate macrophages and therefore provide a good model for studying other T cell factors such as the cell-cell contact (cognate interaction) between T cells and macrophages in macrophage activation.

T\textsubscript{h2} cells were able to activate macrophage nitrite production if recombinant IFNγ was provided in the cultures (Figure 2-4). A similar observation was made previously by Stout and Bottomly (1989) that T\textsubscript{h2} cells induced macrophage cytostatic function in the presence of supernatant from T\textsubscript{h1} cells. They also demonstrated that supernatant from T\textsubscript{h2} cells could not activate macrophage cytostasis. Supernatant from T\textsubscript{h2} cells did not induce any nitrite production from the IFNγ-primed macrophage except in the case of undiluted supernatant which could induce a small amount of nitrite from the macrophages (Table 4). A possible explanation is that the supernatant contained a very low quantity of
certain macrophage activating factor(s) which biological effects could be rapidly diluted out. Since the amount of nitrite induced by the undiluted supernatant was much smaller than the amount of nitrite induced by either LPS or T_H^2 cells, the soluble factors secreted by T_H^2 cells played no significant role in T_H^2 cell-mediated macrophage activation. On the contrary, some of the cytokines secreted by T_H^2 cells such as IL-10 and IL-4 have been known to inhibit the induction of macrophage RNI generation. It is therefore surprising that viable T_H^2 cells, which were presumably activated to secrete the cytokines upon interacting with the antigen-presenting macrophages, could still induce RNI production from IFNγ-primed macrophages very effectively. The most likely explanation of this paradox is that in order to exert inhibitory effects, these cytokines need to be present at the initiation of the cultures containing macrophages and the stimulators. Gazzinelli et al. (1992) have demonstrated that IL-10 must be added before or at the beginning of the culture to inhibit macrophage RNI production. A similar result was obtained by the author using the supernatant of T_H^2 cells in the current study. Addition of T_H^2 supernatant to macrophage cultures after 12 hours of activation could not inhibit the macrophage RNI production (data not shown). Indeed, activated viable T_H^2 cells, which are secreting T_H^2 cytokines, are less effective than viable resting T_H^2 in
Table 9  Comparison of $T_{h1}$ and $T_{h2}$ Clones

<table>
<thead>
<tr>
<th>T Cell Clones</th>
<th>Cytokines Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{h1}$</td>
<td></td>
</tr>
<tr>
<td>J6-19</td>
<td>IL-2, IFNγ</td>
</tr>
<tr>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>$T_{h2}$</td>
<td></td>
</tr>
<tr>
<td>D10.G4</td>
<td>IL-4, IL-5</td>
</tr>
<tr>
<td>AK8</td>
<td>IL-10</td>
</tr>
</tbody>
</table>
inducing cytostatic function in IFNγ-primed macrophages (R. D. Stout, personal communication). The kinetic studies by Abrams et al. (1992) demonstrating that the maximal production of IL-10, a potent Th2-derived inhibitory cytokine, occurs after 24-48 hours of T cell activation further support this interpretation.

It appears that the signal(s) provided by the Th2 cells which synergize with IFNγ in activation of macrophages are mediated mainly by the cognate interaction between T cells and macrophages as evidenced by the ability of paraformaldehyde-fixed T cells or plasma membranes isolated from the T cells to activate the IFNγ-primed macrophages. It has been previously reported that paraformaldehyde fixation of cells could not completely prevent the cells from secreting residual cytokines (Suttles et al. 1991). It could not be determined in the current study if any cytokines were leaked from the fixed activated T cells. However a role of the leaked cytokines, if any, in the fixed Th2 cell-mediated induction of macrophage nitrite production was unlikely because of those reasons discussed above and also because the supernatant harvested from the fixed activated T cells held in medium for 6 hours or overnight contained no macrophage-activating activity in the presence of IFNγ (data not shown). Moreover the fixed activated Th2 cells held in medium for 0, 6 hours or overnight, respectively, to leach out the secreted cytokines, had no
difference in their ability to activate IFNγ-primed macrophages (data not shown). The use of plasma membranes isolated from T\textsubscript{H2} cells further excluded the involvement of any secreted cytokines and established the role of cognate interaction in macrophage activation.

