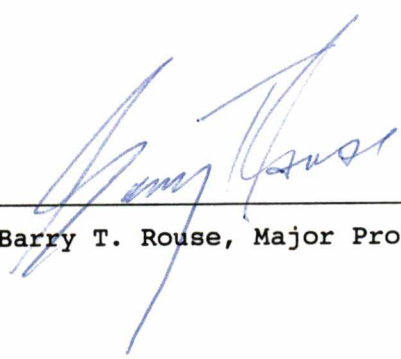


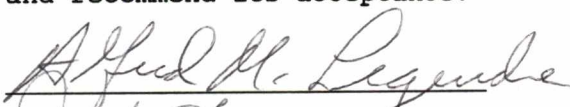
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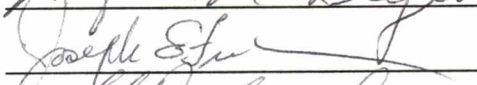
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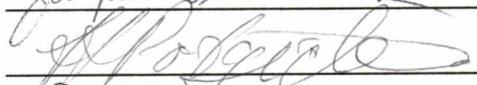


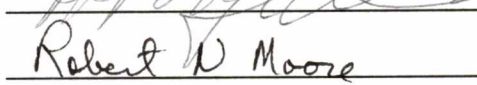
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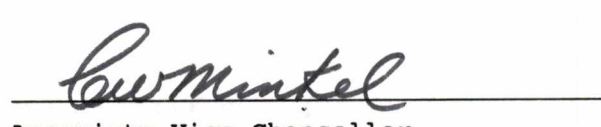








Accepted for the Council:



Associate Vice Chancellor
and Dean of The Graduate School

**FELINE BONE MARROW DERIVED MACROPHAGES:
IN VITRO CULTURING TECHNIQUE, CLONING AND SEQUENCING OF FELINE
TNF ALPHA AND IL 1 BETA CYTOKINES, AND POTENTIAL MODULATION
OF MACROPHAGE GENE EXPRESSION BY FELINE LEUKEMIA VIRUS**

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Degree
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Sandra Leigh Daniel

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ABSTRACT

Investigations of feline macrophage-viral interactions have been hampered due to difficulty in obtaining adequate macrophage numbers from tissues without euthanasia of the cat. We describe a method of small sample bone marrow culture which yields large numbers of macrophages (average 90% population) with residual lymphocytes and stromal cells, and does not require euthanasia. From cultured macrophages, cDNA for feline TNF α and IL-1 β were cloned using degenerate primers and polymerase chain reaction technology. Sequenced feline TNF α cDNA matched 98.6% with coding regions for a genomic clone of feline TNF α , and 90%, 86%, 85%, 82% and 83% overall matches with cDNA for human, porcine, ovine, murine and goat TNF α , respectively. Full length TNF α cDNA was 702 bp with a predicted protein of 233 amino acids and a molecular weight of 25 Kd (precursor form of mature TNF α). Full length cDNA for feline IL-1 β was 804 bp long with a predicted protein of 267 amino acids and a molecular weight of 31 Kd (precursor form). Sequenced feline IL-1 β had 79%, 76%, 77% and 77% overall match with IL-1 β cDNA for human, bovine, rabbit and murine species, respectively.

Bone marrow derived macrophages from normal and FeLV infected cats, were examined for differences in LPS induced TNF α or IL-1 β mRNA expression. TNF α and IL-1 β mRNA levels in macrophages from FeLV positive cats did not undergo modulation with one exception.

For the exception, TNF α mRNA expression was not LPS induced and IL-1 β mRNA level was significantly decreased. Viral antigen was detected in macrophages from this cat, indicating viral replication was occurring within some macrophages. No viral antigen was detectable in macrophages of other FeLV infected cats, virus was present only in residual lymphocytes. These findings suggest FeLV may modulate TNF α and IL-1 β gene expression, but only when macrophages are actively replicating virus. However, one must be cautious in interpretation of these results, since macrophages derived from the bone marrow of random source cats (known to be FeLV positive and FIV negative), may contain other undetected factors which contribute to modulation of gene expression.

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PART I:

LITERATURE REVIEW

1. INTRODUCTION

Since the early days of recorded history, mankind has speculated on the existence of immunity or protection from disease, though often without realizing it. In the days of ancient Greece, philosophers questioned why some people were spared the ravages of epidemic disease while others "were punished" falling ill and in many cases paying the ultimate price, death. The historian Thucydides wrote of the plague of Athens of 430 B.C., "Yet it was with those who had recovered from the disease that the sick and the dying found most compassion. These knew what it was from experience, and had now no fear for themselves; for the same man was never attacked twice - never at least fatally"(170). Immunology, the study of immunity to disease, has progressed greatly since these early days (aptly reviewed by Silverstein (163)), and with each new technological advance science comes closer to understanding, at the cellular and molecular level, the critical role that the immune system plays not only in protection against infectious agents and/or cancer but also equally as important is the potential contributing role that the effector arm of our immune system may play in development of pathological lesions seen concurrently in some disease states.

An exponential increase in information concerning the role of cytokines in immune responses has occurred in the last 10 years. Advancing scientific technology has played a key role in expanding our understanding of the multitude of roles played by cytokines

during immune responses. The marriage of molecular biology and immunology has been instrumental in dissecting out the role cytokines play in immunoregulation. Dr. Steven Gillis (44) eloquently illustrates this point in a recent book chapter: " Our current knowledge base regarding many of the molecules reviewed in this chapter and elsewhere in this volume would be significantly smaller indeed if immunologists and molecular biologists had not joined forces over the past decade to isolate cytokine cDNAs and develop methods for producing these materials in significant quantities so that other investigators could continue to probe their *in vitro* and *in vivo* relevance."

One area of intense immunological research today concerns the potential modulation by infectious agents of immunoregulatory cytokines *in vivo*, with particular attention directed toward understanding the mechanisms by which certain viruses induce severe immunosuppression of the host animal. Viral manipulation of the immune system has been recognized since 1908 when von Pirquet reported that measles virus infection of patients resulted in reduced delayed hypersensitivity responses to tuberculosis antigen (178). Since that time the literature on virus-induced immunosuppression has grown considerably with numerous books and review articles published on the subject (64,94,95,99,153).

While advances in the study of viral immunosuppression of human and murine immune function are proceeding at a rapid rate, similar studies are lagging behind in other species due to lack of appro-

priate antisera and cytokine cDNA clones for use as molecular probes. Unless very low stringency hybridization conditions are utilized, human and murine cDNA probes will not efficiently cross react with mRNA for the same cytokine derived from another species due to lack of extensive sequence homology. Therefore, unraveling potential viral-cytokine interactions, understanding how they occur and relating them to pathogenesis has been difficult in some species such as the feline.

The research from which this dissertation was written was instigated in an attempt to investigate the role of feline leukemia virus (a member of the *Retroviridae* family) at modulating feline macrophage cytokine function. To this end, a technique for growing large numbers of feline macrophages had to be developed as well as the cloning and sequencing of two important macrophage cytokines, specifically tumor necrosis factor alpha and interleukin 1 beta, for use as molecular probes. Finally, comparisons were made between expression of cytokine message in macrophages derived from the bone marrow of normal (no detectable feline leukemia virus antigen) versus persistently infected feline leukemia virus cats.

2. FELINE LEUKEMIA VIRUS: A REVIEW

Feline leukemia virus was isolated from a cluster of cats bearing lymphomas by Jarrett et al in 1964 (69). Since this time many articles have been written on the subject. For extensive reviews, readers are encouraged to read the published reviews by J. Rojko et al (147,150) which are both comprehensive and extensive in nature. This review will serve to familiarize the reader with selected aspects of the biology of FeLV as well as its pathogenic mechanism *in vivo*.

2.1 Introduction

FeLV is an oncogenic C-type retrovirus. Its structure consists of two single stranded, positive sense RNA molecules surrounded by an internal protein core and a host cell derived lipid envelope into which are embedded two virally encoded envelope proteins (reviewed in (113,147,150)). FeLV encodes a reverse transcriptase and is classified as a member of the Oncovirinae subfamily of the Retroviridae family (37,113). FeLV was first isolated in 1964 by William Jarrett et al from a naturally occurring case of feline lymphosarcoma (69). Since its isolation, FeLV has been implicated in a wide range of disease syndromes seen in domestic cats. A recent estimate is that there are one million clinically healthy but persistently infected FeLV positive cats present in the United States (51). It is predicted that over the next three years, 83% of these asymptomatic viremic cats will die of some FeLV related

syndrome, specifically: 5% will die of leukemia or lymphosarcoma, 25% of nonregenerative anemia, 10% of other less frequently associated neoplastic or degenerative diseases and 50% of miscellaneous diseases, due primarily to the severe state of immunosuppression which occurs in cats persistently infected with FeLV (51,147,150). FeLV associated diseases can generally be divided into three main categories: neoplastic or proliferative diseases, degenerative diseases, and diseases which arise due to the profound immunosuppression which occurs in animals persistently infected with FeLV (20,51).

2.2 FeLV Associated Diseases

Proliferative or neoplastic diseases are characterized by neoplastic transformation of the particular cell type in which FeLV has integrated. It is estimated that approximately 33% of all feline cancer deaths are caused by FeLV (20). FeLV causes the majority of both lymphoid (lymphosarcoma, lymphocytic leukemia) and myeloid (granulocytic leukemia, erythroleukemia, erythremic myelosis, reticuloendotheliosis) tumors which arise in cats (20,147,150). Feline lymphosarcoma/leukemia is by far the most common FeLV induced tumor (70% of all cats with leukemia or lymphoma are viremic with FeLV). In one study, these tumors accounted for 90% of all the hematopoietic tumor cases presented in a population of cats (reviewed in (20,147,150)). It is estimated that, 92% of the myeloproliferative diseases in cats are FeLV associated

(50). However, under conditions of natural exposure and infection, the proliferative disease syndromes make up only a small proportion of the FeLV associated diseases seen in clinical situations (147).

Non-neoplastic or nonregenerative diseases constitute a second category of FeLV associated diseases (reviewed in (20,147,150)). These diseases are characterized by FeLV infection of stem cell populations within the bone marrow, resulting in bone marrow suppression, anemia, leukopenia and/or thrombocytopenia. The presenting clinical signs depend upon the lineage commitment of the stem cells infected by FeLV. Nonregenerative anemia, secondary to bone marrow suppression and erythroid aplasia, is the second most frequent disorder associated with FeLV viremia (19). Other disorders that may be grouped under this general classification include a panleukopenia like syndrome and thymic atrophy in kittens (144).

A third group of FeLV related diseases arise due to the profound immunosuppression that occurs at the level of the immune effector cells present in peripheral and secondary lymphoid tissue of FeLV infected-viremic cats (reviewed in (20,147,150)). Immunosuppression is by far the most frequent and devastating manifestation of FeLV viremia. The *hallmark* of immunosuppression in infected cats is an increased susceptibility to opportunistic pathogens. Some clinical disorders associated with FeLV induced immunosuppression include chronic gingivitis and stomatitis, nonhealing

ulcers, abscesses, pyoderma, viral infections with such agents as feline infectious peritonitis (FIP) or feline herpes virus, recurring viral respiratory infections as well as a long list of fungal, protozoan, and rickettsial diseases (reviewed in (20,147,150)). Indeed, the major cause of death in FeLV positive cats is a combination of anemia, leukopenia, and secondary bacterial and/or viral infections which occur as a direct result of FeLV induced immunosuppression (20,147,150). A subsequent section (2.8 and 3.3) will deal with some proposed mechanisms by which FeLV viremia leads to profound immunosuppression.

The type of FeLV-related disease and the time interval before clinical signs ensue are influenced by multiple factors; including, the infecting viral subgroup, its envelope glycoproteins (different subgroups have different cellular receptor specificities so these determine which cells will be infected), and once internalized the site at which the provirus integrates within the cellular genome (i.e. the consequences of tumor formation if provirus integrates near and activates an oncogene)(147). There are three antigenic forms of FeLV major envelope glycoproteins (gp70). These determine the subgroup or serotype of FeLV, which are designated subgroups FeLV-A, FeLV-B, and FeLV-C (150). These envelope proteins determine the viral infectivity, host range properties, and pathogenicity of the three separate subgroups of FeLV (150), and it is the subgroup specificity of the infecting FeLV viral strain which determines when and what type of disease

syndrome is seen clinically (147).

2.3 FeLV Viral Subgroups

Three virus subgroups (FeLV-A, FeLV-B, and FeLV-C) have been identified on the basis of virus neutralization, interference testing, and the ability to replicate in non-feline tissues (156,157). The envelope protein gp70 contains many hypervariable regions and these regions are responsible for determining the virus subgroup reactivity, with each subgroup evoking a unique virus neutralizing antibody (147,157). Specifically, differences in the hypervariable region near the N-terminus of gp70 are responsible for causing differences in the susceptibility pattern of feline cell populations to infection, the subsequent type of disease syndrome which is seen, and the rate of disease progression (147,150,157). Each subgroup binds to a unique subgroup-specific cell surface receptor (147). Receptors for subgroup A are present on the majority of feline lymphocytes and bone marrow cells, subsequently it is the most frequently found in clinical isolates (present in virtually 100% of naturally infected cats) and the most infectious of the subgroups so it is easily transmitted (147). Receptors for FeLV-B are less frequent on cat lymphocytes and the virus replicates less efficiently. In natural infections, FeLV-B, when present, is always found in combination with FeLV-A (approximately 50% of FeLV viremic cats have both subtypes present) (147). Receptors for FeLV-C are present on lymphocytes

in 75% of tested cats but in natural infections FeLV-C is found only 1% of the time and is always in combination with FeLV-A and FeLV-B or FeLV-A subgroups (49). The presence of FeLV-C is frequently associated with the clinical development of nonregenerative anemia (122).

