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T Cell Mediated Induction of Monocyte Il-1 During a Primary Human Immune Response

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T CELL MEDIATED INDUCTION OF MONOCYTE IL-1
DURING A PRIMARY HUMAN
IMMUNE RESPONSE

by

Robert Clive Landis

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
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April

1990

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VITA

The author, Robert Clive Landis, was born on February 19th, 1962, in London, England, to Hilary Blackwell-Landis and Heinrich Johannes Landis.

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Publications

Landis, R. C. 1987. Masters thesis: "Mechanism of action of an immunomodulatory drug, GR1784: Effects on hematopoiesis and lymphocyte mitogenesis."

Landis, R. C., R. I. Fisher, and T. M. Ellis. 1989. Induction of IL-1 α and β mRNA in human monocytes during anti-CD3 mitogenesis. Eur. J. Immunol. (submitted).

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INTRODUCTION

Cellular Cooperation During the Immune Response

The cellular theory of immunity was developed in 1883 by Elie Metchnikoff following his demonstration that macrophages ingested foreign bodies that were introduced into starfish larvae (1, 2). The subsequent discovery, in 1890, of soluble factors in serum that possessed antimicrobial properties (3) led to the identification of antibodies and the development of the humoral theory of immunity (4). These discoveries led to the concept of a dualistic immune response, comprised of cellular and humoral elements.

The involvement of lymphocytes in immunity was first recognized in the 1940s by the demonstration that lymphocytes were involved in antibody formation (5) and that they mediated the adoptive transfer of contact sensitivity (6), the tuberculin (delayed type hypersensitivity) reaction (7) and the allograft response (8). In the late 1950s and early 1960s it was discovered that two distinct types of lymphocytes mediated the humoral and cellular arms of the immune response. Lymphocytes mediating the humoral arm of the immune response were discovered following the observation that bursectomy in

chickens led to the complete elimination of all antibody mediated responses, while leaving the allograft response intact (9, 10). Antibody production and the humoral arm of the immune response was therefore mediated by Bursa derived (B) lymphocytes. The lymphocytes that mediated the cellular arm of the immune response were discovered in similar manner, by the observation that neonatally thymectomized mice were unable to mount an allograft response (11). The cellular arm of the immune response was therefore mediated by thymocyte-derived (T) lymphocytes.

Subsequent studies revealed that the function of these two types of immune cells were not mediated entirely independently of each other. In the first example of cellular cooperation described for the immune response, various researchers in the late 1960s discovered that T cells were required for the optimal generation of antibodies from B cells (12, 13), despite the prior demonstration that T cells by themselves do not secrete antibodies (14). Further investigation of the antibody response led to the discovery that a third cell, the macrophage, participated in this response (15).

A requirement for cellular cooperation with macrophages was also described for the activation of T cells by foreign antigen. In the early 1970s, it became clear that T cells

differed in the manner by which they recognized antigen compared to B cells. Whereas B cells were able to recognize soluble antigen through membrane associated immunoglobulin molecules (16), T cells recognized antigen only when it was associated with class II major histocompatibility (MHC) molecules expressed on the surface of histocompatible macrophages (17). Similar antigen presenting capacity has since been demonstrated for a number of other cell types, including B cells (18), dendritic cells (19), and various members of the macrophage lineage (20, 21). Cells of the macrophage/monocyte lineage have more recently been shown to provide accessory functions during T cell activation distinct from their capacity to act as antigen presenting cells during T cell activation (31, 32).

Further elucidation of macrophage accessory function during T cell activation was made possible following the characterization of the antigen specific T cell receptor (TCR) (22-27). The TCR was shown to be immunoglobulin-like in overall structure, its variable domains simultaneously recognizing both the antigen and self MHC molecules on the appropriate accessory cell (28). Following the characterization of this structure, reagents were developed that were able to dissociate the signalling events mediated via the TCR from the succession of signalling events required for T cells to become fully activated. In the current model

of T cell activation, the first T cell activation signal is provided by the MHC restricted recognition, through the TCR, of foreign antigen expressed on the surface of an antigen presenting cell (17, 29, 30). T cells triggered in this manner undergo necessary physiological changes that accompany the initial phase of T cell activation, although further activation of triggered T cells requires the presence of costimulatory molecules provided by an accessory cell (31, 32). A rich source for a number of costimulatory molecules are cells of the myeloid lineage, such as the macrophage and the blood monocyte. In peripheral blood, monocytes serve as the principal accessory cell during T cell activation. During their antigen specific interaction with T cells, monocytes are capable of providing both signals required for T cell activation. In their dual capacity as antigen presenting cells and rich source of soluble and cell associated costimulatory molecules, monocytes provide both the antigen recognition signal and necessary second activation signal.

Interleukin 1

Interleukin 1 (IL-1) is a polypeptide cellular mediator that is produced after infection, injury or antigenic challenge. IL-1 was initially identified in many laboratories as distinct biological activities, later (34) recognized to be mediated by the same molecule. IL-1 is a polypeptide

hormone that mediates the systemic acute phase response towards microbial infection, inflammation and tissue injury. Within hours after infection or injury, IL-1 gains access to the circulation and acts like a hormone to induce a broad spectrum of changes in such diverse systems as the neurologic, immune and metabolic systems. The neurologic effects of IL-1 include the induction of fever (35), the induction of slow wave deep sleep (36), and the release of hypothalamic releasing factors (37). In the immune system, IL-1 plays an important role in initiating the immune response (31, 32, 38) and promoting tumor killing (39). Included among the metabolic activities of IL-1 are the induced synthesis of hepatic acute phase protein (40), serum metal ion depletion, neutrophilia (41) and fibroblast proliferation (42). IL-1 also induces the synthesis of the vasodilators platelet activating factor and prostaglandins E_2 and I_2 (43). These examples of the cellular effects of IL-1, which represent only a small fraction of its reported properties, demonstrate that IL-1 exhibits pleiotropic effects in many different cell types. An understanding of the entire spectrum of systemic effects mediated by IL-1 has led to a concept of IL-1 as the key modulator of an integrated response by the body towards infectious challenge or inflammation (44).

The principal source of IL-1 during an infectious challenge is the activated blood monocyte (45, 46). Other

sources of IL-1 at the site of inflammation include tissue macrophages (47), vascular endothelial cells (48) and vascular smooth muscle cells (49). The pathways by which monocytes secrete IL-1, although still not completely understood, have become clearer following the recent cloning of the genes encoding IL-1.

A genetic analysis of cloned human IL-1 led to the conclusion that IL-1 was encoded by two distinct IL-1 genes, termed IL-1 α and IL-1 β (50-54). Sequence comparisons between the coding regions of IL-1 α and IL-1 β revealed two remarkable properties of the two encoded IL-1 protein products. First, despite the previously discussed hormonal properties of IL-1, neither IL-1 gene encoded a protein containing a leader sequence common to most secreted polypeptide hormones. Second, IL-1 α and IL-1 β exhibited only 26 % nucleotide sequence homology, yet they were both cloned on the basis of an identical biological activity that they encoded (50). These findings at the genetic level have extensive implications on the mechanisms by which IL-1 is secreted and how IL-1 mediates its biological activities.

At the level of IL-1 secretion, the absence of a leader sequence explained the previous observation that the majority of IL-1 in activated monocytes was present intracellularly (55). IL-1 α and IL-1 β were both shown to accumulate as

biologically active 31 kD IL-1 precursors in the cytoplasm of monocytes activated by the bacterial cell membrane product, lipopolysaccharide (LPS). At this point, the processing steps leading to the formation of mature IL-1 proteins of each species diverged. Mature forms of IL-1 have been detected both in the extracellular compartment and associated with the outer membrane compartment of activated monocytes (56, 57). IL-1 β , which constitutes the predominant secreted form of IL-1 (58), was released from an intracellular membrane compartment to the mature 17 kD secreted product by a poorly understood process that involved proteolytic cleavage by an unknown serine protease, a process inhibitable by the serine protease inhibitor PMSF (59). IL-1 α precursors appeared to be translocated to the cell surface, as 31 kD or 23 kD proteins, by a glycosylation dependent attachment to lectin molecules on outer plasma cell membranes (60). The observed partitioning of mature IL-1 molecules, between membrane and extracellular compartments, suggested that these two products could mediate both short and long range effects. These studies have demonstrated that multiple posttranslational processing steps and multiple levels at which IL-1 may be regulated are involved in the synthesis of mature IL-1 by activated monocytes, as might be expected for a molecule with such potent systemic effects.

IL-1 α and IL-1 β were shown during their cloning to

mediate identical biological activities, despite only 26 % nucleotide sequence homology (50). To examine the mechanism by which two genes with such limited homology could encode products with similar biological activities, both full length genes were transfected and expressed in a mammalian COS cell line (61). Both full length genes encoded protein products with indistinguishable effects on T cells. Deletion mutants demonstrated that truncated proteins from each IL-1 gene, representing a conserved stretch of only 62 out of a total 269 amino acids (23 %), exhibited 50 % of the total biological activity of full length proteins. These findings explained how two proteins with apparently divergent nucleotide sequences could mediate similar biological effects.

The genetic regulation of the IL-1 genes remains poorly understood. A comparison between IL-1 α and IL-1 β gene regulation has not been performed. However, IL-1 β was demonstrated to be the major species of IL-1 induced at the mRNA level in LPS activated blood monocytes, comprising > 90 % of total IL-1 mRNAs (50) and a remarkable 5 % of the total cellular mRNA complement (62). These observations at the level of genetic regulation of IL-1 therefore supported the previous demonstration that blood monocytes were capable of producing large quantities of IL-1 during an infectious challenge.

Role of IL-1 in T Cell Activation

The first signal required for T cell activation involves triggering via the TCR, mediated either through the MHC restricted recognition of antigen on the surface of the antigen presenting cell or, experimentally, by antibodies directed against the TCR complex. Effective TCR triggering initiates a cascade of intracellular activation events, including phosphatidyl inositol turnover (63), the subsequent release of Ca^{2+} from intracellular stores (64), partial protein kinase C (PKC) translocation (65) and IL-2 receptor expression (66). These biochemical events, although required to prime T cells for activation, do not by themselves provide sufficient triggering stimulus for T cells to become fully activated. T cells triggered through the TCR require the presence of the accessory cell derived costimulatory molecules, to provide the necessary second signal for the elaboration of the autocrine growth factor IL-2 and induction of IL-2 dependent clonal expansion (67).

IL-1 was the initial cytokine demonstrated to possess costimulatory properties during T cell activation (68, 69). In these studies, Gery and Waksman recognized that an essential component of macrophage accessory cell function during T cell activation was their ability to secrete the cytokine lymphocyte activating factor (LAF), later renamed

IL-1. Following the initial findings of Gery and Waksman, efforts were directed towards establishing whether an obligatory requirement for IL-1 during T cell activation existed.

Early reports, which demonstrated an obligatory requirement for IL-1 during T cell activation, were performed in experimental systems that were not rigorously depleted of monocytes. In these studies, IL-1 was reported to replace the APC requirement for the induction of IL-2 receptors, IL-2 and T cell proliferation (70, 71). Unacceptably high proliferations induced by monocyte dependent T cell mitogens in these experiments showed that purified T cell populations were contaminated with low numbers of monocytes. The interpretation derived from these studies must therefore be modified from an initial conclusion that IL-1 was an obligate requirement for T cell activation to the conclusion that, in the presence of limiting numbers of accessory cells, IL-1 could reconstitute T cell responses.

Conclusions arguing against an obligatory role for IL-1 in T cell activation were drawn from subsequent reports that demonstrated the IL-1 independent activation of T cells. Accessory cells of the dendritic cell lineage have been demonstrated to provide costimulatory molecules which, in the absence of IL-1, lead to complete activation of T cells (72).

subsequent experiments showed that a requirement for costimulatory signals, including IL-1, could be bypassed altogether in experimental systems in which T cells were activated by solid phase anti-CD3 monoclonal antibody (anti-CD3 mAb) immobilized at high densities to culture plates. In these experiments, human T cells were activated independently of IL-1 and other accessory cell derived costimulatory molecules following prolonged (> 2 h) elevations of intracellular Ca^{2+} levels (73-75). These studies demonstrated that the requirement for a soluble second signal could be obviated in vitro by saturating and cross-linking sufficient TCRs through immobilized anti-TCR complex monoclonal antibodies. In vivo, however, it is unlikely that occupancy of TCRs by foreign antigen ever exceeds 10 %, thus suggesting that there may be an absolute requirement for accessory cell derived costimulatory molecules in vivo (76-78). Although these in vitro experiments excluded an obligate requirement for IL-1 during T cell activation, activation conditions were not necessarily relevant to the activation of T cells under physiological conditions.

More recently, investigators have concentrated on obtaining a clearer understanding of the true costimulatory role of IL-1 during T cell activation under conditions that are physiologically relevant. Such an understanding has been provided by studies which assessed the costimulatory

properties of recombinant IL-1 during the activation of highly purified populations of unprimed human T cells, triggered through the TCR complex with phytohemagglutinin (PHA) or anti-CD3 mAb. In populations of unprimed tonsillar T cells, rigorously depleted of monocytes (< 0.1%), Houssieau and Van Snick (79) demonstrated that IL-6, a second monocyte derived cytokine, dramatically synergized with IL-1 to stimulate proliferation of T cells triggered with PHA. In a subsequent investigation (80), these authors demonstrated that the synergy between IL-1 and IL-6 derived from their complementary effects on the production of and response to IL-2 by T cells. IL-1 played the preponderant role in the induction of IL-2, whereas IL-6 was required, in addition to IL-1, for optimal IL-2 responsiveness. Further investigation by these authors revealed that IL-6 mediated its actions by moving resting T cells from G_0 to G_1 , where they become more responsive to the small amounts of IL-2 induced by IL-1. Similar results were reported by Holsti and Raulet (81), employing highly purified human $CD4^+$ T cells triggered with immobilized anti-CD3 mAb. In this investigation, $CD4^+$ T cells, isolated by cell sorting, were provided with the TCR triggering signal by carefully controlled doses of anti-CD3 mAb. $CD4^+$ T cells triggered in this manner also demonstrated the synergistic induction of T cell proliferation by IL-1 and IL-6, mediated via an induction of IL-2 secretion. These studies have therefore delineated a principal role of IL-1 during the induction of T cells.

Although IL-1 does not mediate its effects alone during T cell activation, it acts synergistically with IL-6 and is required for the critical elaboration of the autocrine growth factor IL-2, resulting in the IL-2 induced clonal proliferation of triggered T cells.

A role for membrane IL-1 in the activation of T cells has been investigated in several previous reports. Initial studies with preactivated, fixed mouse macrophages demonstrated that a membrane component, inhibitable by anti-IL-1 antiserum, was capable of providing the necessary costimulatory signals to antigen primed T cell clones (56). These findings were extended to unprimed human T cells (82), triggered through immobilized anti-CD3, thereby demonstrating that membrane IL-1 α on paraformaldehyde fixed monocytes was capable of providing all costimulatory signals necessary to stimulate proliferation of triggered T cells. An important role for membrane IL-1 as a costimulatory molecule in T cell activation has therefore been demonstrated by experimental observations of anti-IL-1 inhibitable costimulation of T cells by fixed macrophages or monocytes. However, these studies do not rule out the participation of other membrane associated costimulatory molecules in addition to membrane IL-1, such as the cellular adhesion molecules, during T cell activation.

Induction of cellular adhesion molecules may represent

an indirect mechanism by which IL-1 mediates its costimulatory effects during T cell activation. In this model, IL-1 transduces an intracellular signal leading to the induction of adhesion molecules on the monocyte cell surface, or it promotes expression of adhesion molecules on the T cell surface. In support of such a model, the adhesion partners LFA-3 and CD2, on the monocytes and T cell surface respectively, have been demonstrated to exhibit costimulatory properties during T cell activation (83, 84).

Several mechanisms by which IL-1 mediates its costimulatory properties during T cell activation have been identified. Most previous work has concentrated on characterization of the signal transduction pathways stimulated by IL-1 to mediate its effects on IL-2 synthesis, which represents the pivotal event leading to the clonal expansion of appropriately triggered T cells expressing IL-2 receptors. These studies have resulted in the identification of two signal transduction pathways through which IL-1 can mediate its costimulatory effects at the level of IL-2 production in T cells.

A phorbol ester mediated signal transduction pathway has been identified in T cells that stimulates high levels of IL-2 secretion. A number of studies showed that IL-1 did not mediate its costimulatory properties in T cells through the

classic phorbol ester mediated signal transduction pathways, consisting of phosphatidyl inositol hydrolysis and protein kinase C (PKC) activation (85). Although hydrolysis of phosphatidyl inositol has been ruled out as a component of IL-1 signal transduction, several recent studies have reported that IL-1 induced the hydrolysis of another phospholipid, phosphatidylcholine, resulting in the production of diacyl glycerol, the same second messenger generated by phosphatidyl inositol hydrolysis (86). In other parallels to the phorbol ester induction pathway, IL-1 was also shown to mediate its effects on IL-2 induction by stimulating a protein kinase distinct from PKC (87, 88). Experimental evidence therefore supports transduction of IL-1 costimulatory signals by a pathway employing components of the classic phorbol ester signal transduction pathway. IL-1 was demonstrated to stimulate a second, cyclic-AMP (c-AMP) dependent signal transduction pathway (89). These reports demonstrated that IL-1 induced a c-AMP dependent protein kinase, which may represent the same non-PKC protein kinase previously identified to transduce IL-1 costimulatory signals during T cell activation. The c-AMP dependent protein kinase was shown to transduce its IL-2 stimulatory effects at the level of the IL-2 promoter. Recent analysis of the IL-2 promoter region has allowed diverse signals originating from separate receptors and transduced by the separate pathways previously described to be integrated at the level of the IL-2 gene (90).

Direct evidence has been obtained that IL-1 mediates its costimulatory effects at the level of the IL-2 promoter (91). A human IL-2 promoter fused to the chloramphenicol acetyl-transferase (CAT) indicator gene was used to transfect LBRM cells and identify an IL-1 responsive element within the IL-2 promoter. This element contained a 12 bp motif, previously identified as a component of the phorbol ester TPA response element (TRE) (92), that was specifically recognized in gel retardation assays by its appropriate nuclear factor, AP-1. These authors also examined the ability of IL-1 to induce transcription of the two oncogenes, c-jun and c-fos, that encoded the two major protein components of the AP-1 factor (93, 94). These studies revealed that stimulation with IL-1 induced strong transcription from the c-jun and to a lesser extent the c-fos gene, whereas stimulation with PHA induced transcription from the c-fos, but not c-jun, gene. De novo protein synthesis was not required for transcription from either oncogene, indicating that IL-1 induced transcription of IL-2 in T cells triggered with PHA occurred through the de novo protein synthesis of the c-jun component of the AP-1 factor. These observations provided direct evidence for a mechanism whereby IL-1 transduced its T cell costimulatory properties at the level of increased transcription of the IL-2 gene, mediated through the induced binding of AP-1 to the TRE element in the IL-2 promoter.

Indirect evidence also exists that IL-1 may mediate its costimulatory effects by activating the kappa B-like enhancer recently identified within the IL-2 promoter (95). IL-1 was shown to activate the kB-like enhancer in a pre-B cell line via the generation of functional NF-kB DNA binding proteins (96). Activation of functional NF-kB and induction of the kB-like enhancer were dependent upon a cAMP dependent protein kinase activity. IL-1 therefore mediated its kB-enhancer activating properties via its stimulation of the cAMP second messenger pathway. Collectively, these studies suggested that costimulatory effects of IL-1, even when transduced by diverse second messenger pathways, may be integrated at the level of the IL-2 promoter through the TRE and NF-kB response elements.

The TRE and NF-kB response elements have been found in the promoters of many genes in multiple cell types, thereby providing a rationale to explain the spectrum of diverse, systemic cellular effects described for IL-1. In light of the potent effects mediated by IL-1 following an immune challenge, both at the systemic level and at the level of initiation of the immune response, it is particularly important to understand the mechanism by which IL-1 production is initiated and regulated during an immune response.

Induction of IL-1 During an Immune Response

In view of the potent biologic activities previously described for IL-1, it is of interest to understand the mechanisms that regulate its production during an immune challenge. The activated blood monocyte has been shown to be the principal source of IL-1 (45, 46). Circulating blood monocytes have been shown by in situ hybridization analysis not to constitutively express IL-1 mRNA (97). They elaborate IL-1 during phagocytic responses towards any number of particulate stimuli, such as allogeneic erythrocytes or latex beads (44). Monocytes are also stimulated to produce IL-1 in response to bacterial antigens, such as LPS, which possess intrinsic IL-1 inducing properties (98). However, monocytes do not synthesize IL-1 in response to the spectrum of self antigens that they encounter during routine homeostatic functions in peripheral blood (99, 100). Monocytes produce IL-1 in response to soluble antigens only during the course of an immune response to foreign antigen (98). Therefore, a prerequisite for the induction of IL-1 during an immune response is the ability to distinguish self and non-self soluble antigens. However, it has been well established that monocytes lack the ability to distinguish self and non-self antigens, processing and presenting each with equal efficiency (99, 100).

The inability of monocytes to distinguish between self

and non-self antigens during processing has been well documented. Winchester and Mitchison (99) examined the ability of macrophages from two H-2^k mouse strains to process and present strain specific forms of the F protein. F protein occurs in H-2^k mice in two allelic forms, F.1 and F.2, which elicit a strong T cell dependent antibody response in strains exhibiting the opposite F type. These studies demonstrated that macrophages pulsed with either the self or foreign F protein were able to elicit identical responses in T cells from the opposite F type. A second study, by Lorenz and Allen (100), employed T cell hybridomas specific for hemoglobin in the context of I-A^k (Hb/I-A^k) to detect the presence of Hb/I-A^k complexes constitutively expressed on resident peritoneal macrophages. Collectively, these studies demonstrated that monocytes processed and presented self and non-self antigens with equal efficiency and that self antigens were constitutively presented on the monocyte surface in healthy mice.

In view of the inability of monocytes to distinguish between self and non-self proteins, a logical proposal is that the IL-1 inductive signal in vivo is provided by the antigen specific T cell during its interaction with the presenting monocyte. The T cell, which possesses the molecular recognition machinery necessary for self/non-self discrimination (22-27), would signal the monocyte, which lacks

this discriminatory capacity, to elaborate IL-1. According to this hypothesis, recognition through the TCR of foreign antigen triggers the transduction of an IL-1 inductive signal from the T cell to the monocyte, thereby stimulating production of the IL-1 costimulatory molecule and enabling clonal proliferation of the triggered T cell.

The earliest studies investigating IL-1 induction during an immune response were performed in peritoneal macrophages of mice immunized in vivo with infectious agents, particulate substances and T cell mitogens (101, 102). These studies revealed that the secretion of IL-1 by macrophages was enhanced following exposure to soluble products of activated T lymphocytes, thereby identifying the existence of reciprocal stimulatory signals transferred between lymphocytes and macrophages. These findings were subsequently extended in human in vitro cell systems, which demonstrated secretion of IL-1 by the human monocytic U937 line following exposure to cell free supernates of mitogen activated human T cells (103, 104). Dinarello and Kent (105) also reported detection of a soluble IL-1 inducing factor in the supernates of a primary human allogeneic MLR. This observation confirmed that T cells activated directly through their TCR in the MLR response, could generate IL-1 inductive signals during a primary immune activation. A weakness of these early reports, which assessed induction of IL-1 at the secreted level, was that they

utilized the thymocyte comitogenesis IL-1 assay (106), which does not distinguish between IL-1, IL-6 or IL-2 activities.

Recognizing the limitations of the thymocyte comitogenesis assay, subsequent studies by Weaver and Unanue (107) and Wasik and Beller (108) assessed induction of IL-1 at the level membrane associated IL-1 (mIL-1). mIL-1 activity was assessed on paraformaldehyde fixed macrophage membranes and was therefore not subject to the same artifact as secreted IL-1 in the comitogenesis assay. In these experiments, Weaver and Unanue detected a cell contact mediated mIL-1 inductive signal generated by antigen specific T cell clones. The cell contact mediated signal was antigen specific, MHC restricted, and was generated in the absence of de novo protein synthesis. A similar macrophage mIL-1 inductive signal, generated by T cell clones that respond to syngeneic MHC determinants on accessory cells in the absence of antigen, was reported by Wasik and Beller (108). This mIL-1 inductive signal was mediated by direct cell contact, was generated independently of T cell protein synthesis and remained intact following paraformaldehyde fixation. The kinetics of induction of mIL-1 were consistent between these two studies and showed detectable mIL-1 activity as early as 4 h following addition of T cells to macrophages. In addition, through the use of autoreactive T cell clones restricted to class I or class II

MHC molecules on macrophages, Wasik and Beller were able to demonstrate that the IL-1 inductive signal could be transduced by either class I restricted or class II restricted T cell clones. This observation enabled these authors to infer that the T cell IL-1 inductive signal was transduced either by both class I and class II restricted associative recognition elements on the surface of T cells or by non-MHC-restricted cell surface molecules. In summary, these two studies demonstrated that T cell clones generated a macrophage mIL-1 inductive signal, which was transduced through direct cell contact, independent of protein synthesis and was not exclusively mediated during the class II restricted interaction between T cells and macrophages.

A limitation to the studies of Weaver et al. (107) and Wasik et al. (108), was that these investigators examined the IL-1 inductive signals generated by T cell clones which were previously primed and maintained in the presence of antigen. Therefore, although these reports supported a role for T cells in the induction of IL-1 by macrophages, it was not clear from these studies whether previous activation of T cells was required for acquisition of this IL-1 inductive property.

Whether previous activation of T cells is required for the generation of IL-1 inductive signals remains controversial. Bhardwaj and Steinmann (109) have employed

human monocytes to address whether the induction of IL-1 can occur in primary as well as secondary immune responses. These studies supported the specific immunodetection of IL-1 β at the secreted level with assessments of intracellular IL-1 β levels, which provided an early measure of IL-1 production. During primary immune activation towards anti-CD3 mAb, these studies demonstrated inconsistent patterns of induction of intracellular monocyte IL-1 and secreted IL-1 β . However, IL-1 was consistently induced during secondary mitogenic responses, employing anti-CD3 activated T cells blasts isolated from dendritic cell clusters. These authors therefore concluded that induction of IL-1 was a feature of secondary but not primary immune responses. A pattern of IL-1, detectable at the secreted but not intracellular level in response to the anti-CD3 mitogen, suggested technical limitations inherent in these studies for detection of intracellular monocyte IL-1. Furthermore, these studies are difficult to reconcile with the reported induction of IL-1 in other primary mitogenic responses in the human, assessed at the level of secreted IL-1 (110-112) and IL-1 α and β mRNAs (113). Whether or not induction of IL-1 can occur during primary mitogenic responses therefore remains unclear. It should be noted that no prior studies have adequately addressed the question whether previous activation of T cells is required for generation of IL-1 inductive signals, as the mitogenic responses previously utilized stimulate both naive

and previously primed T cells in peripheral blood.

In addition to cell contact dependent signals, a second type of T cell IL-1 inductive signal has been identified which mediates induction of IL-1 through soluble T cell lymphokines. Weaver and Unanue have characterized a soluble mIL-1 inductive signal generated by T cell clones following mitogenic stimulation by concanavalin A (Con A) in the presence of macrophage membranes (114). This T cell signal, designated IL-1 inducing factor (IL-1 IF), was dependent upon protein synthesis for its generation and was shown to be related to the TNF family of lymphokines. Transduction of the mIL-1 inductive signal by IL-1 IF was non-MHC restricted. Several other investigators have confirmed the role of soluble T cell factors during the induction of IL-1 in human blood monocytes. Recombinant preparations of the cytokines IL-2 (115, 116) and TNF- α (48, 117) have been identified to exhibit de novo IL-1 mRNA inductive properties, while IFN- has been shown to augment expression of IL-1 mRNA levels (117). These recent studies have shifted experimental strategy towards measurements of IL-1 induction at the level of mRNA expression. In light of the multiple posttranslational processing steps that have been identified during the production of mature IL-1 protein in monocytes, assessments of IL-1 induction at the mRNA level provide the most suitable level at which to detect early IL-1 inductive signals.

collectively, these studies demonstrated that soluble T cell derived cytokines could induce monocyte IL-1 mRNAs in an MHC unrestricted manner.

The T cell derived cytokines, previously identified to possess IL-1 inductive properties, are unlikely to represent the initial IL-1 inductive signal for the blood monocyte during an immune response to foreign antigen. These IL-1 inducing cytokines are all produced at a later stage of the immune response and are required at the upper end of their physiological dose range. It is more likely that these IL-1 inducing cytokines play a role in the non-specific amplification of IL-1 production from bystander monocytes later in the immune response. The discovery of a pathway that can lead to a non-specific amplification of IL-1 production during an immune response reinforces the need to better understand the events at the site of the antigen specific interaction with T cells and monocytes that initially leads to the induction of IL-1 in monocytes.