The cognate signaling of macrophage activation is neither antigen-dependent nor MHC-restricted. The evidence is as follows. 1). Plasma membranes from preactivated D10.G4 activated MHC-compatible I-A\textsuperscript{k} macrophages regardless of the presence or absence of antigen. 2). Plasma membranes from preactivated AK8 activated MHC-incompatible I-A\textsuperscript{b} macrophages although the T cells are I-A\textsuperscript{k}-restricted and are not alloreactive with I-A\textsuperscript{b} macrophages. 3). Paraformaldehyde-fixed preactivated splenic T cells were able to activate both syngeneic and allogeneic macrophages. Thus the interaction between T cell receptor and antigen-MHC complex is not critically involved in cognate signaling of macrophage activation.

Activation of T cells appears to be a prerequisite for T cell-mediated macrophage activation since only plasma membranes of preactivated but not of resting T cells could induce macrophage activation. The observation that viable resting T cells activated macrophages in an antigen-specific fashion may reflect that the resting T cells need to be activated by the antigen-presenting macrophages prior to activation of the macrophages by T cells. This conclusion
is also supported by the demonstration that the antibody against TcR co-receptor CD4 inhibited the viable T cell-induced macrophage activation but not fixed preactivated T cell-mediated macrophages activation (Fig. 13). A similar result was obtained by Stout and Suttles (1993) using antibodies against class II MHC molecules. CD4 binds to the nonpolymorphic region of class II MHC molecules (Swain 1983, Tite et al. 1986, Doyle and Strominger, 1987). Monoclonal antibodies reactive with CD4 (such as the one produced by GK1.5 hybridoma in this study) and reactive with class II MHC molecules have been demonstrated to block the activation of T cells stimulated by antigen-presenting cells (Swain 1981, Wilde et al. 1983). Therefore the inhibition of viable resting T cell-mediated macrophage nitrite production by anti-CD4 as demonstrated herein is probably due to the inhibition of T cell activation. On the other hand, the failure of anti-CD4 to prevent fixed activated T cells from activating macrophages suggest that binding of CD4 co-receptor to class II MHC molecules is not required for T cell cognate signaling of macrophage activation.

This pattern of T cell-mediated macrophage activation has also been observed in T cell-B cell interactions (Noelle and Snow 1990). It has been demonstrated that T cell-induced B cell proliferation and antibody production also involves the sequential antigen-specific activation of T cells followed by antigen-nonspecific activation of B cells.
by activated T cells. The initial antigen-specific interaction results in the alteration or appearance of activation associated molecules on both the T cell and the B cell, such as p39 and B7, respectively (Noelle et al. 1992, Nabavi et al. 1992, Watts et al. 1993). Both p39:CD40 interactions and CD28:B7 interactions have been reported to signal, in an antigen nonspecific fashion, the proliferation and differentiation of B cells (Noelle et al. 1992, Damle et al. 1991, Owens 1991, Noelle et al. 1991).

It is unknown which surface molecules on activated T cells, and the respective ligands on macrophages (Fig. 1), are involved in signaling of macrophage activation. CD40 has been reported to exist on a number of other cell types including dendritic cells, activated T cells, basophils, mast cells and langerhans cells (Clark 1990, Potocnik et al. 1990, Valent et al. 1990, Romani et al. 1989). Whether or not this molecule is also expressed on monocytes or macrophages and if so, whether it plays a signaling role in macrophage activation remains to be determined. B7 has been demonstrated to be expressed by peritoneal macrophages (Razi-Wolf et al. 1992). B7 has a relative short cytoplasmic tail and may be associated with another molecule for signal transduction (Freeman et al. 1991). It is therefore important to determine if this molecule plays a role in signaling of macrophage activation. It has been reported that binding of purified CD2 and antibodies to LFA-
3, CD44 and CD45 on human monocytes stimulated the production of TNFα and IL-1 by the monocytes (Webb et al. 1990). However, by using intact monoclonal antibodies in that study, the role of Fc receptors in signaling the monocyte activation could not be excluded. Fc receptors are well known signaling molecules on many cell types including monocytes and macrophages (Lynch et al. 1989, Davetch and Kinet 1991). Nonetheless, these adhesion molecules are the possible candidates for delivering the antigen-nonspecific signal for macrophage activation and are therefore worthy of further investigation.

