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I am submitting herewith a dissertation written by Melinda S. Detrick entitled "A Mechanistic Investigation of Inflammatory Murine Monocytopoiesis." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

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A MECHANISTIC INVESTIGATION OF INFLAMMATORY MURINE MONOCYTOPOIESIS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Melinda S. Detrick

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DEDICATION

To my parents and my husband, Robert, with all my love.

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ABSTRACT

During inflammation stimulated host effector and accessory cells release a variety of biological response modifiers (BRMs) that control the immune response. The release of agents such as interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF_α), and nitrogen monoxide (NO) are important not only in protecting the host from invading foreign organisms, but also in pathological conditions resulting from their overproduction. The data generated in this study define another function of these proinflammatory molecules. Using information derived from a murine bone marrow culture system and assays detecting the production of NO, guanosine 3':5' cyclic monophosphate (cGMP), and specific mRNA transcripts, two pathways responsible for augmented production of monocytes and macrophages from bone marrow precommitment progenitors are defined. The pathways are incompatible with each other but individually control a 20-50% augmentation of colony formation in response to macrophage colony-stimulating factor (M-CSF), the macrophage-specific growth factor. One pathway is compatible with mediation by cGMP, the other with mediation by adenosine 3':5' cyclic monophosphate (cAMP). The cGMP pathway is activated by lipopolysaccharide (LPS) and/or host BRMs linked to increased levels of intracellular cGMP, *e.g.* tuftsin, substance P, leukotriene B_4, prostaglandin $F_{2\alpha}$, and atrial natriuretic peptide. Primary stimulation by LPS or the host agents is mediated by TNF_α production and subsequent NO generation. These two agents are necessary in combination for augmented colony formation that is apparently dependent on the production of cGMP from soluble guanylyl cyclase. The cGMP pathway is inhibited by a 30kDa monokine produced by adherent marrow cells and by peptide mimics of the monokine. These inhibitory agents affect the proliferative pathway at a point after NO generation, perhaps by altering NO induced elevation of intracellular cGMP

levels. Stimulation by cGMP is not affected by the inhibitors. In total, these findings describe two pathways capable of augmenting the production of monocytes and macrophages from precommitment bone marrow progenitors during host inflammatory responses. In addition to these findings, evidence is presented in support of an extracellular binding site for cAMP on murine bone marrow progenitors and of a role for the inhibitor in the modulation of endotoxin shock, a pathological condition associated with overproduction of IL-1, TNF_{α}, and NO.

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macrophage colony-stimulating factor	M-CSF
major histocompatibility complex	MHC
colony-stimulating factor	CSF
multilineage colony-stimulating factor	multi-CSF
interleukin	IL
granulocyte-macrophage colony-stimulating factor	GM-CSF
granulocyte colony-stimulating factor	G-CSF
substance P	SP
neurotensin	NT
interferon	IFN
tumor necrosis factor	TNF
prostaglandin E_2	PGE_2
prostaglandin $F_{2\alpha}$	$PGF_{2\alpha}$
lipopolysaccharide	LPS
leukotriene B_4	LTB_4
kilodalton	kDa
nitrogen monoxide	NO
nitric oxide synthase	NOS
N^G-monomethyl-L-arginine	N^GMMA
aminoguanidine	AG
alanine	Ala
polymerase chain reaction	PCR
adenosine 3':5' cyclic monophosphate	cAMP
guanosine 3':5' cyclic monophosphate	cGMP

atrial natriuretic peptide	ANP
sodium nitroprusside	SNP
S-nitroso-N-acetylpenicillamine	SNAP
fetal bovine serum	FBS
ribonucleic acid	RNA
reverse transcriptase	RT
lysine	Lys
proline	Pro
arginine	Arg

CHAPTER 1

INTRODUCTION

The mononuclear phagocyte system, composed of monocytes and macrophages, occupies an integral position in the immune response. Although its primary role in immunity is the nonspecific defense of the host, the mononuclear phagocyte system can also interact with and enhance the specific immune response (1). The multiple roles of this system in the immune response make its study an important and relevant area of research interest. The study of mature cells, the macrophages, in the mononuclear phagocyte system is widespread and prolific (9, 13, 21, 69, 84, 131, 146, 163, 175). So, too, is the study of the monocyte, the bloodborne precursor to the macrophage (9, 21, 69, 84, 131, 146, 163). Also important is the study of the development of monocytes and macrophages (20, 21, 24, 44, 99, 120, 141, 163, 173, 185). All monocytic precursors arise in the bone marrow via a process known as hematopoiesis, or more specifically, monocytopoiesis (1, 21). The research detailed herein focuses on a small population of progenitor cells in a state of precommitment to the monocyte/macrophage pathway as evidenced by their inability to respond to macrophage colony-stimulating factor (M-CSF) alone (124). These cells, unlike committed monocyte/macrophage progenitors, require a nonspecific stimulus prior to or in conjunction with M-CSF in order to undergo clonal proliferation via the monocyte/macrophage developmental pathway. The work presented here describes an intricate system by which the proliferation of these cells is stimulated. This system was defined, in part, with the aid of a specific inhibitor of proliferation active on the precommitment progenitors (96,97). The results of this research project define a system through which an increase in the generation of monocyte/macrophage cells can be

effected. This system may be active during the inflammatory response where additional effector cells are needed. Additionally, the role of the inhibitor in this system may have significant implications not only in monocytopoiesis, but also in hematopoiesis and on the actions of the mature effector cells.

The primary role of cells in the mononuclear phagocyte system exists in their ability to nonspecifically detect and clear foreign particles, effete cells and tumor cells from the host (1). Their effects are manifested in several ways. To begin with, macrophages actively phagocytose and subsequently degrade foreign particles (9, 131). The degradation of the foreign substances involves the formation of phagolysosomes and the action of the enzymes located within. Through similar mechanisms, macrophages can also kill invading microorganisms via the activation of the oxidative burst leading to the generation of reactive oxygen intermediates such as superoxide radical and hydrogen peroxide (69, 84, 146). In addition, the formation of nitric oxide has been implicated in the effective killing of many microorganisms (71, 103, 106). Upon encountering a foreign particle, macrophages are also induced to secrete a variety of cytokines that act in the recruitment of other inflammatory cells, such as neutrophils, to the site of inflammation (125). These cytokines also cause the manifestation of various systemic effects associated with inflammation, for example, fever (125). Macrophages and monocytes have the capacity to monitor and eradicate neoplastic disease from the host as well as foreign particles (71, 131, 188). Grabstein and co-workers, working with the melanoma cell line A375, demonstrated this tumoricidal ability in macrophages activated with granulocyte-macrophage colony-stimulating factor (64).

While the nonspecific defense of the body is important in the first line of protection against foreign microorganisms, the specific response (acquired immunity) of the immune system is valuable in its functions as well. Specific immunity encompasses the host's response to foreign substances; this response is specific for the foreign

substance, *i.e.* the antigen, and increases in magnitude with each successive exposure to that antigen (1). The mononuclear phagocyte system has developed a supportive role in the specific immune response by adopting roles of activator and effector cells. The macrophage becomes an activator cell when it phagocytoses foreign particles (1). After these particles are degraded in the phagolysosome, they are displayed on the cell surface coupled with major histocompatibility complex (MHC) encoded class II molecules (13). Degradation products of foreign particles displayed in this fashion are "seen" by subsets of T lymphocytes. The T lymphocytes are activated by this interaction and secrete cytokines that affect not only other T lymphocytes, but also macrophages (35, 81, 105). These cytokines effectively prime macrophages, which now act as effector cells, to a higher level of activity increasing their capacity to phagocytose, kill, degrade, and present foreign antigens on their cell surface in conjunction with MHC II (1). Macrophages also act as activator cells during the secretion of cytokines stimulated by the presence of foreign substances. As mentioned previously, these cytokines act to recruit other cells of the nonspecific immune response, but they also act to stimulate T lymphocytes (1). Another effector function of macrophages is evidenced by the enhanced phagocytosis associated with the coating of foreign particles, or opsonization, by antibody produced by B lymphocytes specific for that antigen (1). This increase in phagocytosis is effected by the presence of receptors for the F_c (constant) portion of antibody on the surface of macrophages allowing for enhanced binding and subsequent ingestion of the foreign particles (88).

A multitude of cytokines and biological response modifiers active in both the nonspecific and specific immune response are produced by cells of the mononuclear phagocyte system as well as by lymphocytes and other cell types. These cytokines and modifiers are as diverse in their effects as are the cell types responsible for their production. Cytokines and biological response modifiers function to stimulate the

growth and differentiation of bone marrow progenitor cells, the recruitment of infection-fighting cells, the activation of infection-fighting cells, and the enhancement of the immune response activities associated with various cell types as well as in the maintenance of steady-state activities.

The colony-stimulating factors (CSFs), for example, are produced by a variety of cell types. They function as a group to promote the growth and differentiation of bone marrow progenitor cells in steady-state and inflammatory hematopoiesis. The different CSFs affect progenitor cells at various stages of maturation and result in the production of mature cells representing multiple lineages (*e.g.* granulocytes and mononuclear phagocytes). CSFs were named based on their ability to stimulate the formation of colonies in *in vitro* bone marrow culture. Generally the name of the individual CSF represents the type of mature cell present in colonies stimulated from specific bone marrow progenitors.

Multilineage CSF (multi-CSF) or interleukin-3 (IL-3) is a 20 to 26 kilodalton (kD) glycoprotein produced by T lymphocytes expressing the CD4 cell surface marker (1, 3, 37). This cytokine, as implied from its name, stimulates immature bone marrow progenitors (precommitment) and causes the proliferation and differentiation of the immature cells to mature cells representative of all precursor lineages.

Granulocyte-macrophage CSF (GM-CSF), a 22kD glycoprotein, stimulates the growth and differentiation of bone marrow progenitor cells committed to the granulocyte-macrophage pathway of development (1, 3, 37). It is produced by activated T lymphocytes, activated mononuclear phagocytes, vascular endothelial cells, and fibroblasts (1, 3). In addition to its activity on committed granulocyte-macrophage progenitors, GM-CSF has also been reported to affect mature cell populations through the activation of functional activity and the potentiation of the inflammatory response by phagocytes. For example, GM-CSF enhances antibody-dependent cell-mediated

cytotoxicity (ADCC), phagocytosis, killing, and chemotaxis associated with mononuclear cells (60, 64, 174, 178). Bourgoin *et al.* (16), Handman and Burgess (69), Weisbart *et al.* (182), and Reed *et al.* (146) report that GM-CSF is also effective in priming macrophages and neutrophils for subsequent activation of effector functions. GM-CSF is reported to act locally to produce its effects as it is not detected in the circulation. Since cell types responsible for producing GM-CSF are found in both the bone marrow (T cells, stromal fibroblasts, endothelial cells) and peripheral tissues (T cells, macrophages), its ability to produce local effects is based in fact as illustrated by the work of Nicola, Burgess, and Metcalf in which GM-CSF generated by peripheral tissues in endotoxin-treated mice was identical in molecular form and function to GM-CSF purified traditionally from mouse lung (135).

The same cells that produce GM-CSF also synthesize granulocyte-CSF (G-CSF). G-CSF is a 19kD secreted protein that is functional in a dimeric form as are GM-CSF and IL-3 (1, 3, 37). G-CSF unlike GM-CSF is detectable in the circulation and can therefore act on precursors committed to granulocytic development even when produced in peripheral organs in response to inflammatory stimuli (1). G-CSF enhances ADCC by neutrophils and stimulates the generation of superoxide anion in macrophages (108, 193).

Hematopoietic progenitors committed to the B lymphocyte lineage are induced to proliferate and differentiate by interleukin-7 (IL-7; 1). IL-7 is derived from marrow stromal cells and fibroblasts as a 25kD monomer. Another interleukin, IL-5, has been implicated in the development of eosinophils. IL-5 is a 40kD glycoprotein produced by activated mast cells and T lymphocytes of the T_H2 subset expressing the CD4 cell surface marker (1, 37). Due to its eosinophil growth and differentiation activities, IL-5 is also known as eosinophil differentiating factor (37). This interleukin also functions

to activate mature eosinophils as effector cells in helminthic infestations (1). In addition it acts as a costimulator for the growth of antigen-activated B cells (37).

A factor that acts on pluripotent stem cells, not to promote colony formation but rather to make these cells responsive to other CSFs, is the *c-kit* ligand (45, 77). This ligand is also called steel factor based on the absence of its soluble form in a mutant mouse strain called steel and stem cell factor in reference to its effects on stem cells (45, 77). It is produced in two forms by bone marrow stromal cells, a transmembrane, 27kD protein and a secreted, 24kD protein (1, 45). Both forms stimulate the responsiveness of stem cells to the CSFs, but the transmembrane form has been reported to be more active than the secreted form (45). In steel mice only the transmembrane form is produced and these animals are selectively deficient in mast cell and eosinophil production, but normal for other marrow cell-derived populations (1, 45, 77). Complete knockout of the *c-kit* ligand gene is lethal (1, 77).

The last CSF to be discussed is monocyte-macrophage CSF (M-CSF or CSF-1). M-CSF will be referred to throughout this writing as it stimulates the proliferation and differentiation of progenitor cells committed to the monocytic pathway (161). It is produced by macrophages, endothelial cells, and fibroblasts as a 40kD glycoprotein that associates as a dimer in its active form (3). M-CSF, like the other CSFs, has the potential to modulate activities of mature cells. Macrophages stimulated with M-CSF produce prostaglandin E_2, interleukin-1, and gamma interferon (125, 141). In addition, macrophages treated with M-CSF produce enhanced levels of oxygen metabolites (aid in the intracellular killing of phagocytosed foreign organisms) and demonstrate augmented levels of tumor cytotoxicity (187, 188).

The above cytokines exert their main functions on progenitor cells found in the bone marrow. Other cytokines act on mature cell types to produce beneficial and detrimental effects associated with the inflammatory response. These factors exert their effects

through the enhancement of immune system effector activities, activation of immune response cells, and the recruitment of immune response cells to the site of inflammation. The enormity of the factor pool effecting the aforementioned activities is prohibitive to an exhaustive review, therefore only the factors operative in the mononuclear phagocyte system as they pertain directly to the work to be presented in later sections will be discussed.

Tuftsin was identified and isolated in 1970 by Najjar and Nishioka as a bioactive tetrapeptide with the amino acid sequence, Thr-Lys-Pro-Arg (132). Tuftsin is liberated from the CH_2 domain of the F_c region of gamma-immunoglobulin via the action of two enzymes, tuftsin-endocarboxypeptidase and leukokininase (47, 130). Once liberated, the tetrapeptide acts as an immunoregulator of the mononuclear phagocyte system. Its main immunoregulatory function is macrophage activation (160, 173). Macrophages treated with tuftsin are characterized by enhanced phagocytic activity, increased antigen processing and presentation capabilities and augmented superoxide anion generation, and hence, bactericidal activity (47, 131, 160). Tuftsin is also capable of augmenting chemotaxis, the number of antibody-forming cells, and tumoricidal activity associated with macrophages and natural killer (NK) cells (47, 131). Tuftsin's effects are manifested via specific interaction with its cell surface receptor (7, 47). Binding studies demonstrate only one type of binding site for tuftsin with a Kd equal to 1.3×10^{-7} M for polymorphonuclear leukocytes (PMNs) and monocytes and 5.3×10^{-8} M for macrophages exists (9, 47, 160). Tuftsin/receptor interaction on macrophages and PMNs is documented to effect an increase in the intracellular concentration of guanosine 3':5' cyclic monophosphate (cGMP) accompanied by a simultaneous decrease in adenosine 3':5' cyclic monophosphate (cAMP; 57, 160). These effects of tuftsin are in accord with previous observations that exogenous agents of cAMP inhibit chemotaxis

and phagocytosis whereas exogenous agents of cGMP stimulate the same activities (33, 158, 160).

Substance P (SP), Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, is a bioactive polypeptide released from sensory neurons following trauma and is involved in neurogenic inflammation (57, 142, 195). SP contains a tuftsin-like moiety at its amino terminus (SP^{1-4}; 47, 57). Both SP and SP^{1-4} are capable of displacing tuftsin in receptor binding studies (8, 47, 57). SP, like tuftsin, positively regulates the inflammatory activities of macrophages and PMNs by stimulating both the phagocytic and chemotactic properties of these cells (8, 47). The capacity of SP to augment phagocytosis in macrophages and PMNs was shown to reside in the amino terminal tetrapeptide, SP^{1-4} and is presumed to effect its functions via the tuftsin receptor (8). In addition, the amino terminal tetrapeptide has been linked to the histamine-releasing function of SP (142). SP also functions in the activation of lymphocytes and NK cells as demonstrated by the work of Croitoru *et al.* (34). The carboxyl terminus region of SP contains activities that are separate from the amino terminal portion. For example, the carboxyl portion of SP is required for the potent vasodilatory effects of the undecapeptide (25, 142). Traczyk reported in 1977 that SP^{4-11} was twice as potent as SP in affecting rat blood pressure (171) and Bury and Marshford in 1976 demonstrated the carboxyl terminal octa- and nonapeptides of SP are more potent vasodilators than SP in canine hind limb experiments (22). Moreover, removal of the carboxyl terminal amide or substitution of residues at the carboxyl terminal eliminates the vasodilatory activity of SP on rabbit mesenteric vein (32).

Another bioactive peptide with a tuftsin-like moiety is neurotensin (NT). NT, a putative neurotransmitter, is a 13 amino acid peptide with the sequence, pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu (192). NT's tuftsin-like moiety resides in the central portion (amino acids 6-10) of the molecule. The peptide binds two classes of

receptors found on the surface of thioglycollate-elicited macrophages, a minor population of high affinity binding sites and a major population of low affinity sites (10, 57, 59). The high affinity binding sites require an intact amino terminal portion of NT for binding, while the low affinity sites require elements in the carboxyl terminal (57). Tuftsin, SP, SP^{1-4}, the carboxyl 6 amino acids (NT 8-13), and the carboxyl 8 amino acids (NT 6-13) of NT all compete with NT for the binding of the low affinity receptor type (57). In fact, the tuftsin receptor and the low affinity NT receptor may be identical (57). NT, like tuftsin and SP, is a mediator of the inflammatory response capable of stimulating chemotaxis in phagocytic leukocytes, augmenting phagocytosis by macrophages and neutrophils, and enhancing tumoricidal actions of macrophages (10, 57, 58, 94). NT has also been linked to vasodilation resultant from low affinity receptor binding and subsequent increases in intracellular cGMP production (54, 57, 84). NT, like SP, has activities associated with its nontuftsin-like region. For example, the amino terminus of NT is required for binding to the high affinity NT receptor sites (57). High affinity NT receptor sites are G-protein coupled receptors possessing the signature seven transmembrane domains and have been implicated in the control of hypothalamic-pituitary function (119, 179). Interaction of NT with both receptor types induces antagonistic signals resulting in loss of function as shown by Goldman and Bar-Shavit (57) and by Moore *et al.* (126) in phagocytosis augmentation studies and myelopoiesis, respectively.

The interferons are another group of bioactive polypeptides exerting profound effects on the cells of the mononuclear phagocyte system. Their name, interferon, derives from the first reported activity of the group, specifically, the ability of these molecules to interfere with viral replication (1). One of these factors also has the ability to prime immune response cells for subsequent enhanced response to stimulating cytokines or bacterial products. Three interferons have been described, alpha, beta, and gamma.

Each interferon shares activities with the others, but also has functions unique to its identity.

Interferon-alpha (IFN_α) is a 10-26kD polypeptide produced by fibroblasts and B-lymphocytes after stimulation with viruses or nucleic acids (1, 3). *In vitro* IFN_α demonstrates a multitude of functional activities. It inhibits the proliferation of tumor cells, fibroblasts, monocytes, hematopoietic progenitors, and B-cells, while it stimulates the expression of tumor-associated cell surface antigens, the expression of MHC encoded class I and II cell surface markers, the acquisition of fibroblast anti-viral activity, and the differentiation of monocytes and some hematopoietic progenitors (1, 3). In addition IFN_α is capable of inhibiting antibody production *in vitro* (1). *In vivo* IFN_α triggers systemic side effects ranging from fever to muscle pain and anorexia, anti-viral activity and anti-tumor activity, as well as, anti-bacterial and parasitic functions (3).

The second interferon, interferon-beta (IFN_β), is produced by fibroblasts and some epithelial cells as a 20kD glycoprotein with approximately 30% amino acid homology to IFN_α (1, 3). Its production is induced by microorganisms, tumor necrosis $factor_\alpha$ (TNF_α) and interleukin-1 (IL-1) as well as with viruses and double-stranded RNA (3). IFN_β shares *in vitro* anti-viral and anti-proliferative activity with IFN_α, but also induces anti-microbial effects and other immunomodulatory activities including the increased expression of MHC encoded class I cell surface markers, stimulation of NK cell activity, and stimulation of concanavalin A-induced T suppressor cell activity (1, 3). *In vivo* administration of IFN_β has resulted in the increased expression of MHC encoded class I and II cell surface markers, increased NK cell activity and increased ADCC (3).

The third interferon, interferon-gamma (IFN_γ), is the classic macrophage priming agent (1). Its production by T lymphocytes of the T_H1 subset, cytotoxic T lymphocytes and NK cells is induced by antigens, mitogens like phytohemagglutinin, and

interleukin-2 (1, 3). IFN_γ is a 20-25kD glycoprotein whose established effects *in vitro* include anti-viral activity, anti-proliferative activity for tumor cells, upregulation of MHC class I and II surface marker expression, priming of macrophages for activation, enhancement of B cell proliferation and antibody production, activation of NK cells, promotion of the differentiation of T lymphocytes, and synergism with TNF_α in cytotoxic activity and with IFN_α and IFN_β in antiviral activity (1, 3, 24). *In vivo* IFN_γ plays an integral role in anti-viral, tumor, microbial, and parasitic activities as well as in autoimmunity (1, 3). In addition, molecular conjugation of IFN_γ to antigen has been demonstrated to increase the delayed-type hypersensitivity response to the antigen (70). This effect of IFN_γ is being explored for its ability to enhance vaccine immunogenicity.

The prostaglandins represent a broad category of immunomodulatory molecules that act to transduce the actions of hormones, rather than act as hormones themselves. Within this group activities range from the inhibition of various aspects of immune regulation to the stimulation of the same. Prostaglandin E_2 (PGE_2) is a lipid acid produced by monocytes/macrophages via the action of a membrane bound synthetase complex (1). PGE_2 is known to modulate many activities associated with the immune system. In general PGE_2 is believed to downregulate the immune system (78). *In vitro* studies have documented the ability of PGE_2 to inhibit T-cell mitogenesis and IL-2 production and the synthesis of IgM antibodies from B-cells (144). Although PGE_2 has been associated mostly with negative immunomodulatory functions, evidence exists linking PGE_2 to immunoglobulin isotype switching from IgM to IgG1 and IgE and also, to the generation of T helper cells of the T_H2 subset (144, 150). In addition, PGE_2 is believed to play a negative role in hematopoiesis as addition of the lipid acid to cultures containing progenitor cells reduces the responsiveness of the progenitors to M-CSF and GM-CSF (52, 99, 100, 141). Cells of the monocytic lineage are more sensitive to this inhibition than are those of granulocytic lineage (99, 141). The

mechanism through which PGE_2 exerts its effects is still nebulous, but the binding of PGE_2 to its cell membrane receptor is documented to activate adenylate cyclase causing an increase in intracellular concentration of cAMP (46, 52, 74, 82, 114, 136, 144). As the production of cAMP is associated with inhibition of cellular proliferation, this mechanism has been generally accepted by the scientific community (56).

A prostaglandin with effects far ranging from those of PGE_2 is prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Unlike the cAMP-elevating activity of PGE_2, $PGF_{2\alpha}$ is a pharmacologic agent that elevates the intracellular concentration of cGMP as shown in neutrophils by Smith and Ignarro (158). $PGF_{2\alpha}$ also induces calcium- and protein kinase C-independent contraction of vascular smooth muscle; this contraction can be reversed by caffeine, a phosphodiesterase inhibitor responsible for increasing intracellular levels of cAMP (76, 176). In experimental endotoxemia, prostaglandin F metabolite levels are increased as shown by Aiumlamai *et al.* (76). These investigators induced ruminal acidosis in calves, a condition caused by resorption of endotoxin in the ruminant gastro-intestinal tract that mimics, albeit to a lesser degree, endotoxemia, and measured changes in prostaglandin F metabolite levels, body temperature, and other parameters affected during endotoxemia. $PGF_{2\alpha}$ is also active in the corpus luteum, an endocrine structure that secretes progesterone to prepare the uterus for a fertilized oocyte (5, 186). Treatment of large luteal cells with $PGF_{2\alpha}$, however, effects an acute inhibition of progesterone secretion and eventually, cell death (186).

Other cytokines besides the functional groups described above affect the mononuclear phagocyte system as well as other immune response cell types. Tumor necrosis factor-alpha (TNF_α) is one such cytokine. While TNF_α is categorized in a functional group, its counterpart, lymphotoxin (also known as TNF_β), is produced in small quantities, mainly by T lymphocytes, and is not detected in the circulation (1). The amino acid sequence of lymphotoxin is approximately 30% homologous to TNF_α.

Although TGF_β functions similarly, TNF_α is largely responsible for the systemic effects associated with this group (1, 3).

TNF_α was initially described in the serum of Bacillus Calmette Guerin (BCG) infected mice treated with endotoxin (bacterial lipopolysaccharide, LPS; 23). This product, upon injection, induced the hemorrhagic necrosis of solid tumors. TNF_α is composed of identical subunits of 17kD arranged in dimers, trimers, or pentamers (1, 3, 194). Activated macrophages are the major source of systemic TNF_α (1). In addition to its ability to necrose solid tumors, TNF_α also induces the expression of MHC encoded class I and II cell surface markers, hepatic acute phase protein synthesis, the activation of PMNs, T-cells, and B-cells, fibroblast proliferation, and the differentiation of certain myeloid leukemic cell lines (1, 3, 40, 53, 194). TNF_α induces detrimental functions associated with the immune response as well. For example, the production of TNF_α during septic shock is well documented, and injection of TNF_α into healthy animals induces a cascade of features typical of septic shock, including fever, anorexia, hypotension, multiple organ failure, and death (1, 118, 145, 168, 169, 170, 194). TNF_α produces its pleiotropic effects by binding to specific cell surface receptors. Two such receptors are known, TNFRα (80kD) and TNFRβ (60kD; 51). Each receptor type effects functions separate from the other; yet, in some activities, the receptors transduce synergistic signals. Cytotoxic, fibroblast growth stimulating, manganous-superoxide dismutase inducing, antiviral, and endotoxin shock activities of TNF_α have been linked to the activation of the 60kD receptor (14, 73). The role of the 80kD receptor in TNF_α signalling is not as clear, however, it has been definitively linked to thymocyte proliferation (14, 73). Interaction of signals transduced by the receptors has been demonstrated by Higuchi and Aggarwal (73) and Gehr *et al.* (51). Higuchi and Aggarwal determined that both receptor types were necessary to induce DNA fragmentation and cellular differentiation in various human neoplastic cell lines (73).

The report by Gehr *et al.* implicated both TNF_α receptors in the mediation of proliferative signals to phytohemagglutinin in human mononuclear cells (51).

Leukotriene B_4 (LTB_4) is a metabolite of arachidonic acid derived from the 5-lipoxygenase pathway (139). It is produced primarily by phagocytes and exerts potent chemotactic and chemokinetic effects on these cells (139, 149). LTB_4 is a member of a functional group of biological modifiers, the leukotrienes. Members of this group, leukotriene C_4, LTD_4, and LTE_4, are synthesized in mast cells, whereas LTB_4 is a product of phagocytes (1, 139, 149). The other leukotrienes function in the stimulation of smooth muscle cell contraction and are believed to be major mediators of asthmatic bronchoconstriction. LTB_4, on the other hand, induces phagocyte aggregation, degranulation of neutrophils, IL-1 production by macrophages, and enhanced binding of phagocytes to endothelial cells (78, 149). It is found in significant concentrations in inflammatory synovial exudates, psoriatic skin lesions, peritonitis, and inflammatory bowel disease (149). LTB_4 is also capable of augmenting monocyte-mediated cytotoxicity and NK cell activity (78, 149). LTB_4 binds to specific cell surface receptors and has been shown to increase intracellular levels of cGMP, a cyclic nucleotide associated with the upregulation of phagocytic activities (78).

The interleukins are another group of biological modifiers that function in a vast array of immune response and homeostatic mechanisms. As discussed previously, IL-3 is a colony-stimulating factor that functions to stimulate the production mature blood cells representative of all lineages from immature precursors (1). IL-5 is also a CSF responsible for the proliferation and differentiation of mature eosinophils from committed precursors (1). So, too, is IL-7, an interleukin that promotes the proliferation and differentiation of precursor cells committed to the B-cell pathway (1). Along with these ILs are a host of other polypeptides that do not possess CSF activity; one of these, IL-1, is an important modulator of the immune response.

IL-1 is a 17kD polypeptide that exists in two forms, IL-1α and IL-1β (3, 44). The two forms demonstrate less than 30% amino acid sequence homology and are the products of different genes, yet they compete for the same receptors and appear to trigger essentially the same effects (1, 3, 39, 44). Both forms are synthesized as 33kD precursors except the IL-1α precursor is biologically active whereas the IL-1β precursor must be cleaved to attain biological activity (44). IL-1 is produced primarily by activated mononuclear phagocytes and was originally characterized as a co-stimulator of T-cells, *i.e.* IL-1 enhances T-cell responses to antigens (1, 3). IL-1 subsequently was shown to play an integral role in the natural immune response as well as in the specific immune response (1). In fact through the results of various studies, the functional role of IL-1 in the natural immune response has been established as the cytokine's primary regulatory activity. In low concentrations, IL-1 functions to produce local rather than systemic effects on the natural immune response system. The local effects include the mediation of neutrophil emigration, the release of secondary lipid-derived mediators, the stimulation of mononuclear phagocytes and vascular endothelium to synthesize IL-1, and the stimulation of increased expression of surface molecules on endothelial cells that mediate leukocyte adhesion (1, 3, 39, 40). In high concentrations IL-1 becomes an endocrine factor eliciting far-ranging, systemic effects. IL-1 as an endocrine factor is an endogenous pyrogen and can stimulate the synthesis of hepatic acute phase plasma proteins (1, 3, 39). In addition to these effects, IL-1 causes cachexia, T-cell activation, B-cell activation, fibroblast proliferation, and the potentiation of CSF effects on bone marrow hematopoietic progenitors (1, 39, 40, 44). IL-1, like TNF_α, has an integral role in the pathogenesis of septic shock (40). However, unlike TNF_α, IL-1 alone does not cause tissue injury nor is it lethal, rather IL-1 likely acts to enhance the actions of TNF_α (1, 17, 170).

Other ILs elicit valuable immune system functions besides those already described. For example, IL-6 induces hepatocytes to synthesize serum plasma proteins and can act as a growth factor for B cells late in differentiation as well as enhancing clonal proliferation of hematopoietic stem cells stimulated with IL-3 (1, 39, 189). IL-2, the T-cell growth factor, also stimulates the growth of NK cells and enhances their cytotoxic functions (1). In addition, IL-2 stimulates the growth of B-cells and enhances their antibody synthesizing capabilities (1). IL-4, too, is active on B-cells, inducing the production of IgE. It is also operative in stimulating the growth and differentiation of T-cells into the T_H2 subset, the expression of adhesion molecules, and the growth of mast cells and in inhibiting macrophage activation (1, 105). Another IL inhibitory for macrophage functions is IL-10. IL-10 inhibits macrophage cytokine production, cytotoxic activity, and accessory roles in T-cell activation (1, 50, 138). IL-12 functions in natural immunity as a potent activator of NK cell activity. It is also responsible for stimulating the differentiation of $CD8^+$ T-cells into mature cytotoxic T lymphocytes and the differentiation of naive $CD4^+$ T-cells into T-cells of the T_H1 subset (1). Many other ILs have been identified and their roles in inflammation and homeostasis characterized, however, while their roles are equally important in the maintenance of normal host functions and in the acquisition and control of an effective immune response, they do not directly pertain to the system to be described in this treatise and will not be covered.

The last biological response modifier to be detailed is bradykinin (BK). BK is a 9 amino acid peptide produced enzymatically from high molecular weight kininogens at sites of inflammation via the actions of kallikreins (147, 162). It is an activator of prostaglandin synthesis and produces pain (147, 162). BK also affects the vasculature by increasing vascular permeability, inducing smooth muscle contraction and inducing vasodilation (147, 153, 165). This last effect of BK has been linked to the release of endothelium-derived relaxing factor (EDRF, a.k.a. nitric oxide) and subsequent

elevation of cGMP (153). The amino acid sequence of BK is Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg. BK effects its activities through two receptor types, designated B_1 and B_2 (147). The B_2 receptor type has been linked to vascular permeability, smooth muscle contraction, and vasodilation (147). B_1 receptors are stimulated by noxious chemicals and are generally believed silent in *in vivo* systems (147). Interaction of BK with either receptor type is dependent on recognition of intact molecules (147). Moore *et al.*, however, demonstrated that BK is operative in myelopoiesis and this activity can be isolated to the amino terminal tetrapeptide of the BK molecule, Arg-Pro-Pro-Gly (41, 123). This finding demonstrated that BK, like SP and NT, has activities that can be linked to specific portions of the molecule.