It is clear from these previous reports that only limited information is available concerning the signals that regulate induction of IL-1 during an immune response. Heavy reliance has also been placed in previous studies on the use of previously activated T cells or T cell clones. The human system presents the advantage that unprimed T cells may be

isolated from peripheral blood and polyclonally activated through their TCR complex in the well characterized anti-CD3 mitogenesis model (118).

Anti-CD3 as a Model of TCR Triggered T Cell Activation

The anti-CD3 mitogenesis model in humans provides an experimental model that permits the polyclonal activation of peripheral blood T cells in a manner that is functionally analogous to a primary immune response (118). The experimental model employs antibodies directed against the CD3 signalling moiety of the TCR complex to mimic the signal provided by the MHC restricted recognition of foreign antigen through the TCR. The activation requirements of T cells during anti-CD3 mitogenesis are the same as those previously described for an antigen specific immune response (119). Effective triggering through the TCR in this model demonstrates a requirement for monocytes, which cross link CD3/anti-CD3 complexes on the surface of the T cell via monocyte Fc receptors (119). Triggering through the TCR results in the same biochemical events that are mediated by recognition of foreign antigen through the TCR, including elevation of intracellular Ca^+ levels (64), phosphatidyl inositol hydrolysis (63), partial PKC translocation and IL-2 receptor expression (66). Effectively triggered T cells demonstrate a requirement for additional monocyte derived

costimulatory signals before they elaborate IL-2 and undergo IL-2 induced clonal proliferation (67).

This dissertation employed the anti-CD3 mitogenesis model to investigate the T cell mediated induction of IL-1 mRNA and secreted activity in human monocytes.

The major objectives of this investigation were:

- (1) To determine whether induction of human monocyte IL-1 mRNAs occurred during anti-CD3 mediated TCR triggering and
- (2) To characterize the T cell signals required for this induction.

MATERIALS AND METHODS

cdNA Probes

cdNAs for the human IL-1 α , human IL-1 β , human IL-6 and mouse histone H3.3 genes were provided by Dr. Masaaki Yamada of the Dainippon Pharmaceutical Co., Ltd. (Osaka, 541 Japan), Dr. Donald Carter of the Upjohn Corp. (Kalamazoo, MI), the American Type Culture Collection (ATCC; Rockville, MD) and Dr. William Marzluff (Florida State University, Tallahassee, FL) respectively.

The IL-1 α probe was contained in a 1.6 kb EcoRI/PstI fragment in plasmid pHN-02 and included nucleotides 455-872 of the human IL-1 α coding region and 3' untranslated material (53). The IL-1 β probe was contained in a 546 bp PstI fragment in plasmid pIL-1 14 and included nucleotides 357-830 of the human IL-1 β coding region (50). The IL-6 probe was contained in 1.16 kb EcoRI fragment in plasmid pCSF309 and corresponded to nucleotides 1-1120 of the human IL-6 coding region (120). The histone H3.3 probe was contained in a 2.5 kb HindIII fragment in plasmid MH921 and included the entire 405 bp coding region and flanking genomic sequences of the H3.3 mouse histone gene (121).

cDNA inserts were excised from vector DNA by digestion with restriction endonucleases (BRL Life Technologies Inc., Gaithersburg, MD.). For IL-1 β cDNA, for example, 10 μ g pIL-1 14 was digested at 37°C for 1 h with the manufacturer recommended units of the restriction endonuclease PstI. Digested DNA was loaded in the presence of 67 mg/ml sucrose loading buffer and size fractionated by electrophoresis on 1% agarose gels (SeaKem ME; FMC Bioproducts, Rockland, ME). Gels were made up in electrophoresis running buffer, consisting of 40 mM Tris-acetate (pH 6.8), 1 mM EDTA, and 0.5 μ g/ml ethidium bromide. cDNA inserts were visualized on a UV transilluminator, sized with respect to HindIII digested lambda DNA size markers and cut out of the gel. Gel fragments containing cDNAs were weighed, boiled at 0.3 g/ml in H₂O and stored frozen at - 20°C prior to use.

For use as probes in these studies, 25 ng cDNA was labelled with 50 μ Ci α^{32} P-dCTP (3000 Ci/mmol, NEN Research Products, Boston, MA.) employing an oligolabelling kit according to manufacturers specifications (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Specific activities were $> 5 \times 10^7$ CPM/ μ g DNA. The labelling reaction was terminated by the addition of 20 mM EDTA and unincorporated radionuclides were removed by size exclusion column chromatography employing Sephadex G50 (Boehringer Mannheim Biochemicals, Indianapolis,

IN). Incorporation of α -³²P-dCTP into cDNAs was determined by precipitation of 1 ng DNA in the presence of 1 μ g yeast tRNA (BRL Life Technologies, Inc.) with 1 ml 10 % TCA and a 10 min. incubation on ice. The TCA precipitate was harvested onto filters, dried and counted in organic scintillation cocktail (3a20 Complete Counting Cocktail, Research Products International, Mount Prospect, IL) using an LKB 1219 Rackbeta scintillation counter (LKB Instruments Inc., Gaithersburg, MD).

Normal Human Serum

Normal human serum was collected from peripheral blood of 22 normal human donors. Peripheral blood was incubated at 37°C for 1 h, allowed to clot for 16 h at 4°C and serum was collected following centrifugation. Serum was pooled, heat inactivated at 56°C for 30 min and sterile filtered through a 0.45 μ filtration unit (Becton Dickinson Labware, Lincoln Park, NJ). Normal human serum was stored in aliquots at -20°C prior to use.

Cell Lines

The LBRM-33-1A5 mouse thymoma was obtained from the ATCC and was maintained in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 15 % defined FCS (Hyclone Laboratories,

Logan, UT), 2 mM l-glutamine and 25 μ M β -2-mercaptoethanol. The IL-2 dependent cell line CTLL-20.J was maintained in RPMI 1640 containing 10 % FCS (10 % FCS-RPMI), 2 mM l-glutamine and 50 μ M β -2-mercaptoethanol. The KT69-7 hybridoma, secreting the anti-CD4 antibody KT69-7 (122), was provided by Dr. T. Mohanakumar (Washington University School of Medicine, St. Louis, MO). Hybridoma cell lines L243, W6/32, 63D3 and OKT8 were obtained from the ATCC and were maintained in 20% FCS-RPMI, supplemented with 2 mM l-glutamine.

Monoclonal Antibodies

Antibodies were isolated from supernates of the monoclonal antibody secreting hybridomas L243, W6/32, 63D3, OKT8 and KT69-7. Supernates were collected from cultures 3 days after the most recent feeding. Supernates were centrifuged at 10,000 x g for 20 min and adjusted to 40 % (v/v) $(\text{NH}_4)_2\text{SO}_4$ with a saturated solution of $(\text{NH}_4)_2\text{SO}_4$, pH 7.5. Precipitates were harvested after 1 h of incubation on ice by centrifugation at 10,000 x g for 20 min, dissolved in a minimal volume of deionized water and dialyzed extensively against phosphate buffered saline, containing 150 mM NaCl, and 10 mM phosphate (pH 7.2). The concentration of protein in each antibody preparation was determined using the Bradford calorimetric assay (Bio Rad Laboratories, Richmond, CA) (123).

Antibodies obtained commercially included purified MOPC 21 antibody (IgG₁; Sigma Chemical Company), purified UCHL1 antibody (Dakopatts, Glostrup, Denmark), phycoerythrin (PE) conjugated polyclonal goat anti-mouse IgG_{2a} antibody (Fisher Scientific, Pittsburgh, PA) and fluorescein isothiocyanate (FITC) conjugated polyclonal F(ab')₂ goat anti-mouse IgG F(ab')₂ antibody (Organon-Teknika, Cappel Research Reagents, West Chester, PA) were obtained commercially. Anti-CD3 mAb (OKT3, IgG_{2a}; Ortho Pharmaceutical Co., Raritan, NJ) was obtained from the Loyola Medical Center Pharmacy. PE conjugated anti-HLA-DR, anti-Leu M3, anti-Leu 2a and FITC conjugated anti-Leu 3a&b, anti-Leu 4, anti-Leu 5, anti-Leu 18 and unconjugated anti-CD5 (Leu 1), dialyzed in Hanks balanced salts solution (HBSS) to remove cytotoxic preservatives prior to use in cell cultures, were purchased from Becton-Dickinson.

Cell Populations

a. Isolation of Monocytes and T Cells

Leukocytes were obtained from normal volunteer donors by leukapheresis or phlebotomy. Leukapheresis preparations enriched for the mononuclear cell fraction were collected in the presence of citrate as an anticoagulant. Whole peripheral blood was collected in the presence of 10 U/ml preservative

free heparin as an anticoagulant. For the collection of peripheral blood mononuclear cells (PBMCs), leukapheresis preparations or heparinized whole blood were diluted with an equal volume of HBSS and layered onto ficoll-hypaque gradients (LSM, density 1.077 - 1.080 g/ml; Organon Teknika Corporation, Durham, NC). Following centrifugation for 20 min. at 500 x g, the PBMC fraction was isolated from the ficoll interface and washed three times in ice cold HBSS containing 5 % FCS.

For isolation of monocytes and T cells, PBMCs were fractionated by density on continuous Percoll gradients. Continuous Percoll gradients were prepared by centrifugation of 1.075 g/ml iso-osmotic Percoll in 40 ml screw cap polycarbonate tubes at 30,000 x g for 10 min at 22°C. Density marker beads were employed to monitor densities of the resulting Percoll gradients. The densities of resulting gradients ranged from 1.033 g/ml at the top of the centrifuge tube to 1.098 g/ml at the bottom.

PBMCs were fractionated by centrifugation over continuous Percoll gradients (4×10^8 /tube) at 500 x g for 30 min. Monocyte enriched cell preparations were collected from the low density Percoll fraction (1.045-1.062 g/ml), washed three times in HBSS containing 5% FCS and adhered to 150 mm tissue culture dishes (Nunc # 68381, Rupp & Bowman Co., Shaumburg, IL) in 5% human serum-RPMI 1640, at 5×10^7

cells/plate for 2 h at 37°C. Purified monocyte lawns were obtained by removal of non-adherent cells by four vigorous washes with prewarmed HBSS. For double chamber well experiments, monocytes were allowed to adhere to the lower and/or upper chambers of 24 mm Transwells (0.4 μ m pore size, Costar, Cambridge, MA), employing the same wash procedures described above for conventional plates. Purity of monocytes was routinely confirmed by flow cytometric analysis of HLA-DR, CD14 and CD2 cell surface marker expression and reconstitution of functional responses by T cells.

T lymphocytes, collected from the high density Percoll fraction (1.070-1.080 g/ml), were depleted of adherent cells by passage through nylon wool columns. Nylon wool (Cellular Products Inc., Buffalo, NY) was washed by five cycles of boiling for 10 min in deionized H₂O followed by air drying. Combed nylon wool was loosely packed into 60 ml syringes at 3 g/syringe and autoclaved. Prior to use, columns were washed with 100 ml 10 % FCS-RPMI, containing 2 mM l-glutamine and 50 μ g/ml gentamycin, to remove air bubbles. Columns were drained and equilibrated in fresh 10 % FCS-RPMI at 37°C for 1 h prior to the addition of cells. Cells were added to drained columns in a volume of 10 ml, at 10⁸ cells/ml, and incubated at 37°C, 5 % CO₂, for 1 h. Nonadherent cells were collected in the effluent fraction of columns rinsed with prewarmed 10 % FCS-RPMI at a flow rate of 1 drop/second. Nylon wool non-adherent

cells were subsequently incubated with sheep red blood cells (SRBCs) treated with 2-aminoethyl isothiuronium bromide hydrobromide (AET; Sigma Chemical Co., St. Louis, MO) and fractionated over ficoll-hypaque gradients. Erythrocyte receptor positive (E^+) T cells were collected from the erythrocyte cell pellet following lysis of red cells by incubation for 1 min in ice cold lysing buffer, consisting of 150 mM NH_4Cl , 10 mM $KHCO_3$ and 0.1 mM EDTA. Residual contamination by monocytes and granular cells was removed by treatment with a lysosomotropic agent, leucine methyl ester (LME; Sigma Chemical Co.), at 5 mM for 50 min at 22°C. Purity of T cells was confirmed by flow cytometric analysis of HLA-DR, CD14 and CD2 cell surface marker expression and absence of proliferative responses towards either concanavalin A (Con A) or anti-CD3 mitogens.

b. Isolation of T Cell Subpopulations

Negative selection of CD4+ and CD8+ T cell subsets from purified T cells was performed by coating cells with murine monoclonal antibodies followed by magnetic bead removal of unwanted T cell subsets. Purified T cells were coated with saturating amounts of anti-CD8 (OKT8) or anti-CD4 (KT69-7) antibodies for 30 min on ice and washed three times with ice-cold HBSS containing 5% FCS. Murine monoclonal antibody coated T cells were subsequently incubated with goat-anti-

mouse conjugated magnetic beads (Advanced Magnetics, Inc., Cambridge, MA) for 30 min on ice in a 25 cm² flask (Corning Glass Works, Corning, NY) at a bead:cell ratio of 40:1. Cells conjugated to magnetic beads were removed by a 5 min exposure of the flasks to two vertical magnetic separators (Advanced Magnetics, Inc.). The negatively selected cells were collected by pipetting and retreated in an identical fashion with fresh magnetic beads. Purity of negatively selected T cell subpopulations was confirmed by flow cytometric analysis of CD4 and CD8 cell surface markers.

Positive selection of T cell subpopulations was achieved by fluorescence activated cell sorting. CD4⁺ and CD8⁺ T cell subsets were stained with saturating amounts of FITC anti-Leu 3a&b and PE anti-Leu 2a for 30 min on ice, washed three times with ice-cold HBSS containing 5% FCS and sorted using a FACS 420 (Becton-Dickinson Inc.). Likewise, CD45RA⁺ and CD45RO⁺ T cell subsets were stained with saturating amounts of FITC conjugated anti-Leu 18 (IgG₁) and unconjugated UCHL1 (IgG_{2a}) plus PE conjugated goat anti-mouse IgG_{2a}, followed by cell sorting. Purity of T cell subpopulations was confirmed by flow cytometric analysis.

Anti-CD3 Mitogenesis

Anti-CD3 mitogenesis was performed by the addition

autologous T cells and anti-CD3 (50 ng/ml) to plates containing 48 h preadhered lawns of monocytes, at T cell:monocyte ratios of 4:1. T cells were activated in anti-CD3 mitogenic cultures for 1 - 48 h. For double chamber well experiments, T cells and anti-CD3 were added to monocyte lawns in the upper chamber of Transwell plates, employing the same concentrations and culture conditions described above.

All cultures were carried out in culture medium consisting of 5 % normal human serum-RPMI, supplemented with 2 mM l-glutamine and 50 μ g/ml gentamycin. Endotoxin levels of cultures and components were monitored by the chromogenic Limulus Amoebocyte Lysate assay (Whittaker Bioproducts Inc., Walkersville, MD) and were below 5 pg/ml.

For assessments of proliferation, parallel microcultures were established in flat-bottomed 96 well microtiter plates (Falcon, Becton-Dickinson Labware) using 10^5 cells and were incubated for 72 h at 37°C and 5 % CO₂. Cultures were pulsed with 2 μ Ci/well [methyl³H]-thymidine (6.67 Ci/mmol, NEN Research Products) for the final 8 h of incubation. Cells were harvested onto glass filter strips using a PhD cell harvester (Cambridge Technology Inc., Cambridge, MA), baked in an 80°C drying oven for 30 min. and counted in 3a20 organic scintillation cocktail (Research Products International) using an LKB 1219 Rackbeta

scintillation counter (LKB Instruments Inc.).

Con A Mitogenesis

For assessments of Con A induced proliferations, 10^5 cells were incubated at 37°C, 5 % CO₂ for 72 h in 96 well flat bottomed microtiter plates in the presence of 10 µg/ml Con A. Cell culture and assessments of proliferation were carried out as described above.

Immobilized Anti-CD3

Anti-CD3 was immobilized by incubation at 100 µg/well to flat bottomed 24 well plates (Costar) or at 10 µg/well to 96 well plates, in buffer consisting of 50 mM Tris, 150 mM NaCl, 0.02 % (^{w/v}) NaN₃, pH 8.6, for 24 h at 4°C. Prior to cell culture, wells were washed four times with HBSS. Cells were added immediately at 10^6 cells/well to 24 well plates or 10^5 /well to 96 well plates.

Allogeneic Mixed Lymphocyte Response (MLR)

Purified responding T cell populations and purified allogeneic stimulator monocyte populations were isolated from peripheral blood of different normal human donors by the procedures described above. For subsequent isolation and

analysis of RNA, allogeneic MLRs were established by the addition of responding T cells to lawns of stimulator monocytes contained in 150 x 20 mm culture plates at T cell:monocyte ratios of 2.5:1. T cells were activated in allogeneic MLR culture for 12 - 48 h.

Isolation of RNA

a. Cell Harvest

Monocytes and T cells were repurified from anti-CD3 mitogenesis cultures for subsequent isolation of RNA. Monocytes were collected from plates following the removal by vigorous washing of non-adherent cells. Adherent cells were then detached from culture plates by the addition of ice cold Ca^{2+} - and Mg^{2+} - free HBSS, containing 2.5 mM EDTA, for 30 min followed by gentle scraping with a rubber policeman. Contaminating T cells in the adherent fraction of mitogenic cultures were depleted by treatment with anti-CD3 plus complement (3-4 week rabbit; Pel-Freez, Brown Deer, WI), at 37°C for 30 min. Purity of the resulting monocytes prior to RNA extraction was confirmed by flow cytometric analysis of HLA-DR, CD14 and CD2 cell surface marker expression.

T cells were isolated from the non-adherent cell fraction by rosetting with AET treated T cells. Purity of

resultant E⁺ T cells was confirmed prior to RNA extraction by flow cytometric analysis of HLA-DR, CD14 and CD2 cell surface marker expression.

At the time of harvest, all culture supernates were collected, centrifuged and stored frozen at -20°C for subsequent assays of secreted cytokine levels.

b. RNA Extraction

Total cellular RNA was isolated by centrifugation of guanidinium isothiocyanate cell lysates over a cesium chloride cushion (127). Cells were washed once with ice cold HBSS prior to RNA extraction and were > 80 % viable, as judged by trypan blue dye exclusion. Cells were lysed by the addition of 2.5 ml lysis buffer, consisting of 4 M guanidinium isothiocyanate (BRL Life Technologies Inc.), 0.5 % Sarkosyl, 25 mM sodium citrate and 0.1 M β -2-mercaptoethanol. DNA was sheared by passing the cell lysate four times through a 19 gauge needle. Cell lysates were made 2.4 M with respect to CsCl₂ by the addition of 1 g CsCl₂ and loaded onto 1.2 ml cushions composed of 5.7 M CsCl₂ and 0.1 M EDTA in SW40.1 Ti ultraclear ultracentrifuge tubes (Beckman Instruments Inc., Palo Alto, CA). RNA was pelleted through the CsCl₂ cushion by centrifugation in an SW40.1 Ti rotor (Beckman Instruments) at 225,000 x g for 16 h, at 20°C. Following centrifugation, the

guanidinium isothiocyanate layer, containing protein and sheared DNA, was carefully removed and the sides of the tubes cleaned with a sterile cotton-tipped applicator before inversion to remove the CsCl_2 layer. Tubes were cut below the level of the CsCl_2 interface and RNA pellets resuspended by repeated pipetting (> 50 times) in 0.15 ml diethyl pyrocarbonate treated water (DEPC- H_2O). RNA yields were quantitated by absorbance at 260 nm and purities confirmed by $A_{260\text{nm}}/A_{280\text{nm}}$ ratios, which were > 1.80 in all experiments.

Slot Blot and Northern Blot Hybridization

a. Immobilization of RNA

RNA was denatured for slot-blot analysis by heating at 65°C , in the presence of 8 % formaldehyde, 6 X SSC (1 X SSC = 0.15 M NaCl, and 0.3 M sodium citrate; pH 7.2), followed by quenching on ice for 10 min. The RNA was applied under vacuum to nitrocellulose membranes (BRL Life Technologies Inc.) employing a "Hybrislots" manifold (BRL Life Technologies Inc.) at 2.5, 0.5 and 0.1 μg RNA per well. For northern blot analysis, RNA (10 μg) was denatured by heating at 50°C for 1 h in the presence of 1 M glyoxal, 50 % (v/v) dimethyl sulfoxide, and 10 mM phosphate buffer (pH 6.8) followed by quenching on ice for 10 min. RNA was fractionated by size in the presence of 0.1 % bromophenol blue by electrophoresis in

1.4 % agarose (FMC Bioproducts) gels. Gels were made up in running buffer, consisting of 10 mM phosphate buffer (pH 6.8). RNA was transferred to Gene Screen Plus membranes (NEN Research Products) by capillary blotting in 10 X SSC transfer buffer for 18 h and membranes were air dried.

b. Hybridization

Membranes were prehybridized by heating at 42°C for 24 h in prehybridization buffer, consisting of 50 % deionized formamide, 10 % dextran sulfate, 1 % SDS, 5 X SSPE (1 X SSPE = 0.15 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA; pH 7.2), 5 X Denhardt's solution (1 X Denhardt's = 0.02 % (w/v) ficoll, 0.02 % (w/v) polyvinylpyrrolidone, and 0.02 % (w/v) BSA) and 250 mg/ml freshly denatured salmon sperm DNA. Hybridization was carried out for 24 h at 42°C following the addition of 10⁶ CPM/ml ³²P-labelled cDNA, which was heated at 100°C for 10 min and quenched on ice for 5 min prior to addition to prehybridized membranes.

Following hybridization, nitrocellulose and Gene Screen Plus membranes were subjected to two 30 min low stringency washes, performed with 2 X SSPE, and 0.4 % SDS at 22°C. Subsequently, membranes were subjected to two 30 min high stringency washes, performed for nitrocellulose membranes with 0.1 X SSPE and for Gene Screen Plus membranes with 0.1 X SSPE,

and 0.4 % SDS. High stringency washes on both types of membrane were carried out at 56°C for IL-1 α and IL-6 hybridizations and at 60°C for IL-1 β and H3.3 hybridizations. Autoradiography was performed at -85°C in the presence of X-ray film (Kodak XAR-5, Eastman Kodak Company, Rochester, NY) and two intensifying screens (Lightning Plus, NEN Research Products) for the lengths of time indicated in appropriate figure legends.

c. Rehybridization

Rehybridization was accomplished by stripping radiolabelled cDNAs from nitrocellulose by boiling in T.E. (pH 8.0) for 10 min, followed by baking of membranes at 80°C for 2 h. Gene Screen Plus membranes were stripped for rehybridization by boiling in T.E. (8.0), 1 % SDS for 1 h. Membranes were sequentially hybridized with radiolabelled cDNA probes corresponding to the human IL-1 α , IL-1 β and IL-6 genes and the constitutively expressed mouse histone variant H3.3 gene.

d. Data Analysis

Autoradiograms were scanned with an LKB Ultrosan XL Laser Densitometer (LKB Instruments Inc., Gaithersburg, MD).

Hybridization signal intensities were calculated by integration of the densitometric curves and were expressed as the area under a curve in units of A/mm^2 .

To correct for differences in the amounts of RNA bound to membranes and enable quantitative comparisons of IL-1 mRNA expression among samples, data were expressed as an IL-1 mRNA index. The IL-1 mRNA index is represented by the formula:

$$\text{IL-1 mRNA index} = A_{\text{IL-1}}/A_{\text{H3.3}}$$

The IL-1 mRNA index was calculated as the ratio of the hybridization signal intensity obtained with cDNA probes for IL-1 α or IL-1 β ($A_{\text{IL-1}}$) to the signal intensity obtained for the same sample using a cDNA for the constitutive histone variant H3.3 ($A_{\text{H3.3}}$), whose stable mRNA levels have been shown to be independent of the state of differentiation of cells (128) and remain unaffected by changes in cytoskeletal architecture that may accompany the activation of monocytes (118). To allow quantitative comparisons of IL-1 mRNA induction by given stimuli among multiple experiments, each IL-1 species was expressed as a percent IL-1 mRNA index relative to the maximum (100 %) LPS inducible response.

Paraformaldehyde Fixation of T Cells

Prior to fixation, purified T cells were washed twice with HBSS to remove residual serum. Cells were resuspended in 50 ml polypropylene centrifuge tubes (Falcon, Becton-Dickinson Labware) at a final concentration of 5×10^6 cells/ml in HBSS. An equal volume of prewarmed, sterile 1 % ($\frac{1}{10}$) paraformaldehyde (Sigma Chemical Company) was added and the mixture incubated with shaking at 37°C for 5 min. Paraformaldehyde was prepared as a stock 5 % solution in HBSS (pH 7.2) by heating at 65°C for 1 h, followed by sterile filtration through a 0.22 μ filter (Nunc). Stock paraformaldehyde was stored at 4°C and was diluted freshly on the day of fixation in HBSS. Fixation was terminated by the addition of 10 volumes of ice-cold 0.06 % glycylglycine (Sigma Chemical Company) in HBSS (pH 7.2) followed by centrifugation. Fixed cells were washed an additional two times with ice-cold HBSS and two times with ice-cold HBSS containing 5 % FCS. Fixed cells were incubated at 37°C overnight in 10%-RPMI to leach any remaining paraformaldehyde.

Completeness of fixation and maintenance of cell surface integrity following fixation were confirmed by flow cytometric analysis of propidium iodide (PI; Sigma Chemical Co.) vital dye exclusion (124) as well as CD3 and CD8 cell surface marker expression.

Inhibition of Protein Synthesis

T cells were incubated with 0 - 40 $\mu\text{g/ml}$ emetine (Sigma Chemical Company), an inhibitor of de novo protein synthesis, at 5×10^6 cells/ml in 10 % FCS-RPMI for 2 h at 37°C. Following emetine treatment, T cells were washed three times in HBSS containing 5 % FCS, incubated for 1 h at 37°C in 10 % FCS-RPMI and washed an additional two times with HBSS containing 5 % FCS.

Inhibition of protein synthesis was monitored by assessing ^3H -leucine incorporation into T cells, following a 48 h stimulation with immobilized anti-CD3 and 50 U/ml rIL-2 (Cetus Corporation, Emeryville, CA). ^3H -leucine incorporation was performed using 10^6 T cells collected from each treatment group. Cells were washed twice in leucine-free RPMI to remove cold leucine and incubated for 2 h at 37°C in leucine free 10 % FCS-RPMI, containing 25 μCi 1-[3, 4, 5- $^3\text{H}(\text{N})$] leucine (153.0 Ci/mmol, NEN Research Products). Cells were washed four times with HBSS containing 5% FCS to remove unincorporated ^3H -leucine and protein was precipitated from cell pellets by the addition of 50 μl 0.5 % Triton X-100 (Sigma Chemical Company), 50 μl FCS and 1 ml 15 % trichloroacetic acid (TCA). Protein precipitates were formed by incubation for 1 h on ice. Precipitates were washed five times with 15 % TCA to remove unincorporated ^3H leucine and counted in aqueous scintillation cocktail (Ready-Sol, Beckman Instruments Inc.) using an LKB

Rackbeta 1219 scintillation counter (LKB Instruments Inc.).

Assay of Secreted Cytokines

Secreted cytokine levels in culture supernates were determined using commercially available specific enzyme linked immunoassays for IL-1 α (Endogen Inc., Boston, MA) and IL-1 β (Cistron Biotechnology, Pine Brook, NJ) or specific radioimmunoassays for IL-2, IFN- γ and TNF- α (Medgenix Corp., Ventrex Laboratories, Inc., Portland, ME).

LBRM-33-1A5 Conversion Bioassay for Secreted IL-1 Activity

Biologic IL-1 activity was assayed by the LBRM-33-1A5 conversion bioassay, as previously described (125). LBRM-33-1A5 cells (5×10^4), contained in volume of 100 μ l, were cultured with 100 μ l IL-1 containing supernates for 24 h at 37°C and 5 % CO₂ in triplicate wells of round bottomed 96 well plates (Falcon, Becton-Dickinson Labware) in the presence of 0.01% PHA-P (Wellcome Diagnostics, Dartford, England). Supernates (100 μ l) were collected from plates after centrifugation at 400 x g and assayed for IL-2. Control cultures were performed in the absence of PHA to determine intrinsic IL-2 levels in test supernates.

Assays for IL-2 were carried out employing the IL-2

dependent CTLL20.J line (126). CTLL20.J cells (5×10^3), contained in a volume of 100 μ l, were cultured with 100 μ l serial dilutions of IL-2 containing supernates for 24 h at 37°C and 5 % CO₂ in triplicate wells of round bottomed 96 well plates and pulsed with 1 μ Ci ³H-thymidine (6.67 Ci/mmol, NEN Research Products) over the final 8 h of culture. Incorporation of ³H-thymidine into cells was determined as described previously. IL-1 activities were expressed as IL-1 units/ml, where 1 unit of activity corresponded to the dilution of supernate required for 50 % maximal proliferation relative to a mouse recombinant IL-1 control.