It has been demonstrated that stimulating T cells via the T cell receptor led to an increase in the density and the affinity of a number of adhesion molecule such as LFA-1, ICAM-1 and CD2 (Dustin and Springer 1989, Tohma et al. 1992, Springer 1990). The roles of LFA-1 and ICAM-1 in signaling monocyte activation were not demonstrated by the study mentioned above (Webb et al. 1990). However, since macrophage activation is a fairly complex event which reflects the comprehensive tissue distribution of the macrophages and the broad spectrum of homeostatic and defensive functions of the mononuclear phagocyte system (MPS) (Adams and Hamilton 1992, Paulnock 1992, Walker 1976), it is likely that the macrophages used in the current study may not use the same sets of signaling molecules and signaling pathways that other macrophages would use during
the activation. Therefore future studies are warranted to investigate the roles of all these known surface adhesion molecules in signaling of macrophage activation as demonstrated herein.

Another possible signaling molecule involved in macrophage activation is membrane TNFα. It has been reported that TNFα, in the membrane or secreted form, can synergize with IFNγ in activation of macrophage effector functions including cytostatic activity and RNI production (Stout and Suttles 1992, Munoz et al. 1992, Oswald et al. 1992, Sherry and Cerami 1988, Liu et al. 1989). Sypek and Wyler (1991) also showed membrane form of TNFα contributed to activation of macrophage antileishmanial activity by T\textsubscript{h}1 cells. However, it is possible that by selecting membrane TNFα-positive T\textsubscript{h}1 cells in their study, they also selected T\textsubscript{h}1 cells bearing certain components (such as adhesion molecules) which were not present on the surface of membrane TNFα-negative T cells and played a role in activating macrophage effector functions (Sypek and Wyler 1991). In addition, the ability of anti-TNFα to inhibit the T\textsubscript{h}1-mediated activation of macrophages in their study could be due to the neutralization of macrophage-derived TNFα. In the current study, both fresh T cells and T cell clones are used. Since fresh T cells are heterogenous, possibly containing membrane TNFα-positive T cell subsets such as T\textsubscript{h}1 cells, there is a possibility that the membrane form of TNFα
may play a role in fresh T cell-induced macrophage
cytostasis and RNI production. Further study is needed to
resolve this possibility. Membrane TNFα has not been
detected on the Th2 cells (Sypek and Wyler 1991, Birkland et
demonstration that fixed Th2 clones or the plasma membranes
from the Th2 clones could induce macrophage cytostasis and
RNI production in the presence of IFNγ indicates that some
other surface molecule(s) on T cells other than membrane
TNFα is (are) involved in signaling macrophage activation.
The observation that fixed activated Th1 and fixed activated
Th2 cells were equally effective in inducing macrophage
cytostatic function (R. D. Stout, personal communication)
and nitrite production (Fig. 14) support this
interpretation.

It is also noted that although the fixed activated
C57BL/6J or Balb/C splenic T cells induced IFNγ-primed
C57BL/6J macrophages to produce nitrite, they were
significantly less effective than viable Balb/C T cells in
activation of the macrophages (Fig. 9). It is possible that
secreted TNFα or other soluble factors contribute to the
viable fresh T cell mediated-alloactivation of macrophages.
An alternative is that activation associated T cell membrane
molecules involved in macrophage activation are damaged by
the paraformaldehyde fixation and have an impaired function
in signaling of macrophage activation. This latter
possibility is less likely since fixed $T_\text{H}^2$ clone cells are about as effective as viable resting $T_\text{H}^2$ clone cells in inducing macrophage cytostasis and nitrite production.