The production of immunoregulatory cytokines active in the mononuclear phagocyte system is dependent at some stage on the interaction of foreign material with host immune response cells. In most cases, cytokine production is triggered by bacteria, viruses, or a product of these organisms. Following the initial trigger, cytokine production continues as a cascade of events. For example, macrophages stimulated by bacterial lipopolysaccharide (LPS) synthesize and secrete IL-1 and TNF_α, these cytokines induce IL-2 production from T-cells, the IL-2 acts as an autocrine factor and stimulates T-cells to release IFN_γ, which in turn, primes macrophages for increased response to bacterial products. In controlled inflammatory responses, this cascade of cytokine production acts positively to clear the host of infection and repair damage arising from the infection and subsequent response. In some situations, control of the cytokine cascade is skewed and the results can be devastating as evidenced by the pathologies associated with septic shock (170).

Septic shock is a medically important condition characterized by fever, anorexia, cachexia, severe hypotension, multiple organ system failure, circulatory collapse and death (170). In recent years investigators have concentrated tremendous effort towards

understanding the cause(s) of septic shock and developing efficacious treatments to ameliorate the pathologies associated with the condition. Traditionally septic shock is associated with gram negative infection and subsequent invasion of the circulatory system. Bacterial products, most notably LPS, interact with immune response cells and stimulate the production of cytokines. These cytokines stimulate other immune response cells and induce the secretion of new cytokines and continued or increased secretion of cytokines already present. The cascade of cytokine production can be mediated by inflammatory control mechanisms, such as production of steroids that downregulate responses to various inflammatory cytokines, or the effect can spiral out of control (170). In the latter case, septic shock results. The uncontrolled production of cytokines results in extensive tissue damage and the physiological characteristics of shock, including hypotension, vascular collapse and death (170, 194). Two cytokines, TNF_α and IL-1, described in the above paragraphs, are primary effector molecules of septic shock (170, 194). These cytokines have been linked, along with IL-6, to the pathologies associated with the disease state (145, 170, 194). For instance, intravenous infusion of TNF_α in rats produced a sequence of events that mimicked those seen in clinical septic shock, ending in death (168). Tracey *et al.* using anti-TNF_α monoclonal antibodies protected baboons against the development of shock, tissue injury, and death in a model of lethal bacteremia (169). Mathison and co-workers also demonstrated protection by anti-TNF_α antibodies in rabbits during lethal endotoxemia induced by *Salmonella minnesota* (118). As mentioned previously, IL-1 does not by itself produce the tissue injury and lethality associated with TNF_α, but instead functions to enhance these properties of TNF_α (1, 17, 170). The work of Waage and Espevik, in which the administration of low doses of IL-1 with TNF_α potentiated lethality associated with TNF_α alone in mice, illustrates this concept (180).

In the past seven years, researchers have established that TNF_α in the presence of IFN_γ, as well as LPS with or without IFN_γ, induces a small, labile second messenger in stimulated cells (42, 43, 101, 117). This factor was identified as nitrogen monoxide (NO, a.k.a. endothelium-derived relaxing factor; 122). NO was first described as a powerful vasodilator (122) and is produced by the action of an enzyme, nitric oxide synthase (NOS), on L-arginine resulting in the production of NO and L-citrulline (121, 122, 129). NO is produced by two forms of NOS, a constitutive and an inducible. The constitutive form of NOS, cNOS, is a calcium dependent enzyme (122). It functions to produce small quantities of NO (picomolar range) for the regulation of homeostatic vascular tone (122). The inducible form of NOS, iNOS, is a calcium independent enzyme that is only produced after stimulation with certain cytokines and LPS (122). This enzyme functions to synthesize nanomolar quantities of NO for extended periods (122). The induction of iNOS can be inhibited by glucocorticoids (122). Due to its functions as a potent vasodilator and its inhibition by glucocorticoids (act to block pathologies of septic shock if given prior to induction of endotoxemia), the inducible release of NO has been linked to hypotension and subsequent vascular collapse associated with septic shock (86, 122, 157). The administration of N-guanidino-monomethyl-L-arginine (a NOS inhibitor; N^GMMA) ameliorates the hypotension associated with shock resulting in increased survival in experimental models of endotoxemia (86, 133, 134, 143, 157, 164, 167). Doses of N^GMMA capable of inhibiting all NO production (from both the constitutive and inducible forms of NOS) act to potentiate lethality associated with shock demonstrating the importance of low levels of NO in the maintenance of vascular tone (133, 134, 140, 157). The existence of iNOS in the endothelium and vascular smooth muscle presents a mechanism for the widespread pathologies associated with shock rather than localized foci of damage (92, 140, 164). In addition to the effects of NO as a vasodilator, the labile molecule has also

been linked with bactericidal, tumoricidal and parasiticidal activity (11, 43, 71, 103, 122, 129). Liew *et al.* have demonstrated that macrophage killing of *Leishmania* parasites in mice results from the *in vivo* induction of NO synthesis (106). Macrophage-synthesized NO has also been linked to cytostasis of the fungal pathogen *Cryptococcus neoformans* and the parasite, *Toxoplasma gondii* (2, 65, 66, 67). These cytostatic effects were prevented by the presence of N^GMMA. The role of NO in tumoricidal activity has also been documented. Hibbs *et al.* demonstrated the cytotoxicity of activated macrophages against tumor targets was dependent on the presence of L-arginine and N^GMMA inhibited the expression of cytotoxicity (71, 72). Indeed, macrophages have been shown to possess the inducible form of NOS and the promoter for the murine iNOS gene contains elements responsive to IFN_γ and LPS (109, 191).

NO exerts its vasodilating and tissue injury (cytocidal) actions in two ways. First NO acts with iron-sulfur centered enzymes such as complex 1 and complex 2 in the electron transport chain and certain enzymes in the Kreb's cycle (86, 122, 140). The interaction leads to the oxidation of iron and subsequent inactivation of the enzyme resulting in inhibition of oxidative respiration and eventually, cell death (86, 140). NO also interacts with another heme enzyme, soluble guanylyl cyclase (79, 86, 93, 110, 140). Via this interaction, NO activates the enzyme resulting in increased production of cGMP (129). The involvement of overproduction of cGMP in septic shock has been demonstrated in humans by Schneider *et al.* (152). This group measured the plasma cGMP concentrations in patients with septic shock and found that increased cGMP concentrations correlated with the decline in peripheral vascular resistance.

Cyclic GMP and cAMP, like NO, are secondary messengers operative in signal transduction. Since their discovery in 1963 and 1958, respectively, a multitude of studies have revealed their importance in a variety of systems (55, 166). Traditionally,

these molecules act to mediate intracellular cell signalling via the activation of cyclic nucleotide-dependent protein kinases (55). Elevations in the intracellular concentration of cAMP result from the hormonal triggering of cell surface receptors coupled to G protein/adenylate cyclase effector mechanisms (5). For instance, the ability of PGE_2 to bind to its cell surface receptor and activate adenylate cyclase is well documented, as is that of the β-adrenoceptor family (46, 52, 82, 93, 114, 136, 144). The production of cGMP can also be triggered by the binding of ligands to membrane-associated guanylyl cyclase receptors, such as the atrial natriuretic peptide receptor system (49, 93, 190). In addition to the particulate (membrane-bound) form of guanylyl cyclase, a soluble form of guanylyl cyclase exists in the cytoplasm of cells (49, 93). Soluble guanylyl cyclase is composed of heterodimers (α1, 77.5kD and β1, 70.5kD) containing heme as a prosthetic group (49, 93, 190). The soluble form is activated by the labile product of NOS, NO, presumably via the association of NO with the heme moiety inducing a conformational change and subsequent activation (49, 79, 93, 110, 190). Soluble guanylyl cyclase can also be activated by carbon monoxide and the hydroxyl radical (154). Throughout the studies of cAMP and cGMP functions, one concept has always been apparent: the two secondary messengers are antagonist for one another (38, 158). On occasion, this concept has been referred to as the Yin-Yang hypothesis (56). In regards to cell proliferation and other measures of cell activation such as phagocytic capability, cGMP has been linked to positive regulation whereas cAMP has been linked to the negative regulation (33, 38, 83, 158, 175, 184). However, some studies have intimated the effect of the second messengers depends on the phase of cell cycle in which the target cells are exposed (155). Other studies correlate concentration ranges of the second messengers with the stimulation or inhibition of proliferation (112, 148). Interestingly, most of these studies were accomplished with cell membrane-permeable, phosphodiesterase-resistant analogs of the cyclic nucleotides.

Exogenous cGMP and cAMP are considered cell membrane-impermeable and phosphodiesterase-sensitive and as such are ineffective as tools for *in vitro* or *in vivo* studies. Even so a body of literature exists linking cyclic nucleotides to the activation of cell surface receptors, exo-protein kinases and ion channels (95, 98, 102, 104, 113, 115, 148, 151, 177). In these studies exogenous cyclic nucleotides at low concentrations were capable of binding to cell surface receptor-type mechanisms resulting in the transduction of extracellular signals to the cytosol. One of the best characterized systems is present in the slime mold *Dictyostelium discoideum* (90, 91, 95, 104). This organism has cell surface receptors for cAMP (89, 91). Under starvation conditions, waves of cAMP are released from the organisms and they respond to the signal by aggregating and forming a multicellular stalk structure, an example of cooperative activity of an unicellular organism (95). A similar cAMP receptor exists in *Paramecium*, where it functions in chemoreception (177). Reports of cAMP binding sites on spermatozoa and thymocytes also support the concept of cAMP as a first messenger in addition to its duties as a second messenger (113, 115, 148, 151). Cyclic nucleotide-responsive γ-aminobutyric $acid_A$ receptor-mediated chloride ion channels in rat hippocampal neurons have been described as well as the existence of an exo-protein kinase responsive to cAMP (98, 102).

Cytokine-mediated signal transduction is an area of research receiving tremendous effort from a large number of laboratories. The understanding of how signals are transduced across membranes is of vital importance in not only homeostatic regulation of cellular processes, but also in inflammatory regulation and dysfunction of these processes. One area that has received attention is the growth and differentiation of bone marrow progenitor cells. While much is known about the growth regulation of committed progenitors, little is known of the precommitment phase (120). The generation of monocytes and macrophages is a sequential process composed of

proliferative and differentiative stages. With each successive stage, the progenitor cells acquire competence to respond to specific growth factors and their lineage potential decreases, *i.e.* the ability of the progenitor cells to form progeny of different lineages decreases. The formation of lineage-committed progenitors begins with the self-renewing stem cell. This cell upon division forms a daughter stem cell with identical self-renewal capacity and a progenitor cell of myeloid or lymphoid lineage. The multipotential myeloid progenitor has the capacity to produce progeny of the granulocytic, eosinophilic, erythrocytic, basophilic, megokaryocytic, or monocytic lineage. The next defined cell in the monocytic pathway is the granulocyte-macrophage progenitor. After this stage, the committed monocytic progenitor develops. This progenitor is responsive to the growth stimulating effects of M-CSF as evidenced by clonal proliferation in *in vitro* culture. The mechanism by which responsiveness (commitment) to M-CSF is acquired by progenitor cells is unclear, however, Moore *et al.* have described a population of murine bone marrow cells that are in a precommitment phase to M-CSF regulated growth (124). These cells are in the S phase of cell cycle, express Mac-1 antigen, Ia antigen, and asialo-GM_1 on their cell surface and will only respond to M-CSF (evidenced by clonal proliferation) in the presence of a nonspecific activator such as bacterial LPS (124). The precommitment phase progenitors are relatively insensitive to the inhibitory effects of PGE_2 in contrast to their committed counterparts but are inhibited by the presence of a natural protein that does not affect the committed cells (41, 96, 97, 123, 124).

This inhibitor of the precommitment phase progenitors was characterized by Kreisberg *et al.* as a 30kD protein produced by adherent bone marrow cells stimulated with a combination of M-CSF and ferritin, a large iron-binding protein (96, 97). Curiously, the activity of this protein could be mimicked by a 14 amino acid peptide,

Glu-Thr-Val-Ile-Met-Lys-Ala-Lys-Pro-Arg-Ala-Asn-Phe-Pro. This 14-mer represents the carboxyl portion of the initial sequence reported for ferritin (31).

Ferritin, in the early 1980's, was reported to exert an inhibitory effect on hematopoietic progenitors in the S phase of cell cycle expressing MHC II (Ia-like) antigens on their cell surface (18, 19, 20, 185). Moreover, high concentrations of ferritin were detected in the serum of patients with acute or chronic myelogenous leukemia and the cell population reported to be affected by ferritin was not detected (18, 19). The precommitment phase progenitors described by Moore *et al.* correspond to the progenitor cells absent in these patients (124). In an effort to determine the inhibitory effect of ferritin, Moore's group discovered the effect was cytokine-mediated rather than direct (96, 97). Prior to this discovery, the initial reported sequence of ferritin contained a moiety identical to an inhibitory analog of tuftsin, Ala^1-tuftsin (Ala-Lys-Pro-Arg), in its carboxyl terminus (31, 48). Subsequent synthesis and testing of this 14 amino acid carboxyl portion of ferritin proved it to contain inhibitory activity identical to ferritin. This inhibitory activity, however, was not abrogated by polyclonal antibodies directed against ferritin, but was abrogated by polyclonal antibodies prepared against the peptide (96, 97). To further confuse the issue, the initial sequence of ferritin was retracted and replaced with a sequence that no longer contained that portion of the molecule (30). Subsequent work by Moore's group demonstrated the existence of the 30kD protein mentioned above (96, 97). The *in vitro* activity of this protein was abrogated by the polyclonal anti-14-mer antibodies, but not by anti-ferritin antibodies and was mimicked not only by the 14-mer peptide, but also by Ala^1-tuftsin (96, 97).

Currently, research investigating the actions of the inhibitor continue and the protein and peptides have been utilized as aids in defining the proliferative signals necessary for clonal proliferation of the precommitment phase progenitor. This progenitor has been dubbed the 2-signal dependent progenitor due to its requirement for two signals, the

nonspecific signal and M-CSF, before undergoing clonal proliferation along the monocyte/macrophage pathway. Using this system data have been generated that pertain not only to the regulation of the proliferative system of these progenitors, but also to the regulation of inflammatory processes.

Statement of Purpose

The work detailed herein focuses on the regulation of the proliferation of the 2-signal dependent progenitors. The system has been defined in an *in vitro* bioassay using cytokines, bacterial products, hormones, and biochemicals known to produce controlled effects. In the following pages, a proliferative system with at least two branches will be defined and the function of the 30kD inhibitor in this system will be addressed. In addition, the information gleaned from the proliferative pathways of the 2-signal dependent progenitors will be used to demonstrate evidence for the existence of a cell surface binding site for cAMP on bone marrow progenitors and for the efficacy of the inhibitor in the control of mature effector cell-mediated pathologies, *i.e.* in the amelioration of pathologies associated with septic shock.

CHAPTER 2

MATERIALS AND METHODS

Materials

BALB/c female mice, 4-5 weeks of age, were obtained from Harlan Sprague Dawley (Indianapolis, IN). Mice were maintained in a controlled environment facility and were given food and water *ad libitum.*

The M-CSF used had been purified from serum-free tissue culture medium conditioned by murine L_{929} cells as described by Waheed and Shadduck (181). Specific activity of the preparation was 5×10^7 units (U)/mg protein where 1 unit of M-CSF activity is the amount required to stimulate formation of one colony in murine marrow cultures. The growth factor was stored as a stock solution of 4×10^5 U/ml in 0.01M phosphate buffered saline, pH 7.2 (PBS) containing 3,350 molecular weight polyethylene glycol (PEG; Sigma Chemical Co., St. Louis, MO). The M-CSF preparation contained <0.1 ng endotoxin/5×10^4 U as measured by the Limulus amoebocyte assay (156).

Enzyme immunoassays for guanosine 3':5' cyclic monophosphate (cGMP EIA) were obtained from Amersham, Life Science (cat# RPN.226, Arlington Heights, IL). Assay kits were stored at 4°C and the EIA was performed according to the manufacturer's instructions for the acetylation procedure. The sensitivity of the acetylation procedure for cGMP detection was reported by the supplier to be 2-512 fmol/well or 0.7-176 pg/well.

The J774A.1 murine macrophage cell line was acquired from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in Dulbecco's Modified

Eagle's Medium (DMEM; Sigma Chemical Co.) containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), 50 U/ml penicillin G, and 50 μg/ml streptomycin (Gibco BRL/Life Technologies, Grand Island, NY) at 36°C in 8% CO_2. The medium and FBS contained <0.1 ng/ml LPS as provided by the suppliers. Every six days J774A.1 cells were subcultured by removing the spent medium, adding fresh medium, scraping with a sterile rubber policeman (Baxter Scientific Products, Stone Mountain, GA) and spliting into fresh medium at a 1:20 ratio in a clean, sterile T-75 tissue culture flask (Corning, Corning, NY).

The 14-mer peptide (Glu-Thr-Val-Ile-Met-Lys-Ala-Lys-Pro-Arg-Ala-Asn-Phe-Pro) was synthesized by Analytical Services at the University of Tennessee, Knoxville. The peptide was purified by G-10 Sephadex chromatography and amino acid analysis was performed by Analytical Services at the University of Tennessee, Knoxville. The molar ratios of the peptide have been published elsewhere in detail (96).

(Ala[1])-tuftsin (Ala-Lys-Pro-Arg) was synthesized by the Analytical Services at the University of Tennessee, Knoxville. The peptide was purified by G-10 Sephadex chromatography and amino acid analysis was performed.

Recombinant murine TNF_α was purchased from Gibco BRL/Life Technologies. The cytokine had a specific activity of 2 x 10^8 U/mg protein where 1 unit of TNF_α is the amount capable of mediating half-maximal cytotoxicity of murine L_{929} cells in the presence of actinomycin D. TNF_α was stored at a concentration equal to 2 x 10^7 U/ml PBS, pH 7.2 at -20°C. Purity of TNF_α as determined by SDS-PAGE stained with Coomassie blue was > 97%. Endotoxin level was < 10 ng/mg protein.

Recombinant murine IFN_γ was purchased from Genzyme (Cambridge, MA). The specific activity of the preparation was 1 x 10^7 U/mg protein. One unit of IFN_γ bioactivity is defined as the reciprocal of the endpoint dilution which protects 50% of L_{929} cells from destruction post-infection with EMC virus. IFN_γ was stored at -70°C

at a concentration equal to 2 x 10^5 U/ml in PBS plus 0.1% bovine serum albumin (BSA; Sigma Chemical Co.). The preparation as supplied was ≥95% pure as assessed by SDS-PAGE. Endotoxin concentration of the preparation as supplied by the manufacturer's was ≤ 0.01 ng/μg.

Monoclonal rat anti-TNF_α antibodies were purchased from Gibco BRL/Life Technologies. Approximately 10 μg/ml anti-TNF_α antibody was sufficient to neutralize 100 pg TNF_α. The antibody preparation was reported to contain < 0.88 endotoxin units (EU)/mg protein, where one EU is equal to 5 ng endotoxin. Monoclonal anti-TNF_α antibody was stored frozen at -20°C as a 1 mg/ml solution in PBS, pH 7.2.

Polyclonal anti-TNF_α antibodies were obtained from Calbiochem (La Jolla, CA.) and Genzyme. Polyclonal rabbit anti-mouse TNF_α from Calbiochem was stored at -20°C in PBS at a concentration equal to 1 mg/ml. The specific activity of this preparation was 10,000 neutralizing units (NU)/ml where 1 NU was sufficient to neutralize 1-10 U of TNF_α. Endotoxin concentration was reported by the suppliers as 0.13 EU/mg protein. Polyclonal rabbit anti-mouse TNF_α from Genzyme was supplied as hyperimmune neat antiserum. The preparation was stored at 4°C. Neutralization of 1,000 U TNF_α was accomplished with 1 μl antiserum.

Polyclonal rabbit anti-mouse IFN_γ was purchased from BioSource International (Camarillo, CA). The antibody was supplied as neat rabbit antiserum with a concentration ≥ 20,000 NU/0.2ml. The specific activity of the antiserum was 1 x 10^5 NU/ml where 1 NU is the amount of antiserum required to neutralize 1 U of IFN_γ. Antiserum was stored frozen at -20°C.

Oligonucleotide primers for murine TNF_α and murine iNOS were purchased as kits from Clontech (Palo Alto, CA). Both primer sets were stored at -20°C and were supplied at a concentration of 20 μM. The sense, 5', murine TNF_α primer began at

nucleotide 285 of the mRNA sequence and had the following sequence: 5' TTC TGT CTA CTG AAC TTC GGG GTG ATC GGT CC 3'. The downstream, antisense, 3' primer for murine TNF_{α} began at nucleotide 683 of the mRNA sequence and was encoded as follows: 5' GTA TGA GAT AGC AAA TCG GCT GAC GGT GTG GG 3'. The amplified polymerase chain reaction (PCR) product for these primers was 354 base pairs (bp) in size. The primers spanned introns at positions 329, 384, and 432 representing fragments of 500, 200, and 300 bp in size respectively. Murine iNOS primers encoded for a PCR product of 497 bp. The 5', sense primer began at nucleotide 2943 of the mRNA sequence and represented the following sequence: 5' CCC TTC CGA AGT TTC TGG CAG CAG C 3'. The downstream, antisense primer began at position 3440 and represented the following sequence: 5' GGC TGT CAG AGC CTC GTG GCT TTG G 3'. These primers did not amplify iNOS genomic DNA. The primers for aldolase were a generous gift from Dr. Robert J. Klebe at the University of Texas Health Science Center, San Antonio, TX. and are described elsewhere (26).

Lipopolysaccharide (LPS) phenol extracted from *Escherichia coli* 0111:B4 was purchased from Sigma Chemical Co. The preparation was dissolved in deionized water (dH_2O) at a concentration equal to 1 mg/ml and boiled for 30 minutes. LPS was stored at -20°C and was diluted in sterile Hanks Balanced Salts Solution (HBSS; Gibco BRL/Life Technologies) prior to use.

Adenosine, guanosine, adenosine 5' monophosphate (5' AMP), guanosine 5' monophosphate (5' GMP), cGMP, adenosine 3':5' cyclic monophosphate (cAMP), 8-bromoguanosine 3':5' cyclic monophosphate (8-Br-cGMP), N^6, 2'-O-dibutyryladenosine 3':5' cyclic monophosphate (Bt_2cAMP), adenosine 2':3' cyclic monophosphate (2':3' cAMP), guanosine 2':3' cyclic monophosphate (2':3' cGMP), substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), substance P 1-4

(Arg-Pro-Lys-Pro), neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu), atrial natriuretic peptide, prostaglandin $F_{2\alpha}$, leukotriene B_4, L-arginine, aminoguanidine, formamide, formaldehyde, glycerol, sodium acetate, disodium ethylenediaminetetraacetic acid (EDTA), 3-[N-morpholino]propanesulfonic acid (MOPS), sulfanilamide, N-naphthylethylenediamine dihydrochloride, phosphoric acid, N-acetylcysteine, and sodium nitroprusside were purchased from Sigma Chemical Co. Bromophenol blue was purchased from Bio-Rad Laboratories (Hercules, CA). N-guanidino monomethyl-L-arginine (N^GMMA) was purchased from Calbiochem. N(omega)-methyl-D-arginine (MDA) and S-nitroso-N-acetylpenicillamine (SNAP) were purchased from LC Laboratories (Woburn, MA).

Methods

Soft Agar Assay

Six to 8 week BALB/c female mice were sacrificed by cervical dislocation and intact femurs (from hip ball and socket joint to below knee cap) were aseptically excised. Muscle tissue was removed with sterile gauze and cleaned femurs (in 1-2 ml Hanks balanced salt solution; HBSS; Gibco BRL/Life Technologies) were crushed in sterile mortars to extricate bone marrow. The marrow was suspended in sterile HBSS prepared in tissue culture grade, "endotoxin-free" water (Sigma Chemical Co.). Heavy particulate matter was removed by settling and the cells were either centrifuged, counted and added to soft agar culture medium at a concentration equal to 5×10^4 cells/ml for total cell assays. For nonadherent cell assays, the marrow cells were added to Rosewell Park Memorial Institute medium 1640 (RPMI 1640; Sigma Chemical Co.) containing 5% FBS and antibiotics at a ratio of 3-4 femurs per 20 ml medium per 150 cm^2 tissue culture flask (Becton Dickinson, Lincoln Park, NJ),

incubated at 36°C/8% CO_2 for 75-90 minutes, harvested by decanting nonadherent cells in the supernatant, centrifuged, counted, and added to soft agar culture medium at a concentration of 5 x 10^4 cells/ml for nonadherent cell assays. Soft agar culture medium was prepared with either RPMI 1640 or Minimum Essential Medium Eagle, alpha modification (αMEM; Gibco BRL/Life Technologies). Soft agar medium contained base medium, 15% FBS, 1 μM indomethacin (Sigma Chemical Co.), and 0.4% agar (Bacto-agar; Difco Laboratories, Detroit, MI) warmed to 42°C. Soft agar culture medium containing cells was added in 1 ml aliquots to sterile 35 x 10mm petri plates (Becton Dickinson) containing appropriate factors. The agar mixture was allowed to gel at room temperature for approximately 10 minutes, and the plates were incubated for 6 days at 36°C/8% CO_2. Colonies were fixed with 3-4 drops 1% acetic acid (Mallinckrodt, Inc., Paris, KY) and plates were scored for colony formation (>15 cells/proliferative center) at 40x magnification on an Olympus CK inverted microscope (Olympus Corp. of America, New Hyde Park, NY). Results are shown as the means of triplicate cultures plus or minus one standard error. All results shown are representative of the results of a minimum of three experiments.

^{3}[H]-cAMP Binding Assays

Femoral bone marrow was collected from BALB/c female mice. Cells were suspended in HBSS and added to prepared reaction tubes at a final concentration equal to 2-5 x 10^7 cells/assay. Reaction tubes contained [2,8-^{3}H] cAMP (specific activity 33.5 Ci/mmol; Dupont/NEN), 5mM DTT and HBSS at 0°C. The mixture was vortexed and incubated at 0°C for 1 minute. Saturated ammonium sulfate (Mallinckrodt) at 0°C was added simultaneously with 1% BSA at 0°C to each tube. The reaction tubes were vortexed, incubated on ice for 5 minutes, centrifuged 5 minutes at 11,000 rpm, and the supernatants were withdrawn. The pellets were

dissolved in 1M acetic acid and the radioactivity of each pellet was determined in a Beckman LS 7000 spectrometer. The samples were mixed with EcoLite™ scintillation fluid (ICN) prior to determining radioactivity. Nonspecific binding of the radiolabel was assessed by determining the counts per minute (cpm) of samples containing a 10,000-fold excess of cold cAMP. Samples were counted for 30 seconds at 2% error. Results are plotted for specific binding (cpm vs. [2,8-^{3}H] cAMP concentration) and for Scatchard analysis (bound/free vs. bound). Results are representative of a minimum of three experiments.

Nitric Oxide Assay

J774A.1 cells harvested from log phase cultures or nonadherent femoral bone marrow cells were suspended in DMEM without (w/o) phenol red indicator (Sigma Chemical Co.) containing 15% FBS and antibiotics at a concentration equal to 1.5 x 10^6 cells/ml or 2-2.5 x 10^7 cells/ml, respectively. To assay tubes (12 x 75mm snap cap tubes), 200 µl aliquots of the cell suspension were added. Assay tubes contained a final volume equal to 0.6 ml, cells, assay medium, and factors to be tested at appropriate concentrations. The samples were vortexed and 150 µl aliquots were pipetted in triplicate into the wells of a 96-well, U bottom, sterile tissue culture plate (Corning). Plates were incubated for 48 hours at 36°C/8% CO_2. After incubation 100 µl aliquots of supernatant from each well were transferred to the respective well of a clean, 96-well, U bottom plate. To each sample, 100 µl of Griess Reagent (1% sulfanilamide, 1% N-(1-naphthyl)ethylenediamine dihydrochloride, and 2.5% phosphoric acid) was added (68). The reaction was incubated at room temperature for 5 minutes prior to the determination of the absorbance of each well at 540 nm on a microplate ELISA reader, Model EL310 (Bio-Tek Instruments, Burlington, VT). A standard curve was prepared simultaneously with sodium nitrite (Sigma Chemical

Co.). Results are reported as the mean of triplicate values plus or minus one standard error and are representative of at least three individual experiments. The amount of nitrite present in each sample was determined by linear regression of the standard curve for each experiment.

Induction of Endotoxin Shock

Inbred BALB/c females or outbred ICR female mice (Harlan Sprague Dawley) were injected intraperitoneally (i.p.) with *E. coli* LPS 0111:B4 in HBSS. Mice were provided food and water *ad libitum* prior to and during the experimental procedure. Time of death was recorded for 72h. The LD_{50} was determined from death curves obtained with various concentrations of LPS. Factors to be tested for amelioration of shock were injected either i.p. or intravenously (i.v.) via the lateral tail veins at times and concentrations indicated in the results section. Mice receiving the treatments were injected i.p. with 2X LD_{50} LPS. Mice in the final stages of shock, *i.e.* exhibiting partial paralysis, convulsions, and/or unresponsiveness to external stimulation were euthanized by cervical dislocation. Results are reported as the percent survivorship per group of 6-10 animals.

cGMP Enzyme Immunoassay

Samples for the cGMP EIA were prepared by incubating either 5 x 10^5 J774A.1 cells or approximately 5 x 10^6 nonadherent femoral bone marrow cells/ml acetate buffer, pH 5.8 (0.05M sodium acetate containing 0.02% BSA) containing 0.5 mM isobutylmethylxanthine (IBMX; Sigma Chemical Co.) in 1.5 ml eppendorf tubes (Eppendorf, Springfield, MA) for 30 minutes at 36°C/8% CO_2 prior to the addition of cGMP-generating agents. After the preincubation step, sodium nitroprusside (SNP) or acetate buffer, pH 5.8 (control cultures) was added to the assay tubes. Final

concentration of SNP was 10 mM in a final assay volume of 1 ml. SNP spontaneously generates nitric oxide (NO) in mildly acidic solutions (93, 154). The cultures were incubated an additional 45 minutes prior to centrifugation and harvesting the supernatant. Supernatants were stored at -70°C until tested for the presence of cGMP by EIA. If cell pellets were tested, the pellet was resuspended after removal of supernatant in 0.4 ml acetate buffer, pH 5.8. The suspension was boiled for 3 minutes and stored at -70°C until use. Before the cGMP EIA, the samples were thawed and 200 µl aliquots were transferred to clean, 12 x 75mm polypropylene snap cap culture tubes (Becton Dickinson). Assay was run according to manufacturer's instructions for the acetylation procedure. Results are reported as the mean of triplicate samples plus or minus one standard error. cGMP concentrations were interpolated from a standard curve prepared simultaneously with each assay and are reported as fmol cGMP/sample. Results are representative values determined by a minimum of three repetitive experiments.

Isolation of RNA

Nonadherent femoral bone marrow cells were isolated and suspended in RPMI 1640 containing 15% FBS and antibiotics at a concentration equal to 1 x 10^8 cells/ml. Cells at a final concentration of 5 x 10^5 cells/ml were added to T-75 tissue culture flasks containing RPMI 1640 with 15% FBS, antibiotics and factors to be tested. Cultures were incubated at 36°C/8% CO_2 for varying lengths of time and cells were harvested. Cells were centrifuged and pellets were treated with 1 ml TRIzol™ Reagent (Gibco BRL/Life Technologies) for 5 minutes at room temperature. Chloroform (0.2 ml/ sample tube) was added, the mixture was shaken vigorously by hand for 15 seconds, incubated at room temperature for 2-3 minutes, then centrifuged at 4°C for 15 minutes, 12,000 x g. The upper, aqueous layer was withdrawn and 1 ml

anhydrous isopropyl alcohol was added. The sample was mixed and incubated at room temperature for 10 minutes. RNA was pelleted in a Biofuge B at 12,000 x g for 10 minutes at 4°C (American Scientific Products, Stone Mountain, GA), washed once with 70% ethanol prepared in diethyl pyrocarbonate (DEPC)-treated water (DEPC-H_2O; Research Genetics, Huntsville, AL), and dissolved in 10 µl DEPC-H_2O. RNA was stored frozen at -70°C.

Quantitation of RNA

RNA was quantified two ways. First the optical density (OD) at 260/280 nm was determined on a Model DU 650 Beckman spectrophotometer (Irvine, CA) for each sample. For the measurement each sample was diluted 1:1,000 in DEPC-H_2O, transferred to a quartz microcuvette and the absorbance measured at 260, 280, and 320 nm. The ratio of the absorbances at 260 and 280 was determined mathematically for each sample as a measure of purity: a ratio of 1.5 at 260/280nm is optimal for RNA (116). Next, the amount of RNA/sample was determined from the following standard: an OD of 1 at 260 nm is equal to 40 µg/ml RNA (116). Absorbance at 320 nm was used as an additional standard of purity.

Second the quality and relative band density of the RNA was determined by electrophoresis. One µg RNA from each sample (determined above) was mixed with sample buffer (deionized formamide, 10X eletrophoresis buffer, formaldehyde, DEPC-H_2O; final volume of sample, 20 µl), added to 5 µl bromophenol blue solution (glycerol to 5% bromophenol blue, 1.6:1) and run on a 1% agarose gel. The agarose gel was prepared with high melting point agarose (Mallinckrodt) in 1X electrophoresis buffer (EB; 0.02M MOPS, pH 7.0 containing 5mM sodium acetate and 1mM disodium EDTA) by dissolving the agarose, cooling to 45°C, adding formaldehyde and ethidium bromide, and pouring an horizontal gel in a casting tray. The gel was

solidified at room temperature and placed in the buffer tank of a Bio-Rad mini Sub™ DNA cell electrophoresis unit. Buffer reservoirs were filled with 1X EB. RNA samples were electrophoresed at 120 V until the dye front had moved approximately 3/4 through the gel. The gel was viewed under UV transillumination (Fotodyne Incorporated) and photographed. Quality of the RNA was determined by the appearance of two distinct ribosomal RNA (rRNA) bands corresponding to the 28S and 18S rRNA subunits. The relative quantity of each band was judged by eye as a lane comparison. Only RNA samples with clear rRNA bands were utilized for reverse transcriptase- polymerase chain reaction (RT-PCR) experiments.