Though subtype A rarely by itself causes acute disease, its mere presence with circulating p15E (a highly conserved transmembrane spanning envelope protein which mediates many of the immunosuppressive properties of FeLV) causes immunosuppression predisposing the animal to disease by opportunistic pathogens (147). There is an association of FeLV-A subtypes with long periods of relatively asymptomatic viremia with the eventual development of lymphosarcoma/leukemia (147,150).

However, recently a variant of FeLV called variant A was isolated from cats exhibiting signs of profound immunosuppression (72,108,109). In contrast to the relatively noncytopathic FeLV-A strain, the variant A strain of virus replicates extensively with cytopathic effects in intestinal cells, feline bone marrow and lymphoid cells leading to an almost total ablation of lymphoid tissue followed by severe lymphopenia and death within a matter of weeks. Infected cats rapidly develop secondary infections, with cachexia and wasting so severe the animals are described as simply fading away. This syndrome has been termed the feline acquired immunodeficiency syndrome or FAIDS (109). The variant A virus has been molecularly cloned and found to be replication defective

(FeLV variant A lacks a portion of its envelope and polymerase gene which is necessary for replication) and requires exogenous helper FeLV-A virus in order to replicate (108,126). Therefore, variant A is found only in association with FeLV-A during natural infections. Early investigations into the mechanism of variant FeLV-A or FeLV-FAIDS cellular cytotoxicity implicated delayed processing of the viral envelope protein, gp70, through the Golgi apparatus as one factor responsible for increased cellular cytotoxicity (27,138,139). A recent paper (27) has implicated subtle mutational changes within the structure of the gp70 envelope protein as being responsible for T cell destruction. These authors report that these changes are responsible not only for the increased transient time of gp70 through the Golgi complex but also for failure of this viral strain to prevent superinfection of the cell by other retroviruses. Their data strongly suggests that continual reinfection of a cell already producing virus is a critical component of the mechanism of T-cell killing by FeLV-FAIDS (27). Cell killing correlated with the presence of extremely high levels of intracellular unintegrated proviral DNA, due primarily to an inability to prevent continual reinfection of the FAIDS infected cell. Though the exact mechanism by which it is accomplished has yet to be elucidated, superinfection interference is a common mechanism by which retroviruses prevent additional retroviruses from co-infecting a cell which is already infected (84,121). It is of interest that high copy numbers of unintegrated

viral DNA have also been reported in HIV infected cells (129,145).

Natural infections with FeLV-B subtypes are always found in association with the subtype, FeLV-A. FeLV-B subtypes are believed to have arisen through recombination of FeLV-A with endogenous sequences of murine leukemia viruses which are present in feline DNA (166). Upon replication, FeLV-B viral RNA and core structural proteins become encased in a FeLV-A viral envelopes resulting in phenotypic mixing of the viruses (166). In this manner, the phenotypically mixed virus binds to FeLV-A receptors and replicates marginally in feline hematopoietic cells (147). The presence of FeLV-B subtypes in infections increases the percentage of cats which develop viremia (relative to FeLV-A alone), enhances immunosuppression, and some FeLV-B isolates appear to accelerate the development of certain proliferative diseases (myeloproliferative disorders) (147). The appearance of FeLV-B subtype in the bone marrow or plasma of viremic cats often signals the onset of fatal myeloproliferative disease (147).

FeLV-C subtype replicates intermediately, high compared to FeLV-B and low compared to FeLV-A, and in natural infections FeLV-C is always found in association with FeLV A (147). The origin of FeLV-C is uncertain but is believed, based upon molecular sequence comparisons, to arise *de novo in vivo* following exposure to FeLV-A by either mutation or recombination occurring within the gp70 gene (reviewed in (147)). FeLV-C replicates preferentially within erythroid precursors and its emergence from the bone marrow sign-

als the impending development of erythroid aplasia and a rapidly progressive and fatal nonregenerative anemia (122).

2.4 FeLV Encoded Proteins

FeLV resembles murine retroviruses in its genomic structure and replication cycle (reviewed in (113)). In fact, there is a high degree of homology between the viruses of these two groups. The FeLV gag gene encodes for four viral core proteins designated p27(30), p10, p12, and p15c. The major core protein is designated p27(30). The p27(30) protein is responsible for structure of the virion's internal protein shell and contains a group specific antigen which confers antigenic cross reactivity for FeLV subgroups (reviewed in (20,113,147,150)). The p27(30) protein is detectable in circulating neutrophils and platelets by an indirect fluorescent antibody test (IFA) and has a 98% correlation with recovery of infectious virus from plasma (20,150). The presence of p27(30) in circulating leukocytes, indicates that infection of bone marrow cells has already occurred. Soluble p27(30) in the plasma is detectable by means of an enzyme linked immunosorbent assay (ELISA). In a few cases, the IFA will be negative while the ELISA test will be positive, but under most conditions there is a 95% correlation between these two tests (20). Three additional core proteins, designated p10, p15C, and p12, are present. Each core protein has a different role in protecting the tightly coiled viral RNA and reverse transcriptase protein (which are necessary

for the synthesis of a DNA copy of the RNA genome during replication) from degradation by nucleases (reviewed in (113,150)). These proteins are all immunogenic but antibodies against them are not virus neutralizing nor protective (20).

The pol gene encodes for the reverse transcriptase protein (RT) as well as other affiliated proteins which are required for processing of viral mRNAs as well as synthesis of a cDNA copy of infectious FeLV RNA during formation of the provirus structure (reviewed in (113)). It is the provirus which integrates within the host cell genome during infection and replication of FeLV (113,150). Some cats do produce antibodies against RT but viremic cats rarely have high titers against RT and the protective significance of this antibody is unknown at this time (20).

The env gene encodes for two viral proteins, gp70 and p15E, which are present in the viral lipid envelope. The major protein is known as gp70 and its importance in subgroup specificity for FeLV as well as binding to cellular receptors has already been discussed. Antibodies against gp70 are frequently subtype specific and virus neutralizing, resulting in immunity against the appropriate subtype of FeLV (reviewed in (20,150)). The gp70 protein is extensively glycosylated and extends outward from the viral envelope as a projection.

In contrast, p15E is a gp70 associated minor envelope protein, which is principally hydrophobic, and found as a transmembrane protein embedded within the lipid membrane that is picked up at

the time virus buds from an infected cell (113). The p15E protein is highly conserved in structure and differs very little among all the FeLV isolates (16,110,113). Numerous immunosuppressive properties have been attributed to this protein. These will be discussed in detail in subsequent sections (2.7 and 3.3).

2.5 Host Resistance

Multiple factors determine the final outcome of FeLV host-virus interaction and development of disease. These include genetic, environmental, age, and immune status of the cat at the time of exposure (reviewed in (20,147,150)). Some cats are capable of resisting infection even though they are exposed at birth, this resistance maybe related to the absence of FeLV receptors on cells, but the exact mechanism of this resistance is not clear (20,147,150). Age and immune status are closely linked. Cats develop immune competence (mature) by four to six months of age. Immune competence results in the ability to resist persistent productive infections with FeLV. In neonates, 100% of exposed kittens will become viremic and develop disease, 85% of kittens eight weeks old (weanlings) will develop disease, while only 15% of cats older than 4 months of age will develop disease (this is strongly virus strain specific with some strains causing more frequent disease than other strains) (60). Age related resistance to FeLV infection may be abrogated by immunosuppressive treatments and this probably accounts for the infrequent finding that non-

latently infected adult cats may occasionally acquire FeLV infections (147). Age-related increases in immune function are believed to be associated with age related acquisition of macrophage, natural killer cell, complement and antibody function (147). Feline macrophages and FeLV have a critical relationship which seems at least in part to determine whether persistent productive, non-productive infection or immunity to infection occurs (62,147). This concept will be further developed in the next section (2.6).

2.6 Interactions of FeLV and Macrophages

A critical feature that appears to distinguish persistent productive FeLV infections from persistent nonproductive infection is the ability of virus to replicate in differentiated cells of the myeloid lineage, i.e. granulocytes and macrophages, present in bone marrow and peripheral sites (62,147). As previously mentioned, an age-related increase in immune function and refractoriness for FeLV infection appears to correlate with age-related acquisition of macrophage functions (147). Even though the phagocytic capacities of isolated bone marrow or peritoneal derived macrophages from kittens and adults are the same, kitten macrophages have been shown to be five times more susceptible to infection by FeLV than adult cat macrophages (62). Additionally, kitten bone marrow and peritoneal cavity derived macrophages replicate FeLV to a very high titer while adult macrophages from

comparable locations replicate FeLV poorly if at all (62,147).

The refractoriness for FeLV replication in adult feline macrophages may be overcome by *in vivo* or *in vitro* administration of immunosuppressive agents such as glucocorticoids or silica (Silica is toxic for macrophages *in vitro* and *in vivo* causes monocytopenia and neutrophilia.)(reviewed in (62,147)). The *in vivo* treatment of cats believed immune for FeLV with corticosteroids has lead to reactivation of latent FeLV from bone marrow adherent macrophages as well as myelomonocytic precursors. This treatment results in transient FeLV production subsequent to an earlier latent bone marrow infection. This information hints that glucocorticoid treatment enhances retroviral expression by converting the macrophage/FeLV relationship from one of nonpermissiveness for FeLV replication and immunity to a permissive state which is characterized by enhanced viral replication and persistent viremia (147). It is now well established that macrophages participate in the early restriction of viral replication and that persistent replication of FeLV in macrophages leads to continuation of the viremic state (62,147,150)

2.7 Replication of FeLV at the Host Level

Under natural conditions, FeLV infection occurs by the oral route (reviewed in (147,148,150)). Virus first replicates in the tonsillar and pharyngeal lymph nodes for 2 to 4 days post exposure. At this time, a small number of circulating lymphocytes and

macrophages become infected and migrate to lymphoid germinal centers. This migration occurs between 3 and 14 days post exposure, and is the critical point at which the immune system either controls viral replication or infection of bone marrow occurs with persistent infections ensuing. In progressive cases, FeLV will continue to proliferate in lymphoid tissues and viremia ensues. By 7 to 21 days post infection, bone marrow and intestinal cryptal epithelium become infected and FeLV incorporates into hematopoietic precursor cells within the bone marrow. As cells mature and leave the bone marrow, they enter the circulation as virally positive neutrophils and platelets. Thus a state of peripheral viremia is established by 14 to 28 days post exposure. At 28 to 56 days post exposure, FeLV has infected extensive epithelial tissues and is readily shed via saliva, urine, and mucosal secretions thereby establishing a reservoir of infectious material for other cats (reviewed in (147,150)).

In non-progressor or immune cats, the total viral burden must be overwhelmed by a rapid and effective anti-FeLV immune response. In the first 4-6 weeks post exposure, exposed cats must totally eliminate FeLV bearing cells or infection of bone marrow will occur with the development of latency. It is estimated that approximately one-half to two-thirds of FeLV resistant cats probably do eliminate all FeLV infected cells; however, the remaining cats have latent infections since they retain integrated FeLV proviral DNA within myelomonocytic progenitor cells as well as

lymphocytes (91,149). Severe stress in these latently infected cats may alter the viral/host relationship and allow replication of FeLV to reemerge and initiate persistent viremia and disease (147). As previously discussed, the macrophage appears to be a cell which exerts pivotal control over maintaining FeLV in a non-productive state.

2.8 Mechanisms of Immunosuppression by FeLV

As previously discussed, subgroup specificity of infecting FeLV as well as the type of cell and location within the cellular genome where the proviral DNA integrates are all instrumental in determining what type of FeLV-related disease syndrome occurs (147). When proviral integration occurs adjacent to oncogenes, neoplastic conditions such as leukemia or lymphomas are thought to arise (113). The molecular mechanisms by which FeLV leads to neoplastic transformation of cells into which it integrates will not be reviewed at this time, for a detailed discussion of this please refer to the following reviews (113,147,150). Whatever the cell type or location of viral integration, one factor remains consistent, that is the presence in viremic cats of high levels of circulating p15E and a state of profound immunosuppression. Under conditions of natural infections with FeLV, diseases which are associated with immunosuppression cause more deaths than do neoplastic diseases such as leukemia or lymphoma's (52).

Many studies have been directed toward understanding the mecha-

nisms by which FeLV causes immunosuppression in the host. A primary problem appears to be that T cells, particularly helper T cells, fail to function properly in viremic cats (118,147,171). In addition, FeLV replication within the myelomonocytic precursor cells results in functional changes and numerical decreases in circulating neutrophils (79,87). Neutrophils, which have been derived from cats with either productive or latent FeLV infections, are reported to have decreased chemiluminescence responses to phagocytic stimuli (79,87). In addition, transient, persistent or fluctuating neutropenia may occur in both naturally and experimentally induced FeLV infections (52). Neutropenia, accompanied by depressed neutrophil functions, contribute to immunosuppression and interfere with a cat's ability to resist opportunistic infections (52,147).

There have been many reports published implicating FeLV involvement in suppression of cell mediated immunity. Early experiments reported morphological changes including FeLV induction of thymic atrophy, depletion of paracortical lymphoid tissues, as well as the presence of depressed peripheral blood lymphocyte counts in viremic cats (reviewed in (113,118,147)). Most investigations now focus on understanding the mechanisms by which FeLV depresses T and B cell function.

Many reports have been published indicating defective cell mediated immunity in FeLV positive cats. Experimentally infected cats are reported to exhibit prolonged delayed allograft rejection.

tions *in vivo* (61,131) and diminished proliferative *in vitro* responses for T cell specific mitogens (17,18). Additionally, antigen specific humoral immune responses are also depressed in asymptomatic, naturally infected cats (147,172).