Flow Cytometry and Cell Sorting

For analysis of cell surface markers by flow cytometry, 1×10^5 - 1×10^6 cells were stained in 12 x 75 mm polystyrene centrifuge tubes (Falcon, Becton Dickinson Labware In.) with saturating amounts of fluorochrome conjugated antibody for 30 min on ice, followed by three washes in ice-cold HBSS containing 5 % FCS. Cells were resuspended in 1 ml phenol red free HBSS containing 5 % FCS and were analyzed for fluorescence using a FACS Analyzer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The noise threshold was set to exclude volume signals below those of lymphocytes. All analyses were performed using photomultiplier voltages and gain settings that were

standardized using fluorescent calibration beads, and all signals were processed using logarithmic amplification. Fluorochrome conjugated mouse IgG_{2a} monoclonal antibody controls were used to allow for the establishment of markers for distinguishing positive and negative cell populations. Positive staining was defined visually based on natural boundaries between positive and negative cell populations. Cells were sorted using a FACS 420 (Becton Dickinson Immunocytometry Systems) equipped with a 2 W argon laser at rates of 2000 cells/sec with abort circuitry activated.

For analysis of PI uptake, fixed T cells were incubated in a volume of 0.5 ml on ice for 30 min with 50 μ g/ml PI in HBSS. Cells were analyzed for uptake of PI using FL2 photomultiplier voltage settings of 430 V. Unfixed T cells were employed as controls to allow for the establishment of markers for distinguishing viable and non-viable cell populations.

RESULTS

Composition of Purified Cell Subpopulations

The composition and purity of monocyte and T cell populations employed in these studies was confirmed by flow cytometric analysis of cell surface markers and mitogen-induced proliferative responses. The results of flow cytometric determinations of cell surface marker expression are presented in **Table 1**. Evaluations of cell purity were performed on monocyte and T cell subpopulations isolated from peripheral blood mononuclear cells or from in vitro anti-CD3 mitogenic cultures. Purities of monocyte and T cell populations were assessed by expression of the T cell CD2 cell surface marker and monocyte specific CD14 and HLA-DR markers respectively.

Monocytes isolated from PBMCs in 27 separate experiments ($n = 27$) were 77.1 ± 1.6 % positive for expression of CD14 (CD14⁺), 85.9 ± 1.2 % HLA-DR⁺ and 1.9 ± 0.3 % CD2⁺. T cells isolated from PBMCs ($n = 7$) were 3.3 ± 0.9 % CD14⁺, 2.4 ± 0.9 % HLA-DR⁺ and 93.4 ± 0.8 % CD2⁺. Representative contour plots illustrating cell surface marker expression of these cell subpopulations are shown in **Figure 1 of the Appendix**.

Table 1. Cell Surface Phenotypes of Cell Populations Purified From PBMCs and Anti-CD3 Mitogenic Cultures Purity of cell subpopulations was evaluated by flow cytometric analysis of CD14, HLA-DR and CD2 cell surface marker expression. T cells were depleted from the adherent cell fraction of anti-CD3 mitogenic cultures by treatment with anti-CD3 plus rabbit complement. Results are presented as mean \pm S.E.M. percent cells positive for staining with fluorochrome conjugated anti-CD14 (PE anti-LeuM3), anti-HLA-DR (PE anti-HLA-DR) and anti-CD2 (FITC anti-Leu5) antibodies, obtained from multiple experiments (n) as shown. Representative flow cytometric contour plots illustrating cell surface marker expression of these cell subpopulations are shown in **Figures 1 and 2 of the Appendix.**

Cell Surface Phenotypes of Cell Populations Isolated from PBMCs and
Anti-CD3 Mitogenic Cultures

Cells	Source	# of Expts. (n)	% Positive Cells (Mean \pm S.E.M.)		
			CD14+	HLA-DR+	CD2+
Monocytes	PBMCs	27	77.1 \pm 1.6	85.9 \pm 1.2	1.9 \pm 0.3
T Cells	PBMCs	7	3.3 \pm 0.9	2.4 \pm 0.9	93.4 \pm 0.8
Adherent Cells	Mitogenic Culture	10	40.8 \pm 4.4	54.1 \pm 4.7	16.6 \pm 2.3
Adherent Cells Antibody and C' Treated	Mitogenic Culture	10	76.2 \pm 1.5	82.6 \pm 2.2	3.8 \pm 0.8
T Cells	Mitogenic Culture	4	3.9 \pm 0.2	3.6 \pm 0.5	88.9 \pm 3.4

Monocytes and T cells reisolated from anti-CD3 mitogenic culture were assessed for purity prior to RNA extraction and analysis of mRNA content, as shown in Table 1. Monocytes enriched from the adherent fraction of mitogenic cultures (n = 10) contained a significant proportion (16.6 ± 2.3 %) of residual CD2⁺ T cells and non-viable double negative cells. Contaminating T cells in this fraction were subsequently depleted by treatment with anti-CD3 plus complement. Phenotypic analysis of the final cell population, performed in the presence of propidium iodide (PI) in order to exclude non-viable cells from analysis, revealed these cells to be 76.2 ± 1.5 % CD14⁺, 82.6 ± 2.2 % HLA-DR⁺ and 3.8 ± 0.8 % CD2⁺. T cells, repurified from the non-adherent fraction of mitogenic cultures by rosetting with AET treated sheep erythrocytes, were 3.9 ± 0.2 % CD14⁺, 3.6 ± 0.5 % HLA-DR⁺ and 88.9 ± 3.4 % CD2⁺. Representative contour plots illustrating the cell surface marker expression of these cell subpopulations are shown in Figure 2 of the Appendix.

These phenotypic data demonstrated that monocyte and T cell subpopulations combined for mitogenic activation and subsequently reisolated for mRNA analysis contained < 4 % contamination by the reciprocal cell type.

Purity of T cells isolated from PBMCs was also confirmed by assessing their inability to proliferate in

response to the monocyte dependent T cell mitogen, concanavalin A (Con A). In all experiments, Con A induced proliferative responses of isolated T cells, assessed by ^3H -thymidine incorporation, were $< 5 \%$ of unseparated PBMCs. Representative responses from 10 donors demonstrated ^3H -thymidine incorporation by T cells of $1,876 \pm 441$ cpm, compared to $88,413 \pm 13,790$ cpm by PBMC control cultures, or a mean percent response of $2.2 \pm 0.3 \%$. These data demonstrated that the T cells employed in these studies were sufficiently depleted of monocytes that they were unable to respond towards the T cell mitogen Con A, thus confirming the purity of these cells.

The proliferative responses of purified and combined monocytes and T cells towards anti-CD3 mitogen are shown for 4 experiments in Table 2. Whereas purified monocytes and T cells failed to proliferate in response to anti-CD3 antibody (930 ± 141 and 661 ± 148 cpm respectively), combining monocytes and T cells at a ratio of 1:4 completely restored proliferative responses compared to control PBMC cultures ($111,101 \pm 6,519$ vs. $117,643 \pm 15,688$ cpm).

These proliferative data confirmed the purity of isolated cell subpopulations and demonstrated that combined monocyte and T cell mitogenic cultures generated the necessary costimulatory signals to enable T cells to proliferate. These

Anti-CD3 Induced Proliferation of Isolated and Combined
Cell Subpopulations

Cells	Stimulus	³ H-Thymidine Incorporation	
		(Mean CPM ± S.E.M.)	
Monocytes	-	772 ±	21
	Anti-CD3	930 ±	141
T Cells	-	589 ±	145
	Anti-CD3	661 ±	148
Monocytes Plus T Cells (1:4)	-	629 ±	103
	Anti-CD3	111,101 ±	6,519
PBMCs	-	761 ±	61
	Anti-CD3	117,643 ±	15,688

Table 2. Anti-CD3 Induced Proliferation of Isolated and Combined Cell Subpopulations

Proliferation of cell populations was assessed by ³H-thymidine incorporation into cells at 72 h of culture in the presence of either medium or 50 ng/ml anti-CD3. Counts represent the mean ± S.E.M. cpm from four experiments.

cell populations were therefore suitable for the study of signalling events leading to the production of IL-1 during primary immune activation.

Induction of Monocyte IL-1 α and β mRNA Following Adherence to Plastic

Inasmuch as previous studies have demonstrated that IL-1 mRNA levels were induced in monocytes following adherence to plastic, initial experiments were performed to characterize the spontaneous expression of IL-1 α and β mRNAs in monocytes following their isolation from peripheral blood and adherence to plastic.

Freshly isolated monocytes were adhered to culture plates and evaluated by slot blot hybridization analysis, at time points of up to 48 h following adherence, for detection of IL-1 α , IL-1 β and histone H3.3 mRNAs, as shown in Figure 1. To correct for any differences in the amounts of RNA attached to membranes and enable quantitative comparisons of IL-1 mRNA expression among samples, the hybridization signals obtained with cDNA probes for IL-1 α and β were normalized with respect to the signal obtained for the constitutive histone H3.3 gene and were expressed as IL-1 mRNA indices. These results, depicted in Figure 2 as IL-1 mRNA indices, demonstrated that monocyte IL-1 α and β mRNAs were detectable

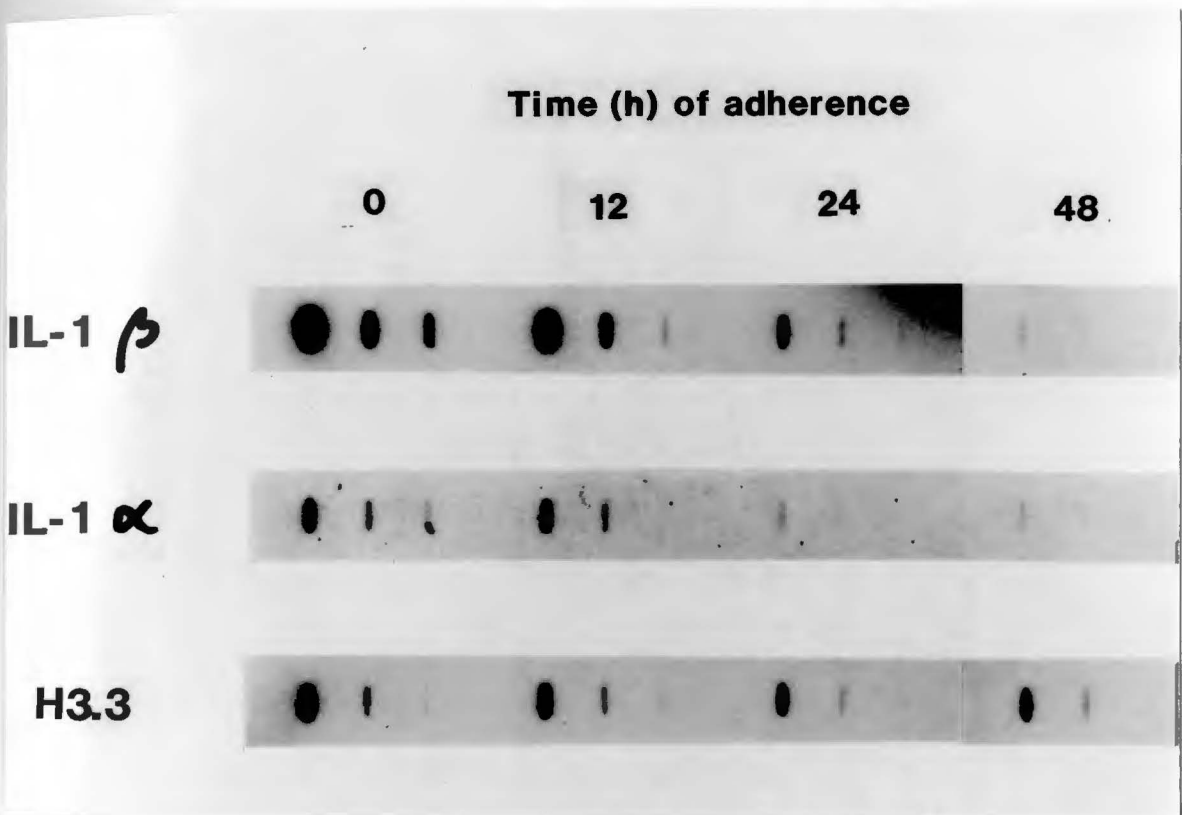


Figure 1. Slot Blot Analysis Depicting Kinetics of Induction of Monocyte IL-1 mRNAs Following Adherence to Plastic Total cellular RNA was extracted from monocytes at the designated time points following adherence and subjected to slot blot analysis as described in Materials and Methods. Membranes were sequentially hybridized with ^{32}P -labelled cDNAs corresponding to the coding regions of human IL-1 β , human IL-1 α , and the mouse histone H3.3 genes. Autoradiography was carried out with two enhancing screens for 18 h for IL-1 β , 40 h for H3.3 and 96 h for IL-1 α . Representative results from one of four experiments are shown.

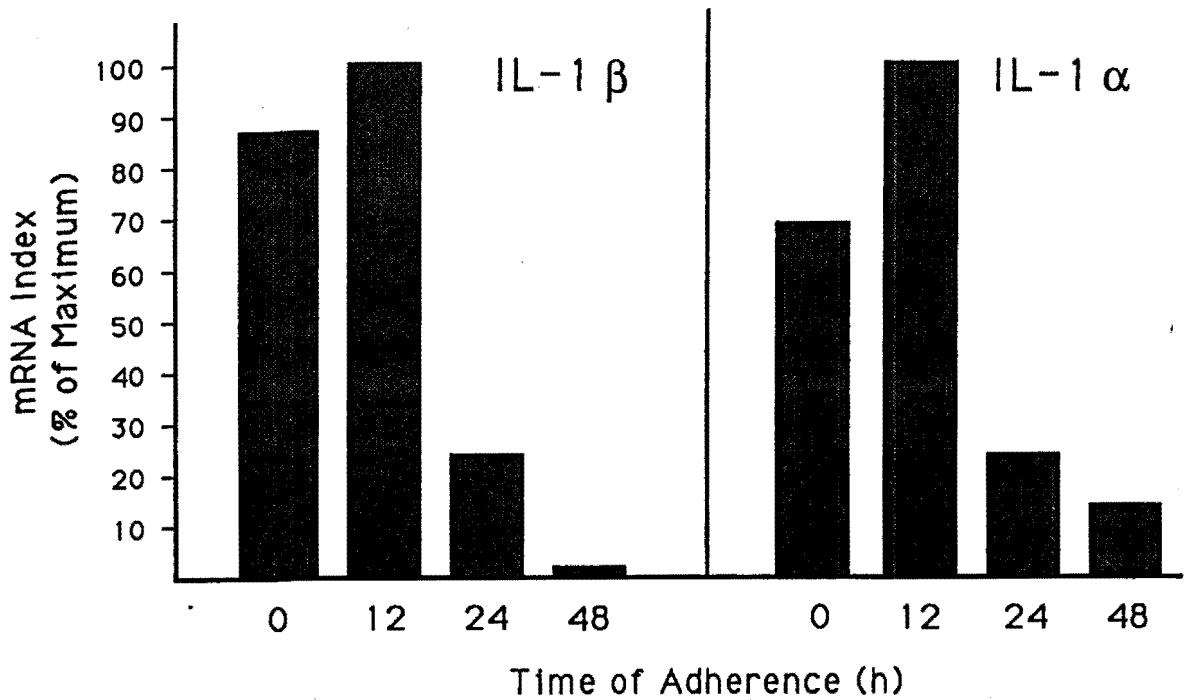


Figure 2. Induction of Monocyte IL-1 mRNAs Following Adherence, Quantitated as IL-1 α and β mRNA Indices
Hybridization signals depicted in **Figure 1** were evaluated by densitometric scanning of the autoradiogram and quantitated as an IL-1 mRNA Index (**Materials and Methods**), expressed as percent maximum IL-1 mRNA Indices for each IL-1 species.

immediately prior to adherence ($t = 0$ h), achieved maximum levels at 12 h following adherence and returned to low or undetectable levels by 48 h.

Inasmuch as previous studies showed that transcription of the histone H3.3 gene remained unaffected by cell cycle or stage of differentiation of cells, this investigation employed H3.3 as a constitutive control gene. To confirm that monocyte levels of H3.3 mRNA remained unaffected following stimulation with LPS or during anti-CD3 mitogenesis, the percent change of H3.3 mRNA levels between stimulated and unstimulated monocytes was determined. As calculated from 21 different experiments, the mean percent change of monocyte H3.3 mRNA levels upon stimulation with LPS was $1.04 \pm 3.7 \%$ and with anti-CD3 mitogenesis was $0.26 \pm 5.1 \%$. These results demonstrated that mean levels of H3.3 mRNA in monocytes were not significantly affected by stimulation.

The results of experiments examining plastic adherence induced monocyte IL-1 mRNAs demonstrated that, following an initial period of mRNA induction, the expression of IL-1 α and β mRNAs in monocytes returned to undetectable levels by 48 h of adherence. Subsequent experiments examining the induction of IL-1 mRNA in monocytes during anti-CD3 mitogenesis therefore employed resting monocytes which had been cultured for 48 h on plastic plates in order to allow spontaneous IL-

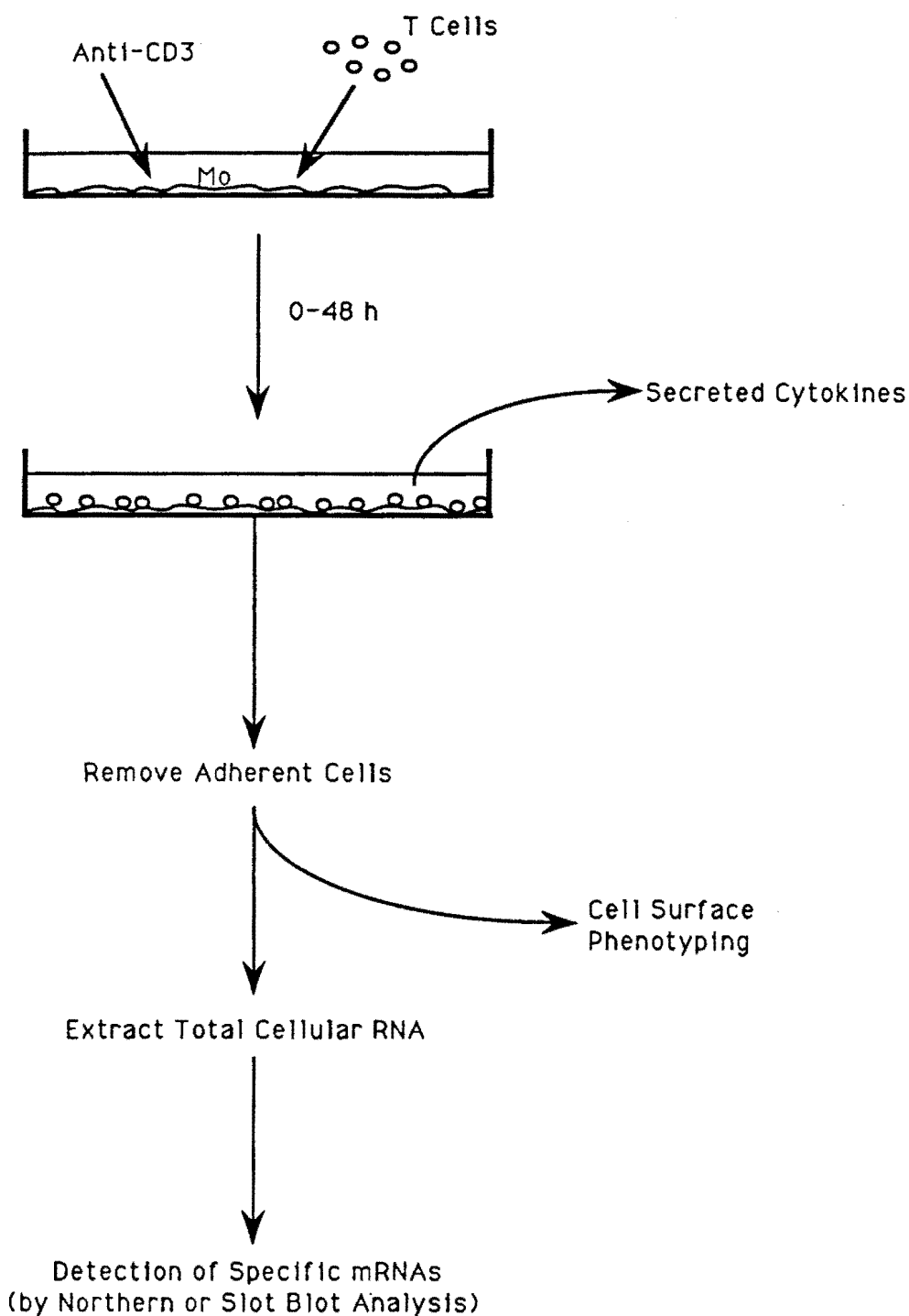
1 mRNA levels to subside.

Monocyte IL-1 α and β mRNAs Are Induced During Anti-CD3 Mitogenesis

Experiments were carried out to determine whether IL-1 α and β mRNAs were induced in monocytes stimulated by the addition of T cells plus anti-CD3. These studies were performed according to the experimental scheme depicted in Figure 3. Purified, 48 h adherent monocytes were incubated with purified autologous T cells plus anti-CD3, using T cell to monocyte ratios of 4:1. Total cellular RNA was extracted from monocytes at 1 - 48 h time points of anti-CD3 mitogenesis and evaluated by northern or slot blot hybridization analysis for detection of IL-1 α and β mRNAs. Culture supernates were collected at 1 - 48 h time points for detection of secreted IL-1 and other cytokines generated during anti-CD3 mitogenesis.

Representative results from one of two experiments examining the induction of monocyte IL-1 mRNA during anti-CD3 mitogenesis are illustrated in Figure 4. Results demonstrated that the predicted 2.3 kb IL-1 α and 1.8 kb IL-1 β mRNAs were both induced in monocytes stimulated by the addition of T cells plus anti-CD3 (lanes 6-10), but not by the addition of T cells or anti-CD3 alone (lanes 13 and 14). No residual

Figure 3. Experimental Scheme Diagram summarizes the experimental steps utilized throughout the course of the investigation, including the addition of anti-CD3 plus purified T cells to lawns of autologous monocytes and the methods employed for the subsequent analysis of IL-1 generated during anti-CD3 mitogenesis.



background IL-1 α or β mRNA expression was detected in monocytes cultured in the presence of medium alone (lanes 11-12). Furthermore, RNA extracted from activated T cells which were purified from anti-CD3 mitogenic cultures at 12 h contained no detectable IL-1 α or β mRNAs (lane 15). IL-1 α and β mRNAs were both induced in monocytes stimulated in control cultures with LPS (lanes 1-5). These results demonstrated that IL-1 α and β mRNAs were induced in monocytes during anti-CD3 mitogenesis and that induction required the presence of both T cells plus anti-CD3. Furthermore, IL-1 mRNA was induced only in the monocyte population. Finally, detection of the expected sizes of mRNA species confirmed the specificity of the IL-1 cDNA probes for use in subsequent studies.

The kinetics of appearance of monocyte IL-1 mRNAs during anti-CD3 mitogenesis (lanes 6 - 10) revealed that both IL-1 α and β mRNAs were detectable in monocytes as early as 1 h following the addition of T cells plus anti-CD3, reached maximum levels at 12 h and declined by 24 h. Similar kinetics of IL-1 mRNA induction were observed in all individuals studied ($n = 9$). In all individuals IL-1 α and β mRNAs were detectable at 1 h of anti-CD3 mitogenesis and decreased by 24 h. In 8 out of 9 individuals, peak induction of monocyte IL-1 α and β mRNA occurred at 12 h, whereas in 1 individual it occurred at 4 h. These data demonstrated that induction of

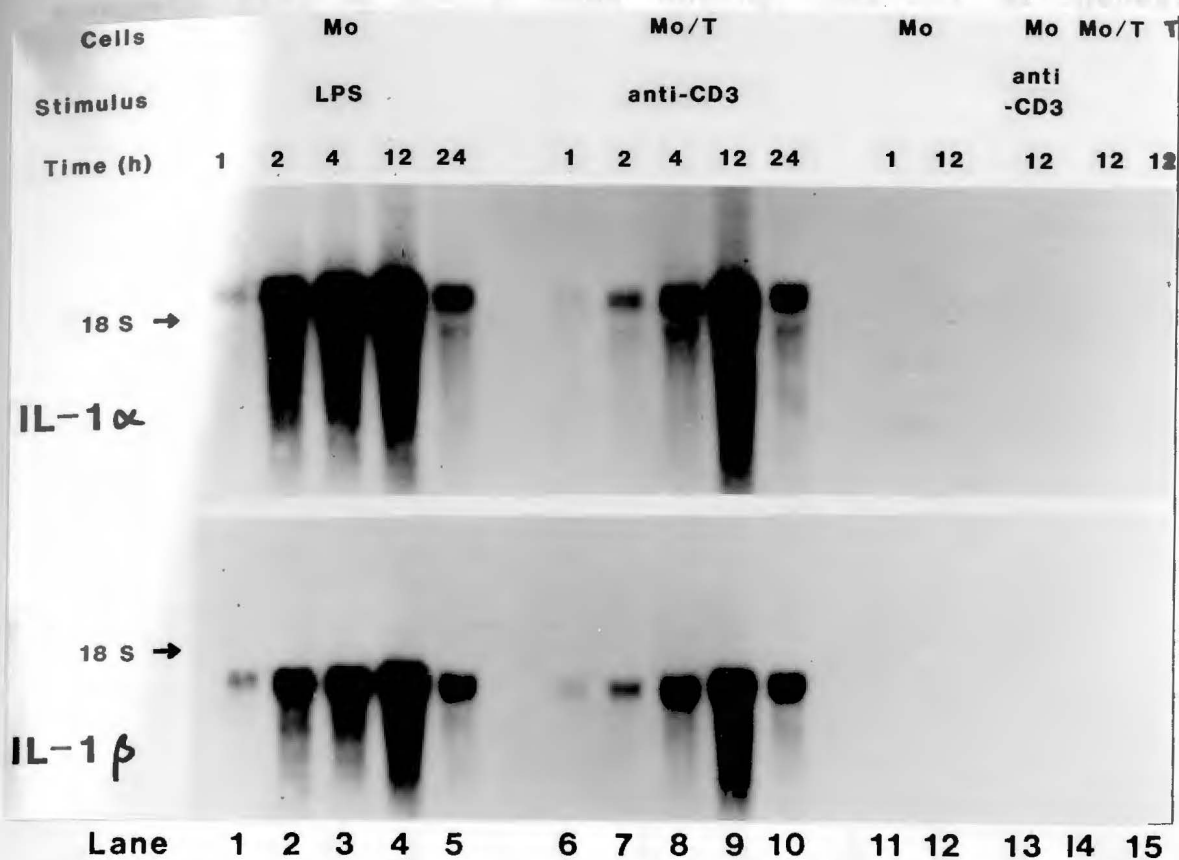


Figure 4. Northern Blot Analysis, Depicting Induction of IL-1 α and β mRNAs in Monocytes During Anti-CD3 Mitogenesis

Northern blot analysis was performed using 10 μ g total cellular RNA extracted from monocytes stimulated with 10 μ g/ml LPS (lanes 1-5), T cells plus 50 ng/ml anti-CD3 (lanes 6-10), medium only (lanes 11-12), anti-CD3 only (lane 13) or T cells only (lane 14). RNA was extracted from activated T cells repurified from anti-CD3 mitogenic cultures (lane 15). The position of the 18 S ribosomal RNA band is designated by an arrow. Autoradiography was carried out in the presence of two enhancing screens for 96 h (IL-1 α) and 18 h (IL-1 β). Representative results from one of two experiments are shown.

monocyte IL-1 α and β mRNA during anti-CD3 mitogenesis occurred by 1 h and peaked at 12 h following the addition of T cells plus anti-CD3.

Comparisons of IL-1 α and IL-1 β mRNAs induced during anti-CD3 mitogenesis revealed that both exhibited similar kinetics of appearance. A comparison of the kinetics of peak IL-1 α and IL-1 β mRNA induction in 9 experiments, demonstrated that peak induction occurred in monocytes at the same time point for both IL-1 species in all individuals tested, whether stimulated by LPS or anti-CD3. These data suggested that induction of IL-1 α and IL-1 β were coordinately regulated.

A comparison between maximum levels of IL-1 α and IL-1 β mRNAs induced during anti-CD3 mitogenesis, relative to the maximum levels inducible by LPS, revealed that relative IL-1 α mRNA levels were higher than relative IL-1 β mRNA levels in 7 out of 9 individuals tested. Expressed as a percent of the LPS inducible response for that IL-1 species, mean percent IL-1 mRNA indices during anti-CD3 mitogenesis were $47.2 \pm 7.4 \%$ for IL-1 α and $35.9 \pm 5.7 \%$ for IL-1 β ($p < 0.05$ by paired t test analysis). These data demonstrated that IL-1 α mRNA levels induced during anti-CD3 mitogenesis, relative to those induced by LPS, were greater than IL-1 β mRNA levels.