Reverse Transcriptase-Polymerase Chain Reaction

RT-PCR was run according to the method of Chen, *et al.* (26). Master mixes for the reverse transcriptase (RT) and PCR steps contained the following:

RT "A mix"*

0.8x RT buffer (Perkin Elmer Corporation, Norwalk, CT)

50mM Tris, pH 8.3 (Sigma Chemical Co.)

5 µg BSA

12.5mM dithiothreitol (DTT; Sigma Chemical Co.)

8mM magnesium chloride (Sigma Chemical Co.)

20 U RNasin (Promega, Madison, WI)

2mM deoxynucleoside triphosphates (dNTPs; Pharmacia, Piscataway, NJ)

1 U avian myeloblastosis virus reverse transcriptase (AMV; USB Life Science, Cleveland, OH)

* stored in 40.6 µl aliquots (6.5 assays/tube) at -70°C

PCR "T mix"*

DEPC-H_2O

0.8x Taq DNA polymerase buffer (Promega)

0.8mM dNTPs (each)

2.5 U Taq DNA polymerase (Promega)

*stored in 507 µl aliquots (6.5 assays/tube) at -70°C

RNA was suspended at 1 µg/13.65 µl DEPC-H_2O in 0.6 ml PCR tubes (PGC Scientific, Gaithersburg, MD). Primer 1 (3' antisense primer, 200 ng) was added to each sample and the tubes were incubated at 68°C for 10 minutes. Upon removal from the water bath, tubes were pulse-centrifuged (Personal micro-centrifuge; PGC Scientifics) to collect contents at the tip and incubated on ice for 5 minutes. "A mix" was added to each tube, 6.35 µl/tube, and samples were vortexed briefly, pulse-centrifuged, and incubated at 42°C for 40 minutes. Samples were again pulse-centrifuged and incubated on ice for 5 minutes prior to the addition of 200 ng Primer 2 (5' sense primer) and 78 µl "T mix" to each tube. Tubes were vortexed and pulse-centrifuged. The contents were overlayed with 3-4 drops light mineral oil (Sigma Chemical Co.) and placed in a Perkin-Elmer Corporation Original DNA Thermal Cycler. The PCR profiles were run according to the following temperature/time protocols:

	Aldolase	TNF	iNOS
denaturation	94°C/20 sec	94°C/20 sec	94°C/20 sec
annealing	58°C/1 min	60°C/1 min	65°C/1 min
extension	72°C/1 min 50 sec	72°C/1 min 50 sec	72°C/1 min 50 sec

RT-PCR Optimal Cycling Determination

Samples were prepared as described above and PCR was conducted for 40 cycles. PCR products were sampled at cycles 20, 25, 30, 35, and 40 by pausing the machine at

the end of the extension phase and withdrawing 10 µl aliquots from each tube. The aliquots were added to clean, 0.6 ml PCR tubes containing 2 µl loading dye (0.25% bromophenol blue and 40% glycerol). After all samples were collected, they were electrophoresed on precast 5% acrylamide gels in Tris/borate/EDTA buffer (Bio-Rad Laboratories) using a Bio-Rad Mini-Protean II vertical electrophoresis chamber at 200 V constant voltage for approximately 20 minutes or until the dye front had reached the far gel edge. The gels were stained with ethidium bromide in TBE, pH 8.0 (10X TBE contained 54g Tris base, 27.5g boric acid and 20 ml 0.5 M EDTA in a final volume of 500 ml) for 10 minutes, viewed by UV transillumination and photographed. Optimal cycling time was determined as the exponential phase of DNA polymerization, *i.e.* cycles covering the time where little to no product is visualized in the gel to peak product production.

RT-PCR: Semi-quantitation

Semi-quantitation of PCR products was performed by the method of Bouaboula *et al.* (15). Briefly, samples were prepared as before except Primer 2 was diluted with an equal volume of α^{32}P-dCTP (Easy-Tides™, 3000 Ci/mmol; Dupont/NEN, Wilmington, DE) prior to the addition to post-RT reaction tubes. Thermal cycling was commenced and 10 µl samples were withdrawn every 2 cycles, beginning with the cycle number at the base of the exponential phase of DNA polymerization and ending with the last cycle of the exponential phase as determined above. Generally, 6 samples were withdrawn from each reaction vial. Samples were combined with loading dye and electrophoresed as described. After photography of stained gels, the bands corresponding to the product of interest were excised from the gel, added to 10 ml UniverSol™ ES scintillation fluid (ICN, Irvine, CA) and counted on a Beckman LS 7000 spectrometer for 10 minutes/sample, 2% error. Values were plotted against

cycle number and the amount of RNA specific for the gene of interest initially present in each sample was determined in relation to the other samples. Results are shown as peak counts per minute (cpm) per sample and are representative of a minimum of three experiments.

Statistical Analyses

Statistical analyses were performed with the Wilcoxon Rank Sum test (183).

CHAPTER 3

RESULTS

Murine bone marrow-derived myeloid progenitor cells cultured with macrophage colony-stimulating factor (M-CSF) alone or in combination with bacterial lipopolysaccharide (LPS) respond by proliferating in soft agar cultures. LPS, which by itself is inactive, enhances the clonal proliferation observed with M-CSF alone (Table 1). Lipopolysaccharide augmentation of M-CSF-stimulated clonal proliferation led initially to the characterization of the small population of progenitor cells responsible for the additional colonies (124). These cells are nonadherent in liquid culture, are uniformly in cell cycle, and express MAC-1 antigen, Ia antigen, and asialo-GM_1 on their surface (124). The cells, due to their requirement for a nonspecific stimulus, *e.g.* LPS, prior to or in conjunction with M-CSF before undergoing clonal proliferation in response to the growth factor, were termed 2-signal dependent progenitors. These 2-signal progenitors are in a state of precommitment to the M-CSF developmental pathway as evidenced by their hyporesponsiveness to the growth factor. Committed progenitors are responsive to M-CSF and form colonies in soft agar culture in response solely to the growth factor (Table 1).

Proliferation of the 2-signal dependent progenitors is inhibited by ferritin, a 14-mer peptide synthesized to mimic the erroneous carboxyl terminus of ferritin, and a tetrapeptide, Ala-Lys-Pro-Arg (Ala^1-tuftsin, AKPR; Table 1). The tetrapeptide is an inhibitory analogue of tuftsin, a naturally occurring macrophage activating peptide (160, 173). This same moiety is present in the erroneous carboxyl portion of ferritin and is represented in the 14-mer peptide, Glu-Thr-Val-Ile-Met-Lys-Ala-Lys-Pro-Arg-Ala-Asn-Phe-Pro. Ferritin, the 14-mer, and AKPR are all inhibitory for 2-signal

Table 1: Effect of ferritin, AKPR, and 14-mer on 2-signal dependent progenitor proliferation costimulated by LPS in total and adherent cell depleted marrow.

	Total marrow cultures[c]				Adherent cell depleted marrow cultures[c]			
treatment	none	+ferritin	+AKPR	+14-mer	none	+ferritin	+AKPR	+14-mer
M-CSF	187 ± 3	186 ± 3[b]	185 ± 4[b]	180 ± 3[b]	190 ± 4	178 ± 7[b]	186 ± 3[b]	187 ± 2[b]
M-CSF + LPS	240 ± 6[a]	185 ± 7[b]	186 ± 5[b]	185 ± 1[b]	234 ± 4[a]	230 ± 7[a]	184 ± 4[b]	188 ± 4[b]

Marrow cells were plated at 5 x 10^4 cells/ml. M-CSF and LPS were added at a final concentrations of 750 U/ml and 0.1 ng/ml, respectively. Ferritin, AKPR, and the 14-mer were added at 10^{-9}M. Data represent the amount of colony formation ± standard error (SE). LPS by itself did not stimulate colony formation (data not shown). Results are representative of triplicate cultures in at least three separate experiments.

[a]Significantly greater than M-CSF control at $p \leq 0.05$.

[b]Not significantly different from M-CSF control.

[c]Results were generated from separate experiments using total femoral marrow or adherent cell depleted marrow.

dependent progenitor proliferation in cultures prepared with total murine marrow (Table 1). In cultures prepared with murine marrow depleted of adherent cells, only the 14-mer and AKPR are inhibitory for 2-signal dependent progenitor proliferation. In either case, the basal, M-CSF-committed population remains unaffected by the inhibitors. The inability of ferritin to abrogate proliferation of 2-signal dependent progenitors in adherent cell depleted marrow cultures led to the discovery of a naturally occurring protein inhibitor whose production is stimulated from adherent marrow treated with ferritin and M-CSF (described in detail in references 96 and 97). The protein inhibitor has a relative molecular mass of 30kDa and is immunologically cross-reactive with the 14-mer peptide.

Cyclic GMP serves as a second messenger for costimulation of 2-signal progenitor proliferation. Previous work had shown that a variety of active molecules can replace LPS as required costimulant for M-CSF-dependent clonal proliferation. These included tuftsin (127), the tuftsin-like neuropeptide substance P (SP; 47, 57, 127), and neurotensin (NT; 126), another neuropeptide containing a tuftsin-related sequence (47, 57, 192). In each case, activity in the 2-signal dependent system was linked to the tuftsin sequence of amino acids. While tuftsin and SP were stimulatory for the 2-signal cells, the neurotensin tuftsin-like sequence, when engaged, negated costimulatory activity of that neuropeptide effected through an unrelated receptor and mechanism (126). An interesting correlation was that LPS, tuftsin, and tuftsin-like peptides are known activators of cytoplasmic guanylyl cyclase (57, 160). Conversely, neurotensin action at its costimulatory concentration is not associated with activation of guanylyl cyclase but at its inhibitory concentration, where it additionally interacts with the tuftsin receptor, the cyclase is activated (54, 57, 84). To test the possibility that cGMP might serve as a second signal for costimulation, three additional stimulators of guanylyl cyclase, leukotriene B_4 (LTB_4; 78), prostaglandin

$F_{2\alpha}$ ($PGF_{2\alpha}$; 158), and atrial natriuretic peptide (ANP; 27, 49, 93) were tested as potential costimulants. As shown in Figure 1, LTB_4, $PGF_{2\alpha}$, and ANP, all at 10^{-9}M promoted 2-signal dependent colony formation equivalent to that promoted by LPS, SP, NT, and tuftsin. As with LPS, SP, NT, and tuftsin, neither of the three stimulated colony formation in the absence of M-CSF (data not shown). An additional interesting point, shown in Figure 2, is the inhibitory peptide AKPR, at 10^{-9}M, abrogated 2-signal dependent colony formation in all cases except when costimulation was mediated by 10^{-9}M NT ($p \leq 0.05$).

Pharmacologic elevators of intracellular cAMP and cGMP costimulate the 2-signal dependent response. Nanomolar concentrations of NT have been shown in other systems to stimulate an increase in intracellular cAMP (119, 179), whereas all of the other costimulants have been shown to effect an increase in intracellular cGMP (57, 78, 158, 160). In particular, the receptor for ANP is a membrane spanning guanylyl cyclase (27, 49, 93). Therefore, the costimulatory effect of pharmacologic elevators of intracellular cAMP and cGMP was examined in soft agar culture. Dose response curves for N^6, 2'-O-dibutyryladenosine 3':5' cyclic monophosphate (Bt_2cAMP) and 8-bromoguanosine 3':5' cyclic monophosphate (8-Br-cGMP) are shown in Figure 3. Both Bt_2cAMP and 8-Br-cGMP were effective as costimulants of 2-signal dependent progenitor proliferation. Maximal stimulation for both cyclic nucleotide analogues was evident at 10^{-9}M ($p \leq 0.05$), with concentrations greater than 10^{-9}M resulting in diminished augmentation. At concentrations greater than 10^{-7}M both analogues effected slight inhibition of the basal, M-CSF-committed population. Neither influenced colony formation in the absence of M-CSF (data not shown).

Costimulation of the 2-signal dependent response by LPS, SP, ANP, LTB_4, Tft, and $PGF_{2\alpha}$ is compatible with costimulation by the pharmacologic agent, 8-Br-cGMP, but incompatible with the pharmacologic agent, Bt_2cAMP.

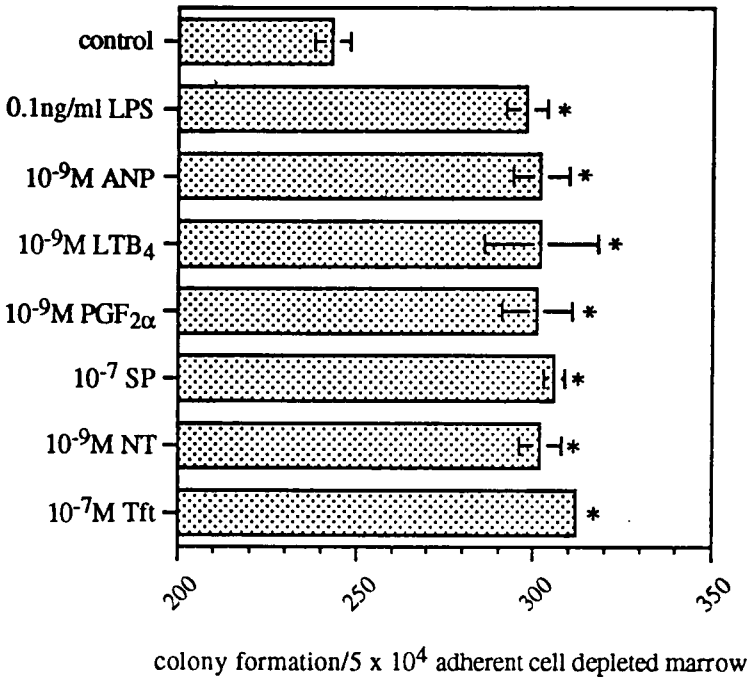


Figure 1: The effect of costimulants of 2-signal dependent progenitor proliferation on colony formation stimulated by 750 U/ml M-CSF. Basal colony formation was 243 ± 5. Results are reported as the mean ± SE and are representative of at least three experiments.

*Significantly greater than M-CSF control at $p \leq 0.05$.

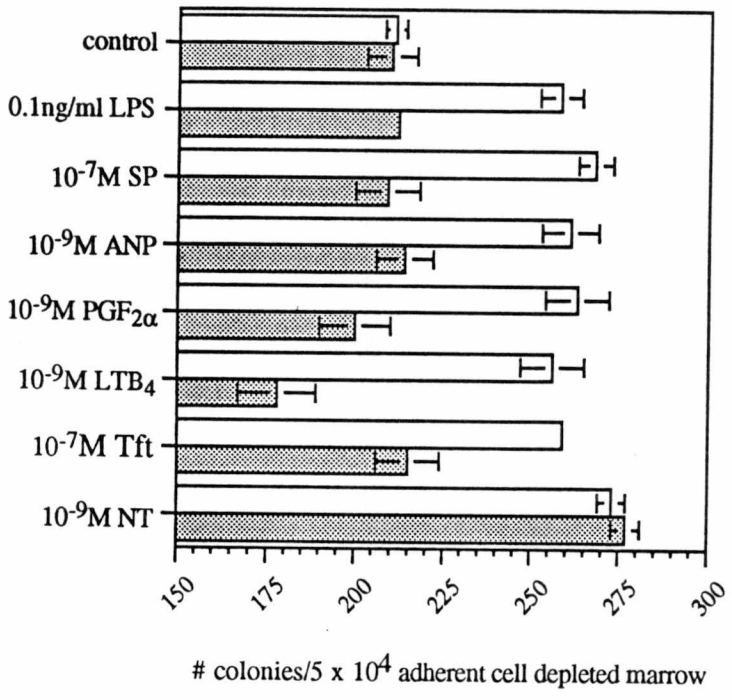


Figure 2: 10^{-9}M AKPR (▨) inhibits 2-signal dependent progenitor proliferation in response to various costimulants. Basal colony formation in response to 750 U/ml M-CSF was 211 ± 3 (control). Results are reported as the mean ± SE and are representative of at least three experiments.

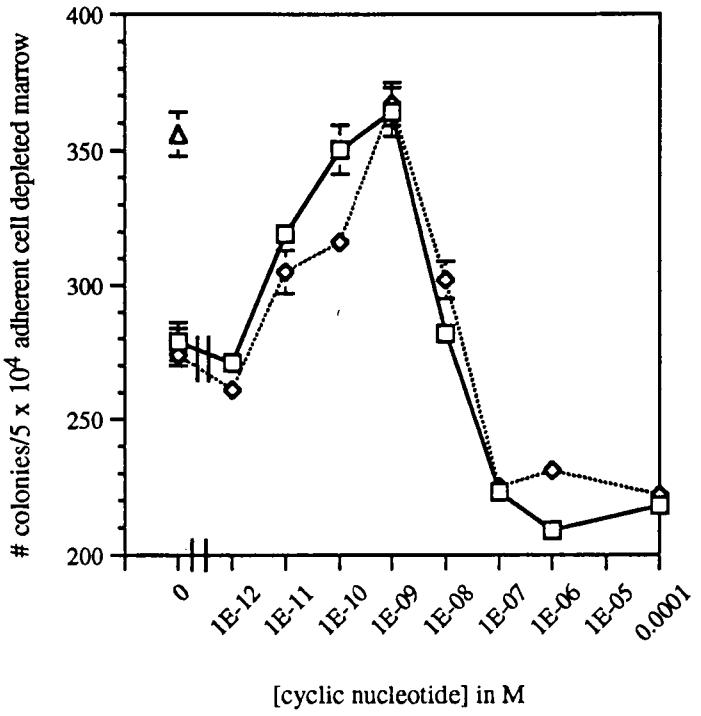


Figure 3: Dose response of Bt_2cAMP (—□—) and 8-Br-cGMP (···◇···) on M-CSF (750 U/ml) mediated colony formation. Basal M-CSF responsive colony formation was 277 ± 7 (○); 2-signal dependent response to 0.1 ng/ml LPS was 356 ± 8 (△). Results are reported as the mean ± SE and are representative of at least three experiments.

Incompatibility between signals mediated by cAMP versus cGMP is documented (38, 56, 158). To ascertain whether 2-signal dependent progenitor proliferation mediated by the costimulants illustrated in Figure 1 is compatible with cAMP- or cGMP-dependent pathways, adherent cell depleted marrow was cultured with M-CSF and the various costimulants with or without Bt_2cAMP or 8-Br-cGMP. As indicated in Table 2, the costimulants reported to act via the generation of intracellular cGMP were compatible with stimulation mediated by the cGMP analogue, 8-Br-cGMP, *i.e.* 2-signal dependent progenitor proliferation costimulated *in vitro* by SP, ANP, LTB_4, Tft, LPS, and $PGF_{2\alpha}$ was neither enhanced nor diminished by the addition of 10^{-9}M 8-Br-cGMP. Proliferation of 2-signal dependent progenitors stimulated by 10^{-9}M NT was abrogated by the addition of 10^{-9}M 8-Br-cGMP. The cAMP analogue, Bt_2cAMP, abrogated stimulation mediated by the putative cGMP elevators, but not that mediated by NT indicating that NT acts via a cAMP-compatible mechanism. Therefore the other costimulants of 2-signal dependent progenitor proliferation effected their actions via cGMP-compatible mechanisms.

Phosphodiesterase sensitive, membrane impermeant 3':5' cyclic nucleotides act as costimulants of 2-signal dependent proliferation. Control soft agar cultures for cGMP- and cAMP-mediated costimulation of 2-signal dependent progenitor proliferation yielded some startling results (Figure 4). The 3':5' cyclic nucleotides (3':5' cGMP and 3':5' cAMP), which are phosphodiesterase sensitive and membrane impermeant in contrast to the analogues (56), were effective at 10^{-9}M in stimulating 2-signal dependent progenitor proliferation in conjunction with M-CSF in direct opposition to the results expected with impermeant molecules. The purine nucleosides, adenosine and guanosine, however, were ineffective as costimulants as were the 2':3' cyclic nucleotides, 2':3' cGMP and 2':3' cAMP. Sodium butyrate (Sigma) at 2 x 10^{-9}M was also ineffective as a costimulant of 2-signal dependent

Table 2: Cyclic nucleotide compatibility of 2-signal dependent progenitor proliferation costimulated by various biological reponse modifiers.

	Colony Response ± SE		
treatment	none	+ Bt_2cAMP	+ 8-Br-cGMP
M-CSF	269 ± 5	325 ± 10[a]	333 ± 10[a]
M-CSF + LPS	325 ± 5[a]	263 ± 7[b]	330 ± 7[a]
M-CSF + ANP	333 ± 10[a]	261 ± 13[b]	323 ± 2[a]
M-CSF + LTB_4	330 ± 11[a]	252 ± 13[b]	325 ± 10[a]
M-CSF + $PGF_{2\alpha}$	333 ± 14[a]	269 ± 5[b]	338 ± 7[a]
M-CSF + SP	336 ± 4[a]	265 ± 12[b]	330 ± 5[a]
M-CSF + NT	330 ± 6[a]	335 ± 5[a]	254 ± 5[b]
M-CSF + Tft	341 ± 2[a]	258 ± 12[b]	332 ± 3[a]

Colony response represents the number of colonies formed from 5 x 10^4 adherent depleted marrow cells/ml in response to 750 U/ml M-CSF ± costimulants. LPS was added to marrow cultures at a final concentration equal to 0.1 ng/ml. SP and Tft were added to cultures at 10^{-7}M; all other costimulants were added at 10^{-9}M. Data are representative of triplicate cultures from at least three separate experiments.

[a]Significantly greater than M-CSF control at $p \leq 0.05$.

[b]Not significantly different from M-CSF control.

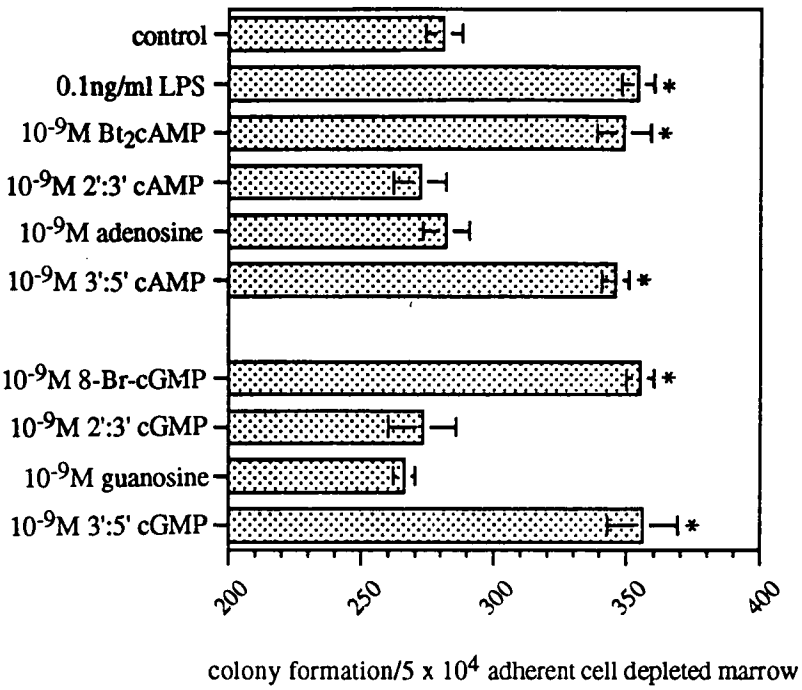


Figure 4: The 3':5' cyclic nucleotides are effective costimulants of 2-signal dependent progenitor colony formation in the presence of 750 U/ml M-CSF. An additional control for Bt_2cAMP, sodium butyrate, was ineffective in costimulating the 2-signal response (M-CSF, 276 $\pm$ 7; 2 x 10^{-9}M sodium butyrate, 274 $\pm$ 5). Results are presented as the mean of triplicate cultures $\pm$ SE and represent results from at least three separate experiments.

*Significantly greater than M-CSF control at $p \leq 0.05$.

progenitor proliferation. Dose response curves for the membrane impermeant, phosphodiesterase sensitive 3':5' cyclic nucleotides revealed that cGMP- and cAMP-mediated costimulation of 2-signal dependent progenitor *in vitro* proliferation was maximal at 10^{-9}M (Figure 5). However, unlike the diminished augmentation demonstrated by the membrane permeable, phosphodiesterase resistant analogues, at concentrations greater than 10^{-9}M (Figure 3) the 3':5' cyclic nucleotides continued to enhance 2-signal dependent progenitor *in vitro* proliferation even to concentrations of 10^{-5}M. Combination of 10^{-9}M 3':5' cAMP or 3':5' cGMP with increasing concentrations of Bt_2cAMP or 8-Br-cGMP, respectively, or *vice versa* indicated the diminished augmentation of *in vitro* 2-signal dependent progenitor proliferation is a property of the cyclic nucleotide analogues (Figure 6). Combination of 10^{-9}M 8-Br-cGMP with increasing concentrations of 3':5' cAMP and 10^{-9}M 3':5' cGMP with increasing Bt_2cAMP concentrations in soft agar culture illustrate the incompatibility of the two pathways (Figure 7, Panel A). So, too, do combinations of 10^{-9}M Bt_2cAMP with increasing 3':5' cGMP concentrations and 10^{-9}M 3':5' cAMP with increasing concentrations of 8-Br-cGMP (Figure 7, Panel B).

The 3':5' cyclic nucleotides are membrane impermeant and phosphodiesterase sensitive. These properties make 3':5' cGMP and 3':5' cAMP inappropriate for use as pharmacologic elevators of intracellular cyclic nucleotides. On the other hand, these properties combined with the ability of nanomolar concentrations of these cyclic nucleotides to costimulate 2-signal dependent progenitor *in vitro* proliferation lead to the possibility of an extracellular binding site(s) for the cyclic nucleotides. This hypothesis was examined in *in vitro* cell surface binding studies using [2,8-^{3}H] cAMP and murine marrow.

Tritiated cAMP binding to murine bone marrow exhibits saturability. In order for binding of [2,8-^{3}H] cAMP to an extracellular binding site to qualify as a receptor

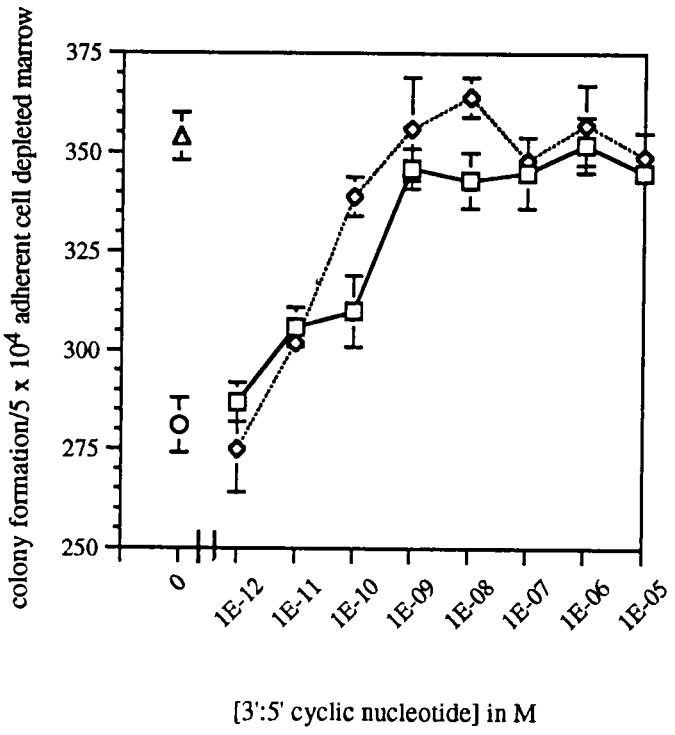


Figure 5: Dose response of 3':5' cAMP (—□—) and 3':5' cGMP (---◇---) on colony formation induced by 750 U/ml M-CSF. Basal, M-CSF responsive colony formation was 281 ± 7 (○); 2-signal dependent response to 0.1 ng/ml LPS was 354 ± 6 (△). Results are presented as the mean ± SE of triplicate cultures and are representative of at least three experiments.

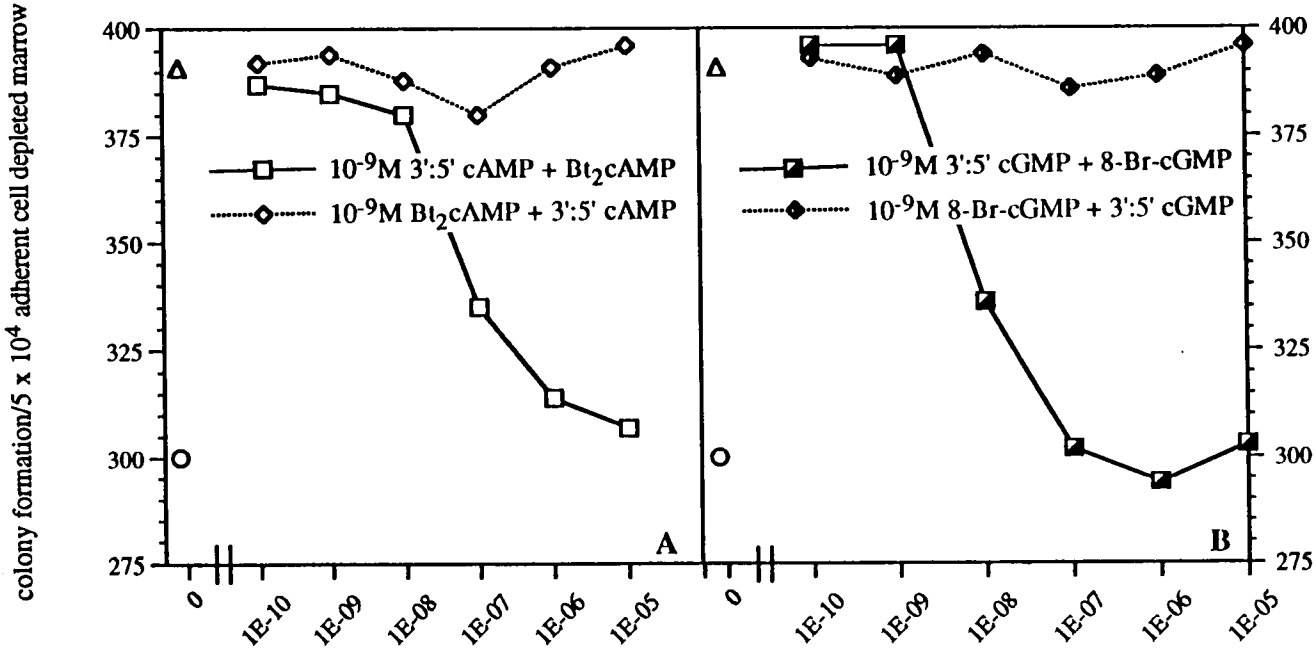


Figure 6: Dose combinations of Bt_2cAMP with 3':5' cAMP (Panel A) and 8-Br-cGMP with 3':5' cGMP (Panel B). Abrogation of 2-signal dependent progenitor response stimulated by cyclic nucleotides is a function of the membrane-permeable, phosphodiesterase-resistant analogues. Basal colony formation in response to 750 U/ml M-CSF was 300 colonies (O); 2-signal dependent progenitor response to 0.1ng/ml LPS was 391 (Δ). Standard error was < 10% of total colony formation for each data set.