Given the pivotal role of cytokines in immunoregulation of T cell, B cell, and macrophage immune function (44), it is not surprising that recent experiments suggest FeLV may cause a general suppression in the production of some cytokines. Ultraviolet light inactivated FeLV has been reported to have an inhibitory effect upon the production of interleukin 2 (IL-2) and macrophage activating factors by Con A stimulated murine splenocytes (123,124) as well as suppression of feline gamma interferon production by Staphylococcal enterotoxin A stimulated splenic and peripheral blood lymphocytes derived from FeLV infected cats (33). Given the central role of gamma interferon as a potent activator of macrophages, natural killer cells, and T cells, it is not surprising that suppression of production of this cytokine would contribute to immunosuppression of the host (44). There is one clinical report of the administration of alpha interferon to anemic, viremic cats leading to a partial remission of viremia and clinical recovery from anemia (22). With regards to the important regulatory cytokine IL-2, Tompkins et al have reported that naturally infected FeLV positive cats are suppressed in their ability to produce IL-2 when their peripheral blood lymphocytes are appropriately stimulated (171).

Many of the immunosuppressive effects caused by FeLV are believed to be mediated by p15E, the transmembrane envelope protein (147). Extensive investigations have been conducted in an attempt to determine the molecular mechanism by which p15E suppresses immune functions. Considerable evidence is amassing to implicate the involvement of cyclic nucleotides and intracellular secondary messengers in the mechanism of immunosuppression by FeLV (reviewed in (147)). It has been hypothesized that incorporation of FeLV p15E into cellular membranes may interfere with calcium/calmodulin or protein kinase C transduction of membrane signals (147,171). Lewis et al (87,88) have shown that the treatment of lymphocytes with FeLV p15E has no measurable effect on cellular prostaglandin E2 or cyclic GMP levels but that it does inhibit cyclic AMP accumulation in the presence of Con A. The direct activation of adenylate cyclase by forskolin will reverse FeLV suppression of lymphocyte function by raising intracellular cyclic AMP levels and restoring blastogenic and capping responses. Forskolin bypasses GTP-binding sites for adenylate cyclase and activates the catalytic site directly. Based on these data, it has been suggested that p15E may function at or in the cell membrane by blocking triggering of calcium/calmodulin dependent activation of adenylate cyclase (147).

Other investigators suggest that p15E functions by blocking

protein kinase C activation (47,171). As previously mentioned, IL-2 production by Con A stimulated peripheral blood lymphocytes from viremic cats may be decreased (171). In a subset population of these viremic cats, the utilization of a calcium ionophore (A21387-A2) and phorbol ester (phorbol myristic acetate-PMA) -which directly activates protein kinase C and increases intracellular calcium levels - will in most cases restore the secretion of IL-2 by Con A non-responder lymphocytes. A2/PMA intercalates within cellular membranes, where PMA directly activates protein kinase C and A2 elevates intracellular calcium levels, thereby bypassing the need for transmembrane signaling. On the other hand, Con A induction of IL-2 production depends upon cell surface receptor stimulation followed by transmembrane signaling. Additionally, a synthetic peptide, CKS-17, which is homologous to a highly conserved region of p15E (16) has been shown to inhibit *in vitro* activation of protein kinase C. Though at odds as to what cyclic nucleotide or kinase enzymes are being depressed, these data support the concept that p15E functions by intercalating within cellular membranes and inhibiting transmembrane signal transduction or the formation of secondary intracellular messengers. The role of p15E in immunosuppression will be discussed in detail in Section 3.

2.9 FeLV Infection: Parallels with Human Immunodeficiency Virus Infection

Although FeLV and HIV infections are clearly caused by separate viruses with unique retrovirus/host relationships, some parallels can be drawn. Both viral agents are transmitted horizontally and require close contact with the exchange of bodily fluids. Following infection there are sequential stages in progression of disease with often long incubation times, and *in vivo* variations occur with tissue cell tropism and pathogenicity depending upon the infecting strain of virus (reviewed in (147,151)). While FeLV does induce the formation of leukemia and lymphomas, it more commonly gives rise to profound immunosuppression characterized by lymphopenia and opportunistic infections (52,147). With human immunodeficiency virus (HIV), though clinical presentation of disease is varied, that most frequently seen is an acquired immunodeficiency syndrome (AIDS) with its attending, usually lethal, opportunistic secondary infections (151). Both HIV and FeLV infections show parallels in that a large percentage of infected individuals do not show clinical signs until a number of years post infection, demonstrating long latency periods (49,151). A large percentage of cats with natural FeLV infections are initially asymptomatic with 83% developing clinical disease within 4 years of infection (49). Though reliable numbers are not yet available for the latency period of HIV, 5 to 10 years may pass from the time of infection until development of AIDS (7,53,151). HIV and

FeLV both infect lymphocytes and macrophages, but the cellular tropism for HIV is more restricted than for FeLV due to the selective distribution of its receptor, the CD4 molecule (147,151).

Other similarities include the type of clinical syndromes seen and the type of immune dysfunctions which occur. A primary target of HIV is the CD4+ T cell and much of the subsequent disease pathology can be directly attributed to failure of CD4+ T cell function (reviewed in (151)). There is an inability of T helper cells from HIV positive patients to produce IL-2 or gamma interferon when appropriately stimulated (81,112). As with HIV, FeLV infection also results in depressed IL-2 and gamma interferon production (33,171). Indirect evidence for T helper malfunction in FeLV positive cats has also been demonstrated by their hyporesponsiveness to T dependent mitogens (17,18), and similar results have been reported for AIDS patients (reviewed in (151)). With respect to humoral responses, parallels are observed here also. Both FeLV and HIV infection may result in hypergammaglobulinemia (4,159,188), but paradoxically, there is an associated decrease in patient ability to respond specifically to synthetic antigens even though increased levels of antigen non-specific immunoglobulin are circulating (reviewed in (147,188,191)).

In conclusion, the similarities which exist between HIV and FeLV suggests possible similarities in pathogenesis. Study of the mechanisms by which FeLV induces immunosuppression will be beneficial not only to the feline population, but may provide informa-

tion which can be applied to its distant relative, the HIV virus. In particular, comparative studies investigating viral modulation of cytokine production and/or function may be especially relevant with respect to dissecting out what roles, if any, cytokine-viral interactions play in the development of disease or immunopathology. The current study was undertaken to investigate whether bone marrow derived macrophages from cats latently infected with FeLV exhibit modulation of selected cytokine mRNA levels whenever stimulated *in vitro* with lipopolysaccharide (LPS).

3. NON-LYTIC MECHANISMS OF VIRAL IMMUNOSUPPRESSION

3.1 Introduction

Viral infection of immune cells may have several outcomes depending not only upon the lytic nature of the virus and cell type infected but also the presence of complicating factors such as opportunistic secondarily infecting agents, environmental and host genetic factors. Apart from the obvious immunosuppression and pathologic lesions which occur when infected immune cells are destroyed by cytolytic viruses, it is becoming increasingly apparent that the host's own immune system may contribute to development of lesions and immunosuppression. This may occur by a number of mechanisms including viral triggering of an uncontrolled immune response which reacts not only against specific viral antigens but also cross-reacting self antigens resulting in tissue destruction and autoimmune disease(158). A current area of extensive research includes investigations into viral interactions with immune cells which result in generation of severe inflammatory responses that are frequently accompanied by dysregulation of cytokine activities (119,151). Viral cytopathic effects in concert with virally induced alterations of immune responses, such as inhibition or excessive release of cytokines may contribute significantly not only to tissue damage and development of clinical signs, but also to development of severe immunosuppression which occurs in certain virally infected individuals (119,151). This review will deal

primarily with non-lytic mechanisms by which viruses induce states of immunosuppression, with special emphasis upon Retroviruses and their interactions with cytokine producing immune cells.

Since the early 1980's, when the causative agent of acquired immunodeficiency syndrome (AIDS) was discovered to be human immunodeficiency virus (HIV), a Lentivirus subfamily member of Retroviruses; investigations into potential mechanisms of virally mediated immunosuppression have escalated. The knowledge that Retroviruses have the ability to cause profound immunosuppression following infection has been appreciated for years from experimental studies with infections with FeLV. FeLV causes leukemia in infected cats but more frequently leads to significant immunosuppression with most cats dying of secondary opportunistic infections, not leukemia (20,147). Recognition of the immunosuppressive properties of FeLV lead investigators to concentrate their search for a retrovirus as the most probable cause of AIDS (49,187). The complex interactions which occur between host and invading pathogens whether viral or otherwise is currently an area of intense research and we are only now beginning to understand these interactions at even the most superficial level (64,94,95,99).

One of the best characterized animal models investigating viral/immune cell/cytokine interactions *in vivo* and the possible role that these interactions play in pathogenesis of disease is a bovine-herpes virus model infection, not a retrovirus. Ohmann et al (119) have recently reviewed the role of inflammatory cytokines

in pathogenesis of respiratory tract infections caused by a cytolytic bovine herpes virus, Bovine Herpes Virus-1 (BHV-1). BHV-1 appears to initiate release of inflammatory cytokines (most notably TNF alpha (TNF alpha) and interleukin 1 (IL-1)) which usually induce self-limiting inflammatory lesions in the lungs of infected cattle. In the absence of complicating secondary pathogens, this process is self-limiting and results in viral containment, recovery and tissue healing. However, in the presence of additional pathogens (*Pasturella haemolytica* in their experimental model) which can significantly augment production of inflammatory cytokine mediators (i.e. IL-1, TNF alpha, as well as gamma interferon), normal immunoregulatory mechanisms are disturbed and severe uncontrolled inflammatory responses ensue accompanied by permanent tissue damage and severe systemic effects. Clinically, the cattle become chronically debilitated and frequently die (119). Ohmann's review (119) is an excellent synopsis of experimental research demonstrating that certain viral agents in combination with secondary infecting pathogens may cause dysregulation of the cytokine network which in turn induces widespread inflammation, scarring, and destruction of host tissue leading eventually to death. To date, this model is one of the best described where studies have focused on cytokine/pathogen interactions *in vivo* and the pathologic consequences which result from this relationship.

With the advent of polymerase chain reaction technology where small numbers of cells can conveniently be scrutinized for multi-

ple mRNA production both *in vitro* (9,10) and *in vivo* (141), we may now conveniently address the question of localized cytokine induction by viral agents *in vivo* especially as relates to lesion formation at sites of infection. From these studies we may begin to understand not only the complexities of normal immune responses but also the pathologic lesions which can ensue whenever dysregulation of these responses occurs. The following is a review of non-cytolytic mechanisms by which viruses may induce immunosuppression with special emphasis upon members of the *Retroviridae* family and their interactions with immune cells which may result in immunosuppression.

3.2 Mechanisms of Viral Immunosuppression: Direct Destruction of Immune Cells

The most obvious mechanism by which viruses may induce immunosuppression is via direct lysis of virally infected immunocompetent cells such as T and B lymphocytes, monocytes/macrophages, and dendritic cells (reviewed in (99,140,153)). This topic will be addressed only superficially and the reader is directed to the above mentioned reviews for extensive coverage. With direct lysis of mature immunocompetent cells or their stem cell precursors, immunosuppression would result due to loss of mature effector cells or their replacements followed by an inability to mount an effective immune response against even the most innocuous organisms. Viral agents may induce cellular destruction via multiple

mechanisms. These include viral interference with host cellular machinery at the DNA, RNA, or protein level resulting in loss of function of vital cellular housekeeping functions, toxicity of viral components for cells, permeability changes in cellular membranes due to budding virions, or lysis of virally infected cells by antigen specific cytotoxic T cells (reviewed in (64,99,140,153)). In HIV infected patients, several mechanisms may be at work. It has been well established that the primary target cell for HIV infection bears CD4+ and for CD4+ T cells at least *in vitro* these cells usually die following a complete replication cycle by HIV (53,151). Therefore immunosuppression appears to be due at least partly to direct T lymphocyte destruction by HIV. However, this does not appear to be the whole story. Other mechanisms of cellular destruction by HIV include host-cell membrane perturbation by the HIV envelope protein (gp120), inhibition of host cell synthetic capacity, the accumulation of unintegrated viral DNA as well as the accumulation of gp160-CD4 complexes around the nuclear pores of HIV infected CD4+ cells (reviewed in (53,74,151)). Additionally, a unique syncytial forming mechanism has been described for lysis of uninfected CD4+ T cells. HIV infected cells which express surface gp120 may cause death of nearby uninfected CD4+ T cells with which they come in contact by fusion (165). *In vitro* studies have shown that one gp120 expressing cell can fuse with a large number of uninfected CD4+ cells generating syncytia which rapidly become nonviable.

With HIV, immune-mediated mechanisms may also be important in causing *in vivo* depletion of CD4+ lymphocytes followed by immunosuppression. *In vitro* experiments have demonstrated that free gp120 binds to CD4 molecules on the surface of uninfected T cells, where it is internalized, processed, and gp120 viral peptides are then presented in the context of MHC class II on the cell surface rendering these cells susceptible to lysis by antigen specific CD4+ cytotoxic T cells (82,162). Since the antigen presenting cell is free of viral infection but is lysed due to its presentation of processed gp120 peptides, this method of lysis is termed the innocent bystander effect (82,162). In this case a soluble viral protein and not the whole virion is all that is required for creating the right conditions for lysis of the functioning non-infected immune cell.

3.3 Retroviral Mediated Immunosuppression: Multiple Mechanisms - From Alteration in Signal Transduction To Activation of Cellular Proliferation

One non-lytic mechanism by which viruses are thought to modulate immune function is through the release of soluble proteins. The immunosuppressive properties of p15E, a transmembrane envelope protein present in many transforming type C retroviruses (including FeLV but not HIV), has been well documented (46,147,150). Cianciolo et al (16) synthesized a 17 amino acid peptide, CKS-17,

which has homology to a highly conserved region of FeLV p15E. This peptide has similar immunosuppressive properties to p15E (reviewed in (46,147,150)). CKS-17 has been shown to suppress *in vitro* synthesis of gamma interferon by peripheral blood lymphocytes (116), depress lymphocyte blastogenesis (16), inhibit interleukin 2 (IL-2) mRNA production as well as cellular surface expression of IL-2 receptors on peripheral blood lymphocytes (PBL) (46,117), and modulate B cell immunoglobulin production(102). In unpublished data, Good et al (46) have recently reported that exposure of monocytes to the CKS-17 peptide results in dramatic changes in both their morphology and distribution. The authors suggest, CKS-17 may be mediating significant immunosuppression through modulation of monocyte/macrophage properties, a cell well known to produce endogenous immunosuppressive factors (prostaglandin E2 and transforming growth factor beta (TGF beta)).