In the study group as a whole, quantitative levels of IL-1 mRNAs induced during anti-CD3 mitogenesis, relative to maximum LPS inducible levels, varied among different individuals tested. Maximum levels of IL-1 mRNA induced during anti-CD3 mitogenesis in 21 different experiments ranged between 15.0 and 76.0 % of maximum LPS inducible levels, with a mean value of 40.4 ± 9.1 %. These data demonstrated that, although qualitative aspects of IL-1 mRNA induction during anti-CD3 mitogenesis were comparable among different individuals, considerable quantitative differences existed. To avoid these quantitative inter-donor variabilities from biasing the results of later experiments, all subsequent experiments examining induction mechanisms of monocyte IL-1 mRNA were therefore presented as results from a single experiment, representative of the number of experiments noted in the text, instead of means from multiple experiments.

Finally, preliminary studies were carried out to determine whether mRNA for IL-6, a costimulatory molecule acting in synergy with IL-1 during T cell activation, could similarly be induced in monocytes during anti-CD3 mitogenesis. The northern blot depicted in Figure 4 was rehybridized to ^{32}P labelled IL-6 cDNA for detection of IL-6 mRNA. Results shown in Figure 5 demonstrated that IL-6 specific mRNA, migrating at 1.1 kb, was detectable at the 4 and 12 h time points of anti-CD3 mitogenesis and obtained maximal levels by 12 h.

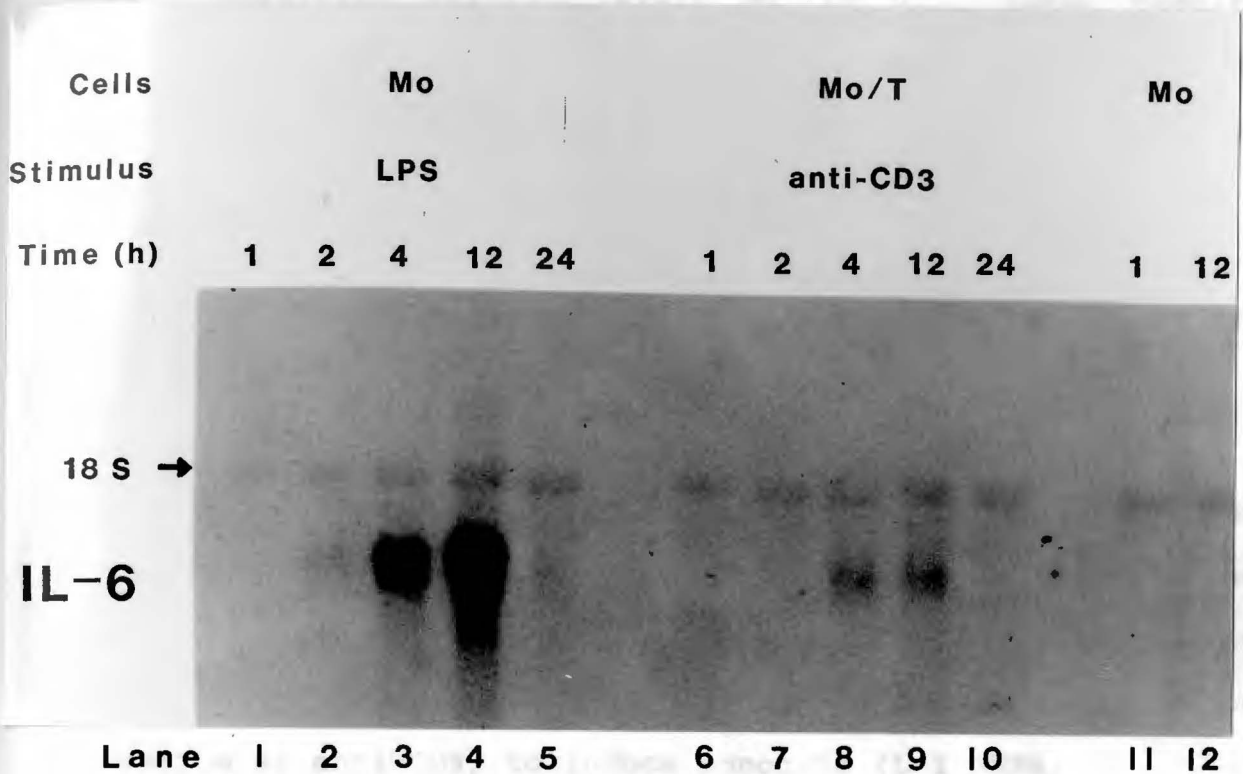


Figure 5. Northern Blot Analysis Depicting Induction of Monocyte IL-6 mRNA During Anti-CD3 Mitogenesis 10 μ g total cellular RNA, extracted from monocytes stimulated for the designated periods of time with either 10 μ g/ml LPS or T cells plus 50 ng/ml anti-CD3, was evaluated by northern blot analysis for detection of IL-6 mRNA. The position of the 18 S ribosomal RNA band is designated by an arrow. Autoradiography was performed in the presence of two enhancing screens for 96 h. Results from a single experiment are shown.

similar to IL-1 mRNA, IL-6 mRNA was not detectable in monocytes cultured in the presence of medium alone, but was detectable at 2-12 h time points in monocytes stimulated with LPS, attaining maximal levels at 12 h. These results demonstrated that mRNAs encoding two costimulatory molecules, IL-1 and IL-6, were both detectable in monocytes stimulated during anti-CD3 mitogenesis.

Induction of IL-1 mRNA in Monocytes by T Cells in the Presence of Anti-CD3 or Anti-CD5

A requirement for T cell activation in the induction of monocyte IL-1 mRNA during anti-CD3 mitogenesis was investigated by determining the ability of T cells plus anti-CD5, a non-mitogenic T cell specific antibody of the same isotype as anti-CD3, to induce monocyte IL-1 mRNA.

In these experiments, monocytes were stimulated by the addition of anti-CD3 or anti-CD5 mAbs, either alone or in the presence of T cells. Total cellular RNA was extracted at 12 h from monocytes stimulated under these conditions and was evaluated by slot blot hybridization analysis for detection of IL-1 α and β mRNAs. A representative slot blot depicting IL-1 β mRNA induction from one of three experiments is shown in Figure 6. The IL-1 mRNA indices for each IL-1 species, relative to the maximum (100%) LPS inducible response, are

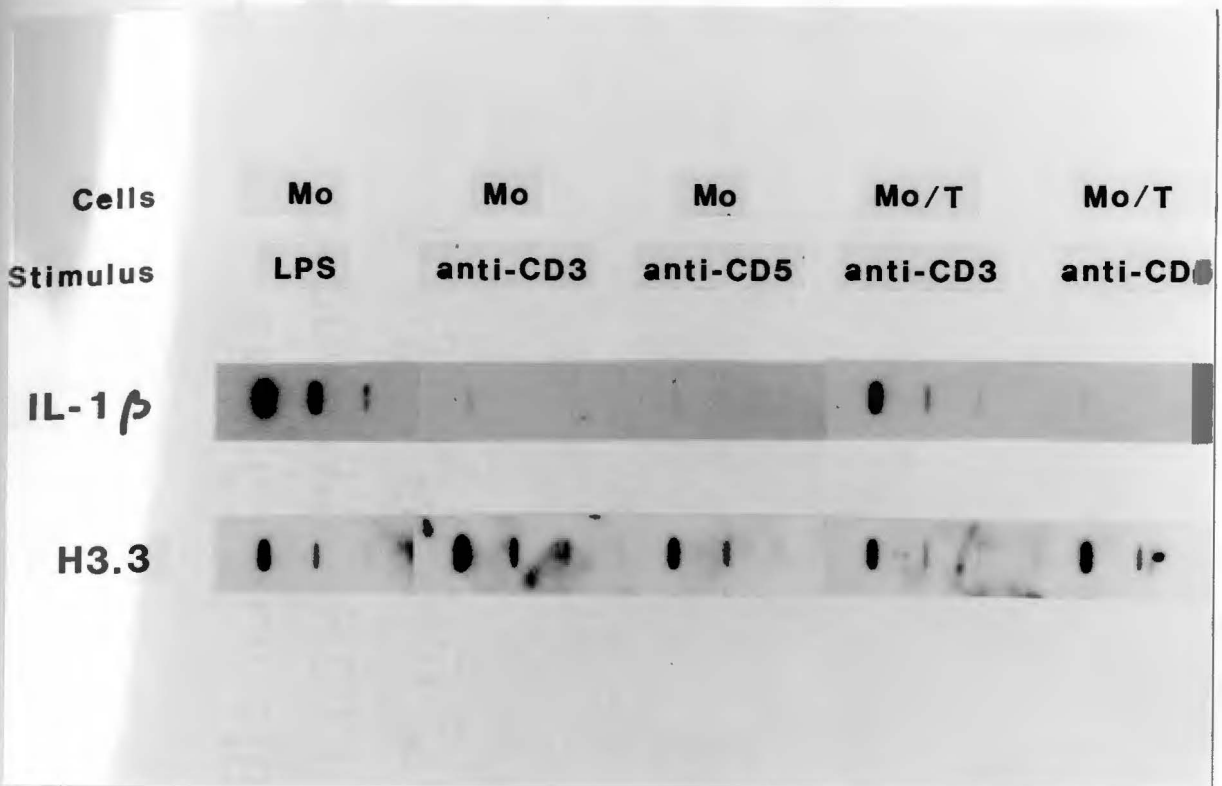


Figure 6. Slot Blot Analysis Depicting the Abilities of Anti-CD3 and Anti-CD5 to Induce T Cell Mediated IL-1 mRNA in Monocytes Monocytes, stimulated for 12 h with 50 ng/ml anti-CD3 or anti-CD5, in the presence and absence of T cells, were evaluated for IL-1 β and histone H3.3 mRNAs by slot blot analysis. Autoradiography was carried out in the presence of two enhancing screens for 36 h (IL-1 β) and 72 h (H3.3). Representative results from one of three experiments are shown.

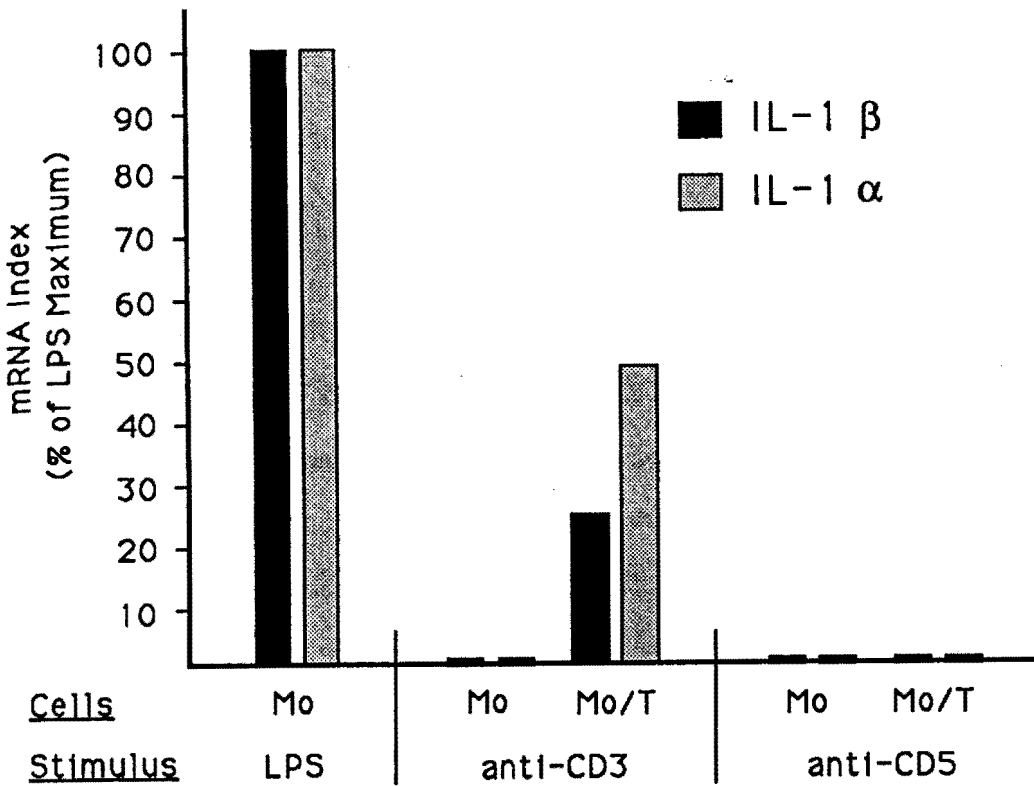


Figure 7. Comparative Abilities of Anti-CD3 and Anti-CD5 to Induce Monocyte IL-1 α and β mRNAs Monocytes, stimulated under the same conditions described in the legend to **Figure 6**, were evaluated by slot blot analysis for IL-1 mRNA content, expressed as percent IL-1 mRNA Indices for each IL-1 species relative to maximum (100 %) LPS (10 μ g/ml) inducible responses. Representative results from one of three experiments are shown.

shown in Figure 7. In contrast to anti-CD3, anti-CD5 was incapable of providing the necessary stimulus for the induction of IL-1 α or β mRNAs in monocytes cultured in the presence of autologous T cells. In control cultures, neither antibody alone induced IL-1 mRNAs when added to monocytes.

Inasmuch as the cell surface density of the CD5 molecule was determined by flow cytometric analysis at saturating concentrations of antibodies to be 3.3 fold less than CD3, additional experiments were carried out to determine whether the inability of anti-CD5 to induce monocyte IL-1 mRNA was due to the lower levels of anti-CD5 binding to T cells. In two independent experiments, monocytes stimulated by T cells plus fivefold greater concentrations of anti-CD5 (250 ng/ml) failed to exhibit IL-1 α and β mRNA induction.

These experiments demonstrated that induction of IL-1 α and β mRNAs during anti-CD3 mitogenesis involved a T cell activation event and was not solely a result of binding of antibody/T cell complexes to monocyte Fc receptors.

Secretion of IL-1 During Anti-CD3 Mitogenesis

Supernates from anti-CD3 mitogenic cultures were evaluated to assess whether the induction of IL-1 at the mRNA level was accompanied by the secretion of IL-1 α and IL-1 β

activities.

In these experiments, supernates collected at various time points from anti-CD3 mitogenic cultures were analyzed for secreted IL-1 α and β activities serologically, using specific immunoassays, and biologically, using the LBRM-33-1A5 conversion assay. Secreted IL-1 β (sIL-1 β) was detected in anti-CD3 mitogenic supernates, using an IL-1 β specific enzyme linked immunoassay, as early as 4 h of culture and increased throughout the 48 h of study, as depicted in **Figure 8** for representative results from one of 4 experiments. Secreted IL-1 α activity was not detected in anti-CD3 mitogenic culture supernates in four separate experiments (0 ± 0 pg/ml) using an IL-1 α specific enzyme linked immunoassay which was sensitive to 10 pg/ml of IL-1 α .

Of interest was the observation that mean levels of secreted IL-1 β induced during anti-CD3 mitogenesis (225 ± 35.8) were equal to or greater than those induced by LPS (184.2 ± 36.9) in the 16 individuals tested, despite the fact that monocyte IL-1 β mRNA levels induced by LPS were, in all experiments, greater than those stimulated by T cells plus anti-CD3.

Biological IL-1 activity in culture supernates, assessed by the LBRM-33-1A5 conversion assay (**Table 3**),

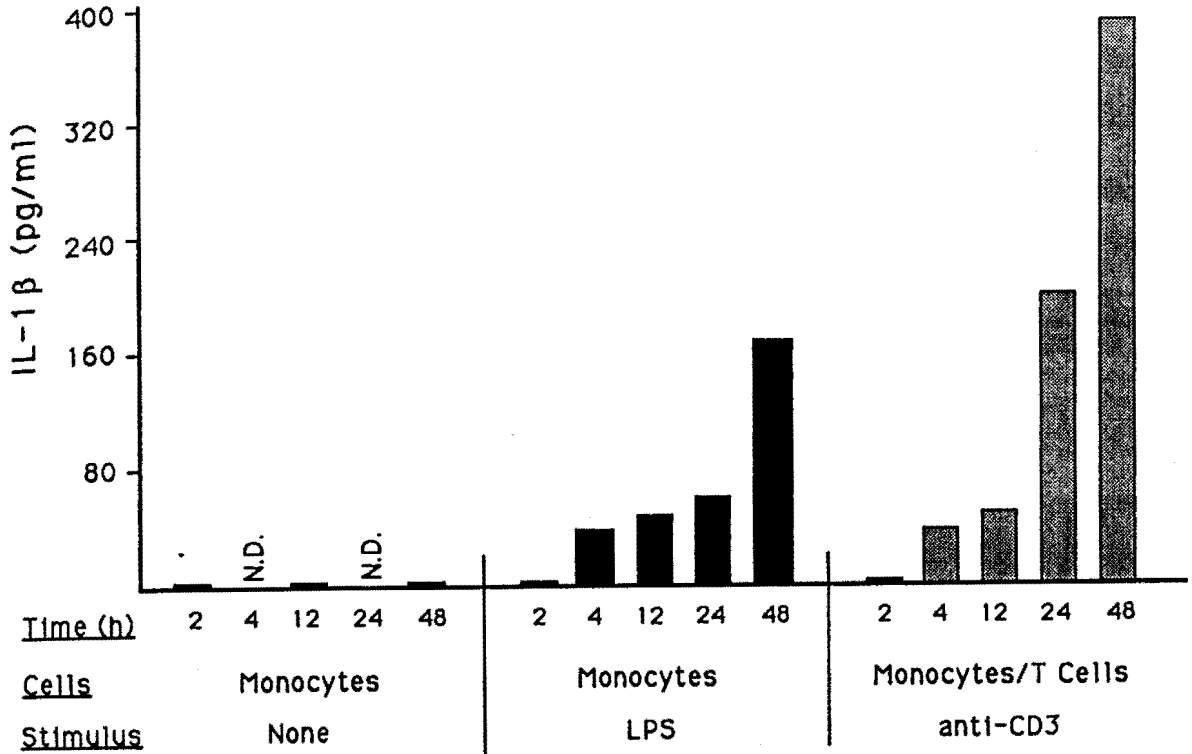


Figure 8. Kinetics of IL-1 β Secretion by Monocytes Stimulated with LPS or T Cells Plus Anti-CD3 Culture supernates, collected at the time points indicated from monocytes cultured in the presence of 10 $\mu\text{g/ml}$ LPS or T cell plus 50 ng/ml anti-CD3, were evaluated for sIL-1 β activities using an IL-1 β specific ELISA. Representative results from one of four experiments are shown. N.D. = Not determined.

Induction of Secreted IL-1 Activity During
Anti-CD3 Mitogenesis

Cells	Stimulus	Time(h)	IL-1 Activity (U/ml)	
			Expt.1	Expt.2
Monocytes	LPS	24	26.7	N.D.
		48	51.2	32.0
Monocytes Plus T Cells	Anti-CD3	12	5.3	N.D.
		24	6.7	0
		48	72.0	64.0

Table 3. Induction of Secreted IL-1 Activity During Anti-CD3 Mitogenesis Monocytes were cultured in the presence of 10 μ g/ml LPS or T cells plus 50 ng/ml anti-CD3 for the designated periods of time and supernates were analyzed for biological IL-1 activity by the LBRM-33-1A5 conversion assay. IL-1 activities were expressed as U/ml, where 1 unit of activity corresponded to the dilution of supernate required for half maximal responses relative to a recombinant IL-1 control. Results from two experiments are shown. N.D. = Not determined.

confirmed the results obtained by IL-1 β specific immunoassay by demonstrating that IL-1 activity was detectable at the earliest time point tested (12 h) and increased throughout the 48 h of study.

These results demonstrated that induction of IL-1 mRNA during anti-CD3 mitogenesis was accompanied by the secretion of biologically active IL-1 β , but not IL-1 α . Furthermore, peak levels of secreted IL-1 β were attained later than peak levels of mRNA induction (≥ 48 h versus ≤ 12 h).

Induction of Monocyte IL-1 Synthesis During Primary Allogeneic Mixed Lymphocyte Responses (MLR)

To confirm the biologic significance of monocyte IL-1 induction during anti-CD3 mitogenesis, subsequent experiments evaluated the induction of monocyte IL-1 during a primary MLR.

Monocytes were stimulated by the addition of allogeneic T cells and evaluated at the designated time points by slot blot analysis for induction of IL-1 α and β mRNAs. Culture supernates were collected for analysis of secreted IL-1 β . Induction of IL-1 was evaluated at the mRNA level by slot blot analysis, expressed as percent IL-1 mRNA indices for each IL-1 species relative to the maximum (100 %) LPS inducible response, and at the secreted level by IL-1 β specific

Table 4. Induction of IL-1 During a Primary Mixed Lymphocyte Response (MLR) Monocytes were stimulated for the designated periods of time by the addition of autologous T cells alone (at a 4:1 T cell:monocyte ratio), allogeneic T cells alone, or T cells plus 50 ng/ml anti-CD3. IL-1 mRNA content of monocytes was evaluated by slot blot analysis and expressed as percent IL-1 mRNA indices of each IL-1 species relative to maximum (100 %) LPS (10 μ g/ml) responses. Levels of sIL-1 β in culture supernates were quantitated by IL-1 β specific ELISA sensitive to 20 pg/ml. Results of a single experiment are shown.

Induction of IL-1 During a Primary Mixed Lymphocyte Response (MLR)

Monocytes Stimulated with:	Time (h)	IL-1 mRNA Index (% of LPS Response)		sIL-1 β
		IL-1 α	IL-1 β	(pg/mL)
Autologous T Cells	12	0	0	<20
	24	0	0	<20
	48	0	0	<20
Allogeneic T Cells	12	12.8	1.7	<20
	24	12.8	4.1	<20
	48	6.4	4.1	195
Autologous T Cells Plus Anti-CD3	12	29.8	47.9	36
	24	N.D.	N.D.	185
	48	N.D.	N.D.	390

immunoassay (Table 4). Results of a single experiment demonstrated that both IL-1 α and β mRNAs were induced in monocytes following the addition of allogeneic, but not autologous, T cells. Whereas IL-1 mRNAs were detected in monocytes at 12, 24 and 48 h time points, secretion of IL-1 β was detected only at the 48 h time point. As a positive control, monocytes stimulated by autologous T cells plus anti-CD3 expressed detectable levels of IL-1 α and β mRNAs and secreted IL-1 β activity at all time points tested.

These results demonstrated that IL-1 was induced at the mRNA and secreted levels in monocytes stimulated during a primary MLR. These results confirmed the previously observed induction of monocyte IL-1, mediated by T cells activated with anti-CD3, in an antibody independent model of primary immune activation, mediated by T cells triggered through their T cell receptors.

Requirement for Cell Contact for Optimal Induction of IL-1 mRNA During Anti-CD3 Mitogenesis

The rapid induction of IL-1 mRNA during anti-CD3 mitogenesis (≤ 1 h), was consistent with a model requiring direct cell contact between T cells and monocytes for induction of monocyte IL-1. A requirement for cell contact between monocytes and T cells in IL-1 mRNA induction was

investigated using double chamber culture wells, as diagrammed in Figure 9. These studies employed double chamber culture plates, in which the upper and lower compartments were separated by a nitrocellulose membrane (0.2 μ pore size). This membrane was freely permeable to soluble factors generated during cell culture, but prevented physical contact between cells contained in either chamber.

In these experiments, adherent monocyte lawns were established in both the upper and lower chambers of double chamber wells. T cells plus anti-CD3 mAb were added to upper chamber monocytes only. Total cellular RNA was extracted at various time points from monocytes in both chambers separately and was evaluated by slot blot analysis for induction of IL-1 mRNAs. Consequently, upper chamber monocytes received both cell contact mediated and soluble IL-1 inductive signals, whereas lower chamber monocytes were exposed only to the soluble cytokines generated during the productive anti-CD3 mitogenesis in the upper chamber.

Representative results from one of two experiments investigating the induction of IL-1 mRNA in monocytes cultured in double chamber wells are shown in Figures 10 and 11. Results were presented as percent IL-1 mRNA indices for each IL-1 species, relative to the maximum (100 %) LPS inducible response. Upper chamber monocytes exhibited a rapid and

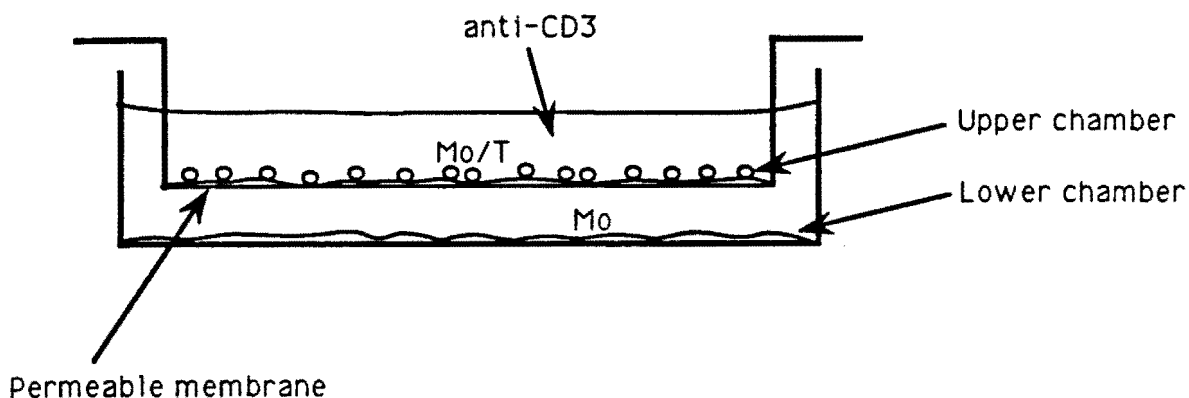
Costar Transwell (24 mm diam.)

Figure 9. Schematic Drawing Depicting Double Chamber Wells Used to Characterize Cell Contact Requirement of T Cell Mediated Monocyte IL-1 mRNA Induction Lawns of adherent monocytes were established in the upper and lower chambers of Costar 24 mm Transwells, in which compartments were separated by a nitrocellulose membrane (pore size 0.2μ) freely permeable to soluble factors generated during cell culture but which prevented physical contact between cells contained in either chamber. The IL-1 mRNA content of monocytes from both upper and lower chambers was analyzed at various time points following the addition of T cells plus anti-CD3 to upper chamber monocytes.

pronounced IL-1 mRNA induction, characteristic of that previously observed during anti-CD3 mitogenesis (see **Figure 4**). IL-1 α and β mRNAs were both readily detectable at 2 h in upper chamber monocytes and attained maximal levels at 12 h. In contrast, lower chamber monocytes exhibited no detectable IL-1 α and β mRNAs at 2 and 4 h time points but did exhibit low levels of IL-1 mRNAs at later (≥ 12 h) time points.

No differences between the kinetics of IL-1 α versus IL-1 β mRNA induction were observed in lower chamber monocytes. The low level of IL-1 α mRNA detectable at 2 h in lower chamber monocytes was not evident at the subsequent 4 h time point and, in two separate experiments, could not be repeated (IL-1 α mRNA index: 0 ± 0 % of LPS inducible response). In control cultures, consisting of lower chamber monocytes stimulated by anti-CD3 mAb alone, no IL-1 mRNAs were detected, thereby indicating that the low levels of IL-1 induction observed in lower chamber monocytes were not due to a residual T cell contamination. In positive control, composed of monocytes stimulated with LPS, the kinetics of induction of IL-1 α and β mRNAs were similar to those previously observed.