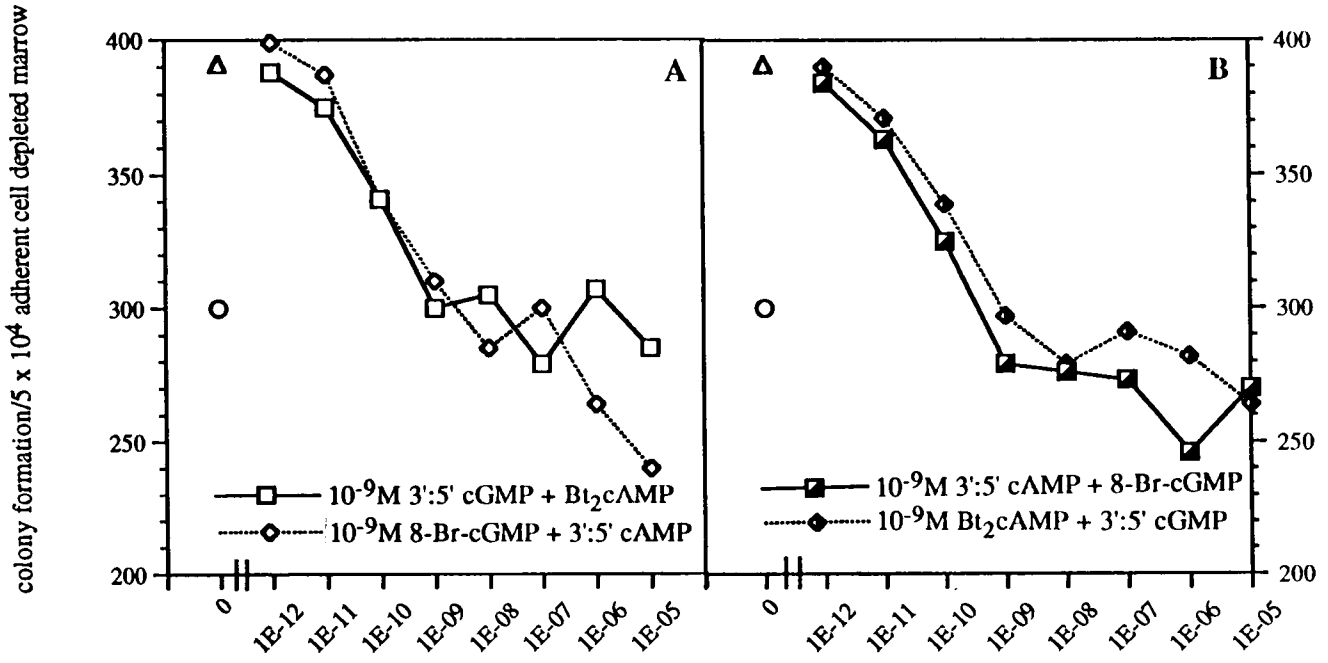


Figure 7: Dose combinations of 3':5' cAMP or Bt_2cAMP with 10^{-9}M 8-Br-cGMP or 3':5' cGMP (Panel A), respectively, and 3':5' cGMP or 8-Br-cGMP with 10^{-9}M Bt_2cAMP or 3':5' cAMP (Panel B), respectively. Basal colony formation stimulated by 750 U/ml M-CSF was 300 colonies (o); 2-signal dependent progenitor response to 0.1ng/ml LPS was 391 colonies (Δ). Standard error was < 10% of total colony formation for each data set.

mediated effect, it should exhibit saturability and specificity, two main tenets of receptor-ligand association. The saturability of [2,8-^{3}H] cAMP binding to murine bone marrow was evaluated by the addition of increasing concentrations of labelled cyclic nucleotide to cell preparations. As shown in Figure 8, [2,8-^{3}H] cAMP was able to bind to bone marrow cells. Binding from approximately 3 x 10^{-9} to 8 x 10^{-9}M was saturable as demonstrated by the plateau effect in the latter region of the curve. Saturable binding was also evident in the concentration region $\leq$ 2 x 10^{-9}M and in various regions $\geq$ 1 x 10^{-8}M, including a plateau effect spanning 1 x 10^{-8} to 6.6 x 10^{-8}M (data not shown). Scatchard analysis of these binding patterns revealed at least 5 binding plateaus with corresponding apparent dissociation constants (K_{Dapp}). Three of these analyses are shown in Figure 9. Scatchard analysis corresponding to the binding plateau in Figure 8 predicted a K_{Dapp} of 2 x 10^{-9}M (Figure 9). Also shown are the Scatchard plots of the binding plateaus spanning [2,8-^{3}H] cAMP concentrations of 10^{-9} to 2 x 10^{-9}M (Line **a**) and 10^{-8} to 6.6 x 10^{-8}M (Line **b**). Analysis of Line **a** predicted a K_{Dapp} of 5 x 10^{-10}M; K_{Dapp} for Line **b** was 8 x 10^{-8}M.

Tritiated cAMP binding to murine bone marrow exhibits specificity. Figures 8 and 9 demonstrated evidence that binding of [2,8-^{3}H] cAMP to the surface of murine bone marrow cells was saturable but the binding also must show specificity to qualify as a receptor-ligand interaction. Therefore, a specificity experiment was performed where tritiated cAMP binding was challenged with 100-fold greater doses of cold (unlabelled) 3':5' cAMP, 2':3' cAMP and 5' AMP (Table 3). As recorded, only 100-fold greater concentrations of 3':5' cAMP were capable of competing with [2,8-^{3}H] cAMP for binding sites demonstrating specificity of the reaction for the 3':5' cyclic nucleotide. This data corroborated that derived from soft agar assays demonstrating 2-signal progenitor response to costimulation by 3':5' cAMP but not to 2':3' cAMP (Figure 4). The data generated from soft agar culture in combination with that derived

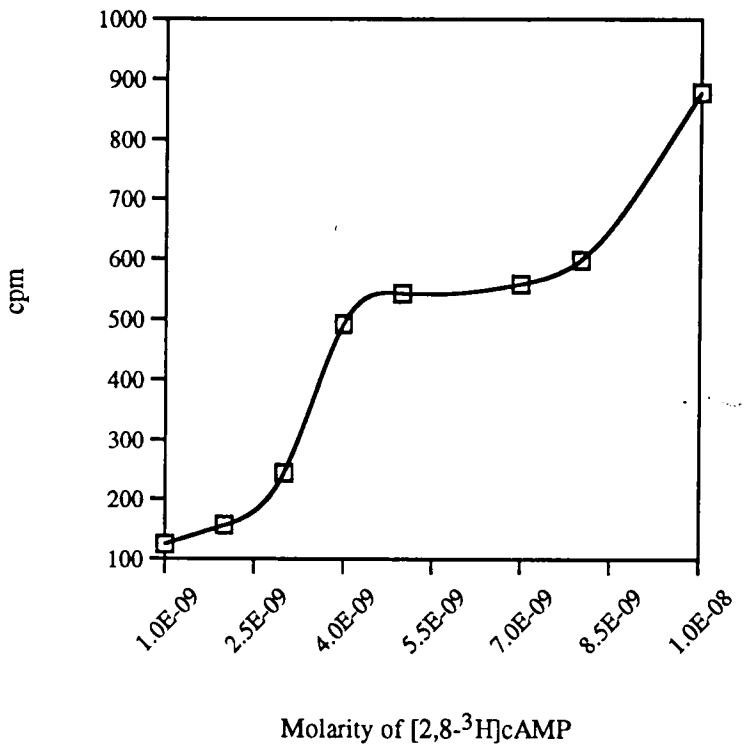


Figure 8: Tritiated cAMP binding to murine bone marrow is saturable. Binding exhibited saturation plateau from 4 x 10^{-9} to 8 x 10^{-9} M [2,8-^{3}H] cAMP. Data are representative of results generated from at least three separate experiments.

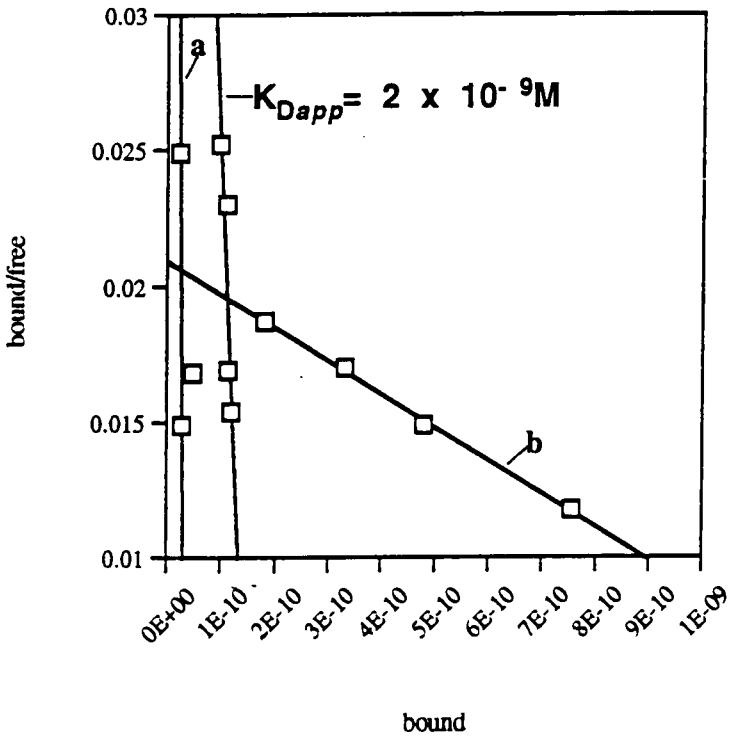


Figure 9: Scatchard analysis of saturation curve for [2,8-^{3}H]cAMP binding to murine marrow defines an apparent dissociation constant (K_{Dapp}) equal to 2 x 10^{-9}M for the plateau spanning tritiated cAMP concentrations from 4 x 10^{-9}M to 8 x 10^{-9}M. Lines **a** and **b** represent Scatchard anaylsis of saturation plateaus for trititated cAMP binding to murine bone marrow spanning concentration ranges from 10^{-9} to 2 x 10^{-9}M (line **a**) and 10^{-8} to 6.6 x 10^{-8}M. K_{Dapp} for line **a** was 5 x 10^{-10}M; for line **b**, 8 x 10^{-8}M.

Table 3: Binding of [2,8-^{3}H]cAMP to adherent cell depleted marrow is specific for 3':5' cAMP.

	[2,8-^{3}H]cAMP Binding in Counts per Minute ± SE			
treatment	none	+ 3':5' cAMP	+ 2':3' cAMP	+ 5' AMP
2,8-^{3}[H]cAMP	3664 ± 19	3120 ± 22[a]	3604 ± 21[b]	3586 ± 19[b]

Binding assays were performed with 4 x 10^7 adherent depleted marrow cells. Tritiated cAMP ([2,8-^{3}H]cAMP) was added to assays at a final concentration of 10^{-8}M. All other factors were added to assays at a final concentration of 10^{-6}M. Data are reported as the mean ± SE of triplicate determinations.

[a]Significantly less than binding control at $p \leq 0.05$.

[b]Not sigificantly different from binding control.

from saturation and specificity binding experiments indicated the action of membrane impermeant, phosphodiesterase sensitive 3':5' cAMP on 2-signal dependent proliferation was a cell surface-associated event.

The 2-signal response costimulated by 3':5' cGMP is not affected by the 30kDa monokine, the 14-mer peptide, AKPR, or ferritin. The 30kDa protein inhibitor (30kDa monokine) was added to soft agar cultures to determine if *in vitro* proliferation of 2-signal dependent progenitors costimulated by 3':5' cGMP or 3':5' cAMP was inhibitable like that costimulated by SP, ANP, Tft, LTB_4, $PGF_{2\alpha}$, and LPS. As expected, based on preceding observations with NT (Figure 2), the inhibitory agent in soft agar culture with adherent cell depleted marrow was not capable of abrogating 2-signal dependent progenitor proliferation costimulated with 3':5' cAMP (Table 4). These results supported those received with NT as the costimulant of 2-signal dependent progenitor proliferation (Figure 2). However enhancement of colony formation mediated by 3':5' cGMP was also unaffected by the inhibitory agent (Table 4). The enhancement of colony formation by 3':5' cGMP was also tested in soft agar culture for inhibition by AKPR, ferritin (in total marrow cultures), and 14-mer (Table 4). As illustrated none of the inhibitory agents were capable of abrogating 3':5' cGMP-mediated augmentation.

TNF_α costimulates the 2-signal response and may mediate costimulation effected by LPS. Inhibition of cGMP compatible 2-signal dependent progenitors by AKPR, 14-mer, and ferritin (ferritin's inhibitory effects only apparent in cultures of total marrow) but not that mediated by 3':5' cGMP suggests the inhibitors act at a step in the signal transduction pathway prior to the elevation of cGMP. Since the costimulants of cGMP-compatible augmentation of 2-signal dependent progenitor proliferation varied in their initial mechanism of action, *e.g.* Tft and SP probably work via the same receptor, but LPS, LTB_4, and $PGF_{2\alpha}$ act via different receptor-mediated

Table 4: Effect of inhibitors of 2-signal dependent progenitor response on proliferation costimulated by 3':5' cGMP or 3':5' cAMP.

	Colony Response ± SE				
treatment	none	+ 30kDa inhibitor	+ AKPR	+ 14-mer	+ ferritin[a]
M-CSF	193 ± 2	198 ± 3[d]	192 ± 3[d]	201 ± 3[d]	201 ± 6[d]
M-CSF + 3':5' cAMP	249 ± 4[c]	246 ± 5[c]	NT[b]	NT[b]	259 ± 5[c]
M-CSF + 3':5' cGMP	246 ± 3[c]	240 ± 8[c]	242 ± 2[c]	242 ± 5[c]	248 ± 7[c]

Colony response represents the number of colonies formed in response to 750 U/ml M-CSF ± costimulants. Adherent cell depleted marrow was plated at 5 x 10^4 cells/ml. The 30kDa inhibitor was added to cultures at a 1:50,000 dilution previously shown to inhibit 2-signal dependent progenitor proliferation without affecting the basal, M-CSF responsive colony formation (EE,J4). AKPR, 14-mer, and ferritin were added at 10^{-9}M, as were the 3':5' cyclic nucleotides.

[a]Ferritin results were generated in cultures of total marrow plated at 5 x 10^4 cells/ml.

[b]Not tested.

[c]Significantly greater than M-CSF control at $p \leq 0.05$.

[d]Not significantly different from M-CSF control.

mechanisms, a common link besides cGMP compatibility in the proliferative pathway was investigated. Reports on the actions of LPS in macrophage systems have demonstrated LPS-mediated increases in tumor necrosis factor-α (TNF_α) production (111, 118). The addition of TNF_α to the *in vitro* soft agar culture system resulted in augmented colony formation in response to M-CSF (Figure 10). Enhancement of 2-signal dependent progenitor response was optimal at 33 U/ml TNF_α. TNF_α-mediated augmentation was abrogated by the addition of monoclonal and polyclonal neutralizing anti-TNF_α antibodies (Figure 11). To investigate the possibility that TNF_α mediates the costimulatory effect of LPS, monoclonal and polyclonal neutralizing anti-TNF_α antibodies were added to soft agar cultures containing M-CSF, LPS and adherent cell depleted marrow (Table 5). Both antibodies were able to abrogate costimulation of 2-signal dependent progenitor proliferation by LPS, suggesting that TNF_α was produced in these cultures and mediated the costimulation by LPS.

Reverse transcriptase-polymerase chain reaction detects increased levels of TNF_α-specific mRNA in adherent cell depleted murine marrow treated with LPS. Macrophages are the predominant source of TNF_α following exposure to LPS (1). The marrow cultures used, however, were depleted of these cells by prior adherence. Thus, if TNF_α is produced in response to LPS in the nonadherent marrow cell cultures, adherent mononuclear phagocytes would not be the source, and the cytokine yield would be predictably minute. This was confirmed when preliminary experiments using a classical L_{929} cell assay for TNF_α (75) failed to detect any TNF_α activity in supernatant medium from LPS-stimulated nonadherent marrow cells. Therefore, to test for TNF_α production following LPS stimulation of adherent cell depleted marrow, a reverse transcriptase-polymerase chain reaction (RT-PCR) for TNF_α mRNA was used. Figure 12 is a photograph of a typical polyacrylamide gel

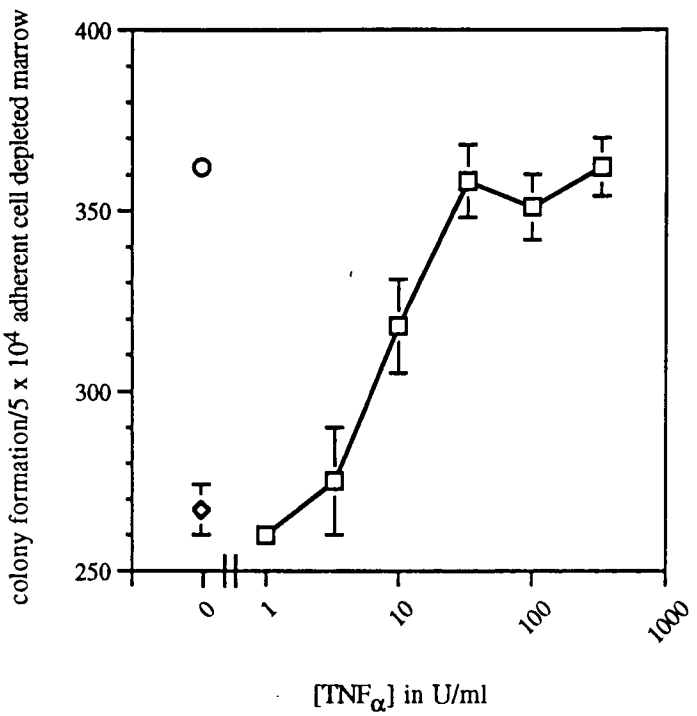


Figure 10: Dose response of 2-signal dependent progenitor colony formation to TNF_α (-□-). Basal colony formation in response to 750 U/ml M-CSF was 267 ± 7 (◇); 2-signal dependent progenitor proliferation in response to 0.1ng/ml LPS was 362 ± 2 (○). Results are reported as the mean ± SE of triplicate cultures and are representative of the values derived from at least three experiments.

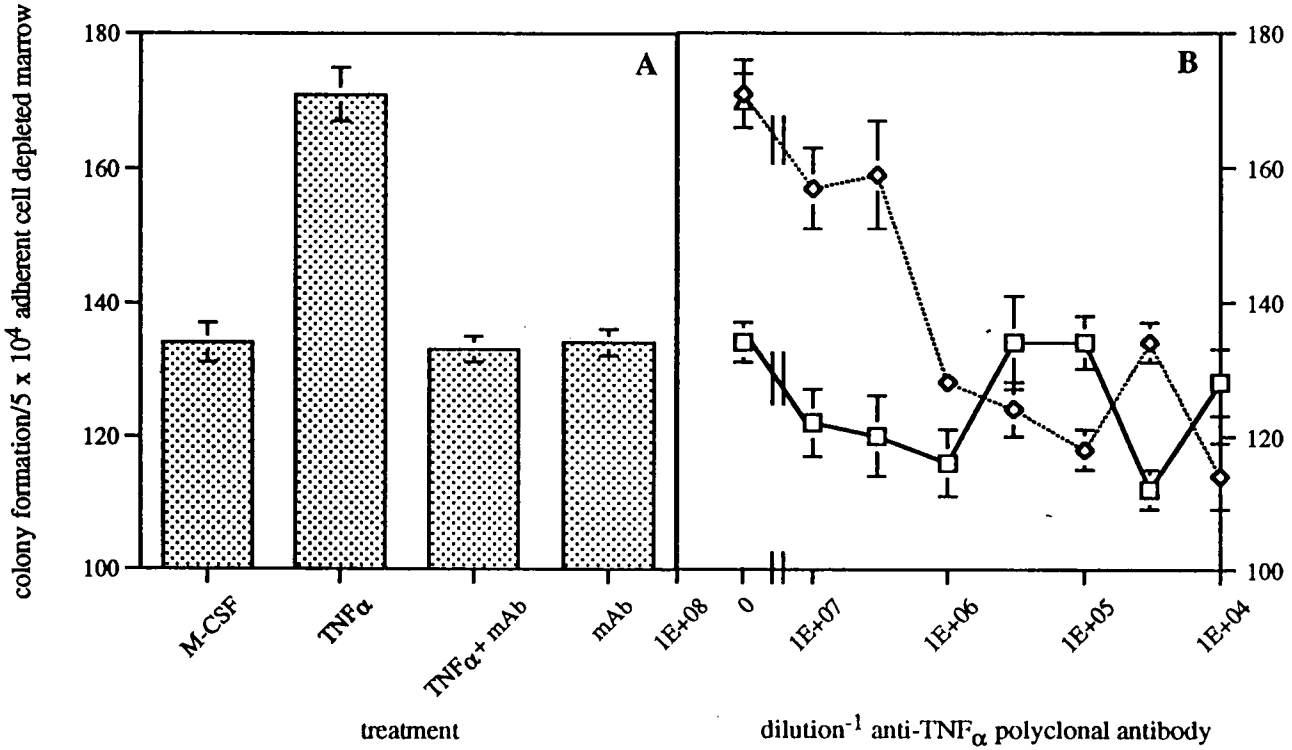


Figure 11: Abrogation of 2-signal dependent progenitor proliferation in response to 750 U/ml M-CSF and 100 U/ml TNF_α by 50 µg monoclonal anti-TNF_α antibody (mAb; Panel A) and polyclonal anti-TNF_α antibody (--◇--; Panel B). Basal colony response to M-CSF was 134 ± 3 (-□- ; Panel B); 2-signal dependent progenitor colony response to 0.1ng/ml LPS was 170 ± 4 (▲; Panel B). Results are reported as the mean ± SE of triplicate cultures and are representative of the values derived from at least three separate experiments.

Table 5: Effect of neutralizing polyclonal and monoclonal anti-TNF_α antibodies on 2-signal dependent progenitor proliferation costimulated by LPS or TNF_α.

	Colony Response ± SE		
treatment	none	+ anti-TNF_α poly[a]	+ anti-TNF_α mAb[b]
M-CSF	143 ± 3	145 ± 3[d]	141 ± 2[d]
M-CSF + LPS	179 ± 4[c]	139 ± 6[d]	140 ± 2[d]
M-CSF + TNF_α	180 ± 5[c]	142 ± 4[d]	140 ± 2[d]

Colony response represents the number of colonies formed in response to 750 U/ml M-CSF ± costimulants. Adherent cell depleted marrow was plated at 5 x 10^4 cells/ml. LPS and TNF_α were added to cultures at 0.1 ng/ml and 100 U/ml, respectively.

[a]Anti-TNF_α polyclonal antibody was added to cultures at a 1:330,000 dilution.

[b]Anti-TNF_α monoclonal antibody was added to cultures at 50 μg/ml.

[c]Significantly greater than M-CSF control at $p \leq 0.05$.

[d]Not significantly different from M-CSF control.

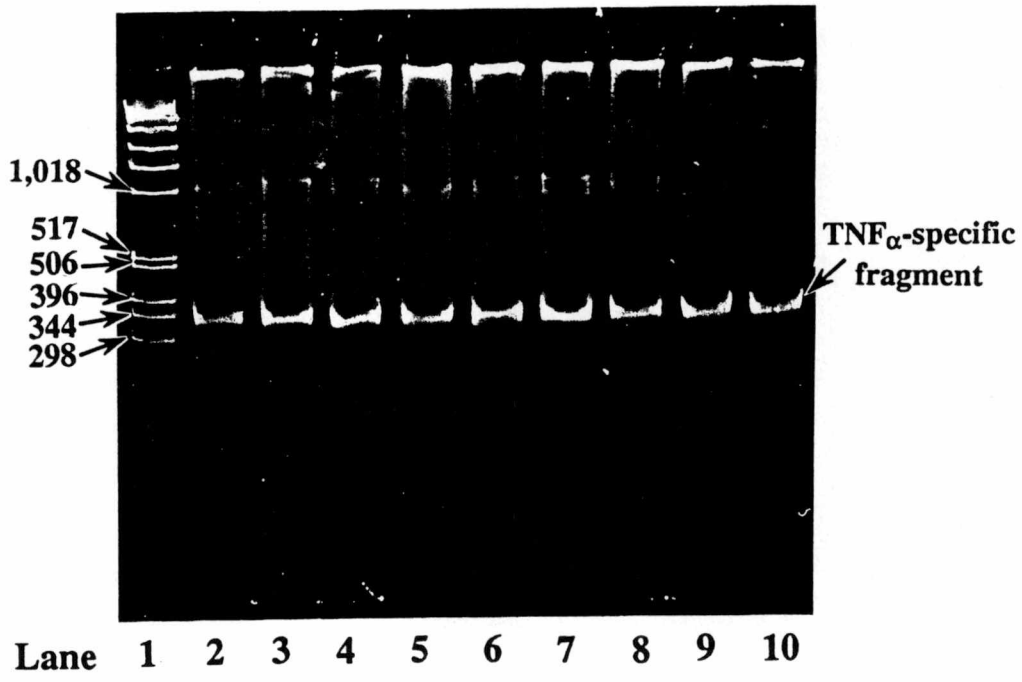


Figure 12: Detection of TNF_α-specific mRNA in adherent cell depleted murine marrow treated with LPS using the reverse transcriptase-polymerase chain reaction (RT-PCR). Amplified TNF_α-specific PCR fragment size is 354 bp. Lane 1 contains 1Kb DNA ladder; lanes 2, 5 & 8 contain TNF_α cDNA at 30, 32 & 34 amplification cycles (x), respectively; lanes 3, 6 & 9 contain M-CSF + LPS, 8h at 30, 32 & 34 x, respectively; lanes 4, 7 & 10 contain LPS, 12h at 30, 32 & 34 x, respectively.

separation of RT-PCR products. Reverse-transcribed and ampliflied TNF_α transcripts yield a 354 base pair PCR product. As can be seen, the reaction was specific for TNF_α message and bands corresponding to a particular sample increase in ethidium bromide fluorescence as a function of increasing PCR cycle number. The data reported from the following experiments were derived from measures of the amount of radioactivity (α^{32}P-dCTP incorporation) in each product band.

RT-PCR results from cultures that were unstimulated, stimulated with LPS, stimulated with M-CSF, and stimulated with LPS plus M-CSF are shown in Figures 13-16, respectively. The data are plotted as counts per minute (cpm) of incorporated α^{32}P-dCTP versus the number of PCR cycles demonstrating increased α^{32}P-dCTP incorporation as a function of initial TNF_α mRNA concentration. As shown in Figure 13, a basal level of TNF_α-specific mRNA was detectable in untreated marrow cultures that did not appreciably change over the 24h sampling period. Panels A and B represent identical data graphed by semi-log (A) or arithmetic (B) methods. The semi-log plot illustrates the amplification of each RT sample was parallel to the others. This property of the PCR reaction allows for the quantification of each sample based on the relative amount of starting material, *i.e.* TNF_α-specific mRNA transcript. The arithmetic plot accentuates the relative differences in starting material at the various collection times. However, as shown in Figures 14 and 15, both LPS and M-CSF stimulated an increase in mRNA transcripts with assessed maximal peaks at 12h for both stimulants. In cultures stimulated with both LPS and M-CSF (Figure 16), apparent maximal TNF_α transcripts were detected between 8 and 12h.

The preceding results are summarized in Figure 17 where peak α^{32}P-dCTP incorporation for the various treatments and culture times are shown. The basal level of TNF_α-specific mRNA transcript in total RNA from adherent cell depleted marrow is represented by the no treatment group. As shown only slight variations in the level

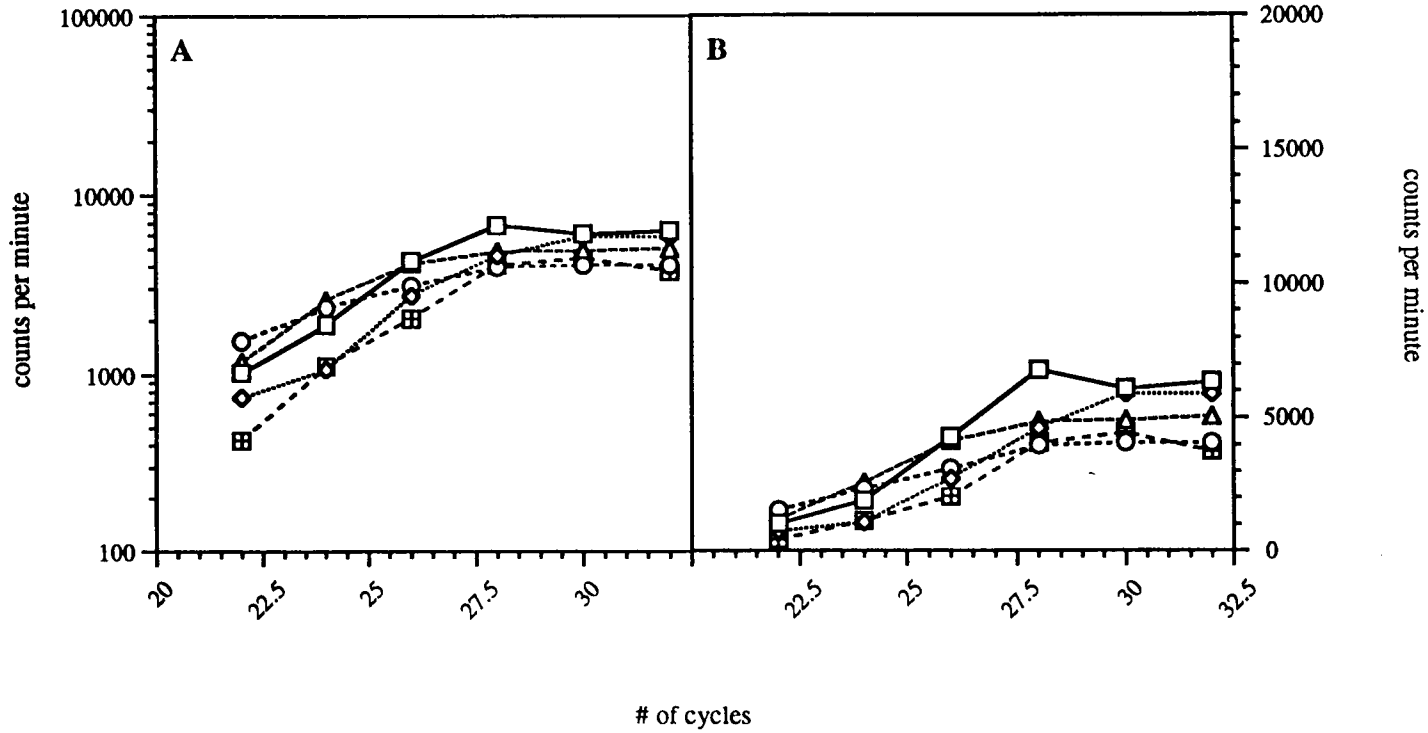


Figure 13: TNF_{α}-specific mRNA transcripts detected in total RNA isolated from adherent cell depleted marrow cultured in RPMI 1640 plus 15% dFBS for 1h (–□–), 4h (--◇··), 8h (··○··), 12h (--△--), and 24h (·-⊞-). Panel A is a semi-log plot of the data representing the incorporation of α^{32}P-dCTP into TNF_{α}-specific mRNA transcripts; Panel B is an arithmetic plot of the same data.

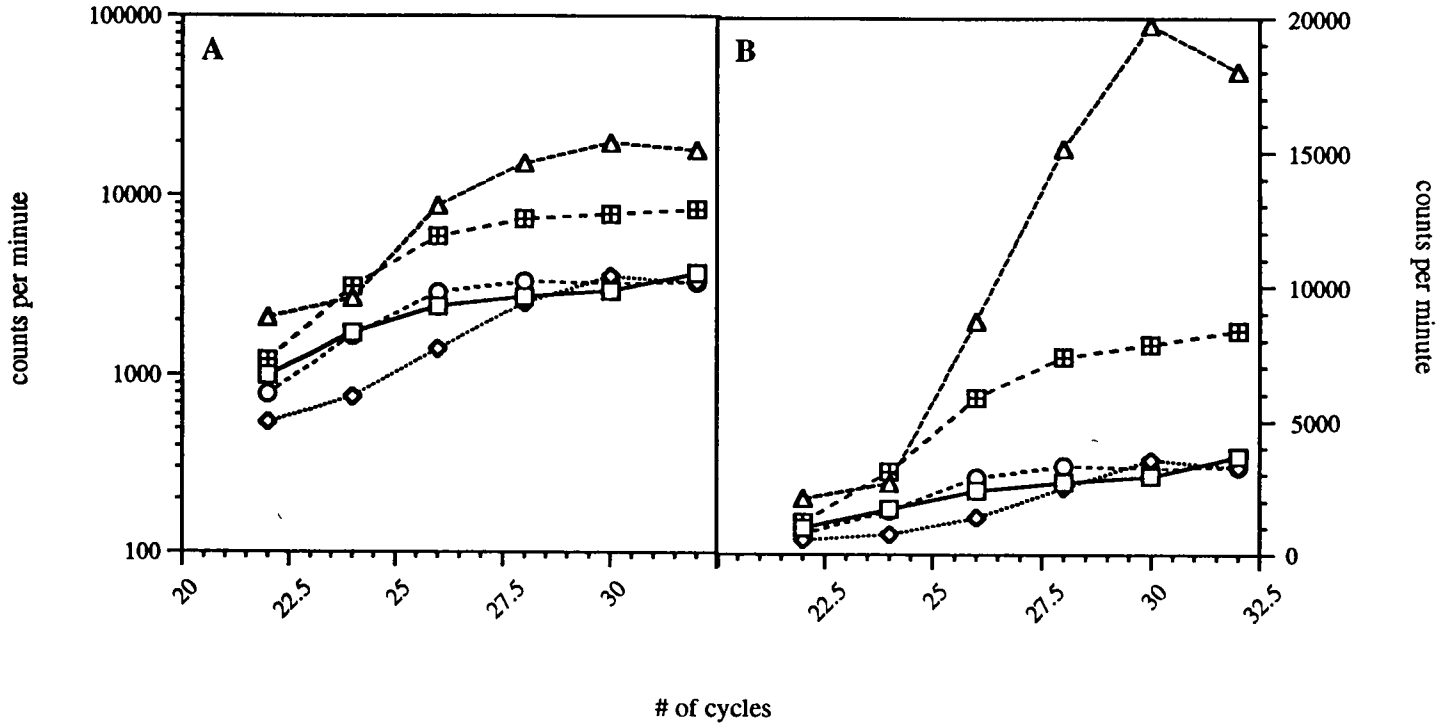


Figure 14: TNF_{α}-specific mRNA transcripts detected in total RNA isolated from adherent cell depleted marrow treated with 0.1 ng/ml LPS for 1h (-□-), 4h (···◇···), 8h (·○··), 12h (--△--), and 24h (-⊞-). Panel A represents a semi-log plot of α^{32}P-dCTP incorporation by TNF_{α}-specific mRNA transcripts; Panel B is an arithmetic plot of the same data.