The molecular mechanism by which p15E or CKS-17 induces immunosuppression is still under investigation. Current research suggests that p15E intercalates within cellular membranes and somehow blocks intracellular signal transduction by interfering with triggering of Ca^{2+} /calmodulin-dependent activation of adenyl cyclase (147,150) or Ca^{+2} mediated protein kinase C activation (24,47,147). Investigators have examined the mechanism by which p15E or UV inactivated FeLV (UV-FeLV) induces immunosuppression as measured by inhibition of chemiluminescence responses within polymorphonuclear leukocytes (PMN) exposed to virus *in vitro* or

PMNs isolated from chronically FeLV infected cats (24,78,79). The chemiluminescence response of PMNs is known to be dependent upon Ca^{+2} mobilization and protein kinase C activation (63,115). These investigations demonstrate that free p15E or UV-FeLV interferes with chemiluminescence responses of *in vitro* exposed PMNs as well as decreased chemiluminescence of PMNs isolated from FeLV positive cats (78,79). However, one group (24) reports that a defect is present in PMNs isolated from viremic cats but they failed to duplicate this defect during *in vitro* exposure of PMNs to UV-FeLV or p15E. These authors reported that suppressed chemiluminescence of PMNs from viremic cats correlated with alteration in PMN signal transduction via unresponsiveness of protein kinase C to activation signals (24). An inability to duplicate these findings upon *in vitro* exposure of normal PMNs to p15E or UV-FeLV has prompted speculation that FeLV may disrupt the gene encoding for protein kinase C or a necessary co-factor gene during proviral integration into bone marrow stem cell precursors for PMNs (77). Since FeLV is known to establish latent infections in myelomonocytic precursor cells present in bone marrow, FeLV integration at critical genomic sites may account for defects present in PMNs as well as monocytic/macrophages throughout long periods of latency (77,149). As PMNs are a critical component of host defense against opportunistic pathogens (100), reduced PMN functions in viremic and non-viremic FeLV positive cats may contribute to FeLV immunosuppression by allowing secondary opportunistic infections to occur

(reviewed in (98,118,147,150)).

It has also been reported that HIV may cause defective neutrophil functions (reviewed in (98)). Though, Lazzarin et al (85) demonstrated that PMNs isolated from HIV infected patients possess phagocytic capability as well as oxidative burst generating capacity, other investigators have reported PMNs obtained from HIV infected individuals do have decreased chemotactic and secretory capacities (98,175). Retroviruses are not the only viruses to cause PMN dysfunction, other reported viruses include: Newcastle disease virus (34), Herpes simplex virus, Vaccinia and Reoviruses (35), as well as Hepatitis B virus (177). Influenza A virus has also been shown to cause PMN dysfunctions including decreased chemotactic activity and adherence (2).

Neutrophils may not be the only cells which lack appropriate signal transduction mechanisms following retrovirus infections. Gottlieb and colleagues (47,48) have reported that CKS-17 peptides inhibit IL-1 mediated responses in murine thymoma cells by interfering with signal transduction through protein kinase C mediated pathways. Tompkins et al (171) have demonstrated *in vivo* T helper cell immunosuppression in naturally infected FeLV positive cats as measured by an inability of mitogens, or Ca^{+2} ionophore/phorbol esters, to induce IL-2 production from PBLs. T helper cell deficiency may correlate with inhibition of signal transduction through a protein kinase C mediated pathway since this pathway is important for activation of IL-2 production in T cells (92,186).

Purified HIV envelope protein, gp120, has also been implicated as causing immunosuppression. Similar to the FeLV system, Oyaizu et al (127) have demonstrated that exposure to purified gp120 results in reduced expression of IL-2 mRNA as well as cell surface IL-2 receptor expression in CD3-Ti driven T cell activation systems. Investigators have previously reported an inability of HIV infected lymphocytes to initiate inositol phospholipid metabolism or Ca^{+2} mobilization (58,89). An inability to generate diacylglycerol, a membrane component which is needed for activation of protein kinase C, would have disastrous effects upon cellular signal transduction. In lymphocytes, one consequence of activation of protein kinase C mediated pathways is the production of IL-2 and IL-2 receptors (92,186). A failure to make IL-2 or produce receptors for it would greatly impair the ability of not only T cells to proliferate and expand when stimulated with antigens but would also have a detrimental affect upon natural killer and B cell activation (75,120,193). Pinching and Nye (132) have reviewed cellular and immunological consequences which occur as a direct result of HIV infection leading to defective signal transduction in a wide variety of infected or HIV exposed cells.

HIV-1 and gp120, cause dysfunction of mononuclear phagocytes (181) as well as T cells (25,127,185). Wahl et al (181) have demonstrated that normal monocytes exposed to purified HIV-1 or gp120 are significantly impaired in their chemotactic and migrato-

ry responses. Impaired chemotactic activity correlated with a decrease in surface receptors for C5a and FMLP, potent chemotactic ligands. Decreased chemotactic activity has also been reported for monocytes derived from HIV positive patients (134,164).

In addition to gp120, two peptides, which are homologous to HIV's gp41 transmembrane glycoprotein, can suppress normal lymphocyte proliferation *in vitro* following mitogen or alloantigen stimulation (13). Cauda et al(12) have shown these same peptides can inhibit natural killer cell activity by some as yet undetermined mechanism which occurs post binding but prior to the lytic hit.

From the above studies, it is apparent that viral proteins, which may be found circulating freely *in vivo* like p15E and gp120, may be responsible for much of the immunosuppression which is seen clinically. The exact molecular mechanism by which immunosuppression occurs has not been elucidated in every case but it appears that modulation of a cell's ability to transmit information from its surface (signal transduction mechanisms) to its nucleus where regulatory proteins selectively turn on or off sets of genes for transcription is a key factor in retroviral immunosuppression.

In addition to gp120, p15E and its homologues, other recently characterized retroviral proteins may play a role in immunosuppression. For example, a non-envelope protein of murine leukemia virus is responsible for the rapid T cell proliferation associated with this infection (67). This aberrant proliferative response is

accompanied by an immune dysfunctional state and ultimately animals succumb to neoplasia. A gag-encoded p30 antigen appears to be responsible for the proliferative activity behaving as a "super-antigen" by activating a subset of CD4+ T cells bearing V beta 5 T cell receptors (67).

For 20 years researchers have investigated the role of minor lymphocyte stimulating (MLS) antigens, now termed "superantigens", in the mouse. These antigens were named for their ability to cause intense proliferation of CD4+T cells in mixed lymphocyte cultures between murine strains that were identical at the major histocompatibility complex site (MHC). It is now clear that these murine alloantigens exert their proliferative effect by binding simultaneously to a MHC class II molecule and to all T cell receptors which bear specific variable beta chain gene segments (55,68). Similar to murine leukemia virus, investigators have recently independently reported that MLS genes or superantigens appear to be encoded by the long terminal repeat of mouse mammary tumor retroviruses (14,32,41,189). A relevant question arises from these findings: What selective advantage would cause murine retroviruses to maintain expression of "superantigens"? Classically, retroviruses are known to infect and replicate primarily in activated cells thus carrying its own superantigen may help these retroviruses gain a foot hold during early infection. In the case of mouse mammary tumor virus, the virus may use this antigen to stimulate T cells which then act as reservoirs for the virus which cannot

infect its primary target tissue, mammary epithelium, until lactation begins (14). Additionally, stimulation *in vivo* with certain V beta selective elements has been reported to cause widespread immunosuppression, leading to speculation that retroviral V beta selective elements may facilitate viral invasion of the host by suppressing host immune responses (55,68). It remains to be investigated as to whether human retroviruses such as HIV encode some type of "superantigen".

A recent paper reports that a variant of simian immunodeficiency virus, which is highly pathogenic *in vitro*, not only replicates in resting cells but also has the ability to induce intense proliferation of both CD4+ and CD8+ simian lymphocytes (42). Whether this virus encodes a "superantigen" is currently under investigation. The acute disease and death which is induced by this viral variant may be related to its ability to replicate in high titer in infected animals, which usually die within 2 weeks of infection (42). Interestingly, in an analogy to HIV, the authors also report high levels of TNF alpha (42) and IL-6 (86) in the serum of animals dying of acute infection. Investigations are underway to determine the significance of these two cytokines for SIV infection and pathogenesis.

3.4 Viral Modulation of Cytokine Activity: Regulation of Interleukin 2 and Gamma Interferon

In recent years, there has been a virtual explosion in re-

search dissecting the role of cytokines during immune responsiveness and regulation. The marriage of immunology and molecular biology has resulted in rapid molecular technology being applied to the field of immunology with special emphasis upon cytokine isolation, characterization, and development of recombinant proteins (44). The availability of recombinant cytokines and specific monoclonal antibodies has contributed to careful dissection of the role that cytokines play during immune responses. These reagents have opened a new era in research into the pathogenic mechanism of infectious agents, namely their ability to modulate cytokines in ways which are beneficial for the infectious agent. The ability of selected viral agents to modulate cytokine activity as well as cytokine modulation of viral replication will be discussed in the following sections. Since cytokines play an integral role in regulation of the type and duration of an immune response *in vivo*, specific disturbances with their production, secretion, or function could result in severe immunosuppression.

As previously discussed, cats naturally infected with FeLV are suppressed in the ability of PBL to produce IL-2 when stimulated with Con A or PMA and a Ca^{2+} ionophore, A23187 (171). Previous investigations had reported that exposure of human or murine T cells *in vitro* to UV-FeLV resulted in inhibition of IL-2, macrophage-activating factor (123,124), and gamma interferon production (33). Given the central importance of both IL-2 and gamma interferon as potent activators of macrophages, natural killer

cells, and T cells, suppression of their production would certainly impact on host immunity and contribute significantly to immunosuppression (44,183).

Though studies concerning modulation of cytokines by FeLV in cats are hampered due in part to lack of appropriate molecular reagents, extensive research is being conducted into cytokine modulation by HIV and the possible implications this has on immunosuppression development. As with FeLV, several reports indicate that lymphocytes derived from HIV infected patients are diminished in their ability to make IL-2 and gamma interferon in response to antigenic or mitogenic stimulation (65,81,112). In vitro exposure of lymphocytes to HIV's envelope glycoprotein, gp120, as well as to synthetic peptides of gp41, inhibit IL-2 mRNA formation, suggesting that the presence of infectious virions are not necessary for IL-2 suppression (127,173). HIV apparently inhibits IL-2 gene expression by interfering with signal transduction and /or secondary messenger formation following activation of the CD3-Ti receptor, thus interfering with IL-2 gene activation (127). Several investigators have demonstrated HIV infected CD4+ T cells are defective in their ability to mobilize calcium following activation through the CD3-Ti complex (58,89,132). Additionally, the 5' regulatory region of the gamma interferon gene has sequence homology with that of the IL-2 gene, perhaps HIV down-regulates gamma interferon gene expression by a similar mechanism as that for IL-2 (169). However, additional experimentation is necessary

for determination of the exact mechanism by which HIV interferes with gamma interferon production.

In contrast, other investigators have reported an increase in the production of gamma interferon by T cells from HIV patients when stimulated *in vitro* with a variety of factors (143,180). The disparity between these results may be accounted for by the method of *in vitro* stimulation used for cytokine induction, i.e. stimulation via antigen receptor, monoclonal antibodies to CD2 or CD3, mitogenic stimulation or more importantly the stage of HIV infection (asymptomatic, symptomatic with secondarily infecting pathogens, or full blown AIDS) of the patient from which the PBL were derived. Researchers report that while *in vitro* proliferation of antigen stimulated HIV infected T cells appear depressed, T cell proliferative responses to certain mitogens are often present in the same population of cells (36,57,81,112).

3.5 HIV Immunosuppression Through Induction of TGF beta:

A Potent Immunosuppressive Cytokine

An endogenous cytokine of potential importance as an agent of immunosuppression is transforming growth factor beta (TGF β). Separate laboratories have reported increased levels of TGF β present in the plasma of AIDS patients (3) or supernates of stimulated HIV infected PBL as compared to controls (71). TGF β supernate levels correlated closely with an inability to activate CD4+T cells with recall antigens (71). The addition of anti-TGF

β neutralizing antibody partially restored the ability of these cells to respond, thus implicating a role for TGF β in suppression of antigen induced proliferation(71).

TGF β is a potent inhibitor of many cellular and humoral immune functions (reviewed in (70,128)). Over-expression of TGF β in HIV positive patients could play an extremely important role in contributing to the profound immunosuppression seen in HIV infected patients (3). Monocytes as well as lymphocytes produce TGF β and immunological functions by both can be inhibited by active TGF β (128). Recently, Wahl and colleagues (182) reported that *in vitro* infection of peripheral blood monocytes with HIV-1 results in increased expression of TGF β genes suggesting that this population of cells maybe at least partially responsible for elevated levels of TGF β in the sera of AIDS patients. Immunological consequences of *in vivo* expression of TGF β is currently an area of intensive research.

A variety of TGF β effects have been noted which can explain how this cytokine mediates immunosuppression. These include TGF β inhibition of expression of interleukin 1 (IL-1) receptors on certain cell populations (28), down modulation of MHC class II antigen expression on antigen presenting cells (23), and inhibition of cytotoxic T lymphocyte induction (CTL's)(40). TGF β inhibition of IL-1 receptor expression would make cells appear to be functionally defective even in the presence of biologically active IL-1 due to a lack of cellular responsiveness for any IL-1 mediat-

ed event (28). IL-1 is well known as an accessory molecule for T lymphocyte activation (186), as a mediator of inflammatory events as well as acting in synergy with other cytokines to induce B cell maturation, proliferation and immunoglobulin production (26,30). A multitude of cells involved in immunoregulatory events as well as homeostasis bear IL-1 receptors, dysregulation of a cell's ability to respond to IL-1 would significantly contribute to an overall picture of immunosuppression.