Like LPS, the T cell-mediated activation of macrophage cytostasis and nitrite production is also dependent on the presence of IFN$\gamma$. Stimulation of macrophages with either LPS alone or T cell plasma membranes alone or IFN$\gamma$ alone induced only background levels of macrophage cytostatic activity and nitrite production. The combination of T cells and IFN$\gamma$ or the combination of LPS and IFN$\gamma$ induced optimal macrophage cytostasis and RNI generation. As was demonstrated before with LPS plus IFN$\gamma$ (Green et al. 1991), the induction of macrophage nitrite production by the synergy of T cells and IFN$\gamma$ can be completely blocked by NG-mono-methyl-L-arginine, an L-arginine analog (Fig. 5), indicating that T cell-mediated RNI production is also mediated via L-arginine-dependent pathway. How this synergy operates at the molecular level inside macrophages is not clear. Studies on macrophage cell line RAW264.7 indicated that LPS and IFN$\gamma$ had a synergistic effect on induction of the mRNA expression for the gene of nitric oxide synthase (Lorsbach et al. 1993, B. Chen, personal communication). This synergistic induction of nitric oxide synthase mRNA was susceptible to cycloheximide inhibition, suggesting de novo protein synthesis is required (Lorsbach et al. 1993). The increase of nitric oxide
production paralleled the increase in macrophage cytotoxicity (Lorsbach et al. 1993).

The macrophage cytostatic/cytotoxic mechanisms comprise a number of factors, such as secretion of IFNα and IFNβ, release of reactive oxygen intermediates and reactive nitrogen intermediates (Stout and Suttles 1992, Auger and Ross 1992, Green et al. 1991). A recent study has suggested that induction of macrophage cytostasis is partly mediated by a TNFα autocrine loop (Stout and Suttles 1992). LPS stimulates macrophages to produce TNFα. TNFα exert autocrine effects on the macrophages via binding to the TNFα receptors which can be induced or up-regulated by IFNγ. One of the TNFα autocrine effects is to induce macrophage IFNβ production (Stout and Suttles 1992). It is possible that the TNFα autocrine loop may also be involved in T cell-mediated induction of macrophage nitrite production. First, it has been demonstrated in this study and in many others (Drapier et al. 1988, Ding et al. 1988, Green et al. 1990) that TNFα can synergize with IFNγ in the induction of macrophage RNI production. Second, the cognate interaction between T_{2} cells and macrophages can stimulate TNFα secretion (data not shown) and TNFα mRNA expression (Fig. 16). However, the TNFα autocrine loop is probably not the sole mechanism underlying the T cell-mediated induction of macrophage RNI production since the level of nitrite induced by rTNFα (ranging from 30 u/ml to 1000 u/ml) plus rIFNγ was
significantly lower than the level of nitrite induced by LPS or cognate T cell-macrophage interaction plus IFNγ as demonstrated herein. In addition, in contrast to LPS, T_{H}2 cells have been repeatedly found to induce much less TNFα secretion from macrophages (data not shown). Yet both LPS and T_{H}2 cells induced equivalent level of macrophage cytostatic activity and RNI production with similar kinetics (Fig. 4, Table 7). The demonstration by several in vitro and in vivo studies (Green et al. 1993, Langermans et al. 1992, Drapier et al. 1988) that anti-TNFα antibodies did not completely abrogate the generation of RNI from macrophages exposed to LPS or other microbial products supports the above view. Apparently, more studies are needed to elucidate the nature of other factors involved in macrophage activation and to gain a clearer understanding of the molecular mechanisms by which cognate T cell-macrophage interaction and IFNγ synergize to activate macrophages.

In addition to inducing macrophage TNFα mRNA expression, the plasma membranes from activated T_{H}2 cells-D10.G4 also induced the expression or augmented the accumulation of mRNA for IL-1α, c-myc, c-fos, JE, IP10 and D3 at as early as 1 hour after stimulation of macrophages (Fig. 16 A). These data clearly demonstrate a role of cognate interaction in intracellular signaling in the macrophages. The demonstration that plasma membranes from resting D10.G4 did not induce the expression of mRNA for all
the genes examined except D3, c-fos and c-myc which were weakly expressed suggests that some activation associated molecules on the T cells are mainly responsible for mediating the intracellular signaling in macrophages. Whereas the repeated observation of weak induction or enhancement by plasma membranes of resting T cells in the expression of mRNA for c-myc, c-fos and D3 indicates that certain surface molecules not associated with T cell activation may also contribute to the signaling events. Since the macrophages used in this study were obtained from I-A^b-positive C57BL/6J mice, plasma membranes from D10.G4 contain T cell receptor reactive with the I-A^b molecules. Therefore a possible candidate is the pair of TcR and ligand MHC molecules.