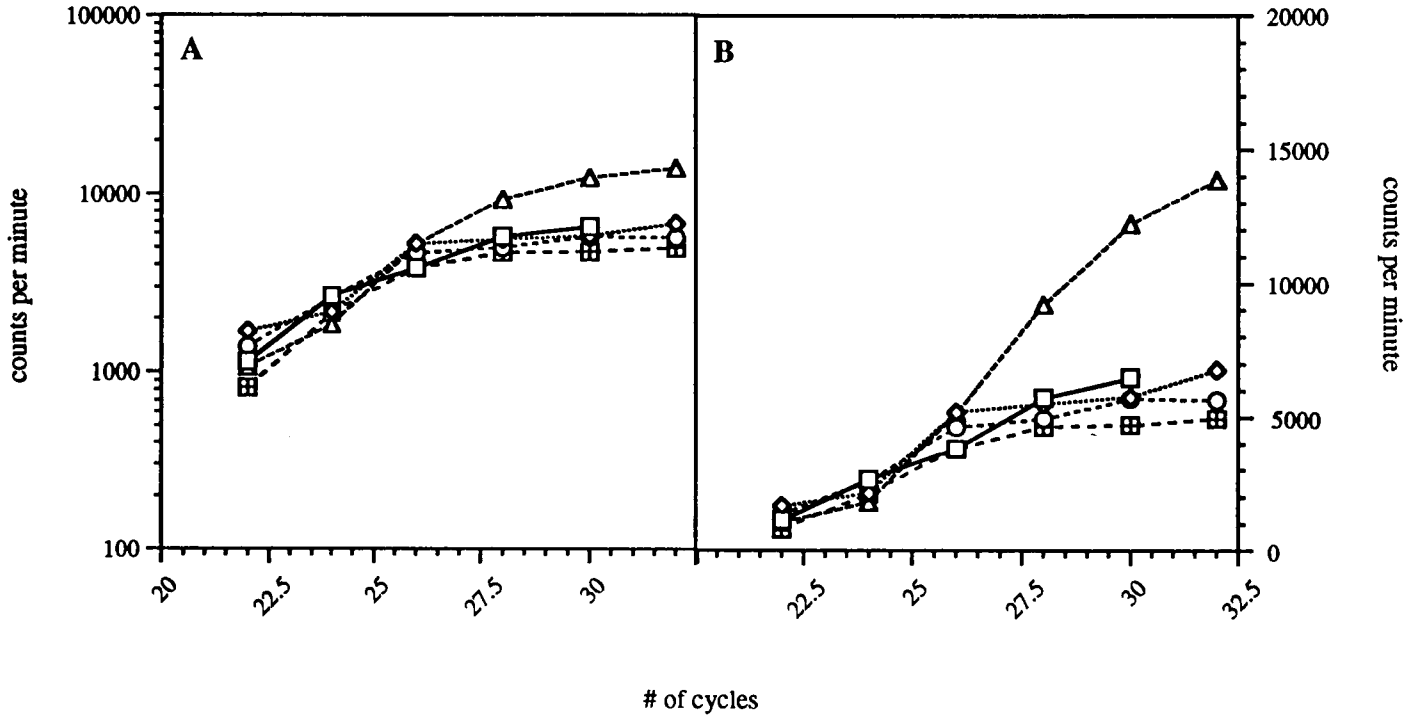


Figure 15: TNF_{α}-specific mRNA transcripts detected in total RNA isolated from adherent cell depleted marrow treated with 750 U/ml M-CSF for 1h (–□–), 4h (··◇··), 8h (··○··), 12h (··△··), and 24h (-⊞-). Panel A is a semi-log plot of α^{32}P-dCTP incorporation in TNF_{α}-specific mRNA transcripts; Panel B is an arithmetic plot of the same data.

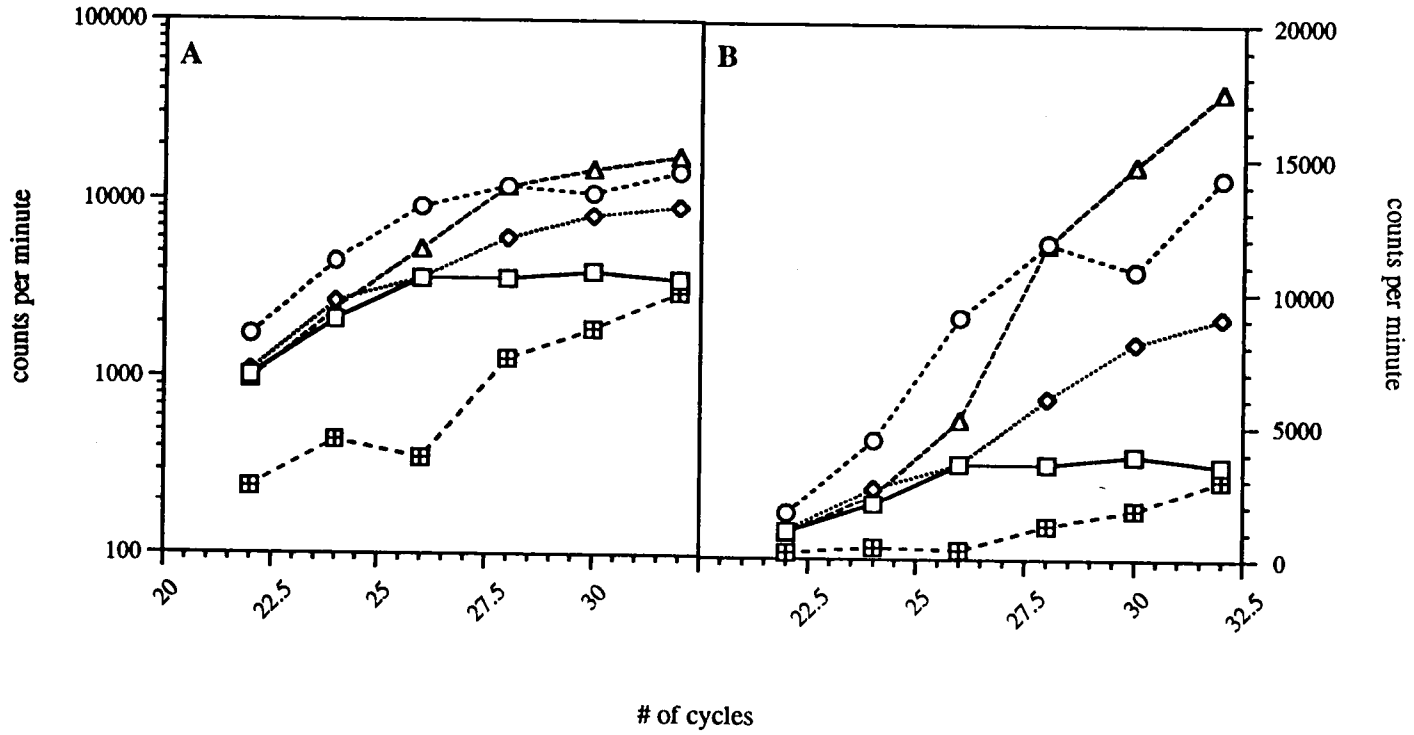


Figure 16: TNF_{α}-specific mRNA transcripts detected in total RNA isolated from adherent cell depleted marrow treated with 0.1 ng/ml LPS plus 750 U/ml M-CSF for 1h (-□-), 4h (···◇···), 8h (·○··), 12h (-△-), and 24h (-⊞-). Panel A is a semi-log plot of α^{32}P-dCTP incorporation in TNF_{α}-specific mRNA transcripts; Panel B is an arithmetic plot of the same data.

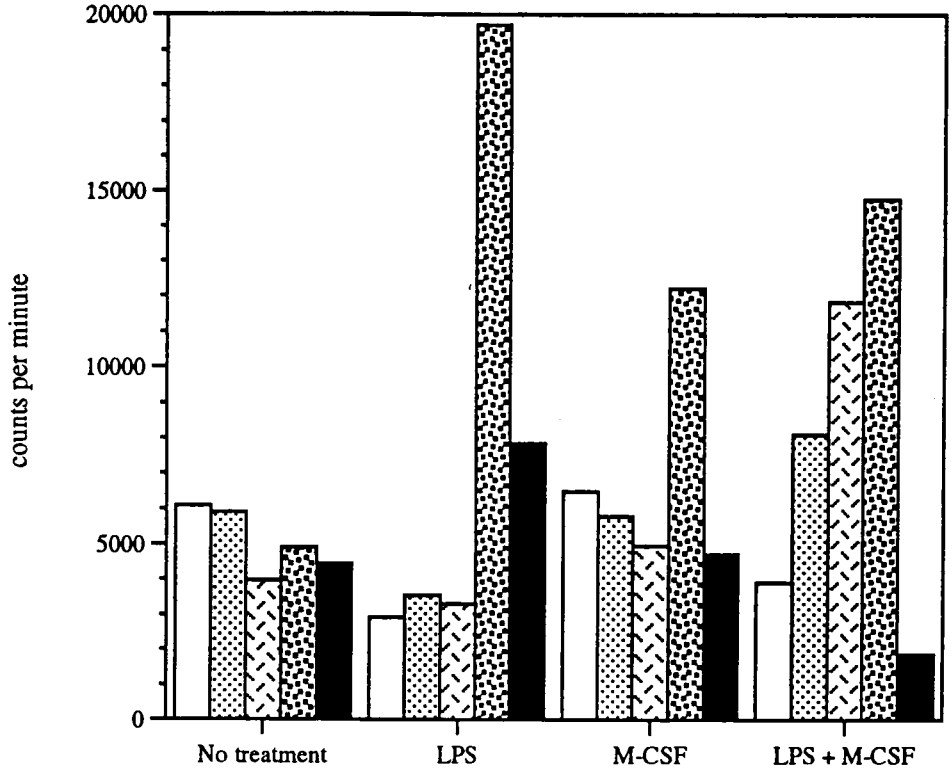


Figure 17: Peak α^{32}P-dCTP incorporation by TNF_{α}-specific mRNA transcripts in total RNA isolated from adherent cell depleted marrow post-stimulation with 0.1 ng/ml LPS, 750 U/ml M-CSF, or M-CSF plus LPS. Basal levels of TNF_{α}-specific mRNA transcript in total RNA is represented by no treatment data. Columns represent peak incorporation at 1h (□), 4h (▨), 8h (▨), 12h (▨), and 24h (■) post-stimulation.

of transcript occurred over the 24h period. The LPS treatment group clearly shows a peak in TNF_{α}-specific mRNA transcript level at 12h post-stimulation, as does the M-CSF treatment group. Adherent cell depleted marrow treated with M-CSF plus LPS demonstrated increased levels of TNF_{α}-specific mRNA transcripts beginning at 4h post-stimulation and peaking at 12h. Transcript levels for LPS or M-CSF stimulated cultures were at or close to basal levels at 24h while that from M-CSF and LPS-treated cultures was below the corresponding unstimulated level.

In Figure 18 peak incorporation of α^{32}P-dCTP by TNF_{α}-specific mRNA transcripts in total RNA isolated from adherent cell depleted marrow is compared for each group at 1, 4, 8, 12, and 24h post-stimulation. Using incorporation levels obtained with total RNA isolated from untreated cultures as baseline, the patterns of incorporation for each group can be compared. At 1h post-stimulation levels of transcript in M-CSF-treated cultures were consistent with basal, untreated levels while those in LPS- of M-CSF plus LPS-treated groups had decreased below baseline. At 4h transcript levels had recovered and begun to increase in M-CSF plus LPS-treated cultures whereas cultures treated with LPS alone still displayed depressed levels of TNF_{α}-specific mRNA transcript. Transcript levels in M-CSF-treated cultures remained at baseline. By 8h the M-CSF plus LPS group demonstrated steadily increasing increasing levels of TNF_{α}-specific mRNA transcript. M-CSF-treated cultures remained near baseline levels of transcript while LPS-treated cultures began to show a reversal of the 1 and 4h observed transcript level depression. At 12h post-stimulation dramatic changes in the levels of TNF_{α}-specific mRNA transcript levels were detected. In comparison to baseline, all three treatment groups demonstrated enhanced levels of specific transcript. The increase in LPS-treated cultures was substantial, from 3317 cpm at 8h to 19720 cpm at 12h. The increases in the M-CSF and M-CSF plus LPS groups were from 4969 cpm at 8h to 12224 cpm at 12h and 11846 cpm to 14761 cpm, respectively.

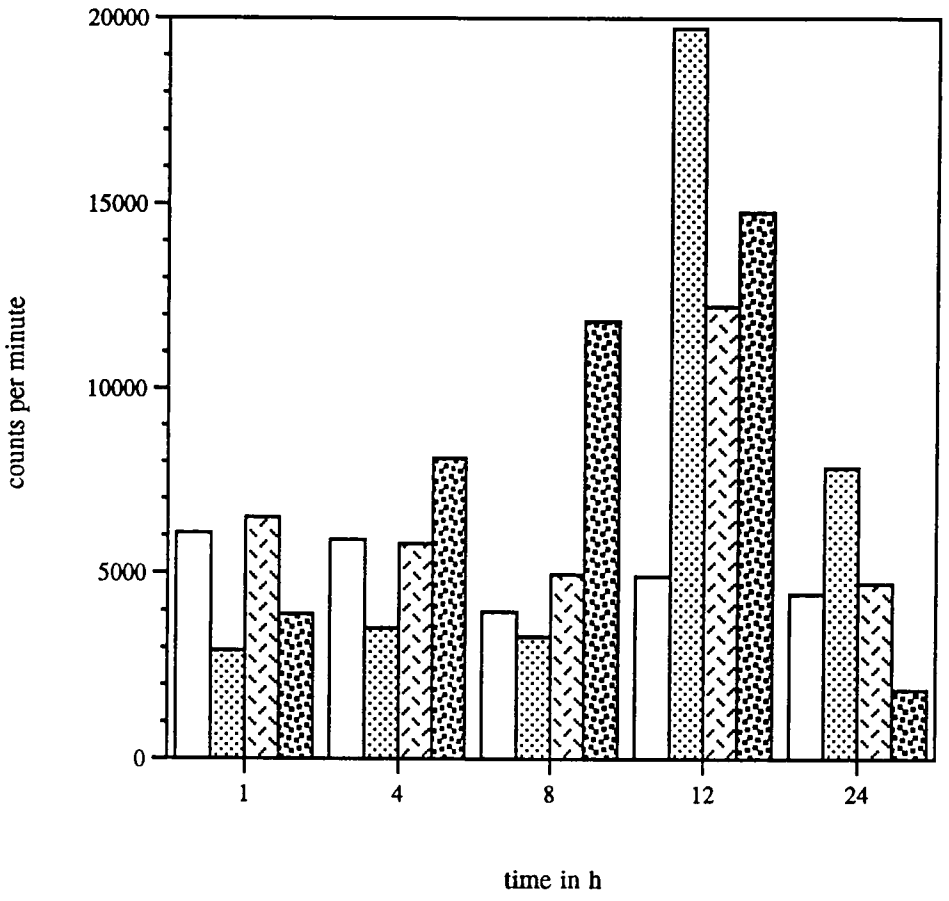


Figure 18: Comparison of peak incorporation of α^{32}P-dCTP by TNF_{α}-specific mRNA transcripts in total RNA isolated from adherent cell depleted marrow post-stimulation with 0.1 ng/ml LPS (▨), 750 U/ml M-CSF (▨), or M-CSF plus LPS (▨). Basal levels of incorporation are represented by TNF_{α}-specific mRNA transcripts in total RNA isolated from untreated adherent cell depleted marrow (□).

Twenty-four hours post-stimulation M-CSF-treated culture TNF_α-specific mRNA transcript levels had returned to baseline; LPS-treated culture transcript levels were still slightly elevated above baseline (LPS= 7854 cpm, baseline= 4451 cpm). Values for TNF_α-specific mRNA transcripts in total RNA isolated from adherent cell depleted marrow treated with M-CSF plus LPS were depressed below baseline levels (M-CSF plus LPS= 1866 cpm, baseline= 4451 cpm).

Costimulation of the 2-signal response by TNF_α is compatible with the cGMP pathway and is inhibited by AKPR, the 30 kDa monokine, and ferritin. The preceding experiments established that TNF_α can serve as a costimulant for the 2-signal progenitors and provided substantial evidence the cytokine is produced by cells in the nonadherent marrow cultures. To further investigate the potential involvement of TNF_α, the enhancement of 2-signal dependent progenitor response by TNF_α was examined for its cyclic nucleotide compatibility. As depicted in Figure 19, the combination of TNF_α with 3':5' cGMP or 3':5' cAMP resulted in augmentation of the response compatible with the cGMP pathway and loss of augmentation compatible with cAMP-mediated mechanisms. Also, enhanced colony formation stimulated by TNF_α in conjunction with M-CSF was abrogated by AKPR, the 30kDa monokine and, in total marrow cultures, by ferritin (Table 6). Thus, TNF_α exhibited properties basically identical to LPS in the 2-signal colony-forming system further suppporting the potential of the cytokine as an intermediate signal in the 2-signal response.

Neutralizing TNF_α antibody blocks costimulation of 2-signal dependent proliferation effected by SP, ANP, $PGF_{2\alpha}$, and LPS. The inhibition of TNF_α costimulation of the 2-signal progenitors by AKPR, the 30kDa monokine and ferritin placed the inhibitory signal at a point in the LPS signal transduction pathway post-TNF_α production, but prior to elevation of cGMP. To further examine this potential pathway, the influence of anti-TNF_α antibodies on costimulation by other cGMP

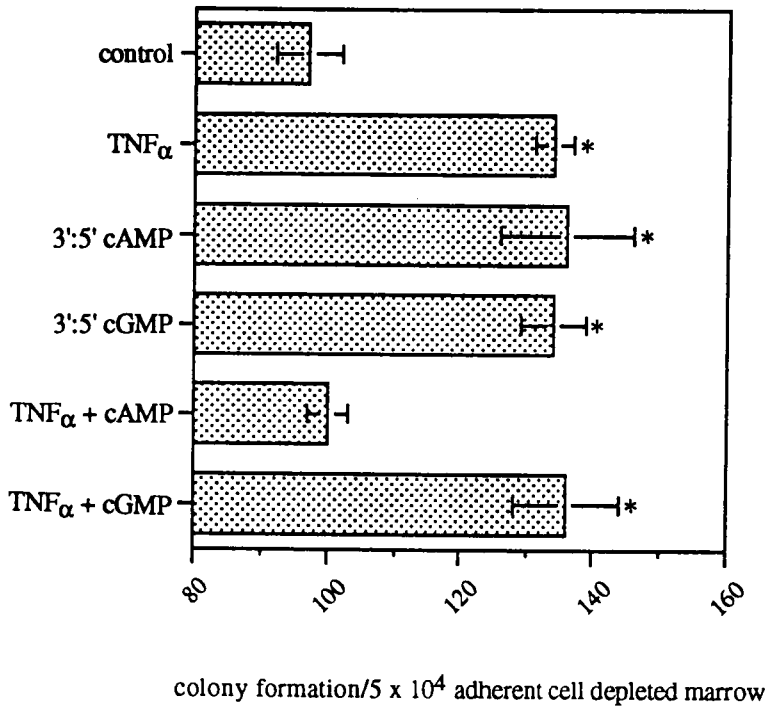


Figure 19: Costimulation of 2-signal dependent progenitor proliferation by 100 U/ml TNF_α is compatible with costimulation mediated by 10^{-9}M 3':5' cGMP, but not 10^{-9}M 3':5' cAMP. Basal colony formation stimulated by 750 U/ml M-CSF was 97 $\pm$ 5. Results represent the mean $\pm$ SE of triplicate cultures and illustrate the data from at least three experiments.

*Significantly greater than M-CSF control at $p \leq 0.05$.

Table 6: Effect of inhibitors of 2-signal dependent progenitor response on proliferation costimulated by TNF_α.

	Colony Response ± SE			
treatment	none	+ AKPR	+ 30kDa monokine	+ ferritin[a]
M-CSF	163 ± 4	161 ± 3[c]	171 ± 3[c]	159 ± 2[c]
M-CSF + TNF_α	214 ± 4[b]	168 ± 3[c]	167 ± 2[c]	162 ± 2[c]

Colony response represents the number of colonies formed in response to 750 U/ml M-CSF ± costimulants. Adherent cell depleted marrow was plated at 5 x 10^4 cells/ml. TNF_α was added to cultures at 100 U/ml. AKPR and ferritin were added at 10^{-9}M; the 30kDa monokine was added at a 1:50,000 dilution.

[a]Ferritin cultures were plated from total bone marrow at a final concentration of 5 x 10^4 cells/ml.

[b]Significantly greater than M-CSF control at $p \leq 0.05$.

[c]Not significantly different from M-CSF control.

compatible mediators was examined. As shown in Figure 20, polyclonal neutralizing anti-TNF_α antibodies abrogated 2-signal dependent progenitor proliferation costimulated by SP, ANP, $PGF_{2\alpha}$, and LPS. Anti-TNF_α antibody had no effect on 2-signal dependent proliferation stimulated by NT, a cAMP compatible costimulant, in combination with M-CSF.

N^G-monomethyl-L-arginine, a nonspecific inhibitor of nitric oxide synthase, blocks the 2-signal response costimulated by TNF_α. Data to this point indicated the cGMP compatible 2-signal progenitor pathway acted via an increase in TNF_α production and the subsequent actions of this biological modifier. Both LPS and TNF_α are known stimulants for inducible nitric oxide synthase (iNOS; 101, 117, 122). Stimulation of iNOS results in the production of nitrogen monoxide, more commonly referred to as nitric oxide (NO; 122), a potent stimulator of cytoplasmic guanylyl cyclase (93, 122, 190). To examine the possibility that TNF_α may effect its proliferative potential via the production of NO, inhibitors of iNOS were added to soft agar culture. As shown in Figure 21, N^G-monomethyl-L-arginine (N^GMMA), an inhibitory arginine analogue for both the constitutive and inducible forms of NOS, inhibited the 2-signal dependent progenitor response to 33 U/ml TNF_α. Concentrations of N^GMMA greater than 1mM inhibited *in vitro* colony formation by basal, M-CSF-responsive progenitors as well, presumably via the inhibition of the constitutive enzyme.

Aminoguanidine, a more specific inhibitor of the inducible form of nitric oxide synthase, blocks the 2-signal response costimulated by TNF_α. N^GMMA and aminoguanidine also block costimulation of the 2-signal response by LPS. As N^GMMA is a nonspecific inhibitor of NOS, a more specific iNOS inhibitor, aminoguanidine (AG; 28), was added to similar cultures. In Figure 22, the dose response of 2-signal dependent progenitor proliferation induced by 33 U/ml TNF_α and

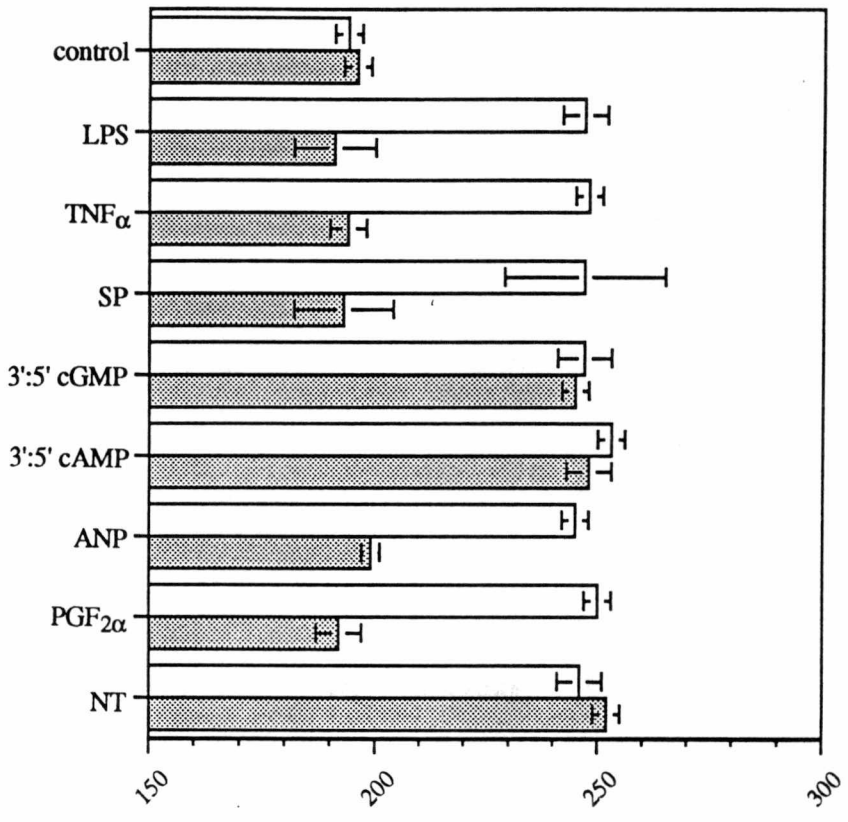


Figure 20: Abrogation of 2-signal dependent progenitor proliferation by anti-TNF_α polyclonal antibodies (▩). Basal colony formation stimulated by 750 U/ml M-CSF was 194 ± 3. LPS, TNF_α, and SP were added to cultures at 0.1ng/ml, 100 U/ml, and 10^{-7}M, respectively. Anti-TNF_α polyclonal antibody was added at a 1:330,000 dilution. All other costimulants were added at 10^{-9}M. Results are the mean ± SE and are representative of at least three experiments.

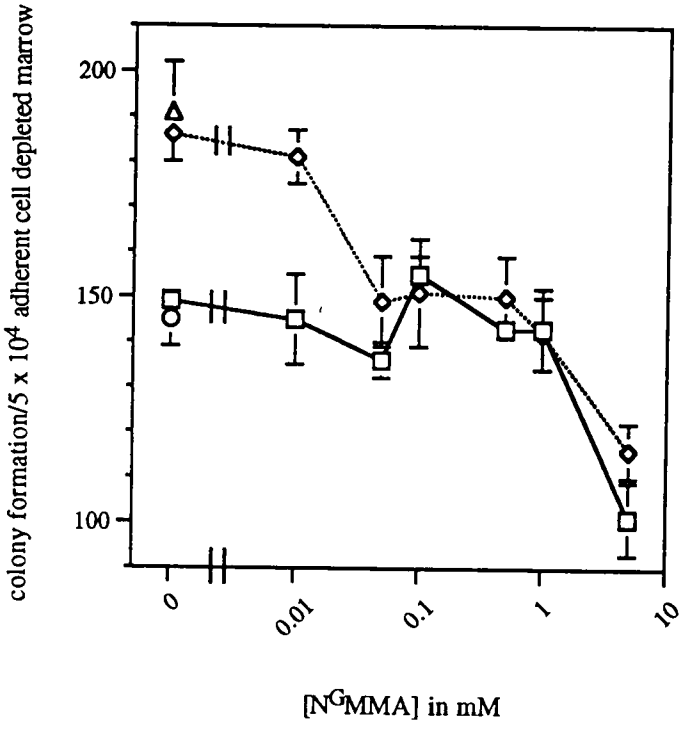


Figure 21: The effect of N^GMMA on 2-signal dependent progenitor proliferation stimulated by 750 U/ml M-CSF and 33 U/ml TNF_α (--◇--). N^GMMA, at concentrations ≤ 1mM, had no effect on colony formation in response to M-CSF (-□-). Basal M-CSF responsive colony formation was 145 ± 6 (O); 2-signal response to 0.1 ng/ml LPS was 191 ± 11 (Δ). Results are presented as the mean ± SE of triplicate cultures. Data are representative of at least three separate experiments.

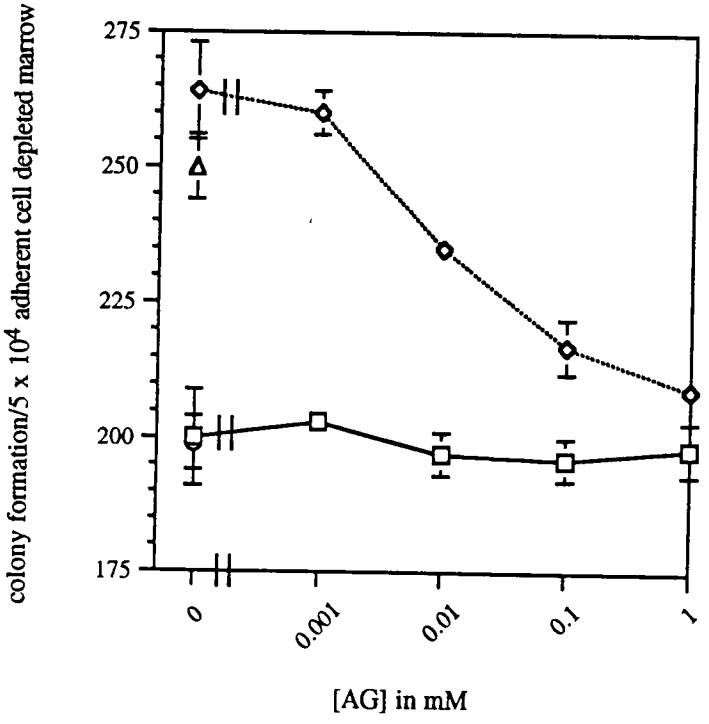


Figure 22: Effect of AG on the 2-signal dependent progenitor response to 750 U/ml M-CSF and 33 U/ml TNF_α (-◇-). AG had no effect on colony formation in response to M-CSF alone (-□-). Basal colony formation in response to M-CSF was 199 ± 5 (O); 2-signal dependent response to 0.1 ng/ml LPS was 250 ± 6 (Δ). Results are presented as the mean ± SE of triplicate cultures and are representative of at least three separate experiments.

750 U/ml M-CSF to increasing concentrations of AG is shown. AG abrogated the 2-signal dependent progenitor response, and inhibition was optimal between 0.1-1mM. To determine if N^GMMA and AG similarly inhibited costimulation by 0.1 ng/ml LPS, the dose response *in vitro* assays depicted in Figure 23 were performed. Inhibition of the response to LPS was optimal at 0.05mM N^GMMA and at 0.1mM AG. Augmentation of the 2-signal dependent progenitor response by LPS was slightly more sensitive to inhibition by both NOS inhibitors than was that mediated by TNF_α. Concentrations of AG and N^GMMA less than or equal to 1mM had no effect on basal, M-CSF responsive colony formation.

The iNOS substrate, L-arginine, reverses the inhibitory effects of N^GMMA and AG on TNF_α or LPS mediated costimulation of 2-signal dependent proliferation. The inhibition of iNOS by N^GMMA and AG is competitive with the iNOS substrate L-arginine (L-arg). Therefore, the inhibition effected by these arginine analogues should be reversible by exogenous L-arginine. Figure 24 illustrates the reversible nature of N^GMMA- and AG-mediated abrogation of TNF_α- (Panel A) and LPS-induced (Panel B) 2-signal dependent progenitor proliferation. L-arg had no effect on basal, M-CSF-responsive colony formation or on 2-signal dependent progenitor proliferation augmented by LPS or TNF_α. Reversal of N^GMMA abrogation of TNF_α or LPS costimulation was complete at 2mM ($p \leq 0.05$) and 4mM ($p \leq 0.05$) L-arg, respectively. Inhibition mediated by AG was reversed by 4mM for cultures costimulated by TNF_α ($p \leq 0.05$) and 2mM L-arg for those treated with LPS ($p \leq 0.05$).

LPS and TNF_α mediated costimulation of the 2-signal response is blocked by L-canavanine. An additional inhibitor of iNOS induction is the amino acid, L-canavanine (12, 122). Inhibition by L-canavanine is selective for iNOS and, if effective in abrogating the response of 2-signal dependent progenitor proliferation,

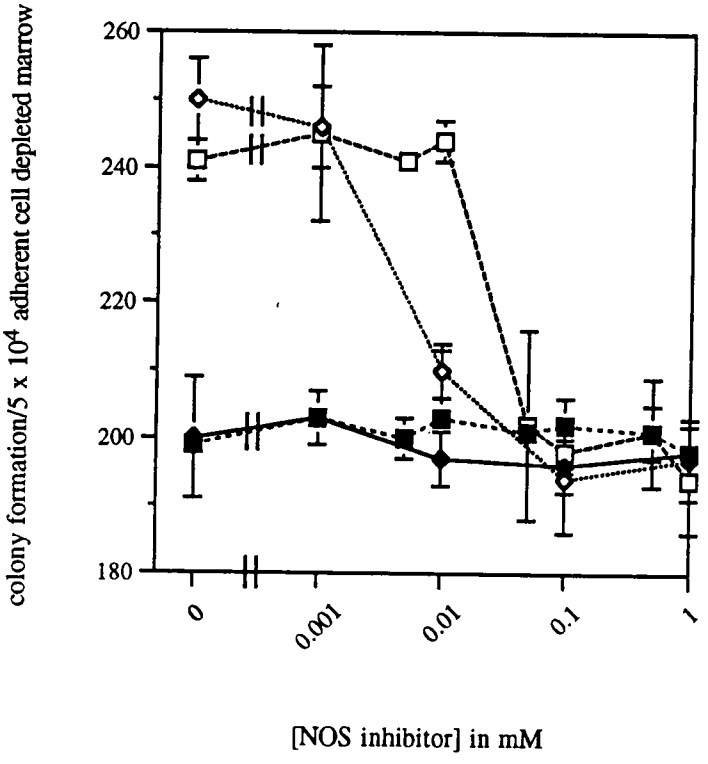


Figure 23: The effect of AG (···◇···) and N^GMMA (--□--) on 2-signal dependent progenitor proliferation costimulated by 750 U/ml M-CSF and 0.1 ng/ml LPS. AG and N^GMMA had no effect on basal, M-CSF responsive colony formation (—◆—,··■··, respectively). Results are presented as the mean ± SE of triplicate cultures and are representative of at least three separate experiments.