Additionally, Fontana et al (40) recently reported with lymphocytic choriomeningitis virus (LCMV) infection of mice, administration of TGF β *in vivo* inhibited the generation of LCMV specific CTL. Whether TGF β functions in a similar fashion in HIV infected patients is unknown. It has been reported that both symptomatic and asymptomatic HIV patients have diminished CTL responses against virally infected autologous cells (160,161), but other investigators have reported the presence of HIV specific CTL activity in the lungs, peripheral blood and cerebrospinal fluid of HIV positive patients (73,133,184). A possible explanation for the discrepancies may involve the stage of HIV infection patients were in when studies were performed. In one study, HIV-specific CTL precursor frequency was found to decrease rapidly in two patients as their disease progressed to full blown AIDS (56). The authors did not investigate whether TGF β over-expression in these patients played a role in down regulating CTL responses.

With respect to down modulation of expression of MHC class II

histocompatibility antigens, TGF β appears to function by decreasing transcription of MHC class II mRNA (23). Given the central role of MHC class II molecules in antigen presentation and immunosurveillance, down modulation of MHC class II antigens on the surface of immunoregulatory cells would result in an inability of antigen presenting cells to present processed antigen to CD4+ immunocompetent cells. A lack of presentation abilities would correlate with decreased cellular activation and proliferation with subsequent immunosuppression due to lack of immune responsiveness.

HIV is not the only retrovirus which has been associated with an overproduction of TGF β . Infection with human T lymphotropic virus type I (HTLV-1), the causative agent of adult T cell leukemia (ATL), also presents a clinical picture of immunosuppression (11,174), and has been linked with *in vitro* overproduction of TGF β (114). Freshly isolated unstimulated ATL cells secreted significantly more TGF β during *in vitro* culture than normal PBLs. The *in vivo* significance of TGF β overproduction by retroviruses must be explored in light of TGF β 's immunosuppressive activities and its potential role in pathogenesis investigated.

Interestingly, TGF β has been reported to suppress HIV replication and expression in a chronically infected promonocytic cell line (U1) but not a chronically infected T cell line ACH-2 or in *in vitro* infected primary T cell blasts (137). Thus TGF β potentially plays a dual role during HIV infections, contributing to

global immunosuppression while simultaneously helping eliminate virus by restricting replication in cells of the monocyte/macrophage lineage. More than likely a delicate balance must exist *in vivo* between inhibition of HIV replication by TGF β and potential enhancement of HIV replication by other cytokines such as TNF α and interleukin 6 (IL-6). These cytokines are elevated in HIV positive patients (8,80,93,142), and are believed to be potent inducers of HIV expression and regulation (135,136). On one hand TGF β with its potent immunosuppressive properties may lead to massive suppression of all aspects of the patient's immune system while simultaneously contributing to elimination of the virus by restricting replication of the virus to only cells of the monocyte/macrophage lineage. The delicate balance between TGF β 's suppressive action on viral regulation and TNF α and/or IL-6's enhancement of viral replication may play important roles in progression of HIV infection to full blown AIDS (137).

3.6 TNF α , IL-6, and HIV Interactions: Do these cytokines enhance HIV replication and thus play a role in pathogenesis?

Both TNF α and IL-6 are present at elevated levels in the serum or tissue fluids at some stages during HIV infection (8,59,80,101), but whether or not they mediate immunosuppression *in vivo* or enhance HIV replication *in vivo* needs to be evaluated. Several investigators have attempted to draw correlations between worsening hematological abnormalities and increasing levels of

circulating TNF α and IL-6 in HIV infected patients (59,101).

Though controversial, both TNF α and IL-6 reportedly affect HIV gene expression and may well serve to regulate HIV's replication state in infected cells (reviewed in (21,151)). *In vitro*, TNF α enhances HIV gene expression by stimulating the promoter region of HIV's long terminal repeat (LTR) via activation of transcription factors which bind to nuclear factor kappa B (NF-kappa B) consensus sequences present in HIV's LTR (29,125). While it is clear that NF-kappa B has a role in HIV transcription (21) and TNF α activates NF-kappa B in cells (29,125), TNF α does not always induce HIV transcription (54,76). TNF α enhancement of HIV replication appears to be cell line specific, occurring in primary monocyte/macrophage cultures, chronically infected monocytic and some T cells, but not all HIV infected clonal T cell lines (76,151,152). Additionally, IL-6, only up regulates HIV expression in chronically HIV infected monocytic cell lines or primary monocyte/macrophages, not T cells (96,135,151). IL-6 appears to enhance HIV replication by some as yet ill defined post transcriptional mechanism (135). The overall importance of TNF α and IL-6 influence on HIV replication and expression *in vivo* is unclear and is an active area of research.

HIV enhancement of monocyte/macrophage production of TNF α and IL-6 *in vitro* is also controversial (103,105,111,155,190) and at least one laboratory has reversed its position following careful control of lipopolysaccharide (LPS) contamination of its media

components from HIV induction of monocytic cytokines (104,105) to a lack of induction (103). In these studies, the presence of even small amounts of contaminating LPS in culture media will skew results as these are potent activators of monocyte/macrophage cytokine gene expression (168).

Though HIV enhancement of TNF α production is controversial (45,111), numerous reports suggest HIV infection or exposure can enhance TNF α production at least *in vitro*, which may then increase HIV replication (125,136,179). HIV regulation of TNF α mRNA levels occurs at the post transcriptional level, since an increased half life for TNF α 's 1.4kb mRNA species has been reported for patients as HIV disease progresses (179). While increased levels of TNF α may play a role in enhanced replication of HIV, the dysregulation of TNF α could also be responsible for many of the clinical signs which are seen in terminal stages of HIV infection.

The cytokine TNF α is extremely pleiotropic and many of its activities may be considered as proinflammatory rather than immunosuppressive but along with IL-1 it is thought to be involved in the severe wasting or cachexia as well as CNS dysfunction which is seen in terminal stages of HIV infected patients (15,191). IL-6, while also a mediator of inflammation, additionally induces differentiation of B cells into antibody secreting plasma cells, which in HIV may contribute to hypergammaglobulinemia and autoantibody formation (reviewed in (97,151,191)), thus contributing to

immunosuppression (30). IL-6 also functions in induction of differentiation and proliferation of CTL (146,167) as well as stimulation of TGF β production, a cytokine known for its potent immunosuppressive actions (183,192). Due to complex interactions occurring between cytokines and the delicate balance which the body must maintain during regulation of an immune response, it is not surprising that immune dysfunction occurs in HIV patients who appear to be experiencing significant modulation of important regulatory proteins.

Co-infection of HIV seropositive individuals with other agents may also contribute to immunosuppression and HIV activation, potentially by modulating cytokine levels. Interestingly, investigators (38) studying the recently reported human herpesvirus 6 (HHV-6), which was first isolated from a patient infected with HIV (154), have reported it may also modulate the production of cytokines in peripheral blood mononuclear cell cultures. HHV-6 can induce the production of IL-1 β and TNF α *in vitro* but not IL-6 (38). UV inactivated, but not heat inactivated, virus retains the ability to induce monokine production by peripheral blood mononuclear cells from normal donors. The presence of neutralizing anti-HHV-6 antibody abrogated its cytokine inducing ability. These observations suggest that cytokine induction by HHV-6 is via a structural viral protein and infection is not necessary. HHV-6 has a tropism for many cells of the immune system, including CD4+T cells, B cells, monocytes/macrophages, and megakaryocytes

(1,90). Infection of these cells could lead to widespread immunosuppression due to disturbances in regulation of cytokine production. It is of particular interest that HHV-6 has been isolated from AIDS patients, since TNF α has been reported capable of up regulating the replication of HIV (29,39,96,136). Thus coinfection in AIDS patients with HIV and HHV-6 could result in an up regulation of HIV replication followed by rapid progression from an asymptomatic state to severe immunosuppression and the development of full blown AIDS. Thus, it appears that HHV-6 infection may be one of many possible co factors that influence the progression of HIV infection.

3.7 Viral Immunosuppression Through Inhibitor Factor Production

Recently examples have been reported in which viruses produce inhibitory proteins which at least in one case shows considerable homology to a host cytokine. Interestingly, the cytokine which the viral protein mimics normally functions to down-regulate immune responses. Epstein Barr virus (EBV) expresses a gene BCRF1 that has considerable homology to a newly described cytokine, interleukin 10 (IL-10) (106).

IL-10, is produced by stimulated TH2 helper cells and is a potent inhibitor of cytokine production by TH1 cells, particularly the production of gamma interferon (107). When the murine cDNA for IL-10 was cloned and sequenced, an unexpected homology with an

uncharacterized open reading frame in Epstein-Barr virus was discovered (106). When murine IL-10 and the Epstein-Barr virus open reading frame (BCRF1) were expressed as recombinant proteins, they were both shown to inhibit the production of gamma interferon by human T cells (66). This finding suggests that the BCRF1 sequence had been acquired by the Epstein Barr virus from the mammalian genome and maintained due to some type of selective pressure. This pressure maybe attributed to the fact that gamma interferon has been reported to inhibit the replication of EBV (176). Since BCRF1 can inhibit the production of gamma interferon it would be advantageous for the virus to maintain this protein to down regulate the host immune response against itself. This modulation of gamma interferon may serve not only to ensure the survival of EBV in an immunologically hostile host, but the effects may also be generally immunosuppressive and favor the survival of other co-infecting agents such as HIV.

Other viruses have also been reported to inhibit host immune responses via the production inhibitory molecules. There has been one report of an IL-1 inhibitor present in the serum of patients with AIDS (6). This inhibitor appeared to be approximately 6000 to 9000 MW and partially masked increased IL-1 in the sera of these patients. This inhibitor may contribute to immunosuppression by tying up IL-1 and thereby blocking IL-1 dependent activation of T lymphocytes in AIDS patients.

3.8 Conclusions

Though this review is far from comprehensive, it is hoped that the reader will appreciate the variety of mechanisms by which viral infections, especially those mediated by retroviruses, may lead to immunosuppression. The specific clinical outcome of viral infection will depend upon a number of factors including the pathogenic nature of the infectious agent as well as the presence of any concurrent opportunistic pathogens or environmental complications.

As we gain better understanding of the molecular mechanisms by which viruses cause immunosuppression, we will be better equipped to design therapies which may specifically block immunosuppression by preventing viral inhibition of cellular functions. Though this promise of future technology is compelling, it must be remembered that even with a viral agent such as HIV which has been one of the most widely studied viruses in the world in the last 10 years, we have a long way to go and much research must be done to further elucidate all the mechanisms by which viruses cause immunosuppression.

With respect to cytokines, we are rapidly approaching a new era in clinical medicine where therapies may be tailored for each individual patient's need. In the golden age of availability of genetically engineered recombinant cytokines, we are on the verge of being able to "reconstitute" a patient's immune system with

those factors which are either no longer produced or functioning inappropriately. In the case of overproduction of important immunoregulatory cytokines, inhibitors may be designed which when properly administered will specifically prevent the target cytokine from reacting with cellular receptors and inducing specific immunological reactions. We are just now beginning to appreciate the presence of biologically active inhibitors of cytokines such as soluble cytokine receptors or specific inhibitory proteins which function during normal immunoregulation of cytokine cascades (5,31,83,107). It seems that certain viral agents such as EBV have already taken advantage of this immunological phenomenon and code proteins that mimic the action of certain biological inhibitors for their own advantage (106). It remains to be seen if immunologists and clinical pharmacologists can achieve the same results but for a therapeutic advantage.

4. RATIONALE FOR THE PRESENT STUDY

FeLV is known to induce a profound state of immunosuppression, which may be at least partially attributed to by the failure of FeLV infected T cells to produce two cytokines, interleukin 2 and gamma interferon. In addition macrophages may play a role in immunosuppression, previous work by Hoover et al (62) established a pivotal role for macrophages in restricting replication of FeLV in acute and persistently infected cats. An age-related refractoriness for FeLV infection was correlated with age-related acquisition of macrophage mediated immune function, Kitten macrophages were shown to be five times more susceptible to infection by FeLV than adult cat macrophages derived from the same tissue source. Additionally, FeLV virus present in bone marrow, was found to resume viral replication whenever macrophage function was disturbed by glucocorticoid therapy or *in vivo* treatments known to suppress macrophage function. While it is clear that myelomonocytic precursors in bone marrow and mature macrophages may replicate FeLV, few studies have addressed the issue of FeLV influence on macrophage function or gene expression.

The experiments planned in this dissertation were aimed at enhancing our understanding of FeLV infection of bone marrow derived macrophages from persistently infected cats, particularly with respect to cytokine production. For this purpose, a technique had to be developed for growth of large numbers of bone marrow derived feline macrophages since no feline myelomonocytic or

macrophage cell lines exists. Additionally, the cDNAs for Interleukin 1 beta and Tumor necrosis factor alpha, two feline monokines, were cloned for use as molecular probes and sequenced. The fourth part of the thesis presents studies with Northern Blot analysis on cytokine production by bone marrow derived macrophages taken from FeLV negative normal cats, clinically normal cats persistently infected with FeLV, and one clinically ill cat with reactivation of FeLV replication within bone marrow derived macrophages. The main goal of these studies was to ascertain whether bone marrow derived macrophages had altered gene expression following reactivation of FeLV replication in cultured cells. These studies suggest that FeLV modulation of gene expression may occur in macrophages which are actively replicating FeLV, but not necessarily in cells that are latently infected.