It is interesting to note that after 4 hours of macrophage activation, the messages for TNFα, IL-1α, JE, IP10, D3 were still present in macrophages stimulated by LPS or plasma membranes (Fig. 16 B). The mRNA for c-myc disappeared in LPS-stimulated macrophages as compared to the continued but decreased presence of the message in plasma membrane-stimulated macrophages. This result suggests that cognate signaling may differ kinetically from LPS-mediated signaling in induction of c-myc expression. A satisfactory explanation for this observation in the current study demands further studies. Data are not available from this study to determine whether the expression of c-fos mRNA is
also altered in macrophages stimulated for 4 hours. The similar observation has been made by other investigators studying the proto-oncogene expression in peritoneal macrophages stimulated by LPS (Introna et al. 1986, Collart et al. 1987). In those studies, it was found that c-myc and c-fos were expressed at 5 to 15 minutes in macrophages after LPS stimulation. Their expression peaked at about 1 hour and disappeared after 2 to 8 hours post stimulation. The difference between the time points of the c-myc mRNA disappearance observed in those studies and in the current study may lie in the different sources of macrophages and different doses of LPS used in the experiments.

It is not clear from the current study whether or not the cognate signaling initiates the transcription of the genes examined or merely stabilizes the existing mRNAs or does both. The products of c-myc and c-fos are nuclear factors which regulate the expression of other genes in macrophages, such as cytokine genes (Crabtree 1989). The products of JE and IP10, like IL-1 and TNFα, are inflammatory proteins (Oppenheim et al. 1991, Rollins et al. 1990, Yoshimura et al. 1987). So is probably the protein encoded by D3 (Tannebaum et al. 1988). Therefore the ability of plasma membranes from activated T cells to induce or augment the expression of these genes further illustrate the significance and importance of cognate T cell-macrophage interaction in mediating macrophage activation in nonseptic
inflammatory reactions.

Since the induction of macrophage cytostasis, RNI production and gene expression by LPS or by T cells is similar in kinetics, in lack of antigen specificity and in dependency on IFNγ, it is extremely important to determine if there is any significant LPS contamination in the medium used in the experiments and particularly in the plasma membrane preparations. Throughout the entire course of this study, the chromogenic endotoxin assay, which is the most sensitive test to date, has been frequently run to monitor the medium and each membrane preparation for LPS contamination. No significant amount of LPS has been detected in either medium or plasma membranes. As demonstrated in this study, the minimum amount of LPS required to synergize with 50 ng/ml IFNγ in induction of nitrite production was >100 pg/ml (Fig. 15) which is 20 fold higher than the concentration of LPS detected in the highest amount of plasma membranes added to the culture. In the plasma membranes used to stimulate macrophage gene expression, the amount of LPS detected is twice less than what is needed (>10 pg/ml) (Anderson et al. 1992) to induce the macrophage and monocyte cytokine gene expression. In addition, the demonstration that the membranes from resting T cells, prepared simultaneously with the membranes from activated T cells, did not activate the macrophages argues against the role of contaminating LPS in the medium or
In summary, the current study provides evidence to support a model for T cell-mediated macrophage activation (Fig. 17). The initial interaction between T cells and antigen-presenting macrophages is an antigen-specific and MHC-restricted event which initiates T cell activation. For example, D10.G4 cells can be induced to proliferate by coculture with antigen presenting I-A\(^k\) macrophages or by coculture with I-A\(^b\) macrophages (Stout and Bottomly 1989). Reciprocal activation of macrophage effector functions does not occur unless rIFN\(\gamma\) is added to prime the macrophage. Unlike the initial activation of the T cells, this reciprocal activation of the macrophages appears to be accomplished by antigen-nonspecific signaling since fixed activated T cells or plasma membranes from activated T cells could activate IFN\(\gamma\)-primed macrophages in the absence of antigen.