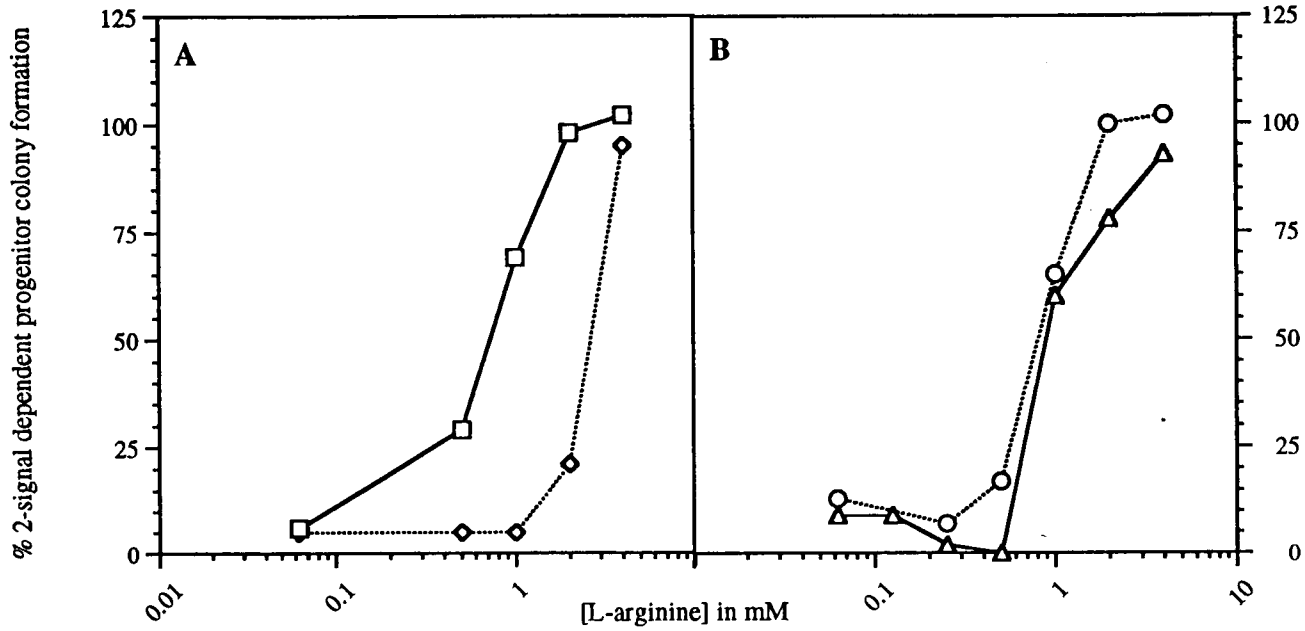


Figure 24: Addition of L-arginine to cultures of 5 x 10^4 adherent cell depleted marrow reverses the inhibition of 2-signal dependent progenitor proliferation associated with N^GMMA and AG. Basal colony formation in response to 750 U/ml M-CSF ± L-arginine was 153 ± 3; 2-signal response to 33 U/ml TNF_α ± L-arginine was 214 ± 5; to 0.1 ng/ml LPS ± L-arginine was 214 ± 2. Two-signal dependent response to TNF_α was abrogated by 0.1mM N^GMMA (150 ± 6;—□—; Panel A) and by 0.5mM AG (153 ± 4;···◇···; Panel A). Augmented colony formation in response to LPS was abrogated by 0.1mM N^GMMA (154 ± 4;—△—; Panel B) and by 0.1mM AG (153 ± 3;···○···; Panel B). Results are based on the mean ± SE of triplicate cultures and represent the results of at least three experiments.

would enhance the evidence gathered indicating that induction of iNOS is necessary for the 2-signal dependent progenitor response to LPS and TNF_{α}. Figure 25 demonstrates the efficacy of L-canavanine in *in vitro* inhibition of the 2-signal dependent progenitor response to 0.1 ng/ml LPS. Inhibition was optimal at 0.5mM L-canavanine.

The D-enantiomer of N^{G}MMA, N^{G}-monomethyl-D-arginine, has no effect on LPS costimulation of 2-signal dependent response. To ascertain that inhibition of the 2-signal dependent progenitor response was a function of analogues of only the L-enantiomer of arginine, N^{G}-monomethyl-D-arginine (N^{G}MDA) as opposed to the L-enantiomer inhibitor N^{G}MMA was added to soft agar cultures containing adherent cell depleted marrow, 750 U/ml M-CSF, and 0.1 ng/ml LPS. Figure 26 illustrates the inability of N^{G}MDA to inhibit LPS costimulation of 2-signal dependent progenitor proliferation. N^{G}MMA at 0.1mM effectively abrogated LPS costimulation of 2-signal dependent progenitor proliferation (750 U/ml M-CSF, 135 ± 1; M-CSF + 0.1 ng/ml LPS, 171 ± 5; M-CSF + LPS + N^{G}MMA, 134 ± 5) in identical *in vitro* cultures.

IL-1 can replace LPS and TNF_{α} as a costimulator of 2-signal dependent proliferation. IL-1 mediated costimulation is compatible with the cGMP pathway and is inhibited by the 30kDa monokine. Another biological response modifier reported to induce the synthesis of NO is interleukin 1 (IL-1; 29, 87). IL-1 is also implicated in the induction of TNF_{α} and vice versa (1). These reported effects of IL-1 intimate the biological modifier may function similarly to TNF_{α} and LPS in the soft agar assay system. Addition of IL-1 to soft agar cultures of adherent cell depleted marrow resulted in augmented colony formation in response to M-CSF (Figure 27, Panel A). The augmented colony response was optimal at 100 U IL-1 ($p \leq 0.05$), was compatible with LPS-mediated costimulation of 2-signal dependent progenitor

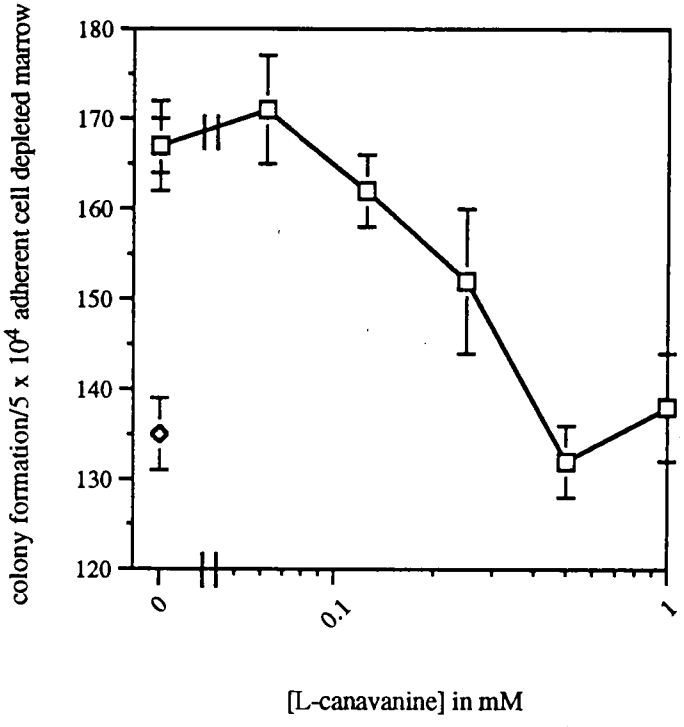


Figure 25: L-canavanine abrogates the 2-signal dependent progenitor response to 750 U/ml M-CSF and 0.1 ng/ml LPS (-□-). Basal, M-CSF responsive colony formation was 135 ± 4 (◆); 2-signal dependent response was 165 ± 3 (○). L-canavanine had no effect on basal, M-CSF responsive colony formation (138 ± 6 at 1mM L-canavanine). Results are presented as the mean ± SE of triplicate cultures and are representative of the results from at least three experiments.

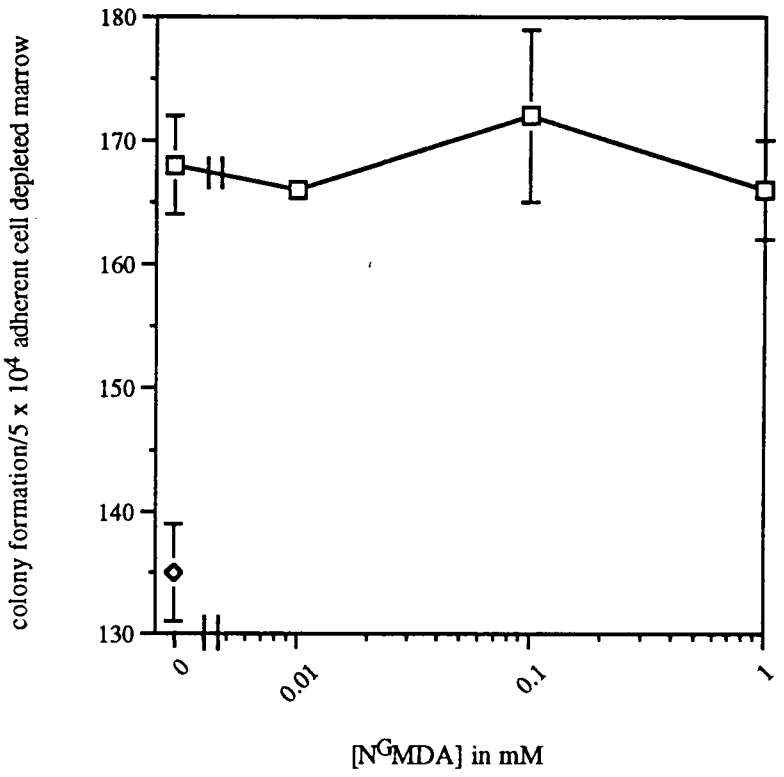


Figure 26: The D-enantiomer of N^{G}MMA, N^{G}MDA, has no effect on 2-signal dependent progenitor proliferation stimulated by 750 U/ml M-CSF and 0.1 ng/ml LPS (–□–). Basal, M-CSF responsive colony formation was 135 ± 4 (◆); 2-signal dependent response was 168 ± 4. Results are presented as the mean ± SE and are representative of the results from at least three experiments.

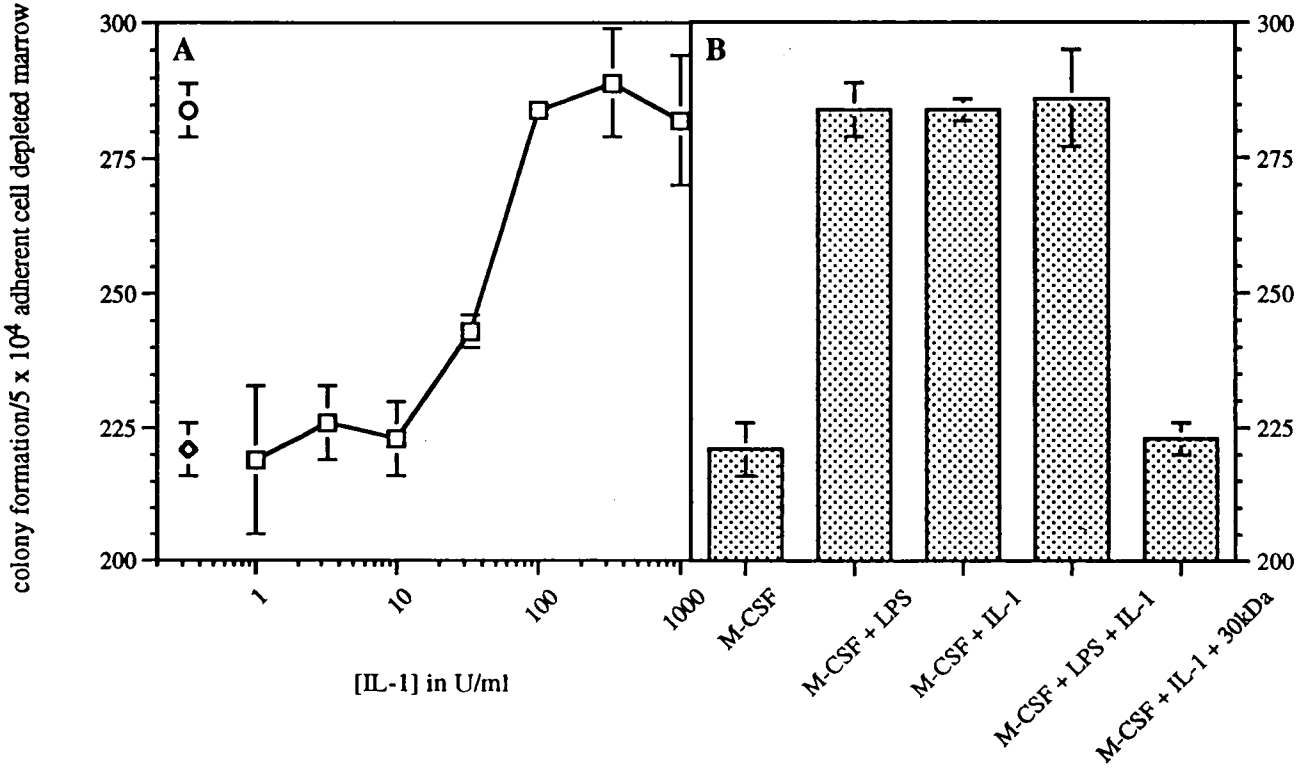


Figure 27: IL-1 enhances colony formation in response to 750 U/ml M-CSF (-□- ; Panel A). Augmentation of colony formation by IL-1 is compatible with LPS-mediated costimulation and is abrogated by the 30kDa monokine (Panel B). Basal, M-CSF responsive colony formation was 221 ± 5 (◇); 2-signal dependent response to 0.1 ng/ml LPS was 284 ± 5 (○). Results are the mean ± SE of triplicate and are representative of the results from at least three separate experiments.

proliferation, and was abrogated by the addition of the 30kDa monokine (Figure 27, Panel B).

NO-generating compounds can serve as costimulators of the 2-signal response. While abrogation of LPS and TNF_α costimulation in soft agar cultures by the NOS inhibitors N^GMMA, AG and L-canavanine and reversal of the NOS inhibitor-mediated abrogation by L-arginine are clear indications of NO involvement in the proliferative pathway, they are indirect. To test directly for involvement of NO in the mediation of LPS or TNF_α costimulation, two NO-generating compounds, sodium nitroprusside (SNP) and S-nitroso-N-acetyl-penicillamine (SNAP), were used in the soft agar assay system. Data shown in Figure 28 illustrate both SNP and SNAP promote 2-signal dependent progenitor proliferation in the presence of 750 U/ml M-CSF. Maximal costimulation of 2-signal dependent progenitor proliferation was observed at 0.125-0.0625mM SNP ($p \leq 0.05$) and 0.031-0.016mM SNAP ($p \leq 0.05$). Both NO-generating compounds were inhibitory for all colony formation at concentrations greater than 0.5mM SNP and 0.125mM SNAP.

Costimulation of 2-signal dependent proliferation by SNP or SNAP is compatible with the cGMP pathway. The cyclic nucleotide compatibility of SNP- or SNAP-mediated costimulation of 2-signal dependent progenitor proliferation in the presence of 750 U/ml M-CSF was tested and the data are provided in Table 7. As expected based on data indicating NO involvement in LPS and TNF_α costimulation, NO-augmented proliferation of 2-signal dependent progenitors was compatible with cGMP-mediated processes, but incompatible with cAMP and cAMP compatible NT.

The 14-mer peptide, AKPR, and the 30kDa monokine inhibit SNP-mediated costimulation. The influence of the 14-mer peptide, AKPR, and the 30kDa monokine on NO-mediated costimulation of 2-signal dependent progenitors was evaluated in soft agar culture using SNP as the NO-generating compound. Dose response curves for

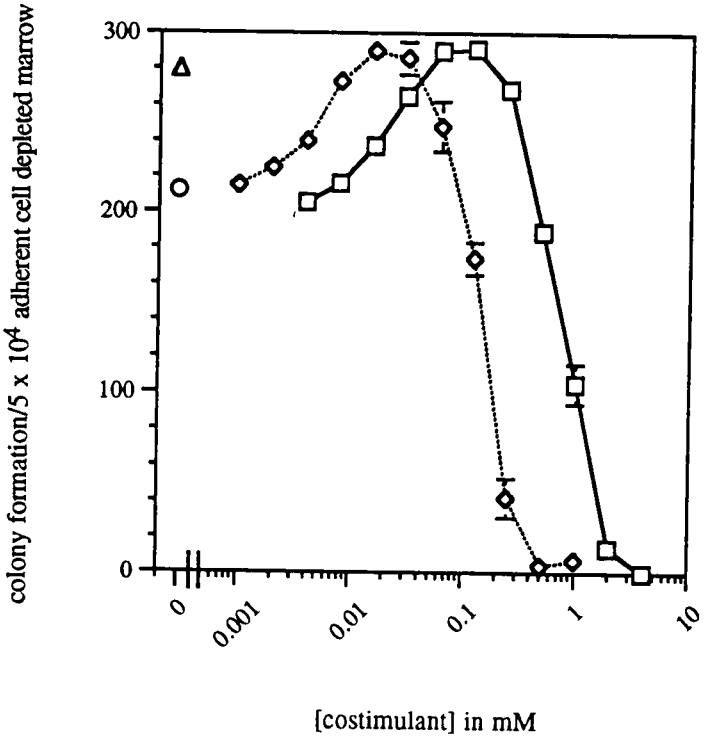


Figure 28: SNP (—□—) and SNAP (···◇···) are costimulants of 2-signal dependent progenitor proliferation in the presence of 750 U/ml M-CSF. Basal, M-CSF responsive colony formation was 212 ± 4 (○); 2-signal response was 280 ± 5 (△). Results are reported as the mean ± SE for triplicate cultures and are representative of the values derived from at least three separate experiments.

Table 7: Cyclic nucleotide compatibility of 2-signal dependent progenitor proliferation costimulated by SNP.

	Colony response ± SE	
costimulant	none	+ SNP
none	220 ± 1	284 ± 11[a]
LPS	275 ± 5[a]	280 ± 7[a]
3':5' cGMP	283 ± 15[a]	294 ± 3[a]
3':5' cAMP	273 ± 5[a]	214 ± 4[b]
TNF_α	283 ± 7[a]	290 ± 4[a]
ANP	277 ± 4[a]	282 ± 4[a]
SP	277 ± 8[a]	288 ± 6[a]
NT	274 ± 6[a]	220 ± 2[b]

Colony response represents the number of colonies formed from 5 x 10^4 adherent depleted marrow cells/ml in response to 750 U/ml M-CSF ± costimulants. LPS, SNP, and TNF_α were added to marrow cultures at a final concentration equal to 0.1 ng/ml, 0.0625mM, and 33 U/ml, respectively. SP was added to cultures at 10^{-6}M; all other costimulants were added at 10^{-9}M. Data are representative of triplicate cultures from at least three separate experiments.

[a]Significantly greater than M-CSF control at $p \leq 0.05$.

[b]Not significantly different from M-CSF control.

inhibition of 2-signal dependent colony formation are given in Figure 29. Panel A depicts inhibition of NO-mediated augmentation by 14-mer peptide and AKPR. Inhibition was maximal at 10^{-9}M for both inhibitory peptides. Basal, M-CSF responsive colony formation was not affected by either peptide inhibitor. Panel B represents the inhibitory effect of the 30kDa monokine. Maximal inhibition was observed at a 1:100,000 dilution of the monokine. Again, basal, M-CSF responsive colony formation was not affected by the inhibitor. A similar dose response experiment was performed with an unrelated tetrapeptide, Gly-Pro-Gly-Gly (GPGG). This tetrapeptide did not inhibit 2-signal dependent progenitor proliferation in response to 0.1 ng/ml LPS or 0.0625mM SNP at concentrations ranging from 10^{-6}M to 10^{-12}M (data not shown). The inhibition by ferritin of 2-signal dependent progenitor proliferation costimulated with SNP was evaluated in total and adherent cell depleted marrow cultures. Ferritin at 10^{-9}M inhibited 2-signal dependent progenitor proliferation in response to 0.1 ng/ml LPS and 750 U/ml M-CSF or 0.0625mM SNP and M-CSF in total cell cultures but not in cultures of adherent cell depleted marrow in contrast to 14-mer-mediated inhibition which was active in both culture systems (Table 8).

NO is generated in adherent cell depleted cultures treated with LPS, LPS plus TNF_α, LPS plus IFN_γ, and TNF_α plus IFN_γ as measured by culture nitrite levels. The soft agar data reported above indicated the inhibitory action of 14-mer, AKPR, the 30kDa monokine, and, in total marrow cultures, ferritin probably are effected post-NO generation. To determine conclusively if the inhibitors influence NOS-dependent generation of NO, a simple chemical assay was performed. The production of NO can be detected in liquid culture by a simple chemical reaction measuring the concentration of nitrite, a stable endproduct of NO synthesis, in the culture supernatant (122). The detection of nitrite in culture supernatant is based on the Griess reaction,

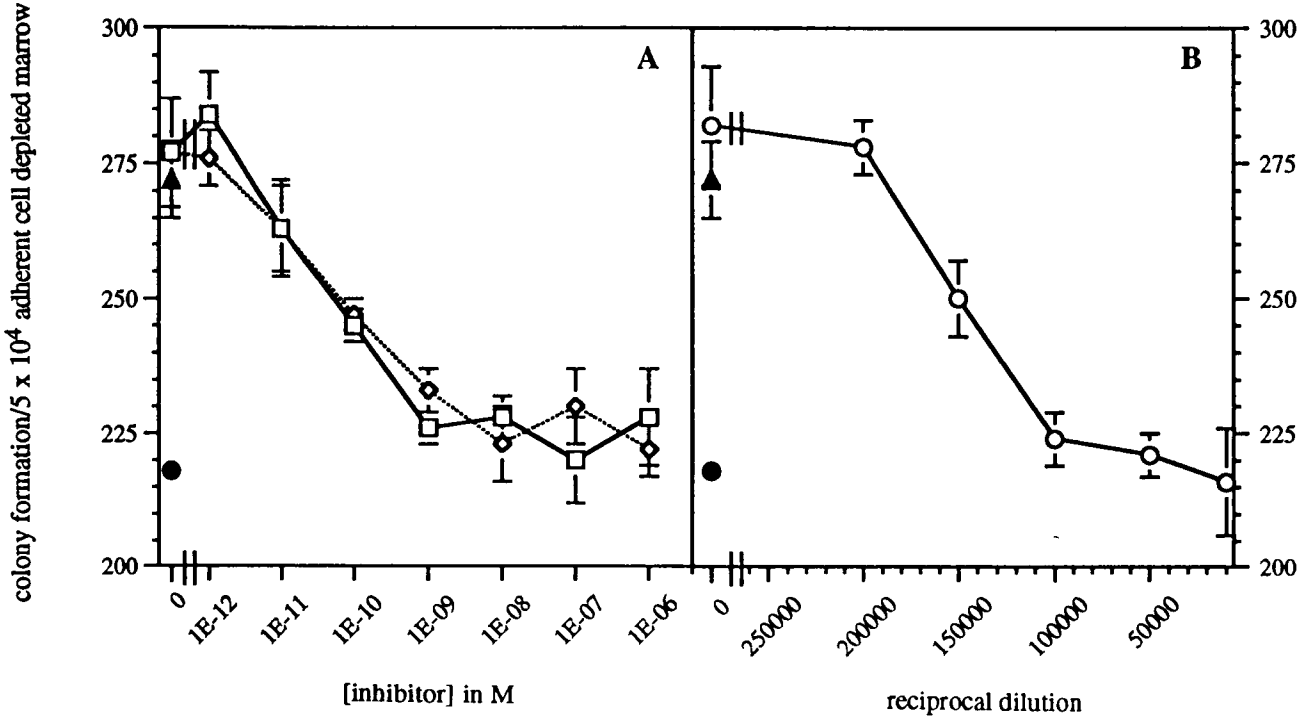


Figure 29: The 14-mer (Panel A;—□—), AKPR (Panel A;--◇--) and the 30kDa monokine (Panel B;—○—) abrogate costimulation of 2-signal dependent proliferation mediated by 0.0625mM SNP in the presence of 750 U/ml M-CSF. Basal, M-CSF responsive colony formation was 218 ± 1 (●); 2-signal response to 0.1 ng/ml LPS was 272 ± 7 (▲). None of the inhibitors affected basal, M-CSF responsive colony formation even at the highest concentration tested (10^{-6}M AKPR, 220 ± 3; 10^{-6}M 14-mer, 217 ± 2; 1/10,000 dilution of 30kDa monokine, 216 ± 3). Results are reported as the mean ± SE for triplicate cultures and are representative of at least three separate experiments.

Table 8: Inhibition of 2-signal dependent progenitor proliferation costimulated by SNP in response to ferritin.

	Total marrow cultures			Adherent cell depleted marrow cultures		
treatment	none	+ferritin	+14-mer	none	+ferritin	+14-mer
M-CSF	179 ± 2	180 ± 1[b]	180 ± 3[b]	188 ± 5	178 ± 7[b]	179 ± 2[b]
M-CSF + LPS	247 ± 7[a]	180 ± 3[b]	184 ± 1[b]	235 ± 1[a]	230 ± 7[a]	178 ± 5[b]
M-CSF + SNP	254 ± 7[a]	198 ± 2[b]	193 ± 3[b]	240 ± 3[a]	240 ± 5[a]	186 ± 4[b]

Marrow cells were plated at 5 x 10^4 cells/ml. M-CSF, LPS, and SNP were added at final concentrations of 750 U/ml, 0.1 ng/ml, and 0.0625mM, respectively. Ferritin and 14-mer were added at 10^{-9}M. Data represent mean colony formation ± SE. Results are representative of triplicate cultures and illustrate data derived from at least three separate experiments.

[a]Significantly greater than M-CSF control at $p \leq 0.05$.

[b]Not significantly different from M-CSF control.

i.e. the formation of aromatic diazonium salts from primary aromatic amines and nitrous acid or other nitrosating agents:

$$2ArNH_2 + N_2O_3 + 2HNO_3 + H_2O \longrightarrow 2ArN_2^+.NO_3^- + 4H_2O$$

This assay was used to measure nitrite levels in liquid cultures of adherent cell depleted marrow. The increased presence of nitrite in culture supernatants from adherent cell depleted marrow would provide further evidence for the involvement of NO in the 2-signal dependent progenitor proliferative pathway. To this end, adherent cell depleted marrow was cultured with increasing concentrations of LPS and the supernatant medium collected 48h later was assayed for nitrite (Figure 30). LPS at 1 and 10 ng/well failed to stimulate nitrite levels above unstimulated controls. LPS at 100 ng/well stimulated a slight but significant ($p \leq 0.05$) increase in culture nitrite accumulation. IFNγ, the classical macrophage priming agent, enhances NO production stimulated by LPS (1). Therefore in an attempt to augment NO production, IFNγ was added to the cultures in addition to LPS (Figure 30). The addition of IFNγ to these cultures enhanced nitrite accumulation stimulated by LPS ($p \leq 0.05$). Optimal nitrite accumulation was detected at 100 ng/well LPS plus 5 U IFNγ. IFNγ by itself was not capable of inducing nitrite accumulation. Similar assays were prepared to determine if TNF$_\alpha$ with or without LPS or IFNγ was capable of inducing nitrite accumulation in culture supernatants from adherent cell depleted marrow. Increasing concentrations of TNF$_\alpha$ did not stimulate the accumulation of detectable levels of nitrite in culture medium (Figure 31, Panel A and B). In the presence of IFNγ, however, TNF$_\alpha$ did induce nitrite accumulation in culture supernatants (Figure 31, Panel A; $p \leq 0.05$). As shown in Figure 30, 100 ng/well LPS stimulated nitrite accumulation in cultures of adherent cell depleted marrow. Combination of LPS with

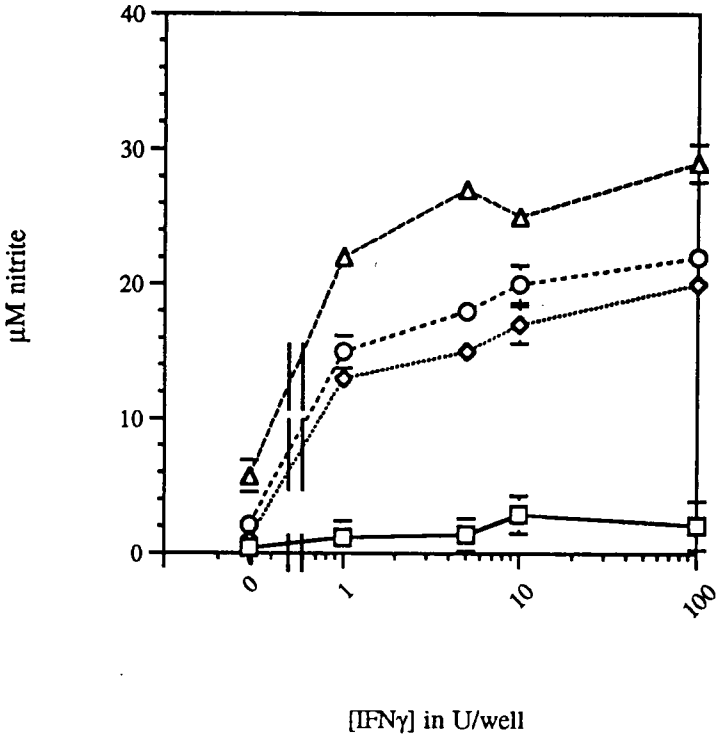


Figure 30: Adherent cell depleted marrow stimulated with LPS and IFNγ produces NO as measured by the presence of nitrite in culture supernatants. IFNγ was not capable of stimulating the accumulation of nitrite in culture supernatant (–□–), whereas 100 ng/well LPS stimulates nitrite accumulation in treated cultures (–Δ–). IFNγ does enhance nitrite accumulation stimulated by LPS in cultures treated with 100 (–Δ–), 10 (··O··), and 1 ng/well (–◇–).

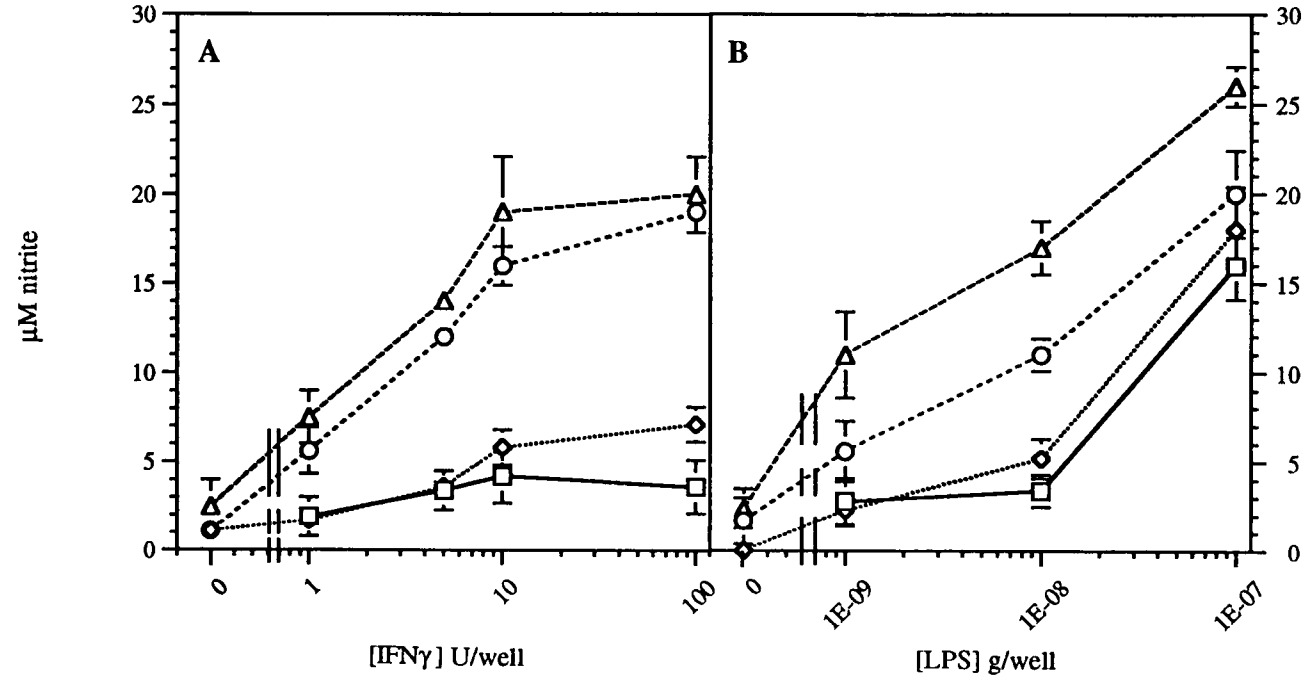


Figure 31: NO is produced by adherent cell depleted marrow in response to TNFα in combination with IFNγ or LPS. NO production was measured as a function of culture nitrite accumulation; nitrite accumulation in response to TNFα was minimal (<2.5 ± 1.5). Addition of IFNγ resulted in enhanced nitrite accumulation at each TNFα concentration tested (Panel A; 10 U, -◇-; 100 U, -○-; 1,000 U, -△-). IFNγ, alone, did not stimulate the accumulation of nitrite (Panel A; □). Addition of LPS to cultures stimulated nitrite accumulation maximally at 10^{-7} g/well (□); this nitrite accumulation was enhanced in cultures treated with 10 (-◇-), 100 (-○-) and 1,000 U (-△-) TNFα simultaneously (Panel B).

TNF_α enhanced nitrite accumulation to levels greater than either stimulant alone (Figure 31, Panel B; $p \leq 0.05$).

The J774A.1 cell line, upon stimulation, produces NO in a manner similar to adherent cell depleted marrow. To confirm the results generated with the NO assay system were not artifactual, a monocyte-macrophage cell line, J774A.1, known to produce NO upon stimulation with LPS was substituted for adherent cell depleted marrow (6). The results recorded in Table 9 illustrate the NO assay system adopted for these procedures detected culture nitrite levels in accord with cell stimulation. These results also demonstrate the similarity in nitrite accumulation profiles generated from adherent cell depleted marrow with those from J774A.1 cells.