However, a secondary and perhaps more important goal has been achieved with the cloning and sequencing of two feline cytokines. These cDNA clones are now valuable reagents which will be useful as molecular probes for exploration of gene modulation by other macrophage tropic feline viruses such as feline infectious peritonitis virus (FIP) and feline immunodeficiency virus (FIV). FIV like HIV is a Lentivirus member of the Retroviridae family and may prove to be an important small animal model for exploring methods for treatment and prevention of AIDS (43,130).

5. SPECIFIC AIMS

1. To investigate whether macrophages derived from the bone marrow of latently infected FeLV positive cats have an altered gene expression for Interleukin 1 beta or Tumor Necrosis Factor alpha when compared to comparable macrophages derived from FeLV negative cats. For this purpose, the following studies had to be completed:

a. A technique was established to grow large numbers of macrophages in bulk culture from feline bone marrow.

b. Using polymerase chain reaction technology, feline Interleukin 1 beta and Tumor Necrosis Factor alpha cDNA were cloned, sequenced, and used as molecular probes for Northern Blot analysis of total RNA obtained from LPS stimulated bone marrow derived macrophages from FeLV positive and negative cats.

c. Optimum conditions were established for Northern Blot analysis of cytokine messages in LPS stimulated bone marrow derived macrophages.

2. Comparison of selected properties of bone marrow derived macrophages taken from clinically normal cats that are FeLV negative, clinically normal cats latently infected with FeLV, and one cat exhibiting illness with reactivation of FeLV replication within bone marrow derived macrophages. The following properties were examined:

a. Latex bead phagocytosis

b. Northern Blot analysis of interleukin 1 beta,

tumor necrosis factor alpha, and beta actin mRNA followed by densitometry evaluation.

c. Immunohistochemistry to determine what cell type or types in the population of *in vitro* cultured cells were replicating FeLV.

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PART II:

**FELINE BONE MARROW DERIVED MACROPHAGES: TECHNIQUE
FOR ISOLATION AND KINETICS OF IL-1 β AND TNF α mRNA INDUCTION
FOLLOWING LPS STIMULATION**

1. ABSTRACT

Macrophages are important immunoregulatory cells but they also are significant factors in pathogenesis of certain viral infections. To date, the study of feline viral-macrophage interactions *in vitro* has been difficult due to lack of established feline macrophage cell lines and difficulty obtaining large numbers of macrophages from bone marrow or other tissues without euthanasia. In this report, we describe a method to culture feline bone marrow cells which yields a large population of quiescent macrophages after 14 days of *in vitro* culture. The cell population averages 90% macrophages with the remaining 10% of cells consisting of lymphocytes, fibroblastic bone marrow stromal cells and an occasional polymorphonuclear leukocyte. This cell population is 93% alpha naphthol butyrate esterase positive and 89% phagocytize latex beads but these values approach 100% when adherent stromal and lymphoid cells are excluded from cell counts. When pulsed with lipopolysaccharide (LPS), biologically active interleukin 1 is present in the supernate at 48 hours and mRNA for tumor necrosis factor α (TNF α) and interleukin 1 β (IL-1 β) is detectable 6 hours post LPS stimulation. TNF α mRNA production is rapidly down regulated and no longer detectable at 24 or 48 hrs post LPS. Cultured macrophages are quiescent until stimulated by LPS or plastic adherence.

2. INTRODUCTION

Virus-macrophage interactions and the consequences of these interactions appear to play important roles in the pathogenesis of retrovirus infections including human immunodeficiency virus (HIV), feline leukemia virus (FeLV), and feline immunodeficiency virus (FIV) (1,16,22,24). While there is a need to specifically study viral feline macrophage interactions *in vitro*, the means to do so has not been readily available due to a lack of feline macrophage cell lines for *in vitro* studies. In past reports, the isolation of large numbers of feline macrophages has necessitated either euthanasia of cats for collection of large amounts of bone marrow, blood, alveolar, splenic or lymph node cells from which macrophages are derived or the use of peritoneal cavity lavage fluids some of which contained irritants to increase cellular yield (12,13,19,21,26). While these techniques are successful, the sacrifice of cats for the sole purpose of macrophage collection is expensive and hard to justify. Under optimal conditions, peritoneal lavage may provide sufficient cells for some immunological studies but there is a question concerning the state of macrophage activation depending upon whether peritoneal irritants are used to increase macrophage yield.

In this report, we describe an approach which yields a large population of quiescent mature macrophages at the end of 14 days of *in vitro* culture. The technique involves aspiration of a small amount of bone marrow from the femur and culturing this *in vitro*

as a suspension culture for a minimum of 14 days prior to use. It is critical that cultures be maintained a minimum of 14 days as large numbers of polymorphonuclear cells (PMNs) will be present in the cultures at day 7. This technique has been successfully used for the last three years to grow large numbers of macrophages from FeLV negative cats for cloning and sequencing of feline monokine cDNAs (3) as well as investigations into monokine mRNA levels from LPS stimulated bone marrow derived macrophages isolated from latently infected FeLV positive cats (4). In addition to being economical, the technique does not require euthanasia of cats and due to the small sample size needed, bone marrow samples may be taken multiple times at different time points from the same cat by alternating bone marrow harvest sites and allowing sufficient time for bone marrow recovery before sampling that bone again. The results presented here show that this technique provides a reproducible method for obtaining a large population of macrophages which may be utilized *in vitro* for biological as well as molecular studies.

3. MATERIALS AND METHODS

3.1 Animals

In all experiments, 8 FeLV sero-negative random source cats (5 females and 3 males) of varying adult ages were utilized as bone marrow donors. Animals were housed separately in cages at the animal facilities of the University of Tennessee School of Veterinary Medicine, Knoxville, Tennessee (a fully accredited AALAC facility) and their treatment, housing and care were in compliance with standards set forth in the "Guide for the Care and Use of Laboratory Animals" (NIH publication no. 85-23). Animals were part of a cat colony maintained by Dr. Al Legendre. Animals were clinically normal and determined sero-negative for FeLV and FIV.

3.2 Cell lines and culturing conditions

P388D1 (a murine macrophage - TIB 63) and LBRM 33-1A5 (a murine thymoma - CRL 8079) were obtained from American Type Culture Collection (ATCC), Rockville, Maryland. P388D1's were cultured in RPMI (GIBCO Laboratories, Grand Island, New York, NY) supplemented with 15% heat inactivated fetal bovine serum (HI-FBS - Hyclone, Logan, UT) plus 2mM L-glutamine (GIBCO), 100 units/ml penicillin (GIBCO), 100 ug/ml streptomycin (GIBCO), and sodium bicarbonate to a pH of 7.2 to 7.3. LBRM 33-1A5's were cultured at a density of $7-9 \times 10^5$ cells per ml in RPMI (GIBCO) plus 15% HI-FBS (Hyclone), 2mM L-glutamine (GIBCO), 100 units/ml penicillin (GIBCO), 100ug/ml streptomycin (GIBCO), 2×10^{-5} M 2-mercaptoethanol (2-ME) (SIGMA

Chem. Co., St. Louis, MO) and sodium bicarbonate to a pH of 7.2 to 7.3. At a density of 1×10^6 cells per ml, P388D1 cells were stimulated in complete medium with 10 ug/ml *E. coli* serotype 0127:B8 lipopolysaccharide (LPS) (SIGMA) for 48 hours for production of murine IL-1 (10). At the end of incubation, supernates were harvested and frozen at -70°C until assayed for IL-1 activity. LBRM 33-1A5 cells were utilized in an IL-1 conversion assay for determination of biologically active IL-1 in supernates (11).

CTLL-2 cells were obtained from the ATCC and cultured in Iscove's Modified DMEM (GIBCO) plus 10% HI-FBS (Hyclone), 10% NCTC 109 (GIBCO), 1mM sodium pyruvate (GIBCO), 2mM L-glutamine, $5 \times 10^{-5}\text{M}$ 2-ME, 1X essential and non-essential amino acids (GIBCO), 100 units/ml penicillin, 100 ug/ml streptomycin, 10 mM HEPES (Res. Organics Inc., Cleveland, OH), sodium bicarbonate to a pH of 7.2 to 7.3 with addition of 3% phorbol myristic acetate (PMA) stimulated EL4.IL-2 supernate for IL-2 growth factor. EL4.IL-2 (TIB 181) cells were obtained from ATCC, cultured in Delbecco's MEM (GIBCO) plus 10% HI-horse sera (GIBCO), 2mM L-glutamine and sodium bicarbonate, and utilized to generate IL-2 for continued growth of the CTLL-2 cell line. When pulsed with 20 ng/ml PMA, EL4.IL2 cells produce a significant amount of murine IL-2 (7).

3.3 Feline bone marrow isolation technique and *in vitro* culturing method

Bone marrow was obtained with cats under general ketamine HCl (Ketalar^R, Parke Davis, Morris Plains, NJ) anesthesia (1 mg/lb IV or 10 mg/lb IM). The sacrolumbar area was clipped and surgically prepped with betadine scrub followed by an alcohol wash. After surgical prep, approximately 0.5 to 1 ml of bone marrow was aspirated by a bone marrow needle from the femur directly into a 10 ml syringe containing 3 ml of Iscove's Modified Dulbecco's Medium (GIBCO) plus 100 units per ml of heparin sulfate (SIGMA Chemical Co., St. Louis, MO) (H-IMDMEM). Samples were thoroughly mixed, placed in sterile 15 ml conical centrifuge tubes (Sanstedt, Inc, Newton, NC) containing 2 ml of H-IMDMEM and held on ice until separation procedure. Cats were allowed to recover and monitored for any adverse reactions. Bone marrow samples from each cat were kept separate, and yields were such that it was never necessary to pool samples.

For separation, bone marrow samples were brought up to 10 ml final volume with H-IMDMEM, vortexed for 20 secs then sat at room temperature for 5 minutes undisturbed. At this time, the bulk of the supernate was carefully removed leaving a 0.5 ml pellet, which contained bone chips and cartilage fragments. Five mls of bone marrow-H-IMDMEM mixture was layered over 3 ml of room temperature Ficoll-Sodium diatrizoate (Histopaque 1.077 - SIGMA, St. Louis,

MO) in a 15 ml conical centrifuge tube. Tubes were centrifuged at 400 xg for 25 minutes at 25°C. Floating fat globules were carefully removed, discarded, and the cellular interface band was removed and washed 2 times with 10 ml of H-IMDMEM, followed by a final wash with non-heparinized IMDMEM. Cells were counted by hemocytometer and viability assessed by 0.2% trypan blue dye exclusion (SIGMA). Viability was usually greater than 97% and never dropped below 95%. Cell concentrations were adjusted to 1.0×10^6 to 2.0×10^6 cells per ml in complete medium consisting of IMDMEM containing 15% HI-FBS (HyClone), 2mM L-glutamine, 1mM sodium pyruvate, 100 units/ml penicillin, 100 ug/ml streptomycin, 10 mM Hepes and sodium bicarbonate to a pH of 7.2 to 7.3. With this suspension culture technique, no additional additives were necessary to provide colony stimulating factors.

Ten mls of cell suspension were cultured in polystyrene 100 mm X 20 mm tissue culture dishes (FALCON, BAXTER, Atlanta,GA) at 37°C, in a humid 6% CO₂ chamber. At the end of one week, 10 ml of complete IMDMEM was added to each plate and incubation continued for an additional 7 days. At the end of 14 days culture time, the cell population consisted of adherent macrophages with some lymphoid and bone marrow stromal cells as well as an occasional polymorphonuclear leukocyte (PMN) or erythroid precursors.

Macrophages which were assessed for interleukin 1 production or harvested for Northern blot analysis were pulsed with 10 ug per ml LPS by one of two methods. Method 1 - For each culture dish,

medium was gently removed and non-adherent cells pelleted by centrifugation. Pelleted cells were resuspended in complete growth medium containing 10 ug per ml of LPS. For each dish undergoing LPS stimulation, 20 ml of medium plus LPS was gently layered over the undisturbed macrophage monolayer and incubated at 37°C, in a humid 6% CO₂ chamber for 6, 24, or 48 hours at which point supernates were harvested, frozen in small aliquots at -70°C for IL-1 biological activity and cells lysed for RNA isolation. Method 2 - Loosely adherent macrophages were gently washed from culture dishes, while adherent cells were removed by incubating at room temperature with Hanks balanced salt solution (Ca²⁺ and Mg²⁺ free) (GIBCO) plus 2.6 mM EDTA (SIGMA) followed by gentle pipetting. Cells were resuspended in complete medium at 1 x 10⁶ cells per ml plus 10 ug per ml LPS. Cultures were incubated in a 37°C, humid 6% CO₂ incubator for 6, 24, or 48 hours at which point supernates were harvested, frozen in small aliquots for IL-1 testing, and cells lysed for RNA isolation. For total RNA isolation, cell pellets were lysed in guanidium isothiocyanate and stored at -70°C until RNA isolation procedure (see below).

3.4 Morphologic and cytochemical characterization of population

Non adherent cells were removed from dishes by gentle pipetting, adherent cells were removed by incubating with 5 ml of Hank's balanced salt solution plus 2.6 mM EDTA. Adherent and non adherent cells were mixed, concentrations determined, and cyto-

centrifuge preparations (Cytospin, Shandon Inc., Pittsburgh, PA) for differential and nonspecific esterase analysis were prepared by spinning 100 μ l of 1×10^6 cells/ml onto glass slides for 5 minutes at 700 RPM. Cytospins were allowed to dry at room temperature and stained with Diff-Quik stain (Difco, Detroit, MI) for differentials or nonspecific esterase stains were done by the method of Li et al (20). Briefly, cytocentrifuge or Lab-Tek slide (Miles Scientific) preparations were dried overnight then prefixed for 30 seconds in Sorensen's buffered formalin acetone (pH 6.6), rinsed in several changes of water, and air dried for 30 minutes. Next they were incubated for 45 minutes in a 37°C water bath with hexazotized pararosaniline solution in Sorensen's buffer (pH 6.3) with alpha-naphthyl butyrate (SIGMA) as the substrate. Slides were water rinsed, drained, counterstained in 0.5% methyl green for 20 minutes at room temperature, rinsed in distilled water, air dried and permanently mounted with Permount (SIGMA). A minimum of 200 cells were counted for differential analysis and esterase determination.