These results demonstrated that the optimal induction of IL-1 mRNA during anti-CD3 mitogenesis occurred in the

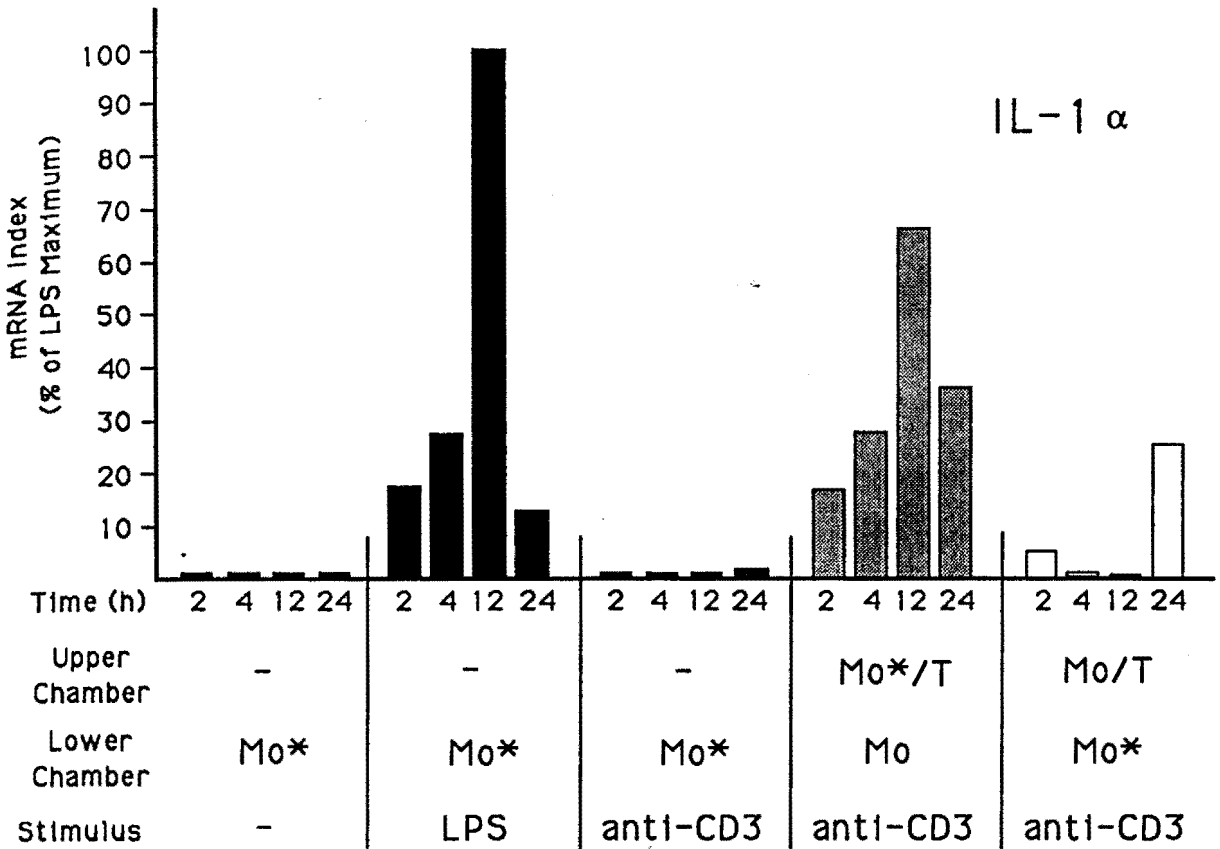


Figure 10. Induction of IL-1 α mRNA in Monocytes Cultured in in Double Chamber Wells Cells were cultured in separate chambers of Transwell plates according to the protocol shown. Total cellular RNA was extracted from the monocyte population designated by the asterisk (*) and subjected to slot blot analysis for detection of IL-1 α mRNA. Control cultures included monocytes plus medium only, monocytes plus 10 μ g/ml LPS, or monocytes plus 50 ng/ml anti-CD3 only. Representative results from one of two experiments are shown, expressed as a percent IL-1 α mRNA Index relative to the maximum (100 %) LPS inducible response.

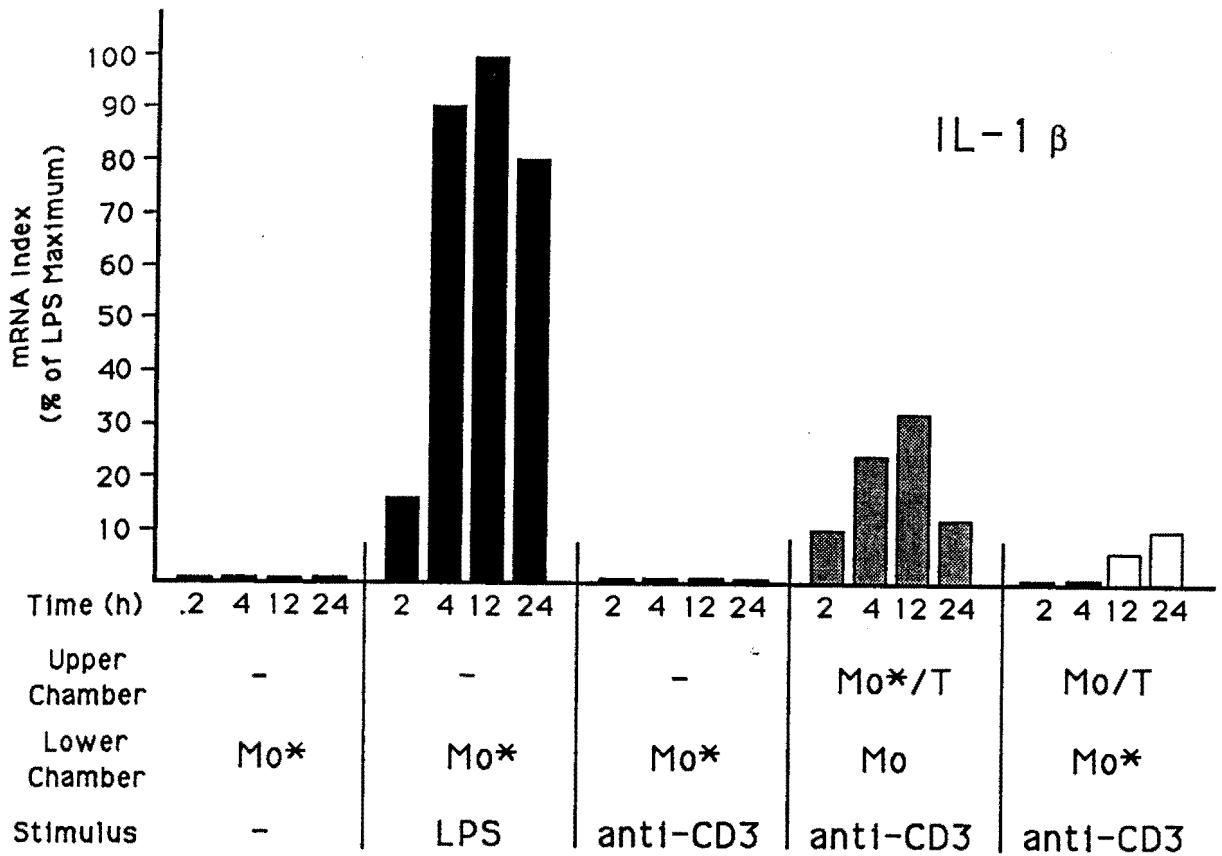


Figure 11. Induction of IL-1 β mRNA in Monocytes Cultured in in Double Chamber Wells. Cells were cultured and analyzed according to the legend to **Figure 10** and results were expressed as a percent IL-1 β mRNA Index relative to the maximum (100 %) LPS (10 μ g/ml) inducible response. Representative results from one of two experiments are shown.

presence of cell contact between monocytes and T cells. A later phase of low level induction of IL-1 mRNA occurred as a result of soluble signals generated during anti-CD3 mitogenesis.

Fixed T Cells are Capable of Mediating IL-1 mRNA Induction in Monocytes: Requirement for Activation of T Cells Prior to Fixation

In order to establish whether both cell contact and soluble factors were required for the induction of monocyte IL-1 mRNA, experiments were performed to determine whether metabolically inactivated T cells, incapable of generating soluble factors, retained their ability to induce IL-1 mRNA via direct cell contact with monocytes.

In these experiments, T cells were metabolically inactivated by fixation with paraformaldehyde and evaluated for their ability to induce monocyte IL-1 mRNA. Three groups of fixed T cells were evaluated in these experiments for their IL-1 inducing abilities:

Group 1: Unstimulated T cells.

Group 2: T cells activated with immobilized anti-CD3.

Group 3: T cells activated with monocytes plus anti-CD3.

Table 5. Confirmation of T Cell Activation Prior to Fixation

The proliferative responses of T cells employed in the fixation studies depicted in **Figure 12** are shown, following activation by medium (Group 1), immobilized anti-CD3 (Group 2) or monocytes plus 50 ng/ml anti-CD3 (Group 3). The incorporation of ^3H -thymidine into cells was assessed at 72 h of culture. Control cultures included T cells stimulated with anti-CD3 alone and T cells stimulated with immobilized anti-CD3 in the presence of 50 U/ml rIL-2. Counts represent mean \pm S.D. cpm from triplicate culture wells and are shown for two representative experiments.

Confirmation of T Cell Activation Prior to Fixation

Group	Cells	Stimulus	3H-Thymidine Incorporation (CPM) (Mean ± S.E.M.)			
			Expt.1		Expt.2	
1	T Cells	-	298 ±	44	570 ±	27
2	T Cells	Immobilized Anti-CD3	5,705 ±	726	3,107 ±	469
3	Monocytes plus T Cells	Anti-CD3	117,127 ±	1,617	74,845 ±	10,579
	T Cells	Anti-CD3	432 ±	97	798 ±	27
	T Cells	Immobilized Anti-CD3 +rIL-2	122,127 ±	5,149	63,159 ±	5,794

Monocytes were stimulated with fixed T cells in the presence of anti-CD3 and evaluated for IL-1 α and β mRNA content. Prior to use in IL-1 mRNA induction studies, fixed T cells from each group were evaluated for effectiveness of activation and completeness of fixation.

Activation of T cells was confirmed by proliferative responses, as shown in Table 5 for representative ^3H -thymidine incorporation data obtained in two of three experiments. T cells activated by monocytes plus anti-CD3 (group 3) exhibited ^3H -thymidine incorporations in two experiments of 117,127 and 74,845 cpm, whereas T cells activated by immobilized anti-CD3 (group 2) exhibited counts of 5,705 and 3,107 cpm, and unstimulated T cells (group 1) demonstrated counts of 298 and 570 cpm. T cells activated by immobilized anti-CD3 plus exogenously added rIL-2, exhibited completely restored proliferative responses of 122,127 and 63,159 cpm. These data demonstrated that activation of T cells by immobilized anti-CD3 alone led to the expression of IL-2 receptors without leading to proliferation. In control cultures, T cell purity was also confirmed by the absence of proliferation in response to anti-CD3, a monocyte dependent T cell mitogen (432 and 798 cpm).

Following fixation in paraformaldehyde, T cells were evaluated by flow cytometric analysis for integrity of cell

Table 6. Effects of Paraformaldehyde Fixation on Cell Surface Marker Expression and Viability Fluorescent staining with PI, anti-CD3 (FITC anti-Leu 4) and anti-CD8 (PE anti-Leu 2b) antibodies are presented for fixed T cells from each of the three activation groups described in the legend to **Table 5**. Results are presented as percent cells positive for staining with PI and each of the anti-CD3 and anti-CD8 antibodies, as shown for two representative experiments. Representative two dimensional histograms illustrating these summarized phenotypic data are shown in **Figures 3 and 4 of the Appendix**.

Effect of Paraformaldehyde Fixation on Cell Surface Marker Expression and Viability

T Cells		% Positive Cells					
		CD3+		CD8+		PI Uptake	
Stimulus	Fixation	Expt.1	Expt.2	Expt.1	Expt.2	Expt.1	Expt.2
Media	-	84.1	68.7	37.5	32.8	5.2	16.0
Media	+	76.8	67.7	40.4	27.4	98.9	98.3
Immobilized Anti-CD3	+	82.4	71.6	46.0	34.8	98.4	98.8
Monocytes plus Anti-CD3	+	78.3	70.0	36.9	30.4	98.9	99.0

surface molecules and completeness of fixation, as summarized in Table 6. In two separate experiments, fixed T cells from all three groups demonstrated complete loss of viability (≥ 98 % PI uptake). Furthermore, fixed T cells demonstrated no changes in the binding of fluorochrome conjugated antibodies to CD3 and CD8 cell surface markers, indicating that these cell surface molecules remained intact. Representative contour plots illustrating the data summarized in Table 6 are shown in Figures 3 and 4 of the Appendix. These data demonstrated that fixation of T cells was complete and maintained the integrity of functional cell surface molecules.

Representative results from one of three experiments investigating the ability of fixed T cells to induce IL-1 mRNA are depicted in Figure 12. Data confirming the activation and fixation of T cells employed in this experiment are shown in Tables 5 and 6 under Expt. 1. Monocytes were cultured with fixed T cells in the presence of anti-CD3 and were evaluated by slot blot analysis for induction of IL-1 α and β mRNAs, expressed as the percent IL-1 mRNA index for each IL-1 species relative to the maximum (100 %) levels achieved by the anti-CD3 mitogenesis response. The data depicted in Figure 12 demonstrated that fixed T cells, activated either by immobilized anti-CD3 (Group 2) or monocytes plus anti-CD3 (Group 3) and subsequently fixed, induced monocyte IL-1 α and β mRNAs to levels comparable to unfixed T cells. In contrast,

Monocytes Activated With Anti-CD3 Plus:

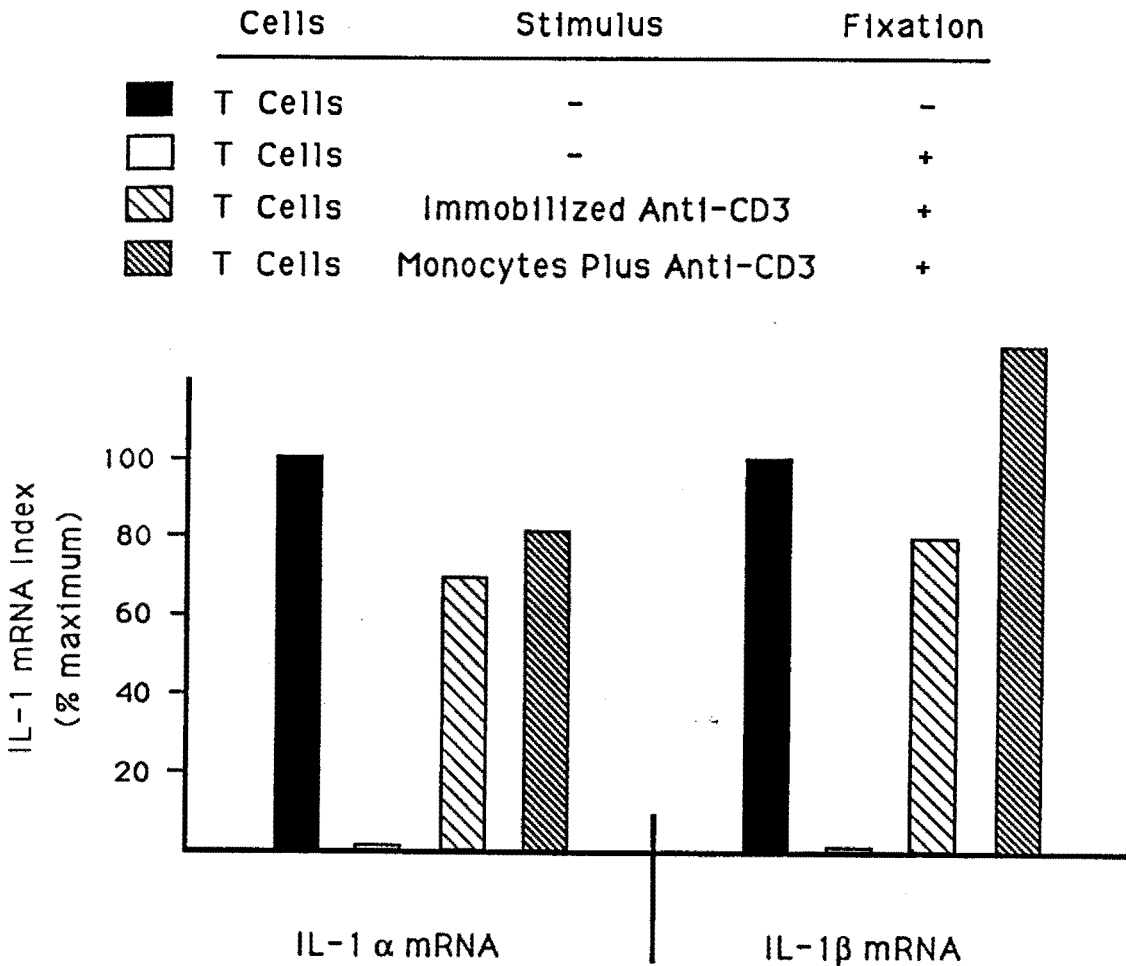


Figure 12. Induction of IL-1 mRNA in Monocytes by Fixed T Cells: Requirement for Activation of T Cells Prior to Fixation. T cells, activated and fixed as indicated, were evaluated for their ability to induce monocyte IL-1 α and β mRNA at 12 h. Results were expressed as percent IL-1 mRNA Indices for each IL-1 species relative to maximum (100 %) responses induced during anti-CD3 mitogenesis. Representative results from one of three experiments are shown.

fixed unstimulated T cells (group 1) did not induce IL-1 mRNAs.

These data demonstrated that metabolically inactivated T cells were fully capable of inducing IL-1 mRNAs in monocytes, but only after activation of these cells. Activation of T cells by immobilized anti-CD3, which led to IL-2 receptor expression but not to proliferation, was sufficient to trigger transduction of this cell contact mediated IL-1 mRNA signal from T cells to monocytes.

T Cell Metabolic Activity Is Required for Induction of IL-1 β Secretion by Monocytes

Inasmuch as metabolically inactivated T cells were capable of inducing monocyte IL-1 mRNAs, subsequent experiments were carried out to determine whether metabolic activity by T cells was required to induce secretion of IL-1 β by monocytes.

T cells from the three fixation groups were compared with unfixed T cells for their ability to induce secretion of IL-1 β by monocytes in the presence of anti-CD3, as shown in Table 7. Representative results from two of three experiments demonstrated that, in contrast to unfixed T cells, fixed T cells from any of the three fixation groups were incapable of

Effect of Fixation on T Cell Mediated Induction
of Monocyte IL-1 β Secretion

Monocytes Stimulated with:			sIL-1 β (pg/ml)	
Cells	Fixation Group	Stimulus	Expt.1	Expt.2
T Cells	-	-	<20	<20
-	-	LPS	330	315
T Cells	-	anti-CD3	265	340
T Cells	1	anti-CD3	<20	<20
T Cells	2	anti-CD3	<20	<20
T Cells	3	anti-CD3	<20	<20

Table 7. Effect of Fixation on T Cell Mediated Induction of IL-1 β Secretion by Monocytes. T cells from the fixation groups indicated were evaluated for their ability to induce IL-1 β secretion by monocytes in the presence of anti-CD3. Supernates, collected at 48 h of culture, were assayed for secreted IL-1 β levels using an IL-1 β specific ELISA and levels were expressed in pg/ml. Representative results from two of three experiments are shown.

inducing detectable levels of secreted IL-1 β by monocytes.

These results demonstrated that fixed preactivated T cells were incapable of inducing IL-1 β secretion by monocytes in the presence of anti-CD3, despite their ability to induce IL-1 β mRNA levels in monocytes. Furthermore, these results demonstrated that metabolic activity by T cells was required for secretion of IL-1 β by monocytes during anti-CD3 mitogenesis.

T Cell Transduction of Monocyte IL-1 mRNA Inductive Signals Does Not Require Protein Synthesis

To determine whether acquisition of IL-1 mRNA inductive capacities by activated T cells required protein synthesis, T cells were treated with a protein synthesis inhibitor and evaluated for their IL-1 inductive abilities.

T cells pretreated with emetine, an inhibitor of de novo protein synthesis, were evaluated for their ability to induce monocyte IL-1 mRNA after extensive washing to remove traces of emetine. Inhibition of de novo protein synthesis in emetine treated T cells was confirmed by monitoring the incorporation of ^3H -leucine into cells following a 48 h stimulation in the presence of solid-phase anti-CD3 and rIL-2, as shown in Table 8. These data demonstrated that

Incorporation of ^3H -Leucine by Emetine Treated T Cells

Emetine ($\mu\text{g/ml}$)	^3H -Leucine Incorporation (CPM $\times 10^3$)			% Inhibition (Mean \pm S.E.M.)
	Expt.1	Expt.2	Expt.3	
0	2,745	980	3,072	
10	154	41	216	94.4 \pm 0.8
20	143	37	164	95.3 \pm 0.5
40	85	31	155	96.2 \pm 0.6

Table 8. Incorporation of ^3H -Leucine by Emetine Treated T Cells Incorporation of ^3H -leucine into T cells are shown following a 24 h stimulation in the presence of immobilized anti-CD3 and 50 U/ml recombinant IL-2. Results of three representative experiments are shown.

inhibition of ^3H -leucine incorporation by emetine treatment ranged between $94.4 \pm 0.8 \%$ and $96.2 \pm 0.6 \%$, compared to untreated cells. To exclude the possibility that emetine treatment effects were reversible during the course of IL-1 mRNA induction experiments, ^3H -leucine incorporations were assessed on cells collected after the completion of the IL-1 induction cultures.

Representative results from one of two experiments examining the effect of T cell protein synthesis inhibition on the induction of monocyte IL-1 mRNAs are shown in **Figures 13 and 14**. Monocytes were cultured in the presence of anti-CD3 plus emetine treated T cells and RNA collected at 4 and 12 h evaluated by slot blot analysis for induction of monocyte IL-1 α and β mRNAs. Data for each IL-1 species were expressed as a percent IL-1 mRNA index relative to the maximum (100 %) levels induced by LPS at 4 h. Emetine treatment of T cells failed to inhibit IL-1 α and β mRNA induction at either the 4 or 12 h time points. Direct effects on monocytes by emetine leaching from T cells were ruled out in control cultures based by the observation that induction of IL-1 by LPS was unaffected by the presence of emetine treated T cells.

These results demonstrated that de novo protein synthesis was not required for T cell induction of IL-1 mRNA in monocytes during anti-CD3 mitogenesis. Furthermore, these

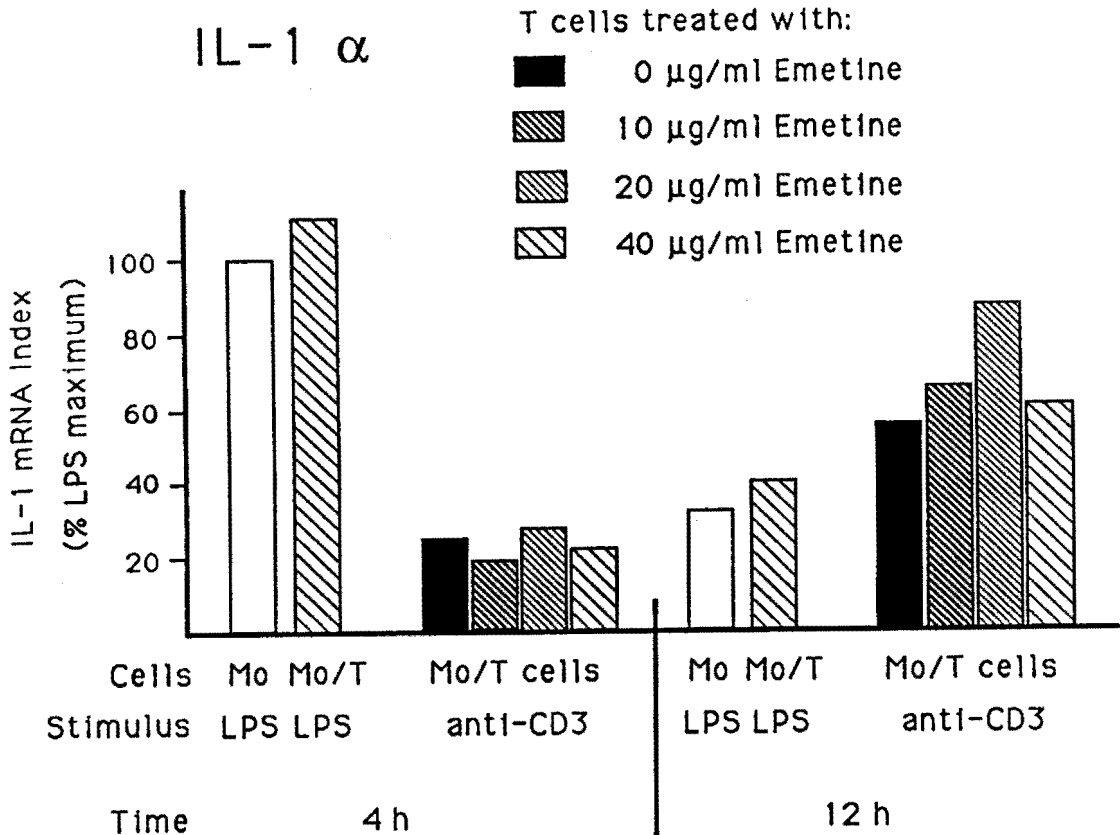


Figure 13. Induction of IL-1 α mRNA in Monocytes Stimulated With Anti-CD3 Plus Emetine Treated T Cells T cells, pretreated at the designated dose with emetine, an inhibitor of *de novo* protein synthesis, were evaluated for their ability to induce monocyte IL-1 α mRNA at 4 and 12 h in the presence of 50 ng/ml anti-CD3. Control cultures included monocytes stimulated with 10 $\mu\text{g/ml}$ LPS, or LPS in the presence of high dose emetine treated T cells. Results were expressed as a percent IL-1 α mRNA Index relative to the maximum (100 %) 4 h LPS inducible response. Representative results from one of two experiments are shown.

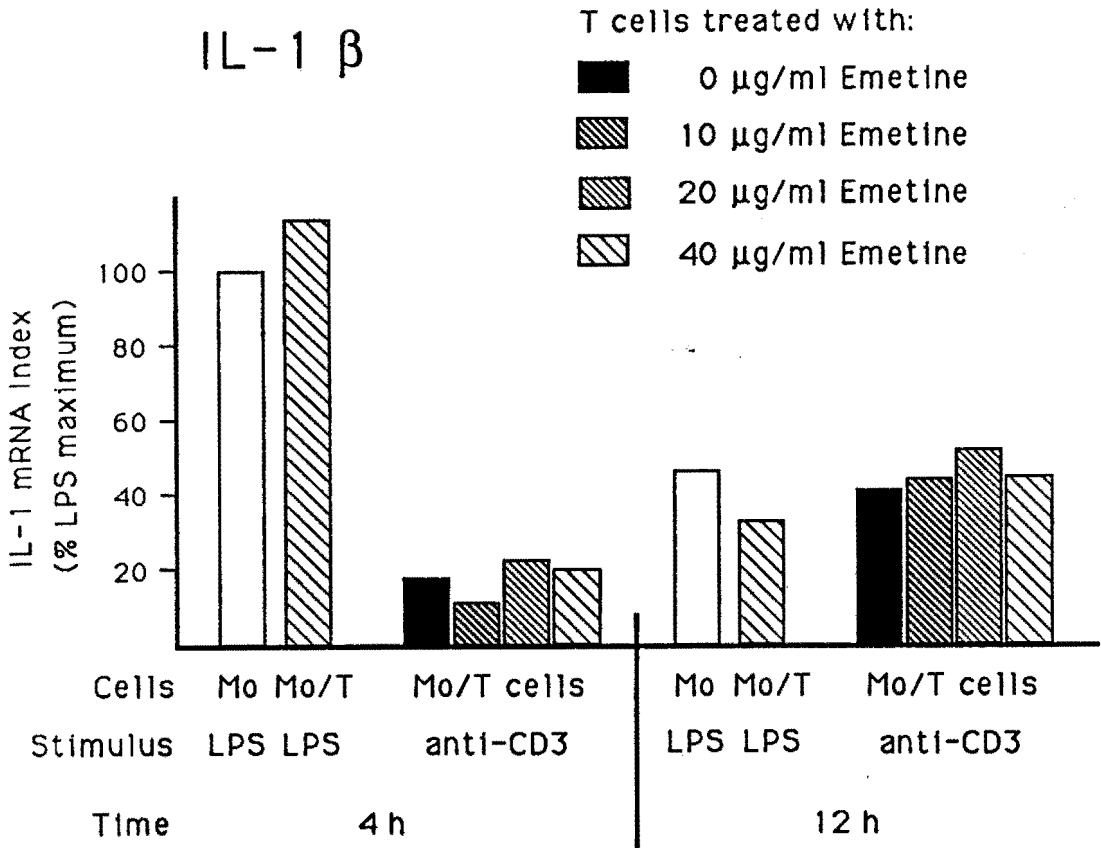


Figure 14. Induction of IL-1 β mRNA in Monocytes Stimulated With Anti-CD3 Plus Emetine Treated T Cells Cells were cultured and analyzed employing the same culture conditions described in the legend to **Figure 13**. Results were expressed as a percent IL-1 β mRNA Index relative to the maximum (100%) 4 h LPS (10 $\mu\text{g/ml}$) inducible response. Representative results from one of two experiments are shown.

results indicated that the acquisition of cell contact dependent IL-1 inducing activity occurred independently of protein synthesis. Finally, these experiments confirmed that the synthesis of soluble T cell lymphokines was not involved in the initial phase of induction of IL-1 mRNA in monocytes.

Protein Synthesis by T Cells Is Required for the Induction of IL-1 β Secretion by Monocytes

Inasmuch as metabolically inactivated T cells were unable to induce secretion of IL-1 β by monocytes during anti-CD3 mitogenesis, experiments were carried out to determine whether T cell protein synthesis was required for the secretion of IL-1 β during anti-CD3 mitogenesis.