Anti-TNF_α antibody decreases NO production in adherent cell depleted marrow cultures treated with LPS, LPS plus TNF_α, and TNF_α plus IFN_γ. The effects of polyclonal rabbit anti-mouse TNF_α antiserum on nitrite accumulation in liquid cultures of adherent cell depleted marrow and J774A.1 cells were tested. Figure 32, Panel A shows the effects of anti-TNF_α antibodies on nitrite accumulation stimulated from adherent cell depleted marrow cultures by 100 ng/well LPS, 1 U IFN_γ plus 1,000 U TNF_α, IFN_γ plus LPS, and LPS plus TNF_α. Nitrite accumulation stimulated by LPS was diminished by anti-TNF_α antibodies from 14.8 ± 0.4 μM nitrite to 4 μM ($p \leq 0.05$) while that stimulated with LPS plus IFN_γ was unaffected. Nitrite levels in adherent cell depleted marrow cultures treated with TNF_α plus IFN_γ or TNF_α plus LPS were also diminished by the addition of anti-TNF_α antibodies (Figure 32, Panel A; $p \leq 0.05$). Addition of anti-TNF_α antibodies to cultures of J774A.1 cells only slightly diminished nitrite accumulation in response to 100 ng/well LPS (26.2 ± 2.5 μM to 21.5 ± 2.5 μM) but completely abrogated the response to IFN_γ and TNF_α (32.5 ± 2.2 μM to 3.3 ± 0 μM, $p \leq 0.05$; Figure 32, Panel B).

Table 9: NO production stimulated from J774A.1 cells treated with IFNγ, TNF_α, LPS or a combination of stimulants.

Treatment	μM Nitrite
None	0 ± 0
1 U IFNγ	1 ± 1
333 U TNF_α	0 ± 0
100 ng/well LPS	48.2 ± 1.7^a
IFNγ + TNF_α	28.4 ± 1^a
IFNγ + LPS	63 ± 1.6^a
LPS + TNF_α	49.6 ± 1.2^a

Nitrite determinations were conducted on 100 μl supernatant generated from cultures of 7.5×10^4 J774A.1 cells. Cultures were incubated with stimulants for 48h prior to determination. Results are reported as the mean ± SE of triplicate cultures and are representative of results generated in at least three separate experiments.

[a]Significantly greater than medium alone control at $p \leq 0.05$.

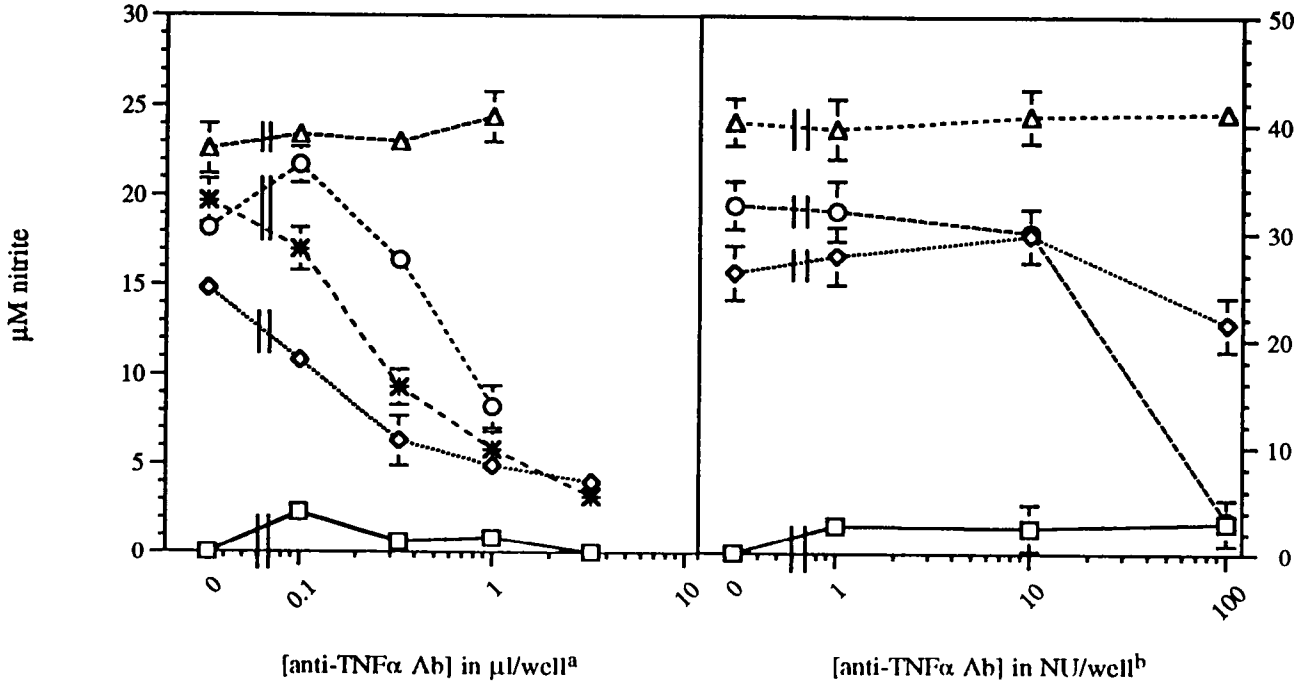


Figure 32: Anti-TNF_α antibodies affect nitrite accumulation in adherent cell depleted marrow (Panel A) and J774A.1 (Panel B) cultures treated with 100 ng/well LPS (--◇--), 1 U IFN_γ plus 1000 U TNF_α (--○--), IFN_γ plus LPS (--△--), and LPS plus TNF_α (--✱--). Anti-TNF_α polyclonal antibodies did not affect basal, unstimulated nitrite accumulation in either culture type (—□—). IFN_γ or TNF_α, alone, did not stimulate the accumulation of nitrite (data not shown). Results are reported as the mean ± SE of triplicate cultures and are representative of at least three separate experiments.

[a]One microliter of antiserum neutralizes 1000 U TNF_α.

[b]One neutralizing unit (NU) of antibody blocks the activity of 1-10 U TNF_α.

Anti-IFNγ antibody abrogates NO production by J774A.1 cells stimulated with IFNγ plus TNF$_\alpha$. Treatment of J774A.1 cultures with polyclonal rabbit anti-mouse IFNγ antiserum completely abrogated nitrite accumulation stimulated by 1 U IFNγ and 333 U TNF$_\alpha$ ($p \leq 0.05$), but had only a minimal effect on accumulation induced by 100 ng/well LPS plus 1 U IFNγ (Figure 33). No inhibitory effect of anti-IFNγ antibodies was detected in cultures treated with LPS or LPS plus TNF$_\alpha$, in fact, a slight increase in nitrite accumulation was evidenced in both of these treatment groups (Figure 33).

The 14-mer peptide and AKPR have no effect on NO production by cultures of adherent cell depleted marrow. Finally, NO assays were performed to determine if the 14-mer peptide or AKPR influenced culture nitrite accumulation. Figure 34, Panel A illustrates the effect of 14-mer on nitrite accumulation stimulated from cultues of adherent cell depleted marrow. No change in nitrite accumulation was detected in cultures treated with 100 ng/well LPS, 100 U IFNγ plus LPS, IFNγ plus 1,000 U TNF$_\alpha$, or LPS plus TNF$_\alpha$ at concentrations of 14-mer tested. Adherent cell depleted marrow cultures treated with IFNγ, TNF$_\alpha$, or 14-mer peptide alone did not produce detectable levels of nitrite. Panel B of Figure 34 depicts the results of a similar NO assay evaluating the effects of AKPR on culture nitrite accumulation. Cultures of adherent cell depleted marrow stimulated with 100 ng/well LPS, 100 U IFNγ plus LPS, IFNγ plus 1,000 U TNF$_\alpha$, or LPS plus TNF$_\alpha$ produced detectable levels of nitrite. Addition of AKPR to these cultures did not reduce nitrite accumulation. Cultures treated with IFNγ, TNF$_\alpha$, or AKPR alone did not produce detectable levels of nitrite.

Exogenous L-arginine does not reverse AKPR-mediated inhibition of 2-signal dependent proliferation costimulated by LPS or TNF$_\alpha$. These results indicated the inhibitor did not influence induced NO production. As an additional test, increasing

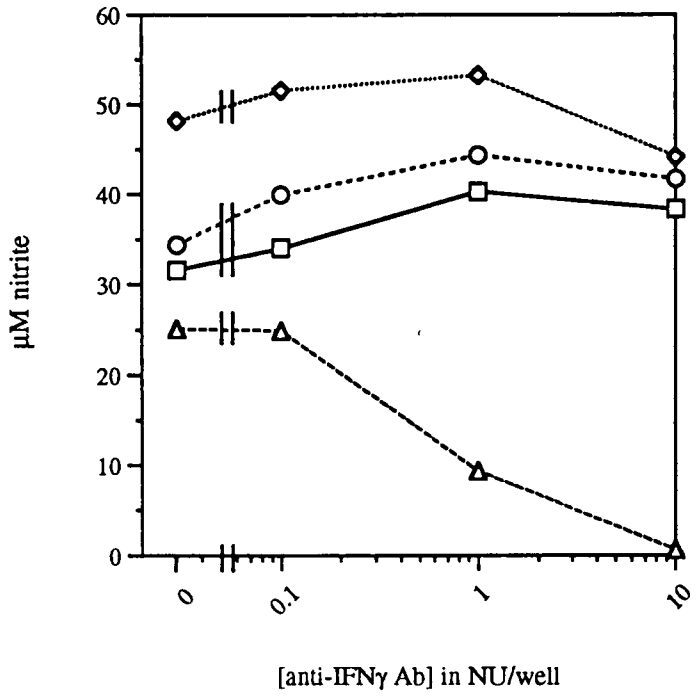


Figure 33: Anti-IFNγ antibodies differentially affect nitrite accumulation in J774A.1 cultures stimulated with 100 ng/well LPS (-□-), LPS + 1 U IFNγ (--◇--), IFNγ + 333 U TNFα (-△-), and LPS + TNFα (--○--). Nitrite accumulation was not detected in cultures treated with antibody, IFNγ, or TNFα (data not shown). Results are reported as the mean ± SE and are representative of values derived from at least three experiments.

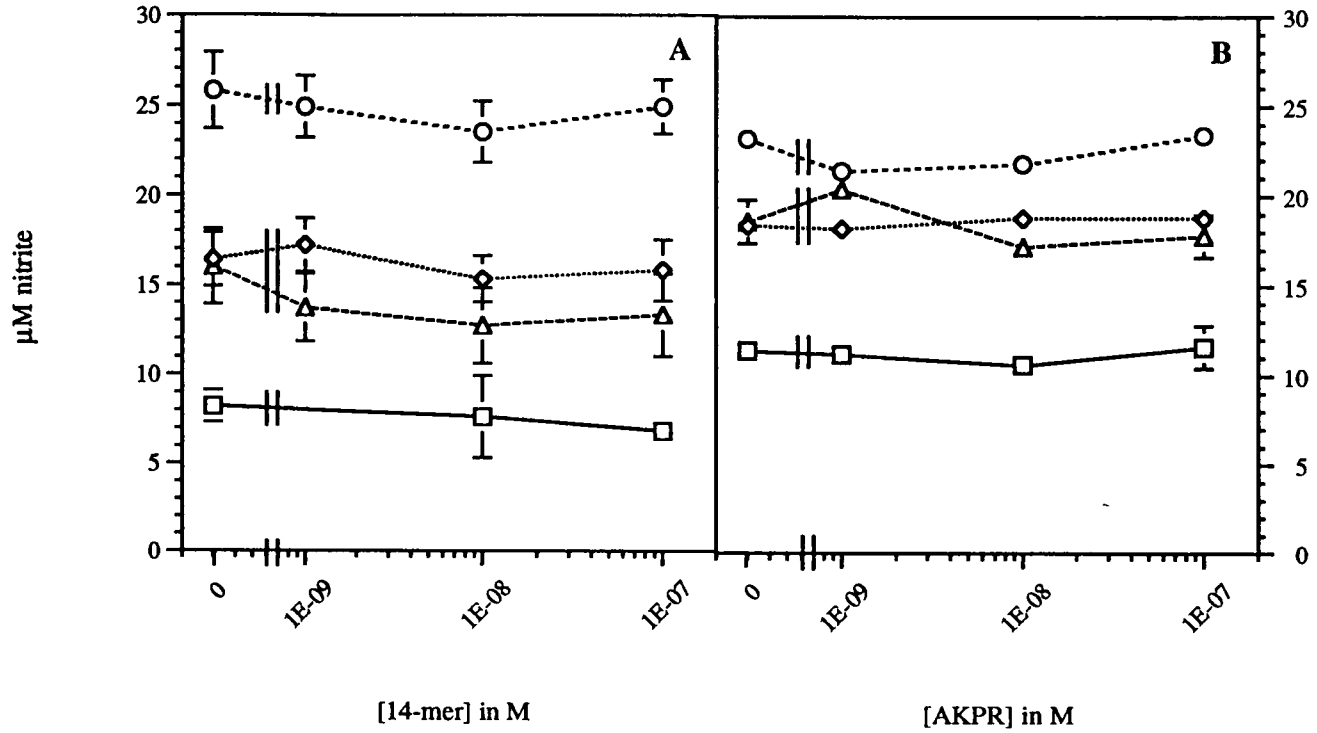


Figure 34: Nitrite accumulation in cultures of adherent cell depleted marrow is not affected by 14-mer (Panel A) or AKPR (Panel B). Cultures were stimulated with 100 ng/well LPS (—□—), 100 U IFNγ plus LPS (··○··), IFNγ plus 1,000 U TNF_α (--◇--), or LPS plus TNF_α (--△--). Cultures treated with 14-mer, AKPR, IFNγ, or TNF_α alone did not generate detectable levels of nitrite. Data represent the mean ± SE of triplicate cultures.

concentrations of L-arginine were added to soft agar cultures of adherent cell depleted marrow to determine if AKPR-mediated inhibition of LPS or TNF_α induced 2-signal dependent progenitor proliferation was reversible in a manner consistent with NOS inhibition (Figure 35). As can be seen, concentrations of L-arginine ranging from 0.125mM to 4mM were not able to reverse AKPR-mediated inhibition of either LPS or TNF_α costimulated 2-signal dependent progenitor proliferation. The addition of L-arginine to cultures stimulated with M-CSF alone or in combination with either LPS or TNF_α had no effect on basal or 2-signal dependent progenitor proliferation.

To summarize the information reported to this point, proliferation of 2-signal dependent progenitors can be stimulated via two identifiable pathways, a cGMP-compatible pathway and a cAMP-compatible one. The cGMP-compatible pathway is effected via a variety of costimulants including LPS. All of the costimulants of 2-signal dependent progenitor proliferation compatible with cGMP-mediated mechanisms can be linked to intermediate signalling by TNF_α through the abrogation of the response by specific anti-TNF_α antibodies. Additionally, increased production of TNF_α-specific mRNA transcript by adherent cell depleted marrow can be detected by RT-PCR in response to LPS stimulation. The cGMP-compatible proliferative pathway can be further traced to the production of NO. The identification of NO as an integral component in the cGMP-compatible pathway derived from assays recording the inhibition of proliferation by the NOS inhibitors, N^GMMA, AG and L-canavanine, the reversal of this inhibition by L-arginine, detection of nitrite in liquid cultures of adherent cell depleted marrow stimulated with LPS and TNF_α, and the ability of NO-generators to stimulate the proliferation of 2-signal dependent progenitors in the presence of M-CSF.

An inhibitor specific for the cGMP-compatible proliferative pathway has also been identified. A 30kDa monokine has been isolated that inhibits 2-signal dependent

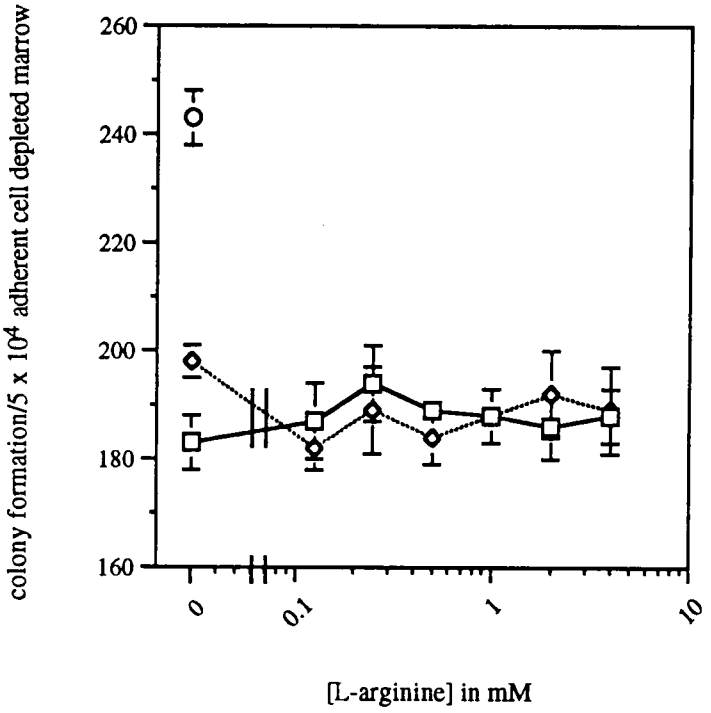


Figure 35: L-Arginine is not able to reverse AKPR-mediated inhibition of 2-signal dependent progenitor proliferation costimulated by 0.1 ng/ml LPS (—□—) or 33 U/ml TNF_α (—◇—). Two-signal dependent progenitor response to 750 U/ml M-CSF plus costimulant was 243 ± 5 (O). AKPR was added to cultures at 10^{-9}M. Addition of 4mM L-arginine to cultures stimulated with M-CSF or M-CSF plus LPS or TNF_α did not affect colony formation (189 ± 3, 245 ± 6, and 241 ± 7 colony forming units, respectively). Data represent the mean ± SE of triplicate cultures.

progenitors proliferating in response to cGMP-compatible costimulants plus M-CSF. In addition, the inhibitory effect of this monokine is mimicked by both the 14-mer peptide and AKPR. Through soft agar assays and NO assays, the action of these inhibitors can be traced to a point in the proliferative pathway post-NO production, but prior to cGMP elevation as 2-signal dependent progenitor proliferation can be abrogated by the inhibitors at each stage, including NO production, however, proliferation costimulated by the addition of cGMP is not affected by the inhibitors.

LPS-induced increases in adherent cell depleted marrow iNOS-specific mRNA were not detectable by RT-PCR. The RT-PCR data showed that 0.1 ng/ml LPS is capable of inducing increased levels of TNF_{α}-specific mRNA transcript. RT-PCR experiments demonstrating the ability of LPS to stimulate the production of iNOS mRNA were unsuccessful. Total RNA samples isolated from adherent cell depleted marrow cultures 1-48h post-stimulation with LPS, M-CSF, or M-CSF plus LPS did not contain message for iNOS detectable by the methods used for TNF_{α} message detection. Control cultures of J774A.1 cells stimulated with 0.1 ng/ml LPS did contain detectable levels of iNOS message and NO assays designed to determine the time course of culture nitrite accumulation demonstrated that adherent cell depleted marrow stimulated with 100 ng LPS produced detectable culture nitrite between 24-48h post-stimulation (24h/0.8μM vs. 48h/15μM, $p \leq 0.05$). Due to the inability to detect iNOS message in adherent cell depleted marrow cultures, several hypotheses of NO involvement in 2-signal dependent progenitor proliferation were proposed. NO may only be required at a minute concentration capable of autocrine stimulation. Therefore, levels of iNOS message in the adherent cell depleted marrow cultures may be too scant for detection by the means utilized. The production of NO might only be necessary for synergistic action with TNF_{α}, the pathway mediator characterized to effect its function(s) via the induction of iNOS.

Suboptimal levels of SNP reverse N^GMMA mediated inhibition of TNF_α costimulated 2-signal response. To test the latter possibility, TNF_α costimulation of 2-signal dependent progenitor proliferation was abrogated by the addition of N^GMMA, the NOS inhibitor. Suboptimal levels of SNP were titrated into the soft agar culture system. The results depicted in Figure 36 demonstrate that suboptimal levels of SNP were sufficient to reverse N^GMMA inhibition of 2-signal dependent progenitor proliferation in response to TNF_α ($p \leq 0.05$). Addition of N^GMMA to cultures of adherent cell depleted marrow had no effect on basal, M-CSF responsive proliferation or on SNP augmentation of M-CSF responsive proliferation. Reversal of N^GMMA-mediated abrogation of 2-signal dependent progenitor response to TNF_α was complete at 0.015mM SNP. SNP, by itself, stimulates optimal augmentation of M-CSF responsive colony formation at 0.0625mM.

Anti-TNF_α antibody blocks SNP costimulation of 2-signal dependent progenitor proliferation. Polyclonal anti-TNF_α antibodies were added to cultures of adherent cell depleted marrow to determine if TNF_α was operative in the costimulation of colony formation mediated by M-CSF plus SNP. Figure 37 illustrates the ability of TNF_α neutralizing antibodies to inhibit 2-signal dependent progenitor proliferation in response to SNP. The neutralizing antibodies did not affect basal, M-CSF responsive colony formation, but were capable of abrogating the 2-signal dependent progenitor response to 100 U TNF_α ($p \leq 0.05$) or 0.1 ng/ml LPS ($p \leq 0.05$).

Levels of SNP unable to costimulate 2-signal dependent proliferation are costimulatory in the presence of suboptimal concentrations of TNF_α. The final soft agar assay to determine if both SNP and TNF_α are necessary for the induction of 2-signal dependent progenitor proliferation in response to M-CSF used suboptimal concentrations of each costimulant to augment M-CSF responsive colony formation

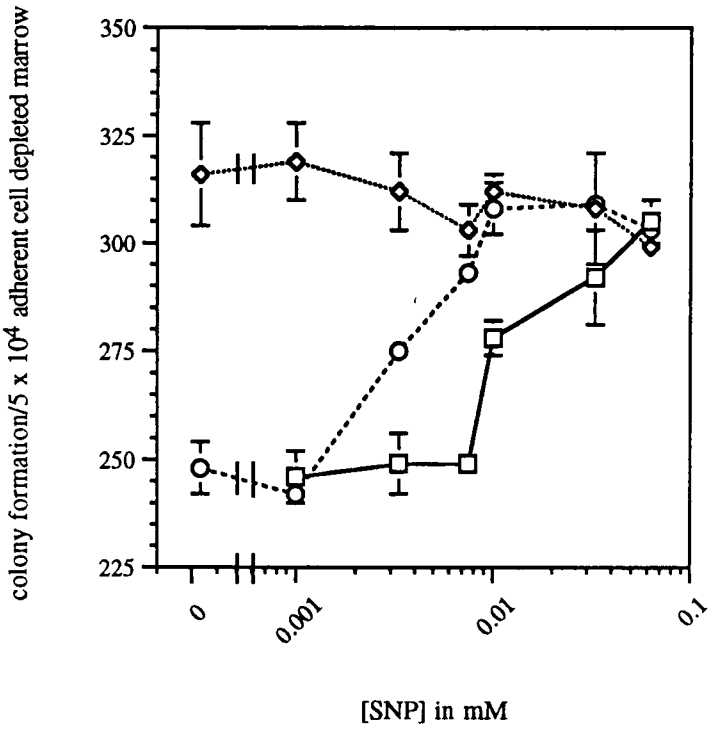


Figure 36: Suboptimal levels of SNP reverse the abrogation of M-CSF and TNF_α-mediated 2-signal dependent progenitor proliferation by 0.1mM N^GMMA (··O··). SNP had no effect on 2-signal dependent progenitor proliferation stimulated by 750 U/ml M-CSF and 100 U TNF_α (--◇--). Optimal 2-signal dependent progenitor proliferation to SNP plus M-CSF was observed at 0.0625mM SNP (-□-). N^GMMA had no effect on basal, M-CSF responsive colony formation (240 ± 5) or on SNP costimulated 2-signal response (311 ± 6). Results are presented as the mean ± SE of triplicate cultures.

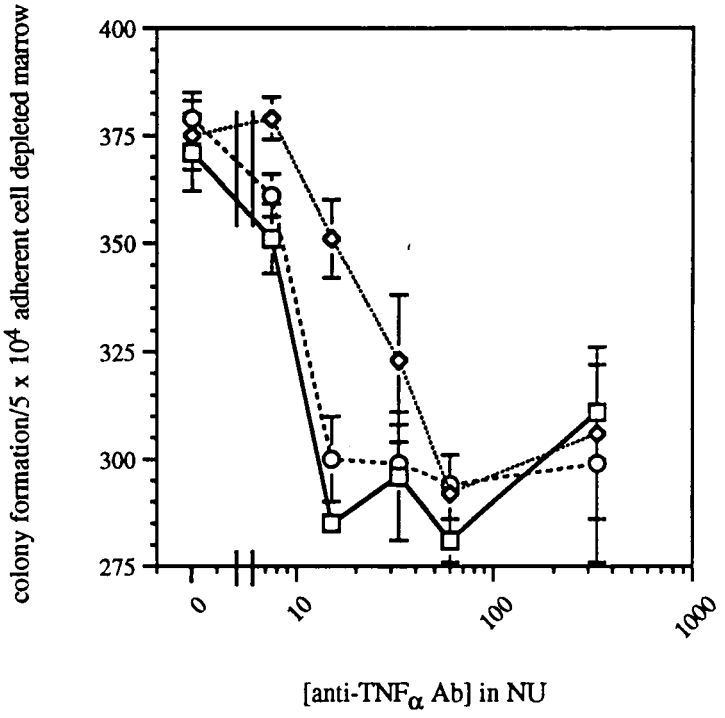


Figure 37: TNF_α neutralizing antibodies abrogate the 2-signal dependent progenitor response to 0.0625mM SNP (—□—). Anti-TNF_α neutralizing antibodies also abrogated the 2-signal dependent progenitor response to 0.1 ng/ml LPS (--○--) and 100 U TNF_α (—◇—). Basal colony formation stimulated by 750 U/ml M-CSF was 302 ± 3. Neutralizing anti-TNF_α antibodies did not affect basal colony formation even at the highest concentration tested, 333 NU (305 ± 2). Data represent the mean ± SE of triplicate cultures.

(Figure 38, Panel A). As illustrated, concentrations of SNP and TNF_α as low as 3.3μM and 1 U, respectively, upon combination were functional as costimulators of M-CSF dependent proliferation of 2-signal progenitors ($p \leq 0.05$). Identical concentrations of either SNP or TNF_α were not able to augment colony formation in response to 500 U/ml M-CSF (Figure 38, Panel B). These data, together, indicate the requirement for NO and TNF_α in the costimulation of 2-signal dependent progenitor proliferation.

Adherent cell depleted marrow produces cGMP in response to the NO-generator, SNP. NO binds to and activates soluble guanylyl cyclase thus stimulating the production of cGMP (93, 121, 122, 129, 190). To determine if adherent cell depleted bone marrow treated with SNP produces detectable amounts of cGMP, an enzyme immunoassay (EIA) was performed. Figure 39 illustrates cGMP production in adherent cell depleted marrow treated for 45 minutes with increasing concentrations of SNP. Concentrations of cGMP greater than those in untreated controls (14 ± 0.4 fmol versus 8.9 ± 0.2 fmol, $p \leq 0.05$) were detected at 10mM SNP.

AKPR, at different concentrations, can either stimulate or inhibit SNP-mediated production of cGMP. The inhibitor of 2-signal dependent progenitor proliferation is effective in inhibiting responses to all costimulants with the exception of cAMP, cAMP compatible NT and cGMP. Experiments with the inhibitor have shown its abrogating effects to occur post-generation of NO, yet prior to stimulation by cGMP. These data suggest the inhibitor may affect soluble guanylyl cyclase. To test this hypothesis, the tetrapeptide, AKPR was added to cultures of adherent cell depleted bone marrow for 45 minutes. Unexpectedly, concentrations of AKPR ranging from 10^{-11}M to 10^{-8}M appeared to effect a minor increase in cGMP in SNP-stimulated adherent cell depleted marrow cells (Figure 40). At 10^{-7}M however, AKPR effected an obvious decrease in the production of cGMP (from 13 ± 0.4 to 10 ± 0.1

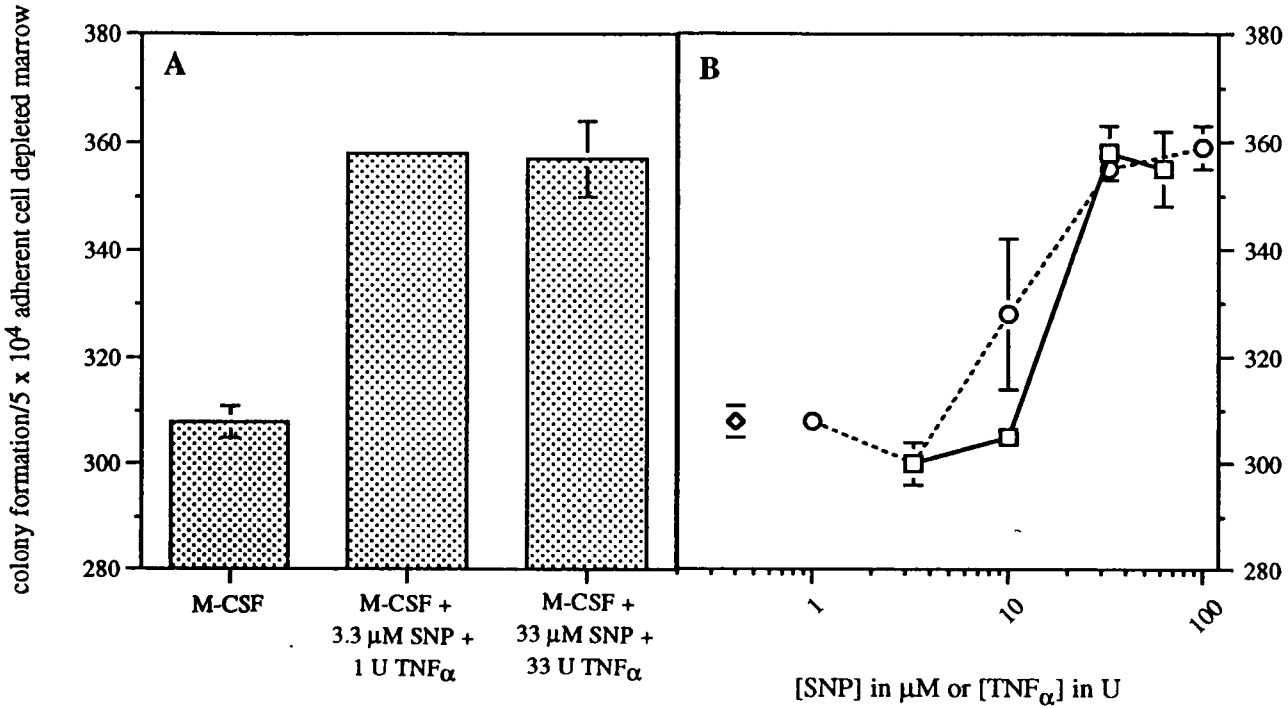


Figure 38: Suboptimal levels of SNP and TNF_α when combined induce augmented colony formation in response to 500 U/ml M-CSF (Panel A). Panel B is a dose response for SNP- (—□—) and TNF_α- (--O--) mediated costimulation of 2-signal dependent progenitor proliferation; optimal response to SNP or TNF_α was observed at 33μM or 33 U, respectively. Basal, M-CSF responsive colony formation was 308 ± 3 (◇).

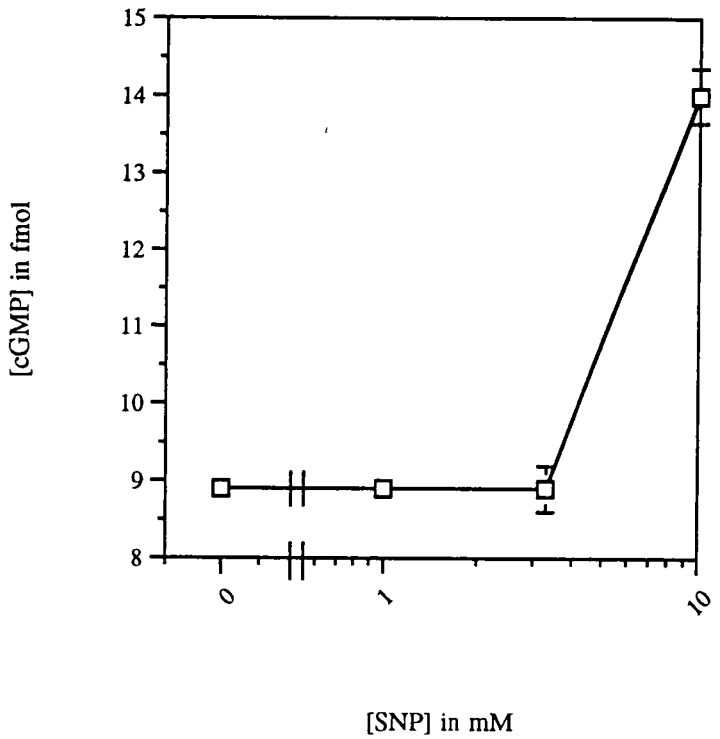


Figure 39: SNP stimulates the production of cGMP from adherent cell depleted marrow. Data represent the mean ± SE of triplicate determinations.