It should be pointed out that although the interaction between TcR and MHC-Ag complex is not essential for signaling activation of macrophage effector functions, this interaction may stimulate macrophage to express other capacities, such as induction or increase of surface molecules which are involved in cognate signaling of macrophage activation. It has been reported that triggering MHC molecules on B cells and macrophages induced expression of B7 (Lidia Koulova et al. 1991, Nabavi et al. 1992) and
Figure 17

Proposed Model of T Cell-Mediated Macrophage Activation
some other adhesion molecules such as LFA-1 (Dustin and Springer 1991). A recent study has demonstrated the induction of B7 in B lymphoma cells by MHC-restricted T cell interaction (Watts et al. 1993). Therefore, the initial antigen-specific interaction between T cells and macrophages may transduce signals bi-directionally, activating T cells and also preparing the macrophages for receiving the subsequent cognate signal(s) from the activated T cells. The ability of plasma membrane of resting D10.G4 to induce or enhance the expression of mRNA for a few "early" genes may be associated with the initial antigen-specific signaling pathway.

T₉₁ cells are known to be more effective than T₉₂ cells in induction of cell mediated reactions and macrophage activation (Mosmann and Coffman 1989, Sher et al. 1992). The demonstration that T₉₂ cells can provide cognate signaling for macrophage activation suggests that the enhanced macrophage activating capability of T₉₁ cells may be a reflection of the ability of T₉₁ cells to produce macrophage activating cytokines such as IFNγ (Langermans et al. 1992, Stout and Bottomly 1989, Sypek and Wyler 1991, Kanazawa et al. 1992, Birkland et al. 1992, Mosmann and Coffman 1989). On the other hand, although it is generally held that T₉₂ cells are predominantly involved in activating B lymphocytes and regulating humoral immune responses, it can be envisioned, based on the data presented in the
current study, that $T_{n2}$ cells may be recruited to participate in a local inflammatory reactions and with the help of $T_{n1}$ cytokines, activate monocytes or macrophages via cognate signaling. Conversely, $T_{n2}$ cells involved in macrophage activation may also be reciprocally activated and secrete inhibitory cytokines such as IL-4 and IL-10 that will dampen the ongoing inflammatory reactions. Therefore $T_{n2}$ cells may play an important role in fine tuning of the inflammatory process through regulation of macrophage activation. Indeed one study has demonstrated that in the inflammatory reactions caused by allograft rejection, both activated macrophages and $T_{n2}$ cells are involved (Fyfe et al. 1993).

The current study establishes a novel mechanism for macrophage activation during the nonseptic inflammatory reactions, such as autoimmune inflammation, tumor immune responses, inflammation caused by graft rejections and atherosclerosis. T cells and macrophages are known to play essential roles in the inflammatory process of all these reactions. An advantage of the antigen nonspecific mechanisms of macrophage activation is that the activated T cells, at a site of inflammation, can induce macrophage cytostatic activity and RNI production in infiltrating monocytes and macrophages that have not yet encountered antigen. The nature of the antigen nonspecific cognate signaling of macrophage activation by T cells is currently
being investigated.


Babbitt, B. P.; Allen, P. M.; Matsueda, G.; Unanue, E. R.


Noelle, R. J.; Daum, J.; Bartlett, W. C.; McCann, J.; Shepard, D. M. 1991. Cognate interactions between helper T cells and B cells. V. Reconstitution of helper T cell function using
purified plasma membranes from activated Th1 and Th2 helper T cells and lymphokines. J. Immunol. 146:1118-1124.


Sambrook, J.; Fritsch, E. F.; Maniatis, T. 1989. Molecular


Tannenbaum, C. S; Koerner, T. J.; Jansen, M. M.; Hamilton, T.


Zullo, J. N.; Cochran, B. H.; Huang, A. S.; Stiles, C. D.
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