Results from two individuals depicting the effect of T cell protein synthesis inhibition on IL-1 β secretion during anti-CD3 mitogenesis are shown in Figure 15. These data demonstrated that emetine treatment of T cells inhibited IL-1 β secretion into culture supernates in a dose dependent manner. Maximal inhibition at the highest emetine treatment dose was 90.4 %. Inhibitory effects due to leaching of emetine from T cells were not responsible for this observation, as demonstrated by the normal induction of IL-1 β secretion by monocytes stimulated with LPS in the presence of emetine treated T cells.

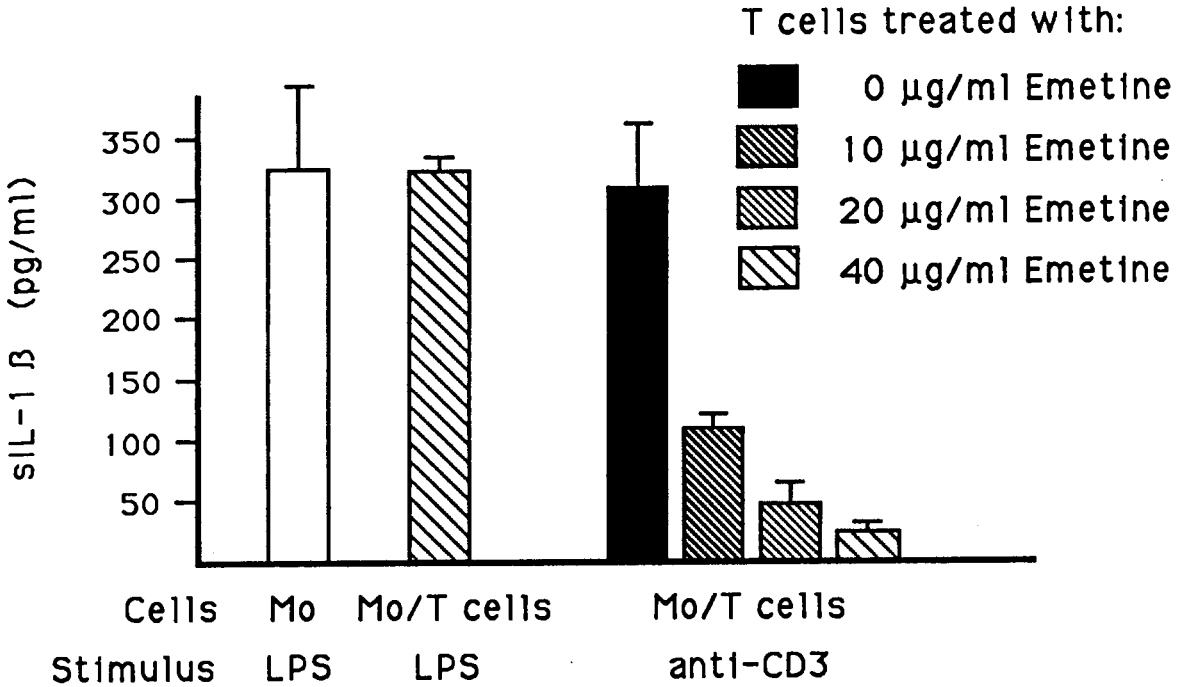


Figure 15. Effect of Protein Synthesis Inhibition on T Cell Mediated Induction of sIL-1 β Cells were cultured for 48 h according to the legend to **Figure 13** and sIL-1 β in supernates was quantitated using a specific ELISA. Results depict mean \pm S.E.M. sIL-1 β levels, expressed in pg/ml, from two experiments.

These results demonstrated that de novo protein synthesis was required by T cells to promote secretion of IL-1 β by monocytes during anti-CD3 mitogenesis. In contrast, protein synthesis by T cells was not required for induction of the initial phase of monocyte IL-1 mRNA.

Induction of Late Phase Monocyte IL-1 mRNA is Associated With the Appearance of IL-1 Inducing Cytokines (IL-2, IFN- γ and TNF- α) in Anti-CD3 Mitogenic Culture Supernates

The results from double chamber well experiments indicated the presence of a second, later phase of induction of monocyte IL-1 mRNA, which was mediated by soluble factors generated during anti-CD3 mitogenesis. Experiments were carried out to determine whether the kinetics of induction of late phase IL-1 mRNA induction correlated with the appearance of cytokines previously reported to possess IL-1 inducing activity (IL-2, IFN- γ and TNF- α).

IL-2, IFN- γ and TNF- α in culture supernates were assessed at different time points of anti-CD3 mitogenesis by specific immunoassays and the results shown in **Figure 16**. These cytokines all exhibited similar kinetics of appearance in culture supernates. Whereas they were not detectable at early 2-4 h time points of anti-CD3 mitogenesis, pronounced levels were detected at ≥ 8 h and reached maximal levels at

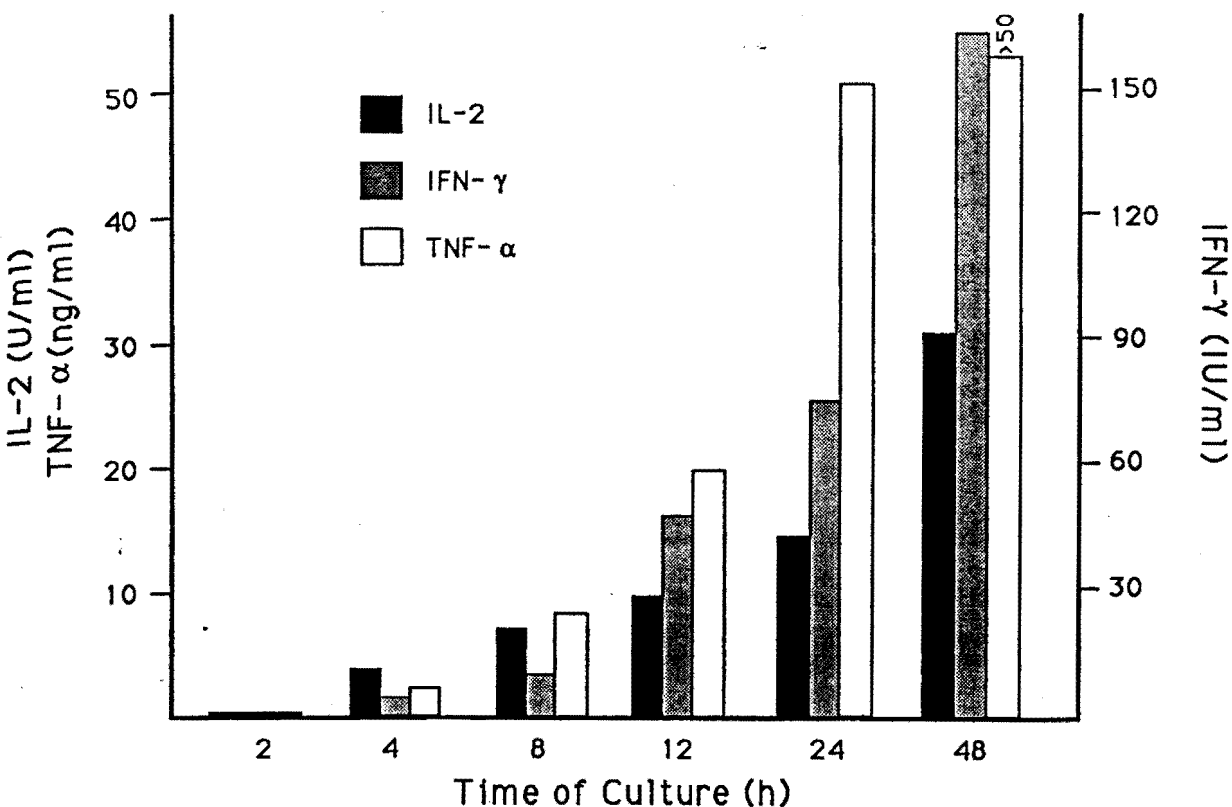


Figure 16. Kinetics of Appearance of IL-2, IFN- γ and TNF- α in Supernates of Anti-CD3 Mitogenic Cultures Cytokines were analyzed in supernates collected at the designated time points of anti-CD3 mitogenic culture by specific radioimmunoassays. Units of IL-2 and IFN- γ activity were calibrated with respect to NIH BRMP IL-2 and NIH IFN-standards respectively. Representative results from one of three experiments are shown. N.D. = Not determined.

48 h.

These results demonstrated that the IL-1 inducing cytokines, IL-2, IFN- γ and TNF- α , were not detectable in culture supernates at a time of anti-CD3 mitogenesis when significant levels of monocyte IL-1 mRNA were detectable (\leq 4 h). The kinetics of appearance of cytokines were, however, temporally associated with the appearance of the late phase of monocyte IL-1 mRNA, at 12 h of culture.

Induction of Monocyte IL-1 by T Cell Subpopulations

In view of previous experiments demonstrating a requirement for direct T cell monocyte contact for the initial induction of monocyte IL-1 mRNA, experiments were carried out to determine whether this IL-1 mRNA inductive signal was transduced during the major histocompatibility complex (MHC) class II restricted interaction between monocytes and CD4⁺ T cells. These experiments compared the ability of class II restricted CD4⁺ and class I restricted CD8⁺ T cell subsets to induce IL-1 mRNA during anti-CD3 mitogenesis.

T cells were fractionated into CD4⁺ and CD8⁺ T cell subpopulations by negative selection employing magnetic beads and purity of final cell subpopulations was confirmed by flow cytometric analysis of CD4 and CD8 cell surface marker

expression, as shown in Table 9. These data, summarized from three separate experiments, demonstrated that CD4⁺ enriched T cells contained 7.3 ± 0.8 % contamination by CD8⁺ cells and that CD8⁺ enriched T cells contained 5.6 ± 2.2 % contamination by CD4⁺ cells. Figure 5 of the Appendix shows contour plots illustrating cell surface marker expression of the CD4⁺ and CD8⁺ T cell subpopulations employed in the IL-1 mRNA induction experiment depicted in Table 10.

Monocytes cultured for 12 h in the presence of anti-CD3 plus CD4⁺ or CD8⁺ T cell subsets were evaluated by slot blot analysis for detection of IL-1 α and β mRNAs, expressed as a percent IL-1 mRNA index for each IL-1 species relative to the maximum (100 %) level induced by LPS, as shown in Table 10. These data, obtained from a single experiment, demonstrated that both CD4⁺ and CD8⁺ T cell subsets were capable of inducing IL-1 α and β mRNAs in monocytes. CD8⁺ cells induced higher levels than CD4⁺ cells of IL-1 α mRNA (149.0 % vs. 45.5 % of LPS inducible response) and IL-1 β mRNA (91.1 % vs. 18.2 %).

These results demonstrated that, although the reasons for the quantitative differences between induction of IL-1 mRNAs by CD4⁺ and CD8⁺ T cells remained unclear, induction of monocyte IL-1 mRNA was mediated by both CD4⁺ and CD8⁺ T cell subsets. Furthermore, these results indicated that

purity of CD4+ and CD8+ T Cells Isolated by Negative Selection

T Cells	% Positive Cells (Mean \pm S.E.M.)	
	CD4+	CD8+
Unseparated	52.8 \pm 5.9	36.7 \pm 1.9
CD4+	63.7 \pm 6.8	7.3 \pm 0.8
CD8+	5.6 \pm 2.2	59.3 \pm 3.8

Table 9. Purity of T cell Subpopulations Isolated by Negative Selection. T cells were negatively selected into CD4+ and CD8+ T cell subpopulations using magnetic beads as described (see **Materials and Methods**). Results are presented as mean \pm S.E.M. percent cells positive for staining with fluorochrome conjugated anti-CD4 (FITC anti-Leu3 a&b) and anti-CD8 (PE anti-Leu2 a), obtained from three experiments. Representative flow cytometric contour plots illustrating cell surface marker expression of T cell subpopulations generated by negative selection are shown in **Figure 5 of the Appendix**.

Induction of Monocyte IL-1 mRNA by CD4+ and CD8+ T Cells

Monocytes plus:		IL-1 mRNA Index (% of LPS response)	
T Cells	Stimulus	IL-1 α	IL-1 β
Unseparated	anti-CD3	75.6	48.0
CD4+	anti-CD3	45.4	18.2
CD8+	anti-CD3	149.0	91.1
-	anti-CD3	0	0

Table 10. Induction of IL-1 mRNA in Monocytes by CD4+ and CD8+ T Cells CD4+ and CD8+ T cell subpopulations were prepared by negative selection as described in the legend to **Table 9** and compared for their ability to induce IL-1 mRNAs in monocytes at 12 h in the anti-CD3 mitogenesis model. The IL-1 mRNA content of monocytes was analyzed by slot-blot analysis, expressed as a percent IL-1 mRNA index for each IL-1 species relative to the maximum (100 %) levels inducible by LPS (10 μ g/ml). Results of a single experiment are shown.

transduction of the cell contact mediated IL-1 mRNA inductive signal was not mediated exclusively through the MHC class II restricted interaction between monocytes and CD4⁺ T cells.

A second series of experiments were carried out to determine whether the IL-1 inductive signal could be generated by virgin, unprimed T cells or was confined to the previously primed T cell subset. These experiments compared the ability of positively selected naive (CD45RA⁺) and memory (CD45RO⁺) T cells to induce IL-1 β secretion by monocytes in a microculture anti-CD3 mitogenesis model. In addition, the initial experiments using negatively selected CD4⁺ and CD8⁺ T cell subsets were repeated in this second series of experiments, employing positively selected cells and secretion of IL-1 β as a measure of IL-1 induction.

T cells were subfractionated into CD45RA⁺, CD45RO⁺, CD4⁺ and CD8⁺ T cell subpopulations by cell sorting and purity was confirmed by phenotypic analysis. Results summarized from three experiments are shown in Table 11. Mean purities of each subpopulation obtained by cell sorting in these experiments was $\geq 98.3 \pm 0.7$ %. Representative contour plots illustrating cell surface marker expression of the T cell subpopulations employed in these experiments are shown in Figure 6 of the Appendix.

Table 11. Purity of T Cell Subpopulations Isolated by Positive Selection. T cells were positively selected by staining with anti-CD45RA (FITC anti-Leu 18) and anti-CD45RO (PE UCHL-1) antibodies, or anti-CD4 (FITC anti-Leu3 a&b) and anti-CD8 (PE anti-Leu 2a) antibodies, followed by cell sorting. Results are presented as mean \pm S.E.M. of the percentage of percent cells positive for staining with each antibody, obtained from three experiments. Representative flow cytometric contour plots illustrating cell surface marker expression of T cell subpopulations generated by positive selection are shown in **Figure 6 of the Appendix.**

Purity of T Cell Subpopulations Isolated by Positive Selection

T Cells	% Positive Cells (Mean \pm S.E.M.)			
	CD45RA+	CD45RO+	CD4+	CD8+
Unseparated	38.1 \pm 6.2	23.8 \pm 5.5	63.4 \pm 3.6	29.4 \pm 4.4
CD45RA+	98.3 \pm 0.3	0	-	-
CD45RO+	0	98.3 \pm 0.3	-	-
CD4+	-	-	99.7 \pm 0.3	0.3 \pm 0.3
CD8+	-	-	0.3 \pm 0.3	98.3 \pm 0.7

Ability of T Cell Subpopulations to Induce
IL-1 β Secretion by Monocytes

T Cells	Monocytes Plus: Stimulus	sIL-1 β (Mean \pm S.E.M.) (% of LPS Response)
-	LPS	100 \pm 0
Unseparated	Anti-CD3	69.0 \pm 7.0
CD45RA+	Anti-CD3	57.0 \pm 11.0
CD45RO+	Anti-CD3	40.0 \pm 6.4
CD4+	Anti-CD3	44.5 \pm 1.5
CD8+	Anti-CD3	46.0 \pm 8.0

Table 12. Ability of T Cell Subpopulations to Induce IL-1 β Secretion by Monocytes CD45RA⁺, CD45RO⁺, CD4⁺ and CD8⁺ T cell subpopulations were obtained by positive selection as described in the legend to **Table 11** and compared for their ability to induce sIL-1 β by monocytes in the anti-CD3 mitogenesis model. Supernates were collected at 48 h of culture for detection of sIL-1 β by specific ELISA. Results depict mean \pm S.E.M. levels of sIL-1 β contained in culture supernates established from two experiments, expressed as a percent of IL-1 β levels induced by LPS (10 μ g/ml).

Culture supernates, collected at 48 h from monocytes cultured in the presence of anti-CD3 plus CD45RA⁺, CD45RO⁺, CD4⁺ or CD8⁺ T cells, were analyzed for secreted IL-1 β levels by IL-1 β specific immunoassay and the results from two experiments are summarized in **Table 12**. These data, expressed as a percent of secreted IL-1 β induced by LPS, demonstrated that CD45RA⁺ and CD45RO⁺ T cells induced comparable secretion of IL-1 β by monocytes (57.0 ± 11.0 % vs. 40.0 ± 6.4 %). These data also demonstrated that CD4⁺ and CD8⁺ T cells induced comparable secretion of IL-1 β by monocytes (44.5 ± 1.5 % vs. 46.0 ± 8.0 %).

These results demonstrated that comparable T cell IL-1 inductive signals were generated by naive and memory T cells. These results also demonstrated that CD4⁺ and CD8⁺ cells were both able to induce secretion of IL-1 β by monocytes, thus confirming previous observations at the mRNA level.

Preliminary Examination of Monocyte Cell Surface Molecules Capable of Transducing an IL-1 mRNA Inductive Signal

Preliminary investigations were performed to characterize molecules on the monocyte cell surface capable of transducing an IL-1 mRNA inductive signal. Initial studies were carried to determine whether monocyte MHC class I and II

Table 13. Induction of IL-1 β mRNA in Monocytes by Monoclonal Antibodies Total cellular RNA was extracted from monocytes stimulated for 4 or 12 h with 10 $\mu\text{g/ml}$ LPS or 80 $\mu\text{g/ml}$ protein containing $(\text{NH}_4)_2\text{SO}_4$ preparations of anti-HLA-DR (L243; IgG_{2a}), anti-HLA-A,B,C (W6/32; IgG_{2a}) or anti-CD14 (63D3; IgG₁) monoclonal antibodies. The IL-1 mRNA content of monocytes was analyzed by slot-blot analysis and was expressed both as an IL-1 β mRNA index and as a percent IL-1 β mRNA index relative to the maximum (100 %) LPS inducible responses. Results of two experiments are shown. N.D. = Not determined.

Induction of IL-1 β mRNA in Monocytes by Monoclonal Antibodies

Stimulus	Time (h)	IL-1 β mRNA Index		% of LPS Response	
		Expt.1	Expt.2	Expt.1	Expt.2
LPS	4	4.6	4.7	100.0	100.0
	12	5.8	N.D.	100.0	N.D.
Anti-HLA-DR	4	9.1	3.9	197.8	83.1
	12	17.9	N.D.	308.6	N.D.
Anti-HLA-A,B,C	4	1.5	N.D.	32.6	N.D.
	12	1.9	N.D.	32.8	N.D.
Anti-CD14	4	1.7	N.D.	37.0	N.D.
	12	0.9	N.D.	15.5	N.D.

molecules could act as IL-1 signal transducing moieties.

In these experiments, anti-class I (HLA-A,B,C) and class-II (HLA-DR) antibodies were compared with LPS for their ability to induce IL-1 β mRNA in monocytes at 4 and 12 h, as shown in **Table 13**. In two separate experiments, anti-HLA-DR antibody (L243 mAb, IgG_{2a}), employed at 80 μ g/ml protein in an (NH₄)₂SO₄ preparation, induced IL-1 β mRNA levels between 83.1 and 328.0 % of LPS inducible levels. Stimulation occurred in the absence of detectable endotoxin levels (\leq 5 pg/ml) in antibody preparations. In a single experiment, an anti-HLA class I framework antibody (W6/32 mAb, IgG_{2a}), employed as an (NH₄)₂SO₄ preparation at the same concentration as L243, induced IL-1 β mRNA at levels between 33.0 and 36.0 % of those induced by LPS. In control cultures, an irrelevant monocyte specific anti-CD14 (63D3 mAb, IgG₁) antibody, employed at the same concentrations as L243, also induced IL-1 β mRNA at levels between 17.0 and 37.0 % of those induced by LPS.

Initial results examining monocyte cell surface molecules capable of transducing an IL-1 mRNA inductive signals demonstrated that any of a number of monocyte surface antigens were capable of transducing an IL-1 β mRNA inductive signal when stimulated by specific monoclonal antibody.

DISCUSSION

The overall objective of this investigation was to study the signals that regulate the induction of IL-1 during an immune response. According to the previously proposed hypothesis, this induction is not constitutive and likely to take place during the antigen specific interaction between monocytes and T cells during an immune response. To address whether T cells induce monocyte IL-1 during a primary immune response, this investigation evaluated the ability of normal human T cells, activated by anti-CD3 antibody in a model of primary immune activation, to induce the production of IL-1 by autologous human monocytes. These studies assessed induction of IL-1 both at the mRNA and secreted levels. Through the use of purified normal cell populations and the analysis of IL-1 at both the mRNA and secreted product levels, this investigation has identified distinct T cell signals which regulate the induction of IL-1 at distinct stages during an immune response.

The purity of the cell populations used represented a critical component for the performance of these studies. Inasmuch as the characterization of T cell signals leading to the induction of monocyte IL-1 necessitated that other cell

types were eliminated from experimental cultures, procedures were required for the routine isolation of highly purified monocyte and T cell populations from human peripheral blood.

T cell populations used in these studies were demonstrated to be pure based on phenotypic analysis. More importantly, purified T cells exhibited only 2.2 % of control proliferative responses when stimulated by the monocyte dependent T cell mitogen, Con A. This provided a more stringent confirmation of T cell purity than phenotypic analysis. Similarly, purified monocyte populations were shown by phenotypic and functional analyses to contain < 2 % T cell contamination and exhibit < 1 % of proliferative responses exhibited by unseparated cells activated by anti-CD3 T cell mitogen. Cocultures of purified T cells and monocytes, however, exhibited completely restored anti-CD3 induced proliferative responses, demonstrating that when added together, highly purified T cells and monocytes were capable of generating the costimulatory signals necessary for the induction of T cell proliferation. Cell populations purified in this manner were therefore suitable for the study of T cell signalling events leading to the production of monocyte IL-1 during a primary immune activation.

To enable resolution of early IL-1 inductive signalling events, particular emphasis was placed in this investigation

on evaluating IL-1 production at the mRNA level. Inasmuch as previous reports have demonstrated the existence of multiple posttranslational processing steps during the production of IL-1 by monocytes, steady state IL-1 mRNA levels provided a more suitable measure than secreted IL-1 levels for the identification of the initial IL-1 inducing signals. Furthermore, induction of both IL-1 species (IL-1 α and IL-1 β) could be unambiguously investigated at the mRNA level without the need to measure secreted and membrane associated IL-1 activities of mature IL-1 α and β protein products.

Levels of monocyte IL-1 α and β mRNAs were quantitated in this investigation through the use of a constitutive control gene, which allowed hybridization signal intensities among samples to be normalized with respect to the quantities of RNA attached to membranes. The mouse histone replacement variant H3.3 gene (121) (98 % homologous to the human H3.3 gene) was chosen as a constitutive control gene for this purpose, as other more conventional constitutive control genes, such as actin and β -tubulin, have been shown to be expressed at elevated levels in activated monocytes as a consequence of the changes in cytoskeletal architecture that accompany monocyte activation (113, 127). The validity of H3.3 as a constitutive control gene in these studies was confirmed by the demonstration that the H3.3 mRNA levels remain unchanged between unstimulated and LPS or anti-CD3/T

cell stimulated monocytes in the study group as a whole. These observations are consistent with a prior report which demonstrated the constant expression of the H3.3 gene, regardless of cell cycle or differentiation state of cells (128).

The study of lymphocyte mediated IL-1 production at the mRNA level necessitated that monocytes expressed no detectable background IL-1 mRNA. Inasmuch as previous reports have demonstrated that adherence to plastic alone stimulated monocytes to express IL-1 mRNA (129) and secreted IL-1 (130), initial experiments were performed to investigate the kinetics of adherence-induced monocyte IL-1 mRNA expression. IL-1 mRNAs were detectable in freshly isolated monocytes prior to adherence. Despite an initial induction of monocyte IL-1 α and β mRNA expression following isolation and/or adherence, IL-1 mRNAs returned to undetectable levels by 48 h of culture. This induction occurred in the absence of detectable endotoxin levels in any of the reagents employed during the purification of monocytes. Previous reports have employed in situ hybridization analysis to demonstrate that unstimulated circulating human blood monocytes do not express IL-1 mRNAs (97). The observed expression of monocyte IL-1 mRNA prior to adherence was therefore most likely stimulated during the extensive cell purification procedures employed in the current studies, similar to the reported Percoll-mediated induction

of the c-fos cellular proto-oncogene in human monocytes (131). The kinetics of adherence-induced monocyte IL-1 mRNAs indicate that monocytes cultured for at least 48 h prior to the addition of stimuli exhibit negligible levels of IL-1 mRNA and are therefore suitable for subsequent studies examining T cell mediated triggering of monocyte IL-1 mRNA.

Inasmuch as previous reports have failed to detect intracellular IL-1 β induced in monocytes during anti-CD3 mitogenesis (109), experiments were performed to determine whether IL-1 α and β mRNAs were induced in monocytes following the addition of T cells plus anti-CD3 in the anti-CD3 mitogenic response. In contrast to a previous report (109), this study shows the rapid and pronounced induction of IL-1 α and β mRNAs in human monocytes stimulated by the addition of T cells plus anti-CD3, but not by the addition of T cells alone or anti-CD3 alone. This observation suggests that T cells activated during anti-CD3 mitogenesis are capable of delivering a monocyte IL-1 mRNA inductive signal. These results also demonstrate that the observed monocyte IL-1 mRNA inductive effects are not mediated directly by the binding of anti-CD3 antibody alone to monocyte Fc receptors. This finding is consistent with a previous report examining direct Fc receptor mediated induction of IL-1 mRNA in human monocytes, which showed that antibody concentrations three orders of magnitude greater than those employed in the current

studies were required to induce IL-1 mRNA in monocytes (132). Previous studies (109) examining the induction of intracellular IL-1 β in human monocytes during anti-CD3 mitogenesis demonstrated that, whereas IL-1 was induced in monocytes following the addition of activated T cell blasts in a secondary anti-CD3 mitogenesis response, no such induction was observed during a primary anti-CD3 mitogenesis, employing the same antibody and model of anti-CD3 mitogenesis as employed in the current investigation. An explanation for the discrepancy in results reported by the previous study and the current investigation, based on a technical difficulty experienced by the previous investigators in determining intracellular IL-1 β levels, was strongly suggested by the inconsistent observation contained in the previous study that IL-1 β was induced in anti-CD3 mitogenesis cultures at the secreted but not intracellular level. In contrast, the current results suggest that the induction of IL-1 mRNA is not confined to secondary immune responses, but can be mediated during the interaction between monocytes and T cells stimulated in a model of primary immune activation. Inasmuch as previously primed T cells in peripheral blood also may participate in anti-CD3 mitogenesis (133), this experiment does not specifically address whether IL-1 inductive signals can be generated by previously unprimed T cells.

Rapid induction kinetics of monocyte IL-1 mRNAs are

observed during anti-CD3 mitogenesis, with detectable levels present as early as 1 h and peak levels present at 12 h following the addition of T cells plus anti-CD3 to monocytes. These rapid induction kinetics are consistent with the assumption that T cells transduce the IL-1 mRNA inductive signal through direct cell contact with monocytes. This conclusion is consistent with the induction kinetics of mRNA previously reported by Wasik and Beller (108), which described a rapid (≤ 4 h), cell contact mediated induction of membrane IL-1 on mouse macrophages stimulated by the addition of autoreactive T cell clones. The rapid induction of IL-1 mRNA during anti-CD3 mitogenesis further suggests that T cells acquire IL-1 inducing capacities very soon after cross-linking and triggering of the CD3/TCR complex. The similar induction kinetics for IL-1 α and IL-1 β , observed throughout this investigation, is consistent with a coordinate regulation of both IL-1 species during anti-CD3 mitogenesis.

The signals leading to the induction of monocyte IL-1 mRNAs during anti-CD3 mitogenesis appear relatively potent, in that they achieve approximately 40 % of the levels of IL-1 mRNA induced by LPS. Relative to the LPS stimulus for each IL-1 species, IL-1 α mRNA levels induced during anti-CD3 mitogenesis were slightly higher than relative IL-1 β mRNA levels. However, consistent with previous reports demonstrating that IL-1 β comprised the major species of IL-

1 induced at the absolute mRNA level in LPS stimulated monocytes (50), an examination of IL-1 β and IL-1 α hybridization signal intensities at comparable exposure times, assuming approximately similar specific probe activities and optimal wash stringencies for each probe, reveals that IL-1 β mRNA appears to be the more abundant mRNA species induced by either LPS or anti-CD3 mitogenic stimuli. Additionally, induction of IL-1 mRNAs during anti-CD3 mitogenesis was limited to the monocyte population. In contrast to previous reports employing activated T cell clones (134), no IL-1 mRNAs were detectable in activated T cells purified from anti-CD3 mitogenesis cultures. Collectively, these observations show that levels of IL-1 α and β mRNA induced in monocytes during anti-CD3 mitogenesis are relatively substantial compared to those induced by LPS.