3.5 Assay for phagocytosis

Cultured bone marrow derived macrophages were harvested, counted and plated 2.0×10^5 cells per ml in complete IMDMEM plus 10 μ g per ml of LPS in 8 chamber Lab-Tek slides. Each well for phagocytosis determination contained 10 μ l of a 1:100 dilution of 0.8 micron Latex beads (SIGMA). Activated macrophages plus beads

were incubated at 37°C in a humid 6% CO₂ atmosphere for 24 hours. After incubation, culture medium was removed, cells fixed and stained with Diff-Quick by carefully dropping fixing and staining solutions into the Lab-Tek chamber. After staining, slides were air dried and permanently mounted. Staining by fixing gently *in situ* without undue disturbance to the cell layer insured loosely adherent cells remained attached for phagocytosis evaluation. A cell was deemed phagocytic if it had phagocytized a minimum of 10 beads, however, phagocytic cells frequently contained so many beads that they were not countable within the cell.

3.6 Interleukin 1 conversion assay for detection of biologically active IL-1

The IL-1 conversion assay was performed by the method of Gillis et al (11). In snap capped sterile 12 x 75mm polystyrene tubes (FALCON), 1×10^5 washed LBRM 33-1A5 cells were added in complete RPMI medium. For quantitation assays, the following was added to each tube: 25% putative IL-1 containing supernate plus 0.75 ug of phytohemagglutinin-P (PHA-P) (SIGMA) in a final tube volume of 300 ul. Additionally, the following controls were run, LBRM cells plus medium alone and LBRM cells plus 25% IL-1 containing supernate in the absence of PHA-P. Samples were run in triplicate, tubes capped and incubated at 37°C in 6% CO₂ for 24 hours. Samples were centrifuged, supernate harvested and frozen in small aliquotes at - 70°C until the CTLL assay for quantitation of IL-2 was done.

until the CTLL assay for quantitation of IL-2 was done.

Step two of the conversion assay was quantitation of IL-2 which will only be present in supernate from LBRM 33-1A5 cells stimulated with both PHA-P and IL-1. Two fold serial dilutions of putative IL-2 containing test medium were prepared in 100 ul of CTLL-2 RPMI growth medium lacking IL-2 supplementation in 96 well round bottom microtiter plates (Corning). CTLL-2 cells were washed twice in serum free RPMI then 5×10^3 cells in 100 ul plated per well. Human recombinant IL-2 (Gibco BRL, Grand Island, NY) was titered in each assay as a control for CTLL-2 proliferation and to provide a means of quantitation. Additionally, supernate harvested directly from stimulated macrophage cultures was tested for the presence of IL-2, produced endogenously by residual lymphocytes. Plates were incubated 24 hours at 37°C in a humid 6% CO₂ atmosphere. Wells were pulsed with 1 uCi of ³H-thymidine for 24 hours then harvested onto Whatmann glass microfiber filters (Baxter) by a PhD automatic cell harvester, air dried overnight and counted in a beta counter (Beckman) following addition of scintillation fluid (Ecolume, Baxter).

3.7 Total RNA isolation procedure and Northern Blot analysis

Cells for total RNA isolation were lysed in a 4M guanidinium isothiocyanate (SIGMA) lysing solution containing 0.5% sodium lauryl sarcosine (SIGMA) , 25mM sodium citrate pH 7.0 (SIGMA), and 47 mM 2-mercaptoethanol. The lysate was centrifuged at 36,000 rpm

for 18 hours in a Beckman ultracentrifuge (rotor - Sorval AH650 rotor) through a 5.7 M Cesium chloride cushion (2).

After phenol/chloroform extraction followed by ethanol precipitation, total RNA was quantitated by spectrophotometry and 10 ug per sample denatured for fractionation in a 1.0% agarose/ 1.1 M formaldehyde gel containing 500 ugs of ethidium bromide for RNA visualization. The 10X running buffer consisted of 0.5M boric acid, 50mM sodium borate, 100 mM sodium sulfate and 10 mM EDTA. For denaturation, RNA samples were dissolved in 20 ul of the following buffer mix: 0.63 ml denatured formamide (BRL), 0.20 ml 37% formaldehyde, 0.063 ml of 10X running buffer, 0.10 ml of 50% glycerol in DEPC treated water, and 0.030 ml of a 0.6% solution of bromophenol blue/ xylene cyanol, boiled for 10 minutes, and placed on ice for 10 minutes prior to gel loading. Gels were electrophoresed at a constant current of 95 volts until the bromophenol blue dye front traveled approximately 14 cm. At this point, gels were visualized with UV for comparisons of equality of RNA loading, soaked briefly to remove gel fragments, then RNA was transferred to nylon filters (Nytran, Schleicher and Schuell, Inc, Keene, NH) overnight by a sponge modification of the capillary transfer method (9).

Following overnight transfer, nylon filters were UV-crosslinked to stabilize RNA (18) (Stratalinker UV Crosslinker, Stratagene) and filters were prehybridized at 60°C for a minimum of 1 hour in 5X SCC, 0.05M sodium phosphate buffer pH 6.5, 5X Denhardt's solu-

tion, 1% SDS, 200 microgram per ml of denatured salmon sperm DNA as a blocker. Following prehybridization, filters were hybridized overnight (18 hours) at 60°C in the same prehybridization mix plus the appropriate ^{32}P labeled nick translated (Amersham kit N5500) cDNA probe. Nick translated probes had a specific activity of 1 to 2×10^7 cpm/ug of DNA specific activity. Post hybridization, filters were washed in the following order: (1) Two times at room temperature for 15 minutes each in 2X SCC plus 0.1% SDS. (2) Two times at room temperature for 15 minutes each in 0.2X SCC plus 0.1% SDS. (3) Once for 20 minutes at 55°C with 0.2X SCC plus 0.1% SDS. (4) Final wash, once for 30 minutes at 60°C with 0.1X SCC plus 0.1% SDS.

Following washes, blots were exposed 70 hours to Kodak XAR 5 film in the presence of Dupont Cronex Lightning plus intensifying screens at -70°C. Following exposure, radioactive cDNA probes were stripped from nylon filters by washing filters in 50% formamide/6X SSPE at 65° C for 30 minutes followed by rinsing briefly in 2X SSPE. Filters were then reprobed with additional radiolabeled cDNA probes. In all studies, the following pattern was maintained: Filters were first probed with TNF α specific probes, blot stripped followed by probing with an IL-1 β specific probe, blot stripped then probed with an actin specific cDNA probe.

Plasmids containing fragments of cDNA for feline TNF α and IL-1 β were prepared in this laboratory and the paper characterizing their preparation and sequencing is in progress (3) (see Part III

of Thesis). A ^{32}P labeled TNF α probe was prepared from the plasmid, pTNF α B9, which contains a 570 bp Eco R1, Hind III fragment of feline TNF α cDNA. A ^{32}P labeled IL-1 β probe was prepared from the plasmid, pIL1#2, which contains a 750 bp, Eco R1, Hind III fragment of feline IL-1 β cDNA. The actin cDNA plasmid (pA1) was a gift from Dr. Janet Lathey (Scripps Institute) and contains a 750 bp Eco R1, Hind III actin cDNA fragment.

4. RESULTS

4.1 Morphological characterization of bone marrow derived population

Using the separation procedure described in the methods section, we routinely harvested an average of 1.5×10^8 nucleated cells (range 2.0×10^7 to 2.8×10^8) from bone marrow. After *in vitro* culture, it was not considered appropriate to harvest cells before 7 days since at this time the cell population was found to be about evenly divided between mature PMNs and immature cells of the myelomonocytic series (Table 2.1). At the end of 14 days in culture, the average number of cells recovered was 2.3×10^7 (range 4.2×10^6 to 1.3×10^8) of which 90% were monocyte/macrophages and 10% were variously composed of lymphocytes, PMNs, immature myelomonocytic or erythroid progenitor cells and an occasional megakaryocyte (Table 2.1). After 14 days in culture, macrophages appeared round, elongated or stellate, differing significantly in the amount of cytoplasmic spreading and ruffling seen (Figure 2.1, Panel A). Macrophage nuclei were frequently kidney shaped or slightly ovoid and eccentrically located with small nucleoli and a large cytoplasm to nucleus ratio (Figure 2.1 Panel B). The predominant cell type was macrophages but a lymphoid cell may be seen nestled against one of the macrophages in Panel B, Figure 2.1.

Table 2.1 Characterization of cell population present following 7 and 14 days of *in vitro* culture of bone marrow cells.

Differential Analysis ^a				
Cell Type	7 day cultures		14 day cultures	
Macrophages	(6 - 11%)	8.5%	(80 - 95%)	90%
Lymphocytes	(4 - 8%)	6.0%	(3 - 9%)	4%
PMN ^b	(37 - 50%)	43.5%	(0 - 17%)	4%
Other ^c	(35 - 49%)	42.0%	(0 - 2%)	1%

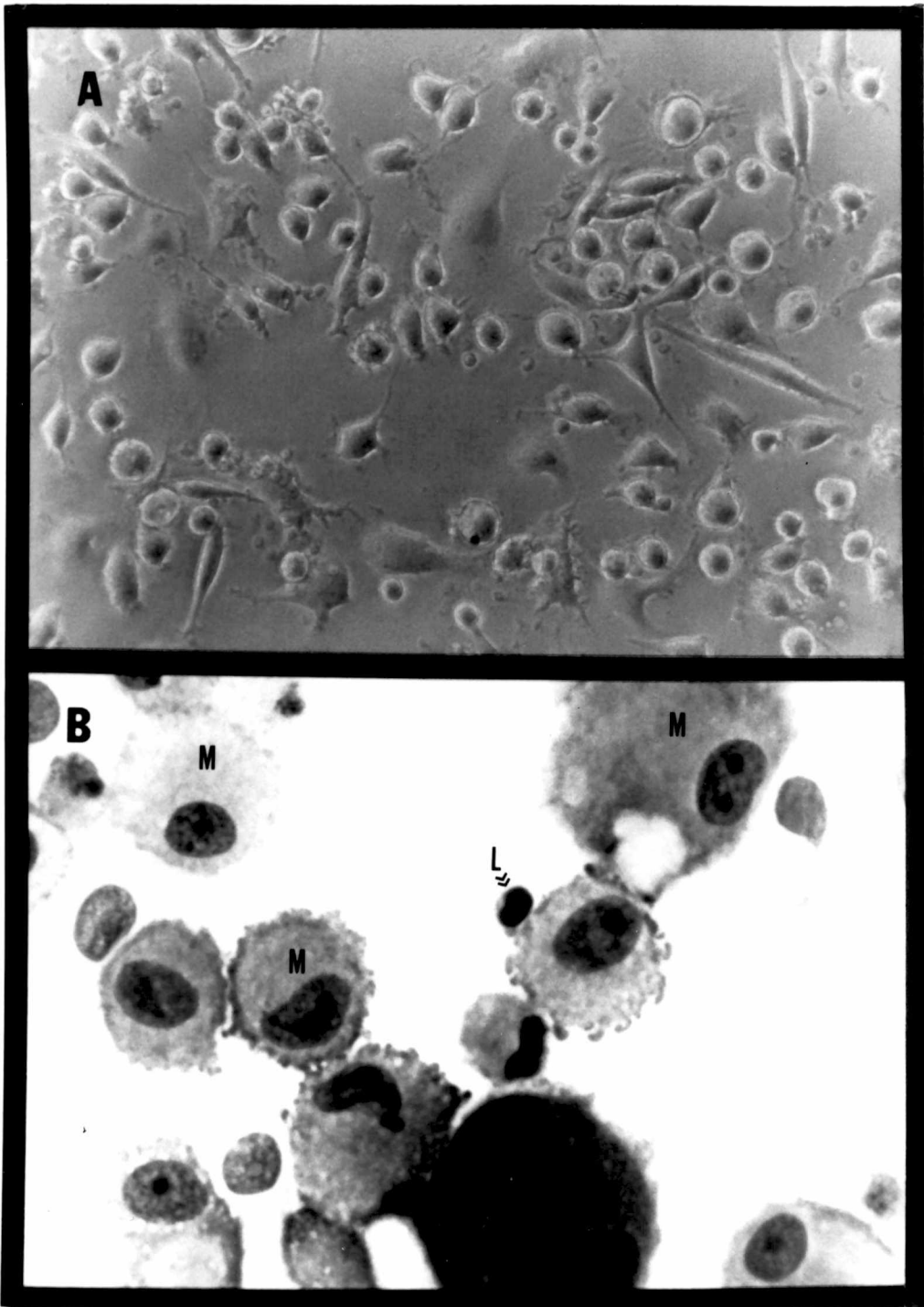
^aDifferential analysis was performed on Diff-Quick stained cytos-
pins as described in the Materials and Methods. Values within the
parenthesis indicate the range observed with the average percent-
age listed to the right of the parenthesis. N = 8 cats