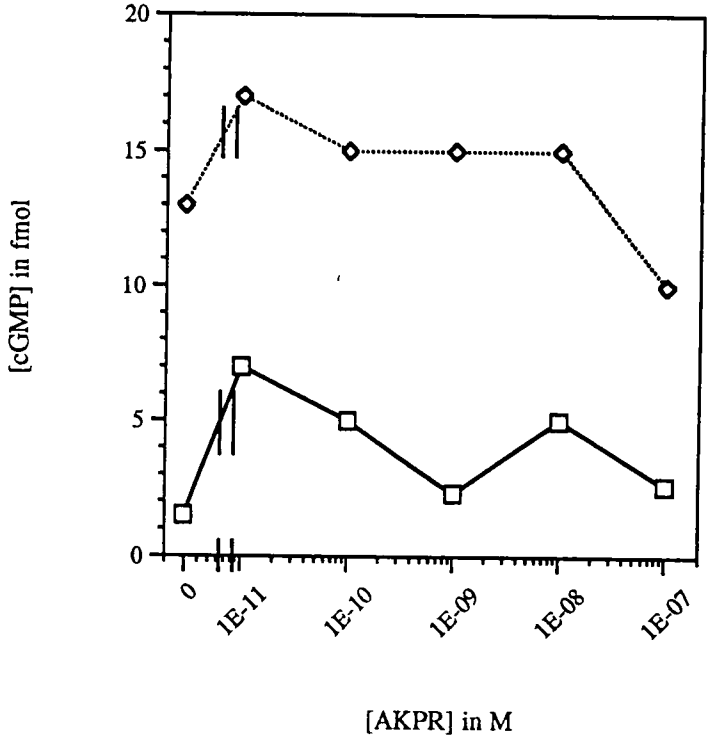


Figure 40: Increasing concentrations of AKPR affect cGMP production by unstimulated (—□—) or SNP-stimulated (—◇—) adherent cell depleted marrow variably. SNP was added to cultures of 4 x 10^6 cells at a final concentration of 10mM. Data represent the mean ± SE of triplicate determinations.

fmol, $p \leq 0.05$). The same concentrations of AKPR affected unstimulated cells as well. At 10^{-11}M, AKPR increased the production of cGMP from unstimulated adherent cell depleted marrow (from 1.5 ± 0.1 to 7 ± 0.1 fmol, $p \leq 0.05$). This increase was diminished at 10^{-10}M and at higher concentrations cGMP concentration fluctuated between the basal level and slight increases.

BALB/c mice with experimentally induced endotoxin shock exhibit increased survival when treated with the 14-mer peptide. Many of the cGMP-compatible costimulants, including LPS, IL-1, TNF_α and NO, are involved in the pathological condition, endotoxin shock (157). In animals with experimentally induced endotoxin shock, serum levels of TNF_α and IL-1 are elevated (170, 194). Evidence exists as well for increases in serum cGMP levels (152). These responses are generally believed to be LPS-mediated. Past studies have indicated the efficacy of iNOS inhibitors in the amelioration of the pathologies associated with endotoxin shock (157). The inhibitor used in this study blocks the actions of LPS, TNF_α, and NO at a point after induction of iNOS. Because these studies have focused on cell responses originally attributed to LPS, *i.e.* the induction of TNF_α and the stimulation of NO production, and the inhibitor blocks these responses, the possibility the inhibitor may block or ameliorate the pathologies associated with endotoxin shock was investigated. The results of an *in vivo* challenge with LPS are reported in Table 10. BALB/c mice were injected intraperitoneally with LPS plus or minus 100 μg 14-mer. At low doses of LPS (100-200 μg), all mice, control and test, survived. LPS at 400 or 800 μg did induce symptoms of shock, including diarrhea, reduced responsiveness to external stimuli, drop in body temperature, paralysis, and death. Mice treated with 14-mer and LPS faired substantially better. At an LPS dose of 400 μg, mice receiving 14-mer intravenously had a survivor rate of 83%. Of those mice receiving a saline injection rather than 14-mer, only 50% survived LPS challenge. The highest dose of LPS

Table 10: The 14-mer peptide increases survival of BALB/c mice with experimentally induced endotoxin shock.

Treatment			
LPS	14-mer	Survivors	% Survival
100 µg	-0-	6/6	100
100 µg	100 µg	6/6	100
200 µg	-0-	6/6	100
200 µg	100 µg	6/6	100
400 µg	-0-	3/6	50
400 µg	100 µg	5/6	83
800 µg	-0-	0/6	0
800 µg	100 µg	3/6	50

Female BALB/c mice at 6 weeks of age were injected intraperitoneally at time 0 with LPS (0.2ml) and intravenously at time 0 with 14-mer or HBSS (0.1ml). Survivorship was determined over a 48h period.

tested, 800 μg, was lethal to all control mice, but to only 50% of the mice receiving the 14-mer peptide.

CHAPTER 4

DISCUSSION

Control of hematopoiesis is an area of intense research interest. While progress has been made towards the understanding of positive and negative regulatory signals involved in early multipotential and growth factor committed progenitor development, the stage just prior to growth factor commitment remains poorly understood, if at all. Commitment of progenitor cells to a specific cell lineage involves the acquisition of lineage-specific growth factor receptors on the progenitor cell surface and subsequent responsiveness to said growth factor (120). What is uncertain, however, is what event(s) triggers the acquisition of growth factor competence by the progenitor cell. In the 1980's a series of reports from Robert N. Moore's laboratory at The University of Tennessee, Knoxville, TN characterized a small population of murine bone marrow progenitor cells that are in a precommitment phase to the monocytic developmental pathway (124). The work presented in the preceding section is a continuation of this work.

The initial indication of the precommitment progenitors occurred when the addition of LPS to bone marrow cultures treated with M-CSF resulted in an 20-50% augmentation of colony formation observed with M-CSF treatment alone (Table 1; 124). This augmentation was repeatable and was not attributable to LPS growth factor-like properties, *i.e.* LPS in the absence of M-CSF did not stimulate colony formation in identical bone marrow cultures. Further investigation of these cells, the 2-signal dependent progenitors, revealed the ability of ferritin to inhibit their proliferation (Table 1). Ferritin is a large iron-binding protein implicated in the inhibition of human and murine myelopoiesis (20). Interestingly, ferritin was capable

of inhibiting 2-signal dependent proliferation only in cultures of total marrow cells; cultures depleted of the adherent cell population were not affected by ferritin (Table 1). The removal of adherent cells from bone marrow cultures did not affect the proliferative capacity of the basal, M-CSF responsive population or the 2-signal dependent one.

The amino acid sequence of ferritin contained a four amino acid moiety in the carboxyl terminus corresponding to an inhibitory analogue of a naturally occurring bioactive peptide, tuftsin (Thr-Lys-Pro-Arg; 31, 47, 48). The inhibitory tuftsin analogue, Ala-Lys-Pro-Arg (AKPR), and the carboxyl fourteen amino acids of ferritin containing the inhibitory analogue, Glu-Thr-Val-Ile-Met-Lys-Ala-Lys-Pro-Arg-Ala-Asn-Phe-Pro, were synthesized. Both of these peptides inhibited augmented colony formation in response to LPS and M-CSF in a manner similar to ferritin in total marrow cultures (Table 1). However in marrow cultures depleted of adherent cells, the peptides, unlike ferritin, remained capable of inhibiting 2-signal dependent proliferation (Table 1). In addition to this contradiction, the original amino acid sequence of ferritin was retracted, and the new sequence no longer contained the AKPR moiety or any of the fourteen amino acids represented by the 14-mer peptide (30). Through a series of experiments (described in detail in references 96 and 97), the inhibitory activity of ferritin was traced to the production of a 30kDa monokine produced by the adherent marrow cell population following exposure to both ferritin and M-CSF. This monokine was immunologically cross-reactive with the 14-mer peptide representing the erroneous carboxyl terminus of ferritin (96, 97) and was inhibitory for 2-signal dependent proliferation in cultures of either total or adherent cell depleted marrow (Table 6).

Tuftsin, a macrophage activator capable of increasing the phagocytosis, antigen processing and presentation, superoxide generation, and bactericidal activity

associated with macrophages (7, 9, 47, 48, 131, 160), was capable of augmenting colony formation in response to M-CSF in a manner similar to LPS (Figure 1). Like LPS-mediated costimulation of 2-signal dependent response, tuftsin augmentation was abrogated by the addition of the inhibitory analogue, AKPR, to cultures of adherent cell depleted marrow (Figure 2).

The addition of two tuftsin-like bioactive peptides, SP (8, 47, 57) and NT (47, 57, 192), to adherent cell depleted marrow cultures produced mixed results. Substance P was effective in augmenting colony formation in response to M-CSF, and this augmentation was blocked by AKPR (Figures 1 and 2). In addition, the portion of SP responsible for costimulation of the 2-signal response corresponded to the first four amino acids, Arg-Pro-Lys-Pro, the tuftsin-like sequence (127). Neurotensin, however, did not augment colony formation in response to M-CSF at concentrations necessary to engage the tuftsin receptor, *i.e.* 10^{-5} to 10^{-7}M (126). At nanomolar concentrations NT did augment colony formation in response to M-CSF, but this augmentation was not affected by the addition of AKPR (Figures 1 and 2). This apparent contradiction was linked to incompatibility of the signals induced by the engagement of NT receptors by NT at 10^{-9}M and engagement of tuftsin receptors by NT at 10^{-7}M (126). Interestingly, activation of the high affinity NT receptor generates an elevation in intracellular levels of cAMP whereas engagement of the tuftsin receptor results in an increase in intracellular cGMP concentrations (57, 119, 160, 179). LPS, like tuftsin and SP, is linked to elevation of intracellular levels of cGMP. Graber *et al.* have demonstrated time-dependent increases in intracellular cGMP concentrations in fetal rat liver and spleen cells treated with LPS (62, 63). This reported cGMP increase parallels increases in the activity of soluble (cytoplasmic) guanylyl cyclase (62).

The incompatibility of cAMP and cGMP systems is widely documented. For example, Cox and Karnovsky reported in 1973 the depression of phagocytosis

associated with cAMP coupled with the stimulation of phagocytosis associated with cGMP (33). The antagonism between cAMP and cGMP in the proliferation of B and T lymphocytes was shown by Diamantstein and Ulmer in 1975 (38). On a more general note, the incompatibility of cAMP with cGMP and *vice versa* was examined and discussed in terms of a Yin-Yang hypothesis by Goldberg *et al.* (56).

Additional studies using the soft agar culture system demonstrated that ANP, LTB_4 and $PGF_{2\alpha}$ were also able to costimulate 2-signal dependent proliferation in the presence of M-CSF (Figure 1). All three of these costimulants increase intracellular levels of cGMP in other systems. Both LTB_4 and $PGF_{2\alpha}$ bind to cell surface receptors and transduce signals that ultimately elevate cGMP via the activation of cytoplasmic guanylyl cyclase (78, 158). ANP, on the other hand, effects its cGMP-elevating properties via a particulate form of guanylyl cyclase rather than the cytoplasmic form (27, 49, 93). ANP is responsible for induction of diuresis, natriuresis, and vasorelaxation and is integral in the control of cardiovascular homeostasis (27). ANP elicits its activities by binding to a specific cell surface receptor. This receptor is a membrane form of guanylyl cyclase; binding of ANP to the membrane receptor domain activates the cytoplasmic guanylyl cyclase domain effecting an increase in intracellular cGMP (27, 49, 93). Augmentation of the colony response by any of these three cGMP-elevators was abrogated by the addition of AKPR (Figure 2). These findings were in accord with data generated from LPS, tuftsin, and SP experiments.

Intracellular levels of cGMP and cAMP can be elevated not only through receptor mediated interactions, but also by exogenous analogues of the cyclic nucleotides. Membrane-permeable, phosphodiesterase-resistant analogues are available as pharmacologic elevators of each cyclic nucleotide. Experiments with such analogues supported previous findings with agents purported to increase levels of cyclic nucleotides via receptor activation and signal transduction. Inclusion of 8-bromo-

cGMP (8-Br-cGMP) in the marrow culture system augmented colony formation in response to M-CSF (Figure 3) as did inclusion of dibutyryl cAMP (Bt_2cAMP; Figure 3). These results further supported roles for cGMP and cAMP in 2-signal dependent progenitor proliferation. Increasing the concentration of either analogue over 10^{-9}M resulted in decreased capacity to augment colony formation and eventual inhibition of basal colony formation (Figure 3). This observation was not surprising as high concentrations of cyclic nucleotide analogues effect decreases in the proliferation of murine thymocytes, bone marrow cells and macrophages (112, 148, 175, 184).

An unexpected but potentially significant finding arising from these experiments was the discovery that 3':5' cGMP and 3':5' cAMP were as effective as their respective analogues in the costimulation of 2-signal dependent proliferation (Figure 4). Even concentrations of the 3':5' cyclic nucleotides exceeding 10^{-9}M were effective in stimulating the 2-signal response (Figure 5). The 3':5' cyclic nucleotides are traditionally not used in pharmacologic studies of the effects of their increased levels on cell proliferation and other aspects of cell function as they are membrane-impermeant and phosphodiesterase sensitive. Therefore, they would not be able to enter the cell and effect increased levels of specific cyclic nucleotides. In our system however, the 3':5' cyclic nucleotides were capable of effecting the same action as their respective analogues; the only difference being the lack of inhibition by either 3':5' cyclic nucleotide at concentrations exceeding 10^{-9}M. In addition the effects of 3':5' cGMP and 3':5' cAMP were specific, that is they were not reproducible by adenosine, 2':3' cAMP, guanosine, or 2':3' cGMP (Figure 4).

These observations led to the theory that 3':5' cAMP and 3':5' cGMP were effecting their costimulatory actions at the cell membrane. Cell surface receptors for cAMP are not unknown, several lower eukaryotes have evolved highly specialized mechanisms of intracellular signalling with cAMP. The slime mold, *Dictyostelium discoideum*, is

one such organism (89, 90, 91, 104). During periods of starvation, the unicellular slime mold organisms aggregate to form a multicellular, sporulating stalk structure. Aggregation of these organisms is mediated by their release of cAMP in pulsating waves creating a concentration gradient. The detection of cAMP via a cell surface receptor induces movement of the organisms up the gradient to the site of stalk formation. *Paramecium spp.* also have a cAMP cell surface receptor that functions in chemoreception. Smith *et al.* (159) and Van Houten *et al.* (177) reported that cAMP serves as a stimulus indicating the presence of food sources for the paramecia. Receptors for cAMP have been detected on higher eukaryotes as well. Cyclic AMP responsive chloride ion channels in rat hippocampal neurons and exo-protein kinases have been described (98, 102). Even more convincing are reports of cAMP binding sites on human spermatozoa by Rosada *et al.* (151) and on rat thymocytes by MacManus and Whitfield (113), MacManus *et al.* (115), and Rixon *et al.* (148). The latter studies indicated cAMP binding sites with apparent dissociation constants in ranges compatible with those reported in the current study.

Binding of tritiated cAMP to adherent cell depleted marrow was specific and saturable (Table 3 and Figure 8). The data generated from these studies was confusing to an extent due to the difficulties inherent in using mixed cell cultures in binding assays. Various saturation peaks were detected over the concentration range of ^{3}H-cAMP tested (10^{-9} to 10^{-6}M), but clear indications of a saturable cAMP binding site in a concentration range comparable to that responsible for induction of maximal costimulation were observed. The binding in this range, upon Scatchard analysis, yielded an apparent dissociation constant equal to 2×10^{-9}M (Figure 9).

A role for cyclic nucleotides in the proliferation of murine bone marrow progenitors is not surprising due to the numerous reports and reviews on cyclic nucleotide involvement in cellular proliferation (55, 56, 114, 115, 137, 172, 184). The

significance of these findings, however, is two-fold. First, this evidence indicates the presence of a cAMP binding site(s) on the cell surface of bone marrow progenitors. While reports of other such sites exists, they have been downplayed and generally ignored (95). The concept that excreted cyclic nucleotides can bind to target cells and elicit specific responses in a complex eukaryotic organism challenges the general belief that cAMP and cGMP function primarily as second messengers in these systems by opening the possibility of a prominent role for the cyclic nucleotides as first messengers.

Second, neither cGMP nor cAMP had been implicated before these studies in the control of myelopoiesis (36). This work not only indicates their involvement in the myelopoietic pathway, but also defines that involvement as two distinct pathways incompatible with one another (Figure 7). Incompatibility between costimulatory signals was also observed when the cyclic nucleotide analogues were combined with agents reported to increase the intracellular levels of the antagonistic cyclic nucleotide (Table 2). Combination of the analogues with costimulatory agents effecting increases in the same cyclic nucleotide resulted in 2-signal responses unchanged from costimulation mediated by either agent alone (Table 2). Besides the incompatibility of costimulatory signals from the two pathways, the cGMP-compatible pathway is blocked by the addition of the 30kDa monokine or either of the two peptides mimicking the monokine (Figures 2 and 27 and Table 6). The cAMP-compatible pathway is not affected by the inhibitors (Figure 2).

The identification of two distinct pathways by which an increase in the number of mature monocytes/macrophages can be effected allows for several interpretations. These pathways offer a mechanism for increased macrophage production during inflammatory responses. Increases in the production of cAMP and/or cGMP during inflammatory responses have been detected. These increases are particularly evident

in studies of endotoxin shock (cGMP; 152), cholera toxin (cAMP; 5) and *Bordetella pertussis* adenylate cyclase-hemolysin (cAMP; 5, 85). The discovery of an inhibitor of cGMP-compatible augmentation of monocyte/macrophage development may allow for preferential activation of one pathway over the other. Although no differences in the extent of augmentation by either pathway have been observed, there may be differences in the rapidity of the response or the activation state of emerging monocytes. As discussed more completely in the latter paragraphs of this chapter, the inhibitor may also be capable of blocking or attenuating macrophage-mediated inflammatory responses detrimental to the host by affecting not only the number of mature macrophages able to participate in the response, but also by affecting the functions of the mature cells.

The cGMP-compatible pathway of augmented monocyte/macrophage development was examined in more detail as the mechanism by which the 30kDa monokine and the mimicking peptides effect their inhibition was unknown. The ability of the inhibitors to block the effects of exogenous cGMP was evaluated first. Intracellular cyclic GMP can activate cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases, and cGMP-regulated ion channels (107). The inhibitory agents could act to block the binding of cGMP to these binding proteins or they could act to block the action of the cGMP-binding protein involved in the proliferative response. However, none of the inhibitory agents affected augmented colony formation stimulated by cGMP (Table 4). These data indicated the inhibition mediated by the 30kDa monokine, the 14-mer peptide, and AKPR occurs at a point prior to cGMP-mediated intracellular effects.

The cGMP-compatible pathway was mediated by a number of agents including LPS, tuftsin, SP, $PGF_{2\alpha}$, and LTB_4 (Figure 2). All of these agents increase intracellular cGMP concentrations presumably via the activation of soluble guanylyl cyclase (57, 62, 63, 78, 158, 160). Because the activation of soluble guanylyl cyclase

by these agents most likely occurs by different mechanisms as each of the agents, with the exception of tuftsin and SP, bind to and activate cells by various means, a common link in these pathways was identified. Both LPS and SP, two mediators of the cGMP pathway effecting their costimulation by different initial mechanisms, have been implicated as stimulators of TNF_α (111, 118). Indeed, tumor necrosis factor alpha (TNF_α) was found to mediate costimulation of 2-signal dependent proliferation by LPS, SP, and $PGF_{2\alpha}$ (Table 5, Figure 20). TNF_α, by itself, was also an effective costimulant of the 2-signal response (Figure 10) and this costimulation was abrogated by the addition of the 30kDa monokine, ferritin (in total marrow cultures), and AKPR (Table 6). Further experiments provided evidence that TNF_α costimulation was compatible with the cGMP pathway (Figure 19). As additional support for TNF_α involvement in 2-signal dependent progenitor proliferation, production of TNF_α mRNA increased in adherent cell depleted marrow cultures post-treatment with LPS as detected by reverse-transcriptase polymerase chain reaction (Figures 12-18).

Recent reports have placed LPS and TNF_α as stimulators of inducible nitric oxide synthase (iNOS; 42, 61, 101, 117, 122). Inducible nitric oxide synthase is responsible for the prolonged inflammatory production of nitrogen monoxide (nitric oxide, NO; 121, 122). Nitric oxide, in turn, binds to and activates soluble guanylyl cyclase (93, 121, 122, 129, 190). Induction of iNOS by TNF_α and subsequent production of NO would provide a mechanism for the activation of cytoplasmic guanylyl cyclase and subsequent production of cGMP associated with each of the cGMP-compatible costimulatory agents.

As illustrated in the preceding chapter, both LPS and TNF_α were capable of stimulating the production of NO from adherent cell depleted marrow (Figures 30 and 31). Two of the inhibitors of 2-signal dependent proliferation, the 14-mer peptide and AKPR, were not able to block or attenuate NO production from adherent marrow cells

treated with LPS and TNF_{α} (Figure 34). These results indicate the production of NO by adherent cell depleted marrow and the inability of the peptide inhibitors to block that production, but not NO involvement in 2-signal dependent proliferation.

Involvement of NO in the 2-signal response was shown by several means. First, 2-signal dependent proliferation was inhibited by the iNOS inhibitors, N^{G}-monomethyl-L-arginine (N^{G}MMA), aminoguanidine (AG), and L-canavanine (Figures 21-23 and 25) and this inhibition was reversed by the addition of the iNOS sustrate, L-arginine, to the cultures (Figure 24). Second, two NO-generating compounds, sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP), were added to cultures of adherent cell depleted bone marrow and they, too, were capable of costimulating the 2-signal response (Figure 28). This NO-mediated costimulation of 2-signal dependent proliferation was compatible with the cGMP pathway (Table 7) and was abrogated by each of the inhibitory agents (Figure 29 and Table 8). These results placed the actions of the 30kDa monokine, the 14-mer peptide, and AKPR at a point in the pathway post-NO generation and prior to cGMP elevation. The ability of NO to bind and activate the haem-containing soluble guanylyl cyclase via a putative conformational change (122) suggests the 30kDa monokine and the mimicking peptides somehow inactivate guanylyl cyclase or make the NO-binding sites on the enzyme unavailable.

To determine if the inhibitors did affect soluble guanylyl cyclase, cGMP enzyme immunoassays were performed on marrow cultures. The NO-generator, SNP, was able to stimulate increases in cGMP production as expected (Figure 39). AKPR, however, only inhibited this increased production at the highest concentration of AKPR tested, 10^{-7}M (Figure 40). At lower concentrations, AKPR appeared to increase rather than decrease cGMP production (Figure 40). This finding at first seemed discouraging, however, it did not actually disprove the theory that AKPR, the

14-mer peptide and the 30kDa monokine were affecting guanylyl cyclase, it only changed the interpretation of the type of interaction. First, the decrease of cGMP production observed at high concentrations of AKPR might actually be the situation in the soft agar culture system. The difference in the concentrations of AKPR used in the soft agar system versus the enzyme immunoassay may be artifactual due to technical differences between the two assay types. In order to detect cGMP production by the marrow cells in response to SNP, the concentration of SNP had to be increased to 10mM- much higher than that used in the soft agar culture system to promote 2-signal dependent colony formation (Figure 28). This fact alone indicates the cGMP enzyme immunoassay is not sensitive enough to detect the increases in cGMP production associated with the 2-signal response at lower concentrations of SNP or the method used to stimulate the production of cGMP was not as effective as the soft agar culture system.

Second, the increased production of cGMP detected at low concentrations of AKPR may be capable of inhibiting 2-signal dependent proliferation. Just as concentrations of the cGMP analogue, 8-Br-cGMP, greater than 10^{-9}M inhibited the 2-signal response (Figure 3), AKPR-induced cGMP increases could shut down the proliferative response. An increased intracellular cGMP concentration may allow for cross-activation of cAMP-dependent protein kinase as well as cGMP-dependent protein kinase creating incompatible signals and cancelling the proliferative response. Cross-activation of the cyclic nucleotide-dependent protein kinases in not unknown. Jiang *et al.* demonstrated in pig coronary arteries that elevations of intracellular cAMP were capable of cross-activating the cGMP-dependent protein kinase (80). With either interpretation, the ability of AKPR to interfere with the production of cGMP by NO-activation of guanylyl cyclase is reinforced.

Even though the evidence generated in this study pointed to a proliferative pathway mediated by LPS stimulated production of TNF_α followed by NO production and guanylyl cyclase activation, a discrepancy existed. ANP effects increases in intracellular cGMP via binding to a receptor guanylyl cyclase (27, 49, 93). This mechanism of action would appear to negate the need for TNF_α production and subsequent NO generation. However, this was not the case. Neutralizing antibodies against TNF_α were capable of inhibiting the 2-signal response to ANP (Figure 20) indicating the need for TNF_α production even when elevation of cGMP was a direct result of primary ligand binding. Two-signal response to SNP, the NO generator, was also presumed to occur without TNF_α production as NO activation of guanylyl cyclase occurred post-TNF_α generation. In cultures of adherent cell depleted marrow, neutralizing anti-TNF_α antibodies inhibited 2-signal dependent proliferation costimulated by SNP, disproving the hypothesized step-by-step pathway (Figure 37). Considering both the ANP data and the SNP data, a new hypothesis was formed. Perhaps both TNF_α and NO were necessary to propel the 2-signal dependent progenitors into M-CSF responsive proliferation. Subsequent studies of the 2-signal response proved this was the case. Suboptimal levels of SNP were capable of reversing the inhibitory effects of the iNOS inhibitor, N^GMMA, on 2-signal dependent proliferation costimulated by TNF_α (Figure 36). In addition, suboptimal levels of SNP when combined with suboptimal levels of TNF_α were fully costimulatory for 2-signal dependent proliferation (Figure 38). Therefore, the original hypothesis of LPS-mediated costimulation of 2-signal dependent progenitor proliferation was altered to include the duality of TNF_α and NO in the costimulatory pathway.

To this point, these studies have only considered the driving force(s) behind 2-signal dependent progenitor M-CSF competence acquisition. During the course of these studies, an inhibitor of this 2-signal response was used to enable the breakdown

of the cGMP-compatible proliferative pathway into sequential steps. The 30kDa monokine and its peptide mimics have been shown in a collaborative effort with Hal E. Broxmeyer at the Indiana State University Medical School to affect other bone marrow progenitors in the S-phase of cell cycle (96, personal communication). These characteristics may allow the use of the peptides or the monokine as chemotherapeutic agents. The ability of these agents to block the proliferation of S-phase progenitors may protect these same progenitors from the cytotoxic activities of agents used to kill actively dividing cancer cells increasing the recovery rate and decreasing the time of recovery for cancer patients. These suppositions are also supported by evidence of high levels of ferritin in patients with acute or chronic myelogenous leukemia (18, 19, 20). The high ferritin levels may be responsible for depressing the normal cell population thereby allowing the cancer cells to gain a stronghold in the bone marrow compartment. The high levels of ferritin may also be indicative of the host's attempt to control proliferation of the cancerous cells by the production of the 30kDa monokine. In these systems, the monokine may not be effective against the cancer cells or may not be produced, thus allowing the outgrowth. In these same patients, a cell population corresponding to the 2-signal dependent progenitors is absent (18, 19, 20). Further studies in *in vivo* systems are necessary before real progress can be made in understanding the 30kDa monokine's role in controlling the hematopoietic process.

In addition to the inhibitor's effects in hematopoiesis, indications for a role for the 30kDa monokine and the peptide mimics in endotoxin shock were derived from the current studies. The ability of the inhibitory agents to block the effects of LPS, TNF_{α}, and NO suggested a possible role in the control of macrophage inflammatory responses. *In vivo* tests of the 14-mer peptide in a murine model of endotoxin shock reinforced this concept. Intravenous administration of the 14-mer peptide to BALB/c mice receiving intraperitoneal injections of lethal concentrations of bacterial LPS

improved survival by approximately 50% (Table 10). Although more conclusive studies are necessary, these data indicate the ability of the 14-mer peptide to ameliorate to some degree the pathologies associated with endotoxin shock. This information is potentially significant to the medical industry as endotoxin shock is a high risk factor associated with immunocompromised patients, both old and young (128, 134, 143, 170). Death rates attributed to endotoxin shock in hospital conditions range from 50-75% (143). Although no single therapy has yet been shown to be effective in the treatment of endotoxin shock (including administration of iNOS inhibitors), the possibility that combined treatments, including antibiotic therapy, may be the most effective in lowering the mortality associated with endotoxin shock is encouraging (128, 133, 134, 143, 157, 164, 167, 169). The addition of the 30kDa monokine or its mimicking peptides to the list of putative endotoxin shock reducing agents would allow for an even greater possibility of identifying an effective treatment for the potentially lethal condition.

In conclusion, these studies have outlined a pathway for increased production of monocytes and macrophages that is cGMP-compatible (Figure 41). The increased production of monocytes and macrophages arises from the stimulation of a population of progenitor cells in a precommitment phase to M-CSF responsiveness. Identification of this system is the first complete study of the events triggering the acquisition of lineage-specific competence by precommitment phase hematopoietic progenitors. In addition a second pathway stimulating commitment of these progenitors exists and is compatible with cAMP mediation. An inhibitor of the 2-signal dependent progenitors has been identified and two peptide mimics have been synthesized. These inhibitory agents were utilized to define the cGMP-compatible pathway of M-CSF competence acquisition. The cGMP-compatible pathway when initiated by LPS stimulation includes the production of TNF_{α}, the stimulation of iNOS

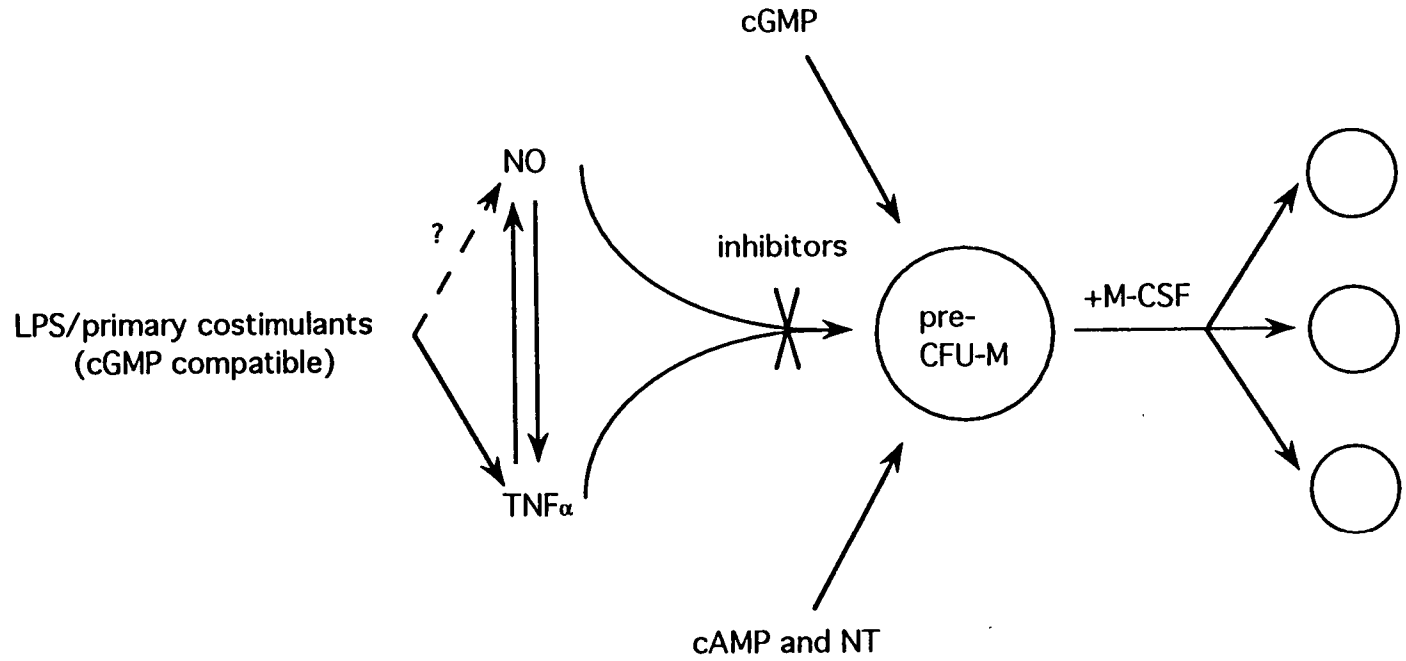


Figure 41: Schematic of proposed pathways for 2-signal dependent progenitor proliferation. LPS or costimulatory agents compatible with cGMP costimulate the 2-signal response via production of TNF_α and NO. NO can also stimulate the production of TNF_α. Both TNF_α and NO are necessary for costimulation of the 2-signal response. Costimulation of the 2-signal response by cGMP compatible mechanisms is inhibited by AKPR, the 14-mer, and the 30kDa monokine. Cyclic GMP also mediates costimulation of the 2-signal response as does cAMP and a putative intracellular cAMP elevator, NT. Costimulation of 2-signal dependent proliferation by these agents is not affected by the inhibitors, however, the two pathways (cGMP compatible vs. cAMP compatible) are mutually incompatible.

and subsequent production of NO, and ultimately the activation of soluble guanylyl cyclase. The inhibitory agents appear to affect the production of cGMP by guanylyl cyclase, but rather than inhibiting the production, the inhibitors may actually increase the cGMP concentration to a level inhibitory to cellular proliferation. Finally, preliminary data supporting a role for the inhibitory agents in the amelioration of the pathologies associated with endotoxin shock was generated. These studies opened the possibility of cyclic nucleotide action at the level of primary message rather than secondary, of new therapeutic agents in the fight against the lethality of endotoxin shock, and of a cell population to use as a tool in studying growth factor precommitment in hematopoietic progenitors.

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