Inasmuch as previous reports have established an obligatory role for monocyte Fc receptors during the cross linking of CD3/anti-CD3 complexes in anti-CD3 mitogenesis (119), the possibility that induction of IL-1 mRNA was mediated through the binding of anti-CD3/T cell complexes to monocyte Fc receptors was examined. To determine whether induction of IL-1 during anti-CD3 mitogenesis was mediated through the binding of antibody/T cell complexes to monocyte Fc receptors or occurred instead through the required activation of T cells, experiments were performed in which a

non-mitogenic T cell specific anti-CD5 antibody, of the same IgG_{2a} isotype as anti-CD3, was substituted in place of anti-CD3. Results show that anti-CD5 is incapable of providing the necessary stimulus for the induction of monocyte IL-1 mRNA in the presence of T cells, indicating that IL-1 mRNA induction during anti-CD3 mitogenesis requires a T cell activation event and is not mediated solely a result of binding of antibody/T cell complexes to monocyte Fc receptors.

In view of previous observations that monocytes can transcribe IL-1 mRNA in the absence of IL-1 secretion (59), it was necessary to determine whether the observed induction of monocyte IL-1 mRNA by T cells plus anti-CD3 was associated with secreted IL-1 activity. These experiments show that serologic IL-1 β activity is present as early as 4 h and peaks at \geq 48 h in anti-CD3 culture supernates. Detection of biologic IL-1 activity in supernates, assessed by the LBRM-33-1A5 conversion bioassay (125), confirms that the induction of IL-1 mRNA during anti-CD3 mitogenesis is accompanied by the secretion of a biologically active product.

Secreted IL-1 α activity was not detected in anti-CD3 mitogenesis culture supernates. This observation is consistent with a large body of evidence (56, 57, 60) which demonstrates that mature IL-1 α is not secreted extracellularly but remains associated with the monocyte cell

membrane. Membrane IL-1 activities of monocytes stimulated during anti-CD3 mitogenic culture were not determined in the present studies. Thus although soluble IL-1 α was not detected, it remained possible that IL-1 α activity was induced during anti-CD3 mitogenesis but remained associated with the monocyte cell membrane.

A significant lag between peak induction of IL-1 β mRNA at 12 h and peak secreted IL-1 β levels at ≥ 48 h is observed during anti-CD3 mitogenesis. Previously discussed reports have demonstrated that multiple posttranslational processing steps are involved in the secretion of IL-1 from LPS stimulated monocytes (55, 59, 127, 135). Consistent with these reports, the observed lag between induction at the monocyte IL-1 mRNA level and secreted IL-1 β level may be explained by a model in which IL-1 β mRNA, initially induced during anti-CD3 mitogenesis, is translated into the 31 kD IL-1 β precursor and stored in the cytoplasm until the monocyte receives sufficient activation signals to process the precursor to the secreted 17 kD IL-1 β molecule.

Interestingly, despite the fact that, in all experiments, levels of IL-1 β mRNA induced by LPS were greater than those induced by anti-CD3 mitogenesis, levels of IL-1 β secreted into culture supernates during anti-CD3 mitogenesis are comparable to levels secreted by LPS stimulation of

monocytes. These observations suggested that anti-CD3 activated T cells or their soluble products may be involved in promoting secretion of IL-1 β from monocytes containing IL-1 mRNA transcripts.

To determine whether the observation of IL-1 induction during anti-CD3 mitogenesis could be repeated in an antibody-independent model of an immune response, experiments were performed to measure the synthesis of IL-1, both at the mRNA and secreted levels, during a primary allogeneic mixed lymphocyte response (MLR). The allogeneic MLR represents a model of a primary immune response in which T cells are activated directly through their antigen receptors by recognition of allogeneic class II MHC antigen on the cell surface of allogeneic monocytes. The allogeneic MLR therefore provides an antibody-independent model of immune activation.

The results show that induction of IL-1 occurs both at the mRNA and secreted levels during an allogeneic MLR, thereby providing separate evidence in an antibody-independent model of an immune response to support the earlier induction of monocyte IL-1 observed during anti-CD3 mitogenesis. Previous reports examining the induction of IL-1 in human monocytes during a primary allogeneic MLR have yielded contradictory results. Whereas Dinarello and Kent (105) reported induction of secreted IL-1 activity in primary allogeneic MLR cultures,

Bhardwaj and Steinman (109) failed to observe induction of secreted IL-1 during a primary MLR, but did observe induction of IL-1 during a secondary MLR. These authors use this observation to support their previous view, obtained in murine anti-CD3 mitogenesis responses in the mouse, that induction of IL-1 is not a component of primary immune responses. The current findings support the previous detection of secreted IL-1 during a primary human MLR reported by Dinarello et al., but contradict the inability to detect IL-1 during a primary MLR reported by Bhardwaj and Steinman.

To determine whether the monokine IL-6, which acts synergistically with IL-1 to costimulate T cell activation, was coinduced with IL-1 during anti-CD3 mitogenesis, an initial experiment was carried out to examine the induction of IL-6 mRNA in monocytes during anti-CD3 mitogenesis. The results demonstrate that IL-6 mRNA is coinduced with IL-1 during anti-CD3 mitogenesis. These results demonstrate that IL-6 mRNA may require the same T cell signals as IL-1 for induction. Furthermore, these observations are consistent with a model in which the synergistically acting costimulatory molecules, IL-1 and IL-6, are simultaneously induced at the site of an immune response during the antigen-specific interaction between T cells and monocytes during an immune response.

Having established that signals from activated T cells mediate the induction of monocyte IL-1 mRNAs and secreted IL-1 β , subsequent experiments were designed to characterize the nature of the T cell derived stimulatory signal.

The rapid induction kinetics of IL-1 mRNA observed during anti-CD3 mitogenesis were consistent with a requirement for cell contact between T cells and monocytes for transduction of the IL-1 inductive signal. A cell contact requirement was therefore examined through the use of double chamber culture wells, in which monocytes cultured in separate chambers were exposed to two different sets of signals. Monocytes exposed to these two sets of signals exhibit strikingly different kinetics and magnitude of IL-1 mRNA responses. Monocytes cultured in upper chambers, which received signals obtained through direct cell contact with T cells, demonstrate a rapid and pronounced induction of IL-1 mRNA, detectable at 2 h and peaking at 12 h, characteristic of the response previously observed during anti-CD3 mitogenesis. In contrast, monocytes cultured in lower chambers, which were exposed only to the soluble signals generated during anti-CD3 mitogenesis exhibit no detectable IL-1 mRNA at early 2 and 4 h time points, but do exhibit low levels of IL-1 mRNA at ≥ 12 h. Control experiments verified that the low levels of IL-1 mRNA induction observed in lower chamber monocytes were not caused by an anti-CD3 mitogenesis

response with residual T cells contaminating this chamber.

Two distinct phases of induction of IL-1 mRNA during anti-CD3 mitogenesis were also identified by these studies. First, a pronounced initial phase, which occurs in the presence of cell contact between T cells and monocytes, and second, a low level late phase, which occurs as a result of soluble signals generated during anti-CD3 mitogenesis. The initial triggering phase of monocyte IL-1 mRNA, which occurs in the presence of cell contact between T cells and monocytes, has been extensively characterized in this investigation and will be discussed later. The late phase of induction of IL-1 mRNA, mediated by soluble factors generated during anti-CD3 mitogenesis, was further investigated. Experiments were performed to determine whether the kinetics of induction of late phase monocyte IL-1 mRNA correlated with the appearance in culture supernates of the late phase cytokines IL-2, IFN- γ and TNF- α , which have been previously recognized to possess IL-1 inducing activities (115-117).

The IL-1 inducing cytokines, IL-2, IFN- γ and TNF- α , all exhibit similar kinetics of appearance in anti-CD3 mitogenic culture supernates. Whereas they are not detectable at early 2 - 4 h time points, pronounced levels are detected at ≥ 8 h and reach maximal levels at 48 h. These results show that the appearance of IL-1 inducing cytokines is temporally

experiments were designed to evaluate the ability of metabolically inactivated T cells to induce IL-1 mRNAs through direct cell contact with monocytes.

Results of these experiments show that T cells, metabolically inactivated by paraformaldehyde fixation, are fully capable of inducing both IL-1 α and β mRNAs in monocytes in the presence of anti-CD3, but only when these cells had been preactivated. These observations demonstrate that cell contact alone with appropriately activated T cells is required for the initial induction of monocyte IL-1 mRNA during anti-CD3 mitogenesis. Furthermore, these results definitively rule out the involvement of soluble T cell factors in this initial triggering of IL-1 mRNA. An evaluation of T cell fixation conditions verified that paraformaldehyde fixation resulted in complete loss of cell viability, as judged by flow cytometric analysis of PI vital dye exclusion.

Direct effects of T cell fixation upon monocytes were ruled out by the inability of fixed, unstimulated T cells to induce monocyte IL-1 mRNAs in the presence of anti-CD3. The inability of fixed, unstimulated T cell to induce monocyte IL-1 mRNA also supports the conclusion of a previous experiment employing non-mitogenic anti-CD5 antibody, that induction of IL-1 mRNA during anti-CD3 mitogenesis occurs through a required T cell activation event. Whereas fixed,

unstimulated T cells do not induce monocyte IL-1 mRNA in the presence of anti-CD3, fixed preactivated T cells are capable of induction of monocyte IL-1 mRNA. These results are consistent with previous results obtained using T cells plus a non-mitogenic anti-CD5 antibody and demonstrate that induction of monocyte IL-1 mRNA during anti-CD3 mitogenesis occurs as a result of activation of T cells and not through the binding of antibody/T cell complexes to monocyte Fc receptors.

The ability of fixation to maintain cell surface determinants at a given stage of T cell activation was utilized in these studies to characterize the activation signals required for acquisition of IL-1 inducing properties at the T cell surface. Three groups of fixed T cells, differing in their activation state prior to fixation, were investigated for their ability to trigger IL-1 mRNA in monocytes. These groups consisted of unstimulated T cells, T cells activated by immobilized anti-CD3 for 24 h and T cells activated by monocytes plus anti-CD3 for 24 h. Results show that fixed T cells, preactivated either by immobilized anti-CD3 or monocytes plus anti-CD3, induce monocyte IL-1 mRNA, whereas fixed, unstimulated T cells do not. Proliferative responses demonstrate that T cells activated with monocytes plus anti-CD3 exhibit the complete proliferative capacity expected of combined monocyte, T cell populations activated

in an anti-CD3 mitogenesis. T cells in this group had therefore received the TCR complex triggering signal and all monocyte derived costimulatory signals. T cells which were stimulated by immobilized anti-CD3 alone, exhibited < 5 % of the anti-CD3 mitogenic response. However, in the presence of exogenously added IL-2, proliferative responses of these T cells were completely restored. This observation demonstrates that T cells activated by immobilized anti-CD3 had received sufficient activating signals to express IL-2 receptors, but insufficient signals to generate IL-2 and become fully activated. Previous reports have demonstrated that TCR complex triggering culminates in the expression of IL-2 receptors (66) and that soluble costimulatory signals are required for elaboration of IL-2 and subsequent clonal proliferation of triggered T cells (67). Proliferative responses of T cells stimulated by immobilized anti-CD3 therefore indicate that these cells had received the TCR triggering signal only. In conclusion, experiments examining the activation signals required by T cells for generation of IL-1 mRNA inductive capacity demonstrate that TCR triggering alone provides sufficient stimulus for transduction of the IL-1 mRNA inductive signal.

Early acquisition of T cell IL-1 mRNA inductive properties following the TCR triggering signal alone is consistent with the rapid induction at ≤ 1 h of monocyte IL-

1 mRNA during anti-CD3 mitogenesis. These rapid induction kinetics preclude the possibility that T cells must receive monocyte derived costimulatory signals and become fully activated prior to transduction of the IL-1 mRNA inductive signal. In support of this notion, the induction of IL-1 mRNA at 1 h precedes the earliest reported detection of IL-2 during anti-CD3 mitogenesis by 3 - 7 h (67, 136). The signalling requirements for T cells and the kinetics of transduction of the IL-1 mRNA inductive signal are also consistent with the previously reported and well established sequence of signalling events required for the complete activation of T cells during an antigen specific immune response (63-67). As previously discussed, IL-1 plays its preponderant costimulatory role during the activation of human T cells at the level of IL-2 production (80, 81). Early transduction by the T cell of the IL-1 mRNA inductive signal, before the production of IL-2, is therefore chronologically consistent with a subsequent role for IL-1 in the synthesis of IL-2.

Despite being fully capable of inducing monocyte IL-1 mRNA, fixed, preactivated T cells are incapable of inducing secretion of IL-1 β by monocytes. These results demonstrate that metabolic activity by T cells is required to induce IL-1 β secretion by monocytes. Furthermore, this observation is consistent with a requirement for soluble T cell factors to induce secretion of IL-1 β by monocytes. This significant

finding is discussed in greater detail following an evaluation of the protein synthesis requirement of T cell mediated induction of IL-1 β secretion.

Inasmuch as earlier experiments employing fixed T cells have demonstrated that novel IL-1 mRNA inducing properties are generated at the T cell surface following triggering of the TCR complex, experiments were performed to determine whether de novo protein synthesis was required for acquisition of this IL-1 mRNA inductive property. These experiments evaluate whether the IL-1 mRNA inductive signal is generated de novo or whether it preexists at the T cell surface, requiring only modulation or conformational change for transduce a signal. The protein synthesis requirement of the IL-1 mRNA inductive property was investigated by evaluating the ability of T cells, pretreated with the de novo protein synthesis inhibitor emetine, to induce monocyte IL-1 mRNA during anti-CD3 mitogenesis. Results show that T cells pretreated with emetine are fully capable of inducing monocyte IL-1 α and β mRNA in the presence of anti-CD3. These studies demonstrate that de novo protein synthesis is not required by T cells to transduce the cell contact mediated IL-1 mRNA inductive signal to monocytes during anti-CD3 mitogenesis. Furthermore, these observations support the conclusion from T cell fixation studies that synthesis of soluble T cell lymphokines is not involved in the initial phase of induction of monocyte IL-1

mRNA. Finally, these findings suggest that the IL-1 inductive molecule possesses a slow turnover rate. Completeness of de novo protein synthesis inhibition was confirmed in these studies by assessments of ³H-leucine incorporation, which demonstrated that emetine treated T cells exhibited between 94 - 96 % inhibition of de novo protein synthesis. Previous studies have reported that IL-1 production by monocytes is superinduced in the presence protein synthesis inhibitors (137). However, direct effects on monocytes by emetine leaching from T cells were ruled out in the current experiments by control cultures, which demonstrated the normal induction of monocyte IL-1 mRNA by LPS in the presence of emetine treated T cells. In summary, de novo protein synthesis was not required for transduction of the cell contact mediated IL-1 mRNA inductive signal by T cells.

The characteristics of the T cell IL-1 mRNA inductive signal identified in this investigation suggests that this signal represents the initial IL-1 inductive stimulus provided to the monocyte during an immune response. According to the previously proposed hypothesis, the initial IL-1 inductive signal during an immune response to foreign antigen is thought to be transduced by the T cell during its antigen specific interaction with the presenting monocyte. The basic components of the T cell IL-1 mRNA inductive signal described in this investigation, including rapid kinetics of

transduction, requirement for cell contact, acquisition following TCR triggering alone and independence of de novo protein synthesis, are all consistent with this model of initial IL-1 induction during an immune response. This investigation is the first to report the induction of IL-1 mRNA in monocytes mediated by a T cell signal requiring cell contact. The similar induction requirements for IL-1 α and IL-1 β mRNA species observed in these studies, suggests that the initial inductive signals for each species is the same. The basic characteristics of the IL-1 mRNA inductive signal identified in this investigation are consistent with two previous reports in the literature, which have described cell contact mediated mIL-1 inductive signals generated by antigen specific or autoreactive mouse T cell clones, similarly insensitive to protein synthesis inhibitors (107, 108). A comparison between the T cell mIL-1 inductive signal reported by Wasik and Beller (108) and the IL-1 mRNA inductive signal reported in this investigation suggests that these signals may be the same. The requirements of these two T cell signals are identical with respect to cell contact, de novo protein synthesis and metabolic activity. Whereas the use of mouse T cell clones in the previous investigations left unresolved whether IL-1 inductive signals could be transduced by normal polyclonal cell populations, the current studies have demonstrated that normal human T cells are capable of transducing the initial IL-1 inductive signal to monocytes

through direct cell contact. Importantly, the T cell activation signals required for transduction of this signal have also been defined in this investigation. Whereas the current investigation has determined that TCR triggering alone is a sufficient stimulus for T cells to transduce the IL-1 mRNA inductive signal to monocytes, the two previous studies were unable to resolve the activation signals required at the level of the T cell, as they employed T cell clones constitutively maintained in the presence of stimulating antigen and feeder cells to provide costimulatory molecules. This investigation has therefore characterized several novel features of the T cell signal that mediates the initial induction of IL-1 mRNA in monocytes.

Inasmuch as experiments in the current investigation demonstrated that protein synthesis was not required by T cells for induction of IL-1 β mRNA, a requirement for protein synthesis for T cell mediated induction of IL-1 β secretion by monocytes was investigated. Experiments were therefore performed to evaluate the ability of T cells, pretreated with the protein synthesis inhibitor emetine, to induce IL-1 β secretion by monocytes in the anti-CD3 mitogenesis model.

The results demonstrate that, despite the presence of IL-1 β mRNA transcripts in monocytes, secretion of IL-1 β requires the presence of a T cell product which is dependent

upon de novo protein synthesis for its generation. A direct effect on monocytes by emetine leaching from T cells was ruled out by control cultures, which demonstrated that the LPS induced secretion of IL-1 β by monocytes was unaffected by the presence of emetine treated T cells. The presence of low levels of IL-1 β secreted independently of protein synthesis in T cells could not be completely ruled out by these studies. At the highest dose of emetine, which inhibited 96 % of T cell de novo protein synthesis, residual secreted IL-1 β levels of 10 % were detected. The observation that T cell protein synthesis is required for induction of secreted IL-1 β by monocytes confirms and enlarges upon the previous observation of this current investigation that metabolically inactivated T cells were incapable of inducing IL-1 β secretion despite inducing monocyte IL-1 β mRNA. Furthermore, these observations suggest that activated T cell lymphokines are required to induce secretion of IL-1 β by monocytes. This possibility is consistent with previous reports which have identified IL-1 inducing properties exhibited by the late phase cytokines IL-2, IFN- γ , TNF- α and IL-1 IF (115-117).

Simultaneous analysis of IL-1 at the mRNA and secreted levels has led to the identification of two distinct stages of IL-1 production during anti-CD3 mitogenesis, each requiring a separate T cell inductive signal. First, the initial triggering of monocyte IL-1 mRNA is induced by a cell contact

mediated T cell signal generated independently of protein synthesis. Second, secretion of IL-1 β by monocytes is induced by a T cell product whose generation is dependent upon de novo protein synthesis. Characteristics of the initial IL-1 mRNA triggering signal have been previously discussed.

Several properties of the secreted T cell product that is required for IL-1 β secretion by monocytes, have been characterized by earlier experiments in this investigation and by previous reports in the literature. Experiments which showed a requirement for de novo protein synthesis to generate this T cell factor, suggest that this T cell product is a lymphokine, although the possibility of a de novo induced cell surface determinant cannot be excluded in these experiments. However, studies employing fixed, preactivated T cells argue against this second possibility, as the expression of such a cell surface determinant would be expected to be preserved following fixation. In support of this assumption, fixation did not alter the ability of T cells to transduce the cell associated IL-1 mRNA inductive signal to monocytes. The lymphokine hypothesis is consistent with previous reports in the literature, which have identified numerous lymphokines, including IL-2, IFN- γ , TNF- α and IL-1 IF, which possess secreted IL-1 inducing properties (114-117). Synthesis of all of these lymphokines has been shown to be dependent upon de novo protein synthesis and, therefore, any combination of

these lymphokines represent potential candidates as the secreted IL-1 inducing T cell factor identified by this investigation.

The activation requirements for synthesis of this secreted IL-1 β inductive T cell factor are currently unknown. Kinetic studies of IL-1 β secretion during anti-CD3 mitogenesis show that secreted IL-1 β is first detectable in culture supernates at 4 h. These kinetics argue neither for or against a requirement for TCR triggering in the absence of costimulatory molecules. Indirect evidence from the literature suggests that T cells require a minimum of TCR triggering plus the presence of monocyte membranes for synthesis of soluble IL-1 inducing lymphokines. With one exception, all previously identified IL-1 inducing lymphokines are late phase cytokines, which require both TCR triggering and costimulatory molecules for synthesis. The exception noted concerns the recently identified membrane IL-1 inducing lymphokine, IL-1 IF, which requires only mitogen and the presence of fixed macrophage membranes for its release from T cell clones (114). Therefore, although no direct evidence exists to indicate what signalling requirements are necessary for release of the T cell factor which induces IL-1 β secretion from monocytes, indirect evidence suggests that it requires both TCR triggering plus costimulatory signals for its generation.

The ability to resolve early and late events during IL-1 synthesis in these studies has enabled the identification of a T cell factor required for the secretion of IL-1 β from monocytes. Thus, despite the presence of IL-1 β mRNA transcripts in monocytes, secretion of IL-1 β requires the presence of a soluble T cell factor. This novel finding suggests that T cells regulate IL-1 β synthesis in monocytes at two discrete levels: At the level of initial induction of monocyte IL-1 mRNA and at the level of secretion of IL-1 β .

In the final phase of this investigation, the cell surface molecules involved in transducing the initial IL-1 mRNA triggering signal from T cells to monocytes during anti-CD3 mitogenesis were studied. The possibility that triggering of IL-1 mRNA during an immune response occurred exclusively during an MHC class II restricted interaction between monocytes and CD4⁺ T cells was examined by comparing the IL-1 inductive abilities of CD4⁺ and CD8⁺ T cells in the anti-CD3 mitogenesis model. In these experiments, CD4⁺ and CD8⁺ T cell subpopulations isolated by negative and positive selection were evaluated for their IL-1 inductive abilities at the level of IL-1 mRNA and secreted IL-1 β respectively.

Results show that CD8⁺ T cells are at least as potent

as CD4⁺ T cells in the induction of monocyte IL-1 mRNAs and IL-1 β secretion. These results demonstrate that the IL-1 inductive signal is not transduced exclusively during an MHC class II restricted interaction between monocytes and CD4⁺ T cells. Furthermore, these observations suggest that triggering of monocyte IL-1 mRNA during an immune response is either transduced by MHC non-restricted elements on the surface of T cells or can be transduced by both CD4 and CD8 associative recognition elements. No hypothesis exists to suggest an *in vivo* role for CD8⁺ cells during the induction of IL-1 by monocytes. The possibility that induction of IL-1 is mediated by MHC non-restricted elements on the T cell surface is therefore favored as a more physiologically relevant interpretation of the observed results. These conclusions are the same as those previously reported by Wasik and Beller, who demonstrated that both class I restricted and class II restricted autoreactive T cell clones induced macrophage membrane IL-1 (108). These conclusions, however, are in contrast to another report in the literature, which concluded that alloreactive T cell clones induce intracellular monocyte IL-1 by "MHC-specific contact" with monocytes of the haplotype to which the clones were originally sensitized (109). The experimental system employed in reaching this conclusion, however, was unable to distinguish between events required for MHC restriction and those required for IL-1 triggering.

Inasmuch as it remained unclear from previous studies whether IL-1 inductive signals may be generated by unprimed T cells, purified naive and memory T cells were evaluated for their ability to induce IL-1 β secretion by monocytes during anti-CD3 mitogenesis. Studies with naive T cells directly address the critical question whether the initial IL-1 triggering signal can be transduced by naive T cells in an experimental model of an afferent immune response. Results of these experiments show that virgin (CD45RA⁺) T cells are equally capable of inducing IL-1 β secretion by monocytes as memory (CD45RO⁺) T cells. These observations demonstrate that purified cells exhibiting a naive T cell phenotype are capable of transducing the initial IL-1 mRNA triggering signal to monocytes during anti-CD3 mitogenesis. These studies further show that induction of IL-1 is a feature of the afferent limb of the immune response. No previous report exists in the literature to address whether naive T cells in the afferent arm of the immune response are capable of generating an IL-1 inductive signal. Previous studies have examined whether induction of IL-1 can be observed during primary and secondary polyclonal T cell responses. Based on a demonstration of IL-1 induced during secondary but not primary polyclonal responses, previous authors have suggested that induction of IL-1 is a feature of the efferent but not afferent arm of the immune response (109, 138). However, in view of the fact that memory cells participate in mitogenic responses, these

previous reports did not, in fact, address whether induction of IL-1 occurred during the afferent arm of an immune response. The current investigation therefore provides the first evidence that IL-1 inductive signals can be transduced by virgin, unprimed T cells in an afferent model of an immune response.

The mechanism by which the initial IL-1 mRNA inductive signal is transduced to the monocyte during antigen presentation is suggested by studies carried out in this investigation. Studies employing fixed T cells showed that T cells acquire a novel IL-1 inducing characteristic at the cell surface following activation through TCR triggering. Kinetic studies of IL-1 mRNA induced during anti-CD3 mitogenesis show that acquisition of this cell surface entity is rapid, occurring at < 1 h. Furthermore, acquisition of this cell surface moiety does not require de novo protein synthesis. When considered together, these findings suggest a number of cell surface molecules through which T cells may trigger IL-1 mRNA in monocytes. In the absence of a de novo protein synthesis requirement, it must be proposed that the IL-1 inductive molecule exists at the surface of unactivated T cells. For signal transduction to occur the cell surface molecule subsequently requires either a conformational change induced by TCR/CD3 cross-linking or comigration with the TCR/CD3 complex to the site of monocyte-T cell contact.

A candidate molecule for the initial IL-1 mRNA inductive signal at the T cell surface is the T cell associative recognition element, CD4. According to the mechanism proposed, transduction of the IL-1 inductive signal may occur through the MHC class II molecule on monocytes following comigration of CD4 with the TCR/CD3 complex to the site of monocyte-T cell interaction or following conformational change induced by CD3/TCR cross-linking. Previous reports in the literature and preliminary studies contained in this report, suggested that MHC class II molecules could potentially act as IL-1 signalling molecules on monocytes, based on the ability of antibodies directed against these molecules to induce IL-1 synthesis (139, 140). However, experiments demonstrating that both CD4⁺ and CD8⁺ T cells could induce monocyte IL-1, argued either for the involvement of both CD4 and CD8 molecules in the signal transduction or for the involvement of an MHC-unrestricted element. Inasmuch as no hypothesis exists to suggest an in vivo role for CD8⁺ cells during the induction of IL-1 by monocytes, these results favor the explanation that transduction of the IL-1 inductive signal involves a moiety other than an MHC molecule. Several other cell surface adhesion molecules may represent alternative candidate molecules, including the T cell/monocyte cell surface molecules CD2/LFA-3, LFA-1/ICAM-1 or LFA-1/ICAM-2. Indirect

evidence from the literature is consistent with the involvement of CD2 or LFA-1 T cell molecules in transduction of the IL-1 mRNA inductive signal. CD2 has been shown to be physically associated with the CD3/TCR complex and is therefore susceptible to potential conformational change following TCR triggering (141). LFA-1 has been shown to be rapidly converted from a low avidity to a high avidity form following TCR cross-linking, in a process that requires metabolic energy but no de novo protein synthesis (142). The high avidity form of LFA-1 is capable of binding transiently and reversibly to ICAM-1 on monocytes in a cycle of < 1 h duration. Transduction of an IL-1 mRNA inductive signal through LFA-1 is therefore consistent with a model of IL-1 induction by T cells that involves comigration or focussing of the IL-1 triggering entity at the site of monocyte-T cell contact. The alternative possibility exists that the IL-1 triggering entity on the surface of T cells represents a hitherto undescribed cell surface molecule.

A mechanism by which the second T cell IL-1 inductive signal, which is required for secretion of IL-1 β by monocytes, is transduced to the monocytes is suggested data obtained in this investigation. Secretion of IL-1 β by monocytes containing IL-1 β mRNA transcripts is dependent upon a T cell product which requires protein synthesis for its generation. Previous reports have demonstrated that secretion

of IL-1 β from the intracellular 31 kD precursor pool in monocytes is dependent upon proteolytic cleavage by a serine protease (59, 143). The T cell factor may therefore induce release of mature IL-1 β by stimulating the production or activity of the serine protease processing enzyme in monocytes.