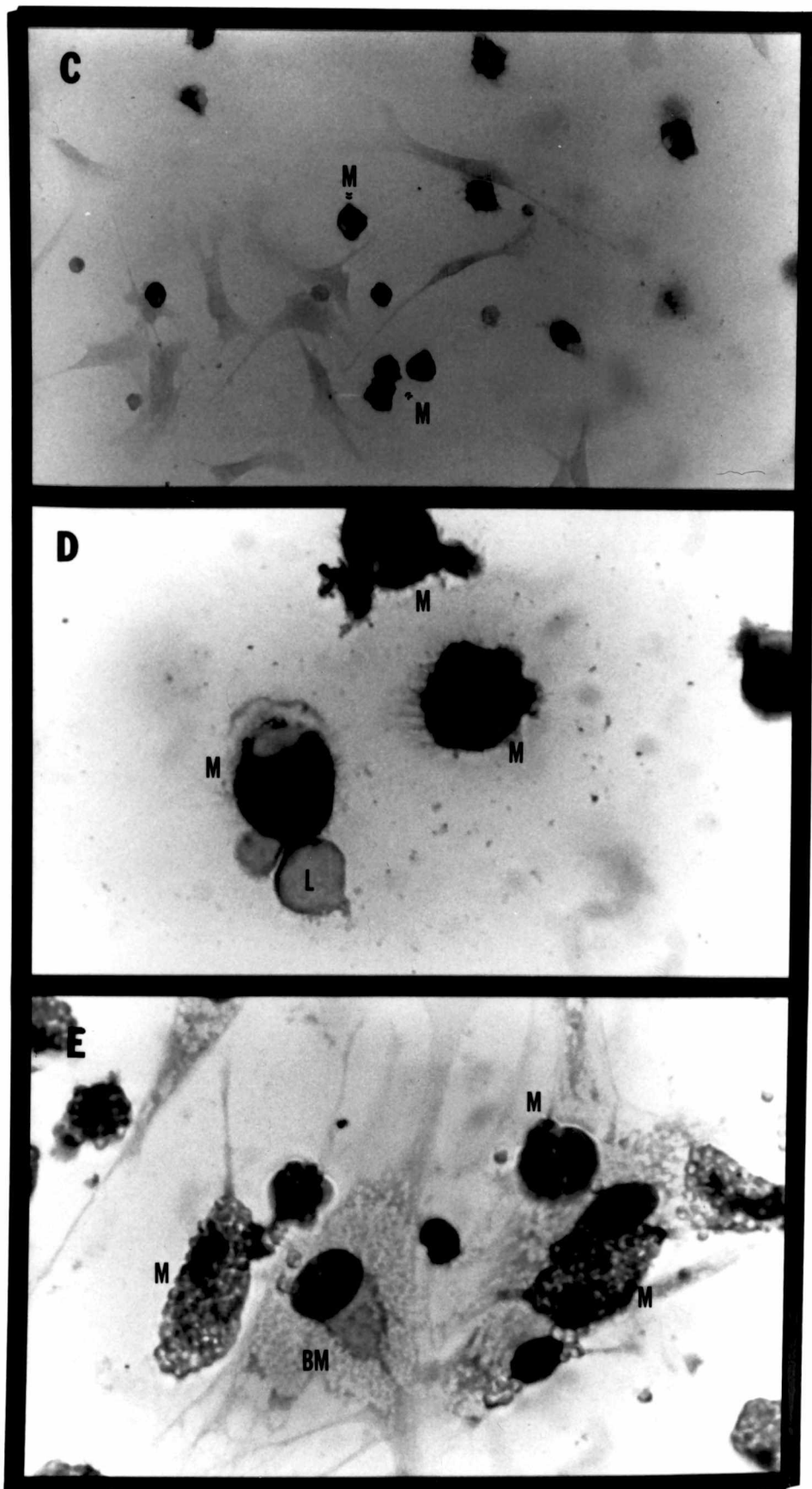
^bPMN = polymorphonuclear leukocytes

^cOther cell types included granulocyte/macrophage progenitor
cells, erythroid precursors or megakaryocytes.

Figure 2.1. Identification of bone marrow derived cells as macrophages based upon morphology, histochemistry, and phagocytosis assays. Cytospins of bone marrow cells cultured *in vitro* for 14 days were made, stained by Diff-Quick (Panel B) and evaluated morphologically. Lab-Tek chamber slide cultures of bone marrow cells were evaluated for alpha naphthol butyrate esterase activity (Panels C and D), while latex bead phagocytosis was evaluated following 10 ug/ml LPS stimulation (Panel E). Panel A is representative of the typical appearance of bone marrow derived macrophages after *in vitro* culture for 14 days - magnification 66X. Panel B is a representative Diff-Quick stain of the cultured population - magnification 400X. In Panels C and D, the darkly staining cells are positive for esterase activity - magnification 100X Panel C and 400X Panel D. In Panel E, the phagocytic macrophages have been stained with Diff-Quick allowing visualization of the nucleus. Extensive phagocytic activity is readily apparent, massive numbers of latex beads are visible within the cytoplasm of many cells. A non-phagocytic bone marrow stromal cell as well as a lymphocyte are visible in the center of the micrograph. Magnification 400X.

M = macrophage L = lymphocyte BM = bone marrow stromal cell





Fourteen day old bone marrow cultures were examined for esterase and phagocytic activities (Table 2.2) The majority of cells (average 93%) were positive for butyrate esterase activity (Table 2.2 and Figure 2.1 Panel C and D). There was variability in the amount of enzyme activity with some cells staining intensely dark while others stained lightly for esterase activity. In Figure 2.1 Panel C, numerous darkly stained rounded macrophages are readily visible while esterase negative adherent bone marrow stromal cells and lymphocytes are present in the background. Occasional esterase positive stellate or elongated cells could be seen suggesting that these were of the macrophage lineage, but the predominant esterase negative cell had long cytoplasmic extensions with a triangular shaped body similar to that reported for stromal cells. In Panel D of Figure 2.1, two esterase negative lymphocytes may be seen nestled against a macrophage.

Macrophages from 14 day cultures displayed significant phagocytosis for latex beads (Table 2.2 and Figure 2.1, Panel E). Over a 24 hour period, LPS activated macrophages engulfed significant numbers of latex beads, often leading to cellular distortion and obscuring of the nucleus. On the average, 89% of the population present on day 14 phagocytized latex beads. Non-phagocytic cells were adherent, triangular shaped cells with long cytoplasmic extensions, having the appearance of bone marrow stromal cells but an occasional non-phagocytic lymphocyte was also present (Figure 2.1, Panel E).

Table 2.2 Evaluation of cells from 14 day old *in vitro* bone marrow cultures for esterase and phagocytic activity.

<u>Alpha Naphthol Butyrate Esterase</u> ^a	
Positive	Negative
(79 - 99%) 93%	(1 - 12%) 7%

<u>Latex Bead Phagocytosis</u> ^a	
Positive	Negative
(78 - 97%) 89%	(3 - 22%) 11%

^aAlpha naphthol butyrate esterase and phagocytosis assays described in Materials and Methods. Values in parenthesis represent the ranges seen for all cats studied and the average percentage listed to the right of the parenthesis. N = 8 cats.

4.2 Presence of biologically active IL-1 in LPS stimulated bone marrow derived macrophage cultures

Cells present at the end of 14 days of *in vitro* culture are esterase positive and phagocytize latex beads, properties characteristic of macrophages. We next examined whether these cells could be stimulated to produce interleukins, specifically IL-1 and TNF α . At the onset of these studies, species specific cDNA probes for IL-1 β and TNF α were not available. Therefore, we examined LPS stimulated macrophage cultures for the presence of biologically active IL-1 by means of an IL-1 conversion assay. IL-1 conversion assays measure the ability of IL-1 in the presence of PHA-P to convert the murine thymoma cell line, LBRM 33-1A5 from a non-IL-2 producer into an IL-2 producing cell. IL-2 present in LBRM supernates was quantified by means of an IL-2 dependent CTLL-2 proliferation assay. The LBRM 33-1A5 cell line requires the presence of both IL-1 and PHA-P in culture before it will produce IL-2. The absence of either component negates the ability of this cell line to be converted to an IL-2 producer.

Biologically active IL-1 was clearly detectable in the supernates of bone marrow derived macrophages which had been stimulated for 48 hours with 10 ug/ml of LPS (Table 2.3 and Figure 2.2). Supernate from LPS stimulated macrophages for cats 1 and 2 contained biologically active IL-1 which converted LBRM cells to IL-2 producing cells. In fact, their levels were comparable to that produced by the P388D1 cell line. Cats 1 and 2 IL-1 containing

Table 2.3 BM macrophages produce biologically active IL-1

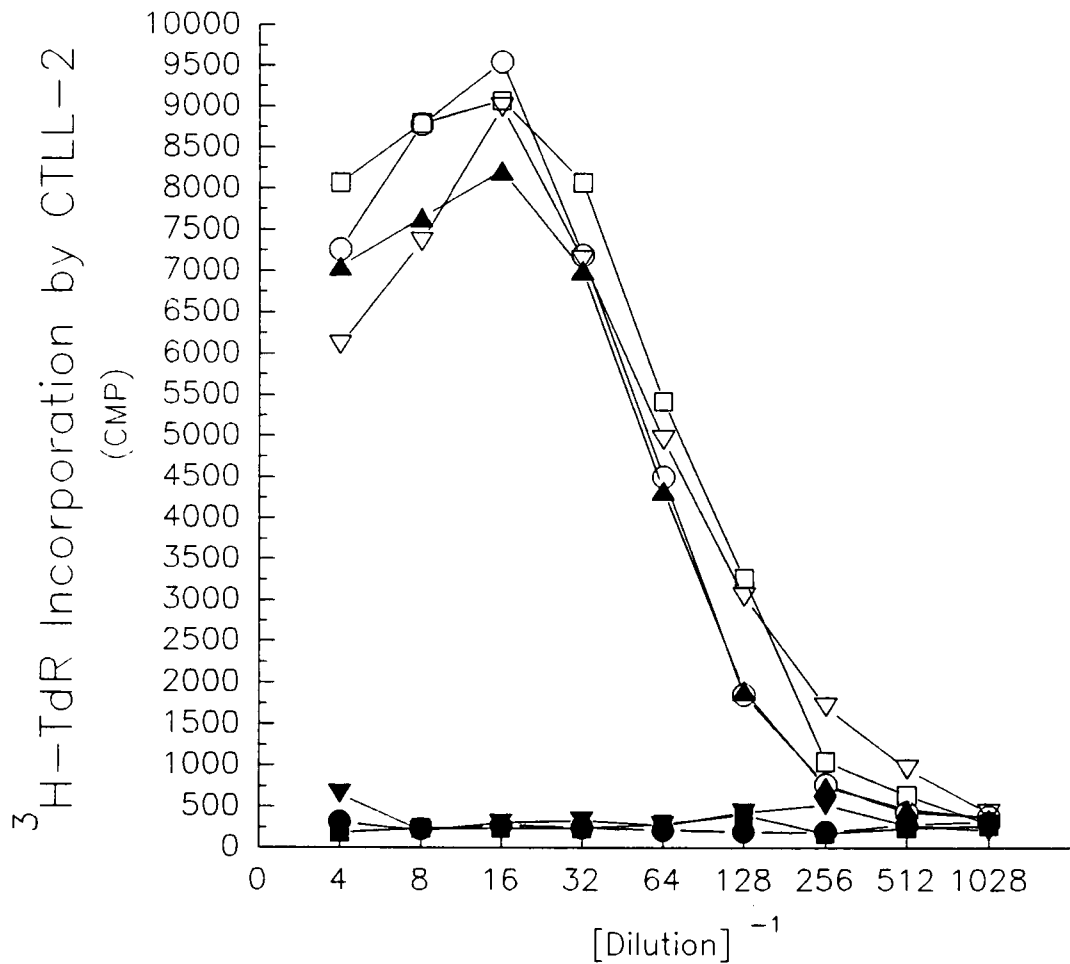
IL-2 production by LBRM 33-1A5 cells stimulated with IL-1 present in supernate of LPS stimulated cat or murine macrophages^a

LBRM cells stimulated with		IL-2 present in 24 hour
test supernate	PHA-P	supernate, units/ml
Medium	None	0
Medium	+	0
P388D1 (murine)	None	0
P388D1 (murine)	+	40
Cat 1	None	0
Cat 1	+	40
Cat 2	None	0
Cat 2	+	50

^aIL-1 conversion assay and IL-2 detection described in Materials and Methods. Units of IL-2 determined by comparison to a human recombinant IL-2 standard curve. Supernates from LPS stimulated macrophages were negative for endogenously produced IL-2. Supernates from LBRM 33-1A5 cells treated with medium only, medium plus PHA-P, or test medium containing IL-1 without the addition of PHA-P were also negative for IL-2 production.

Figure 2.2 IL-1 conversion assay : Proliferation of CTLL-2 cells as a consequence of IL-2 production by LBRM-33 1A5 cells cultured in the presence of IL-1 and PHA-P.

IL-1 Conversion Assay



- ▽ - Cat 1 Supernate IL-1 + PHA
- ▼ - Cat 1 Supernate IL-1 Alone
- - P388D1 Supernate IL-1 + PHA
- - P388D1 Supernate IL-1 Alone
- - Cat 2 Supernate IL-1 + PHA
- - Cat 2 Supernate IL-1 Alone
- ▲ - Human Recombinant IL-2 Std

supernate caused production of 40 and 50 units/ml of IL-2 by LBRM cells over a 24 hour period, respectively. Supernates taken directly from LPS stimulated macrophages were negative for endogenously produce IL-2, suggesting IL-1 conversion of LBRM 33-1A5 cells to IL-2 producers was the source of IL-2 and not the residual lymphocyte population contaminating the macrophages. Supernates from LBRM 33-1A5 cells that had previously been exposed to medium only, medium plus PHA-P only, or test medium containing IL-1 without the addition of PHA-P were also negative for IL-2 production, indicating the LBRM 33-1A5 cells were not producing IL-2 unless properly stimulated with IL-1 and PHA-P (Table 2.3). Figure 2.2 is a graphic representation of ³H thymidine incorporation by the CTLL-2 cells which showed that optimal IL-2 induced proliferation occurred at a 1:16 dilution of supernates generated from LBRM cells previously exposed to IL-1 derived from P388D1, Cat 1, or Cat 2 LPS pulsed cells. This level of proliferation corresponds to approximately 40 to 50 units per ml of the human recombinant IL-2 used in these studies (Table 2.3).

4.3 Northern blot analysis of LPS stimulated macrophages for IL-1 β and TNF α mRNA