The proposed mechanism by which IL-1 β secretion is induced by the T cell factor may also explain how LPS or phagocytic stimuli are capable of inducing IL-1 β secretion by monocytes in the absence of T cell products. Previous observations have demonstrated that bacterial products and particulate antigens have an intrinsic ability to stimulate intracellular monocyte proteases (144). It was interesting to note from the present studies that despite inducing higher levels of IL-1 β mRNA in all experiments, LPS was less effective than the anti-CD3 stimulus at inducing IL-1 β secretion. These results suggest that secretion of IL-1 β by monocytes is more efficient in the presence of activated T cell products. An implication of the proposed mechanism governing IL-1 secretion from monocytes, is that effective secretion of IL-1 β , regardless of the nature of the IL-1 mRNA triggering stimulus, requires the recruitment of activated T cells and their products.

Based on the results contained in this report, a brief

model of IL-1 induction during an immune response may be proposed. T cells are initially triggered through their TCR by recognition of specific foreign antigen expressed in conjunction with self class II MHC molecules on the surface of monocytes. Within 1 h following triggering, T cells transduce a cell contact mediated IL-1 mRNA inductive signal to the monocyte participating in the antigen specific T cell-monocyte interaction. This IL-1 mRNA inductive property is acquired at the cell surface of triggered T cells in the absence of de novo protein synthesis. Initial induction of monocyte IL-1 mRNA occurs in the absence of soluble T cell signals. Monocyte IL-1 α and IL-1 β genes are transcribed, specific mRNAs are translated and a fraction of immature cytoplasmic IL-1 precursors are translocated to the exterior cell membrane of monocytes, with little or no concomitant secretion of IL-1. Appropriately triggered T cells initially receive IL-1 costimulatory signals, either in the form of membrane IL-1 or in the form of modest levels of secreted IL-1. IL-1 may exert its costimulatory properties in synergy with IL-6 and other monocyte derived soluble or cell associated costimulatory molecules. T cells that have received the necessary costimulatory signals elaborate IL-2 and other late phase lymphokines which by themselves possess IL-1 inducing properties. The major phase of IL-1 β secretion by monocytes requires the presence of a T cell factor that is dependent upon de novo protein synthesis for its generation.

This T cell factor is most probably a soluble lymphokine, or combination of lymphokines. Multiple late phase lymphokines with IL-1 inducing properties participate in an amplification loop, in which initially modest levels of late phase lymphokines induce the secretion of IL-1 by monocytes, thereby further activating T cells and enhancing additional secretion of late phase lymphokines. Steady state levels of monocyte IL-1 mRNAs are maintained by soluble IL-1 mRNA inductive or augmenting cytokines generated during the course of an immune response. These IL-1 inducing cytokines induce further IL-1 production non-specifically in bystander monocytes during the immune response.

This investigation has identified novel T cell mediated signals that regulate two important stages of IL-1 synthesis by human blood monocytes during an immune response. First, a T cell signal that initiates induction of monocyte IL-1 mRNAs through direct cell contact. Second, a soluble T cell factor that is required for secretion of IL-1 β from monocytes. During the initial phase of an immune challenge, unprimed T cells provide the specificity of the IL-1 response to soluble antigen by transducing a rapid IL-1 mRNA inductive signal to monocytes participating in an antigen specific interaction with T cells. At a subsequent stage of the immune response, activated T cells amplify this specifically induced IL-1 response at multiple levels, including induced IL-1

secretion by monocytes, maintenance of steady state IL-1 mRNA levels and induced IL-1 production by antigen non-specific monocytes. In humans, T cells therefore control both the specificity and the amplitude of IL-1 synthesized by monocytes in response to an immune challenge.

SUMMARY

1. Freshly isolated human blood monocytes expressed IL-1 α and β mRNA immediately prior to adherence, which peaked at 12 h following adherence to plastic culture plates and diminished to undetectable IL-1 mRNA levels at 48 h of adherence.
2. IL-1 α and β mRNAs were induced in 48 h adherent monocytes following the addition of T cells plus anti-CD3 antibody, but not following T cells or anti-CD3 alone. A non-mitogenic anti-CD5 antibody, of the same isotype as anti-CD3, was incapable of providing the necessary stimulus for the induction of monocyte IL-1 mRNAs in the presence of T cells. Monocyte IL-1 α and β mRNAs were detectable as early as 1 h following the addition of T cells plus anti-CD3, peaked at 12 h and declined by 24 h. Induction of IL-1 mRNA during anti-CD3 mitogenesis was confined exclusively to the monocyte population; no IL-1 mRNA was detected in the activated T cell population.
3. Similar induction kinetics for IL-1 α and IL-1 β mRNA were observed throughout this investigation. Relative

to LPS inducible levels for each IL-1 species, levels of IL-1 α mRNA induced during anti-CD3 mitogenesis were greater than relative levels of IL-1 β mRNA.

4. Induction of monocyte IL-1 mRNA during anti-CD3 mitogenesis was associated with secreted IL-1 β , but not IL-1 α , which was detectable as early as 4 h and reaching maximum levels at 48 h. Biologic activity was detectable at 12 h, the earliest time point tested, and attained maximal levels at 48 h of study.
5. IL-6 mRNA was coinduced with IL-1 mRNA in monocytes during anti-CD3 mitogenesis.
6. IL-1 synthesis was induced in monocytes during the primary allogeneic mixed lymphocyte response.
7. The initial and major phase of induction of monocyte IL-1 mRNA occurred in the presence of cell contact with T cells plus anti-CD3. A later, low level of induction of IL-1 mRNA occurred in monocytes receiving only soluble factors generated during anti-CD3 mitogenesis.
8. Induction of the late phase of IL-1 mRNA in monocytes, which occurred independently of contact with T cells, was temporally associated with the appearance of the IL-1

inducing cytokines, IL-2, IFN- γ , and TNF- α in anti-CD3 mitogenic culture supernates.

9. Metabolically inactivated T cells were capable of inducing IL-1 mRNAs in monocytes, but only if T cells had been preactivated. Metabolic activity by T cells was required for the secretion of IL-1 β by monocytes.
10. Induction of monocyte IL-1 mRNA during anti-CD3 mitogenesis occurred independently of de novo protein synthesis by T cells, although de novo protein synthesis was required by T cells for secretion of IL-1 β by monocytes.
11. Induction of IL-1 synthesis in monocytes during anti-CD3 mitogenesis was mediated equally well by both MHC class II restricted (CD4⁺) and MHC class I restricted (CD8⁺) T cells and by both naive (CD45RA⁺) and memory (CD45RO⁺) T cells during anti-CD3 mitogenesis.

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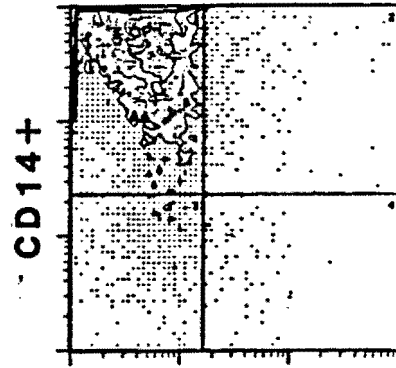
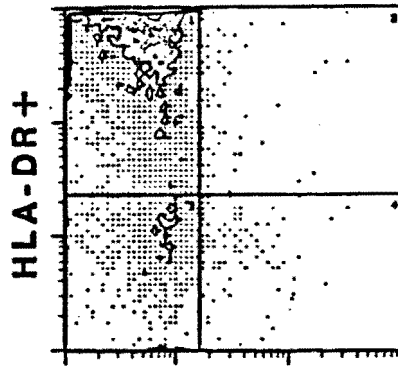
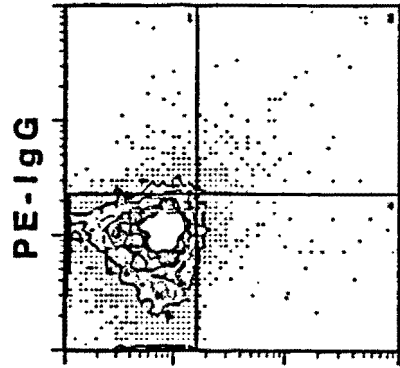
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APPENDIX

Figure 1. Flow Cytometric Contour Plots Depicting CD14, HLA-DR and CD2 Cell Surface Marker Expression by Monocyte and T Cell Populations Purified from PBMCs Monocyte and T cell populations isolated from PBMCs were labelled with anti-HLA-DR (PE anti-HLA-DR), anti-CD14 (PE anti-Leu M3) and anti-CD2 (FITC anti-Leu 5) antibodies and analyzed by flow cytometry. Figure depicts flow cytometric contour plots from a representative experiment.

Monocytes



T Cells

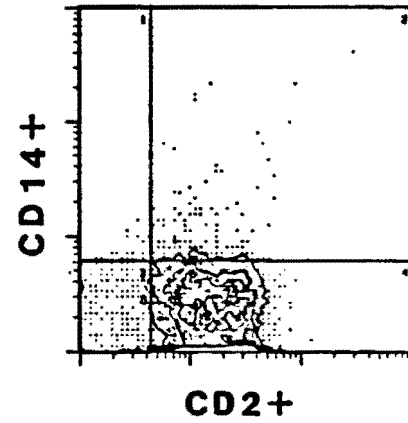
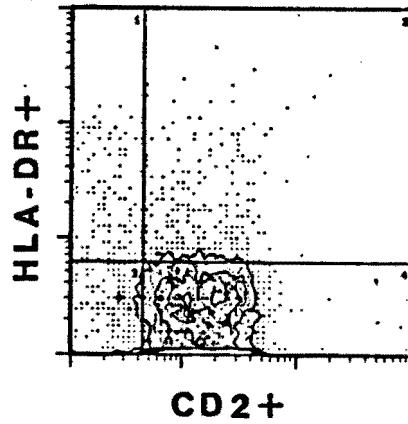
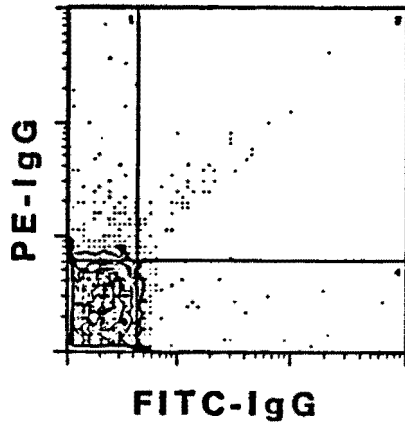


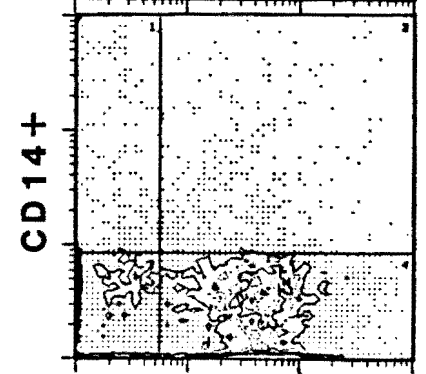
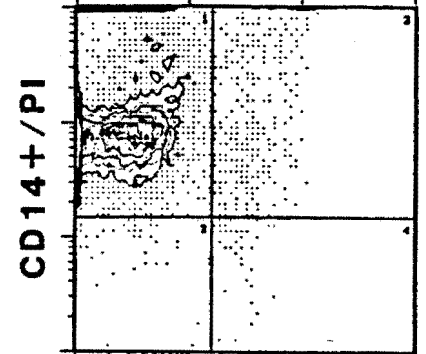
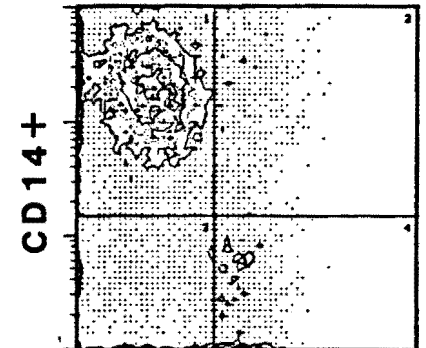
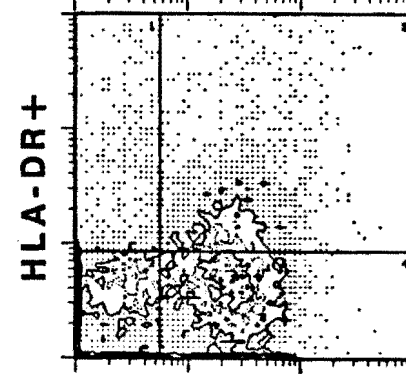
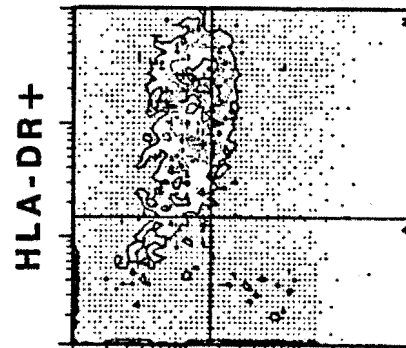
Figure 2. Flow Cytometric Contour Plots Depicting CD14, HLA-DR and CD2 Cell Surface Marker Expression by Monocyte and T Cell Populations Isolated From Anti-CD3 Mitogenic Cultures

Monocytes isolated from the adherent fraction of anti-CD3 mitogenesis cultures were depleted of contaminating T cell blasts by anti-CD3 dependent complement mediated lysis. Final monocyte populations were analyzed in the presence of PI to exclude non-viable cells from analysis. T cells were isolated from the non-adherent cell fraction by rosetting with AET treated SRBC. Resultant cell populations were labelled with anti-HLA-DR (PE anti-HLA-DR), anti-CD14 (PE anti-Leu M3) and anti-CD2 (FITC anti-Leu 5) antibodies and presented as flow cytometric contour plots.

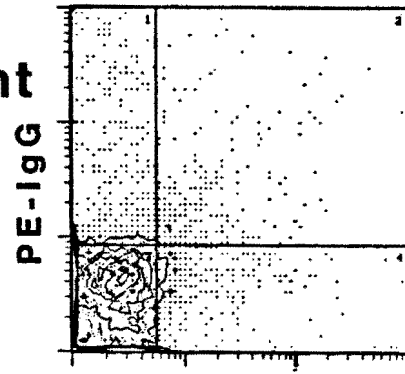
**Adherent
Cells**



**Following antibody/C'
depletion of T Cells**



**Non-adherent
E+ Cells**



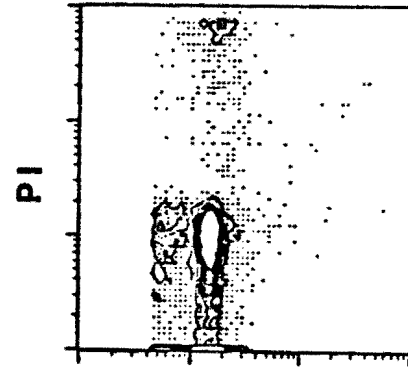
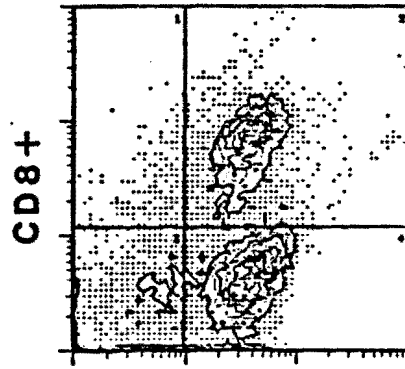
FITC-IgG

CD2+

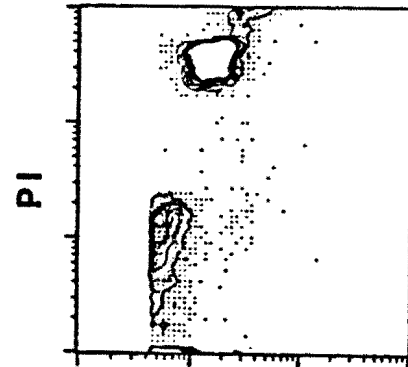
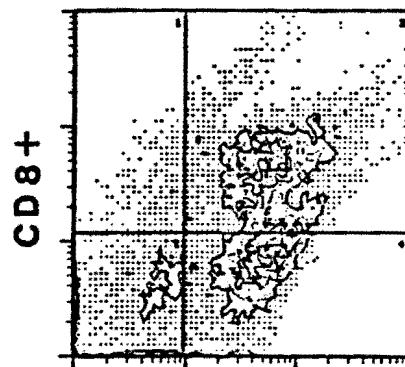
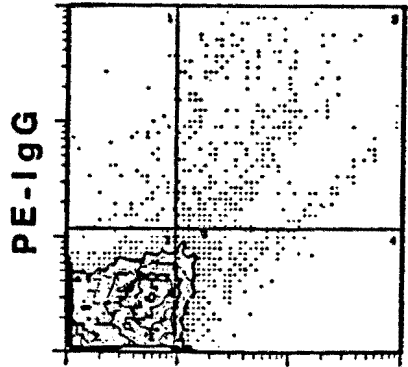
CD2+

Figure 3. Flow Cytometric Histograms Depicting CD3 and CD8 Cell Surface Marker Expression and PI Uptake by Fixed and Unfixed T Cells Fixed and unfixed unstimulated T cells were stained with PI or anti-CD3 (FITC anti-Leu 4) and anti-CD8 (PE anti-Leu 2a) antibodies and analyzed by flow cytometry. T cells shown in this figure were employed in the fixation studies investigating monocyte IL-1 mRNA induction by fixed T cells, depicted in **Figure 12**.

Unfixed T Cells



Fixed T Cells



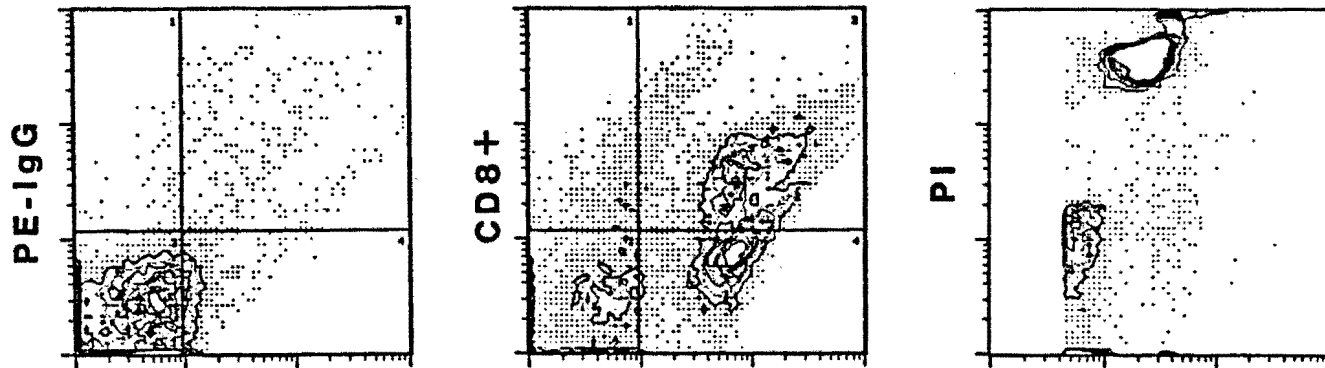
FITC-IgG

CD3+

Volume

Figure 4. Flow Cytometric Histograms Depicting CD3 and CD8 Cell Surface Marker Expression and PI Uptake by Fixed and Unfixed T Cells Fixed T cells, preactivated with immobilized anti-CD3 (Group 1) or monocytes plus anti-CD3 (Group 3), were stained with PI or anti-CD3 (FITC anti-Leu 4) and anti-CD8 (PE anti-Leu 2a) antibodies and analyzed by flow cytometry. T cells shown in this figure were employed in the fixation studies investigating monocyte IL-1 mRNA induction by fixed T cells, depicted in **Figure 12**.

**T Cells fixed following stimulation with:
Solid-phase anti-CD3**



Monocytes plus anti-CD3

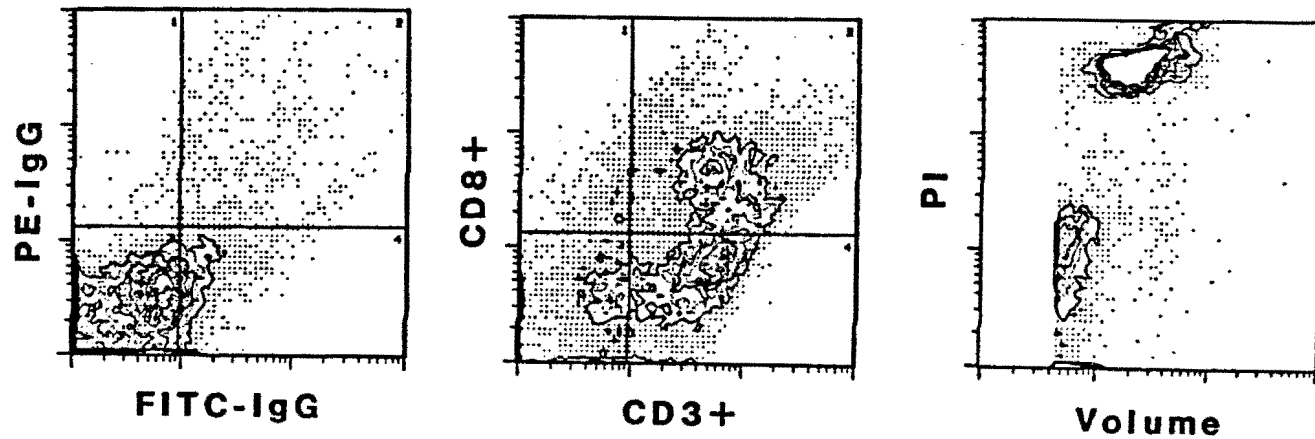
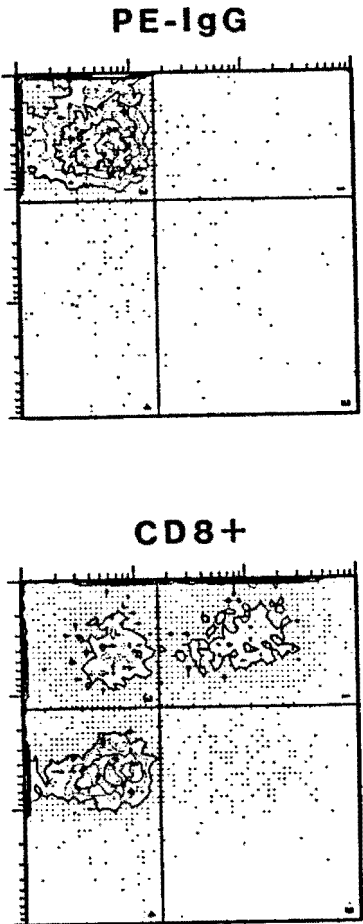
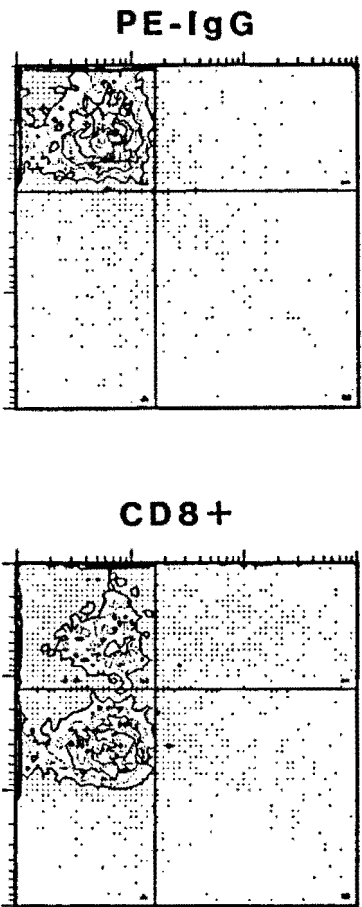


Figure 5. Flow Cytometric Contour Plots Depicting CD4 and CD8 Cell Surface Marker Expression by T Cell Subpopulations Obtained by Negative Selection T cells, separated into CD4+ and CD8+ enriched T cell subpopulations by negative selection, were labelled with anti-CD4 (FITC anti-Leu 3a&b) and anti-CD8 (PE anti-Leu 2a) antibodies and analyzed by flow cytometry.

Unseparated T Cells



CD4+ selected



CD8+ selected

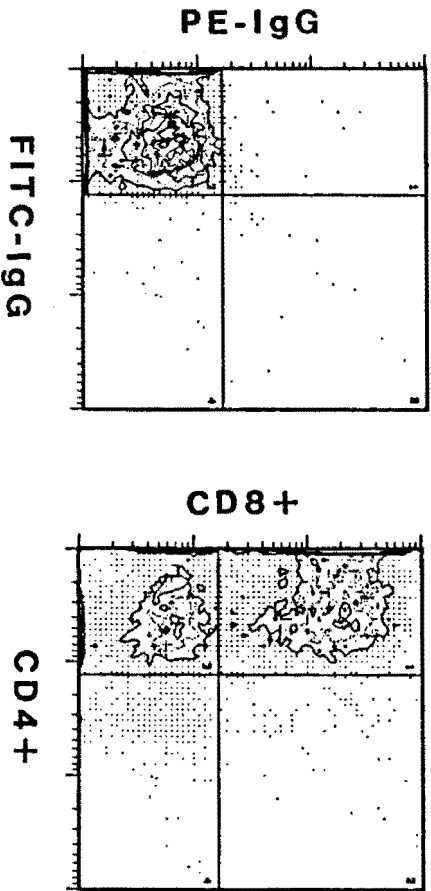
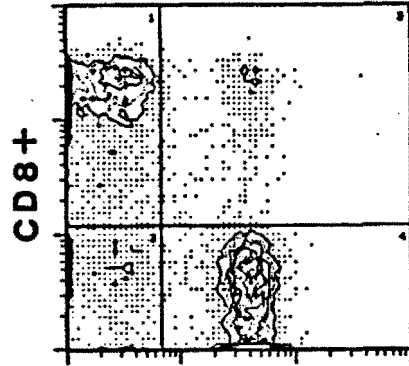


Figure 6. Flow Cytometric Contour Plots Depicting CD4, CD8, CD45RA and CD45RO Cell Surface Marker Expression by T Cell Subpopulations Obtained by Positive Selection T cells, labelled with anti-CD4 (FITC anti-Leu 3a&b) plus anti-CD8 (PE anti-Leu 2a) antibodies (panel A.) or anti-CD45RA (FITC anti-Leu 18) plus anti-CD45RO (PE UCHL-1) antibodies (panel D.), were sorted into CD4⁺ (Panel B.), CD8⁺ (C.), CD45RA⁺ (E.) and CD45RO⁺ (F.) T cell subpopulations. Figure depicts flow cytometric contour plots from a representative experiment.

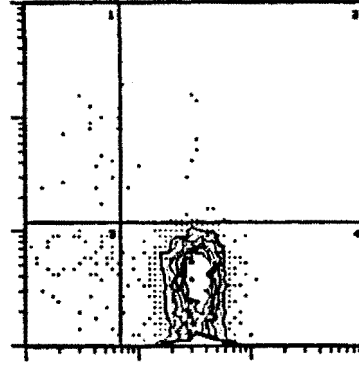
FACS purified T Cell subpopulations

A.



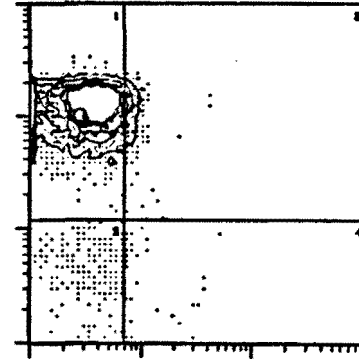
CD4+

B.



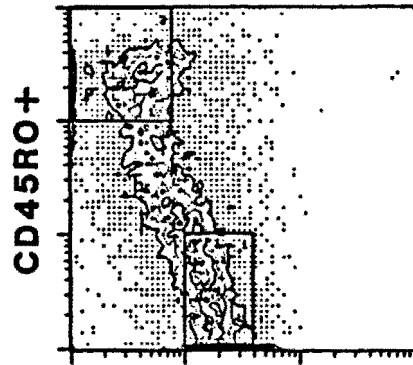
CD4+

C.



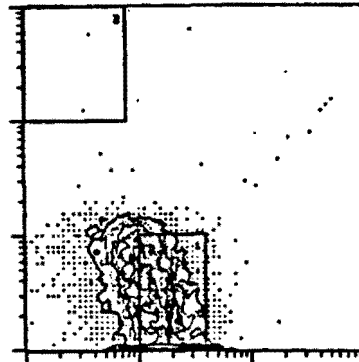
CD4+

D.



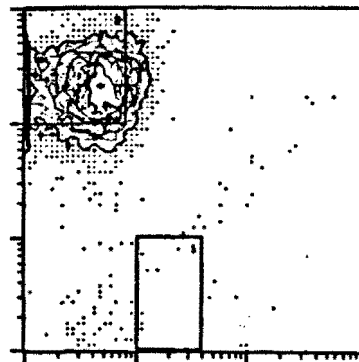
CD45RA+

E.



CD45RA+

F.



CD45RA+

APPROVAL SHEET

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

April 16, 1990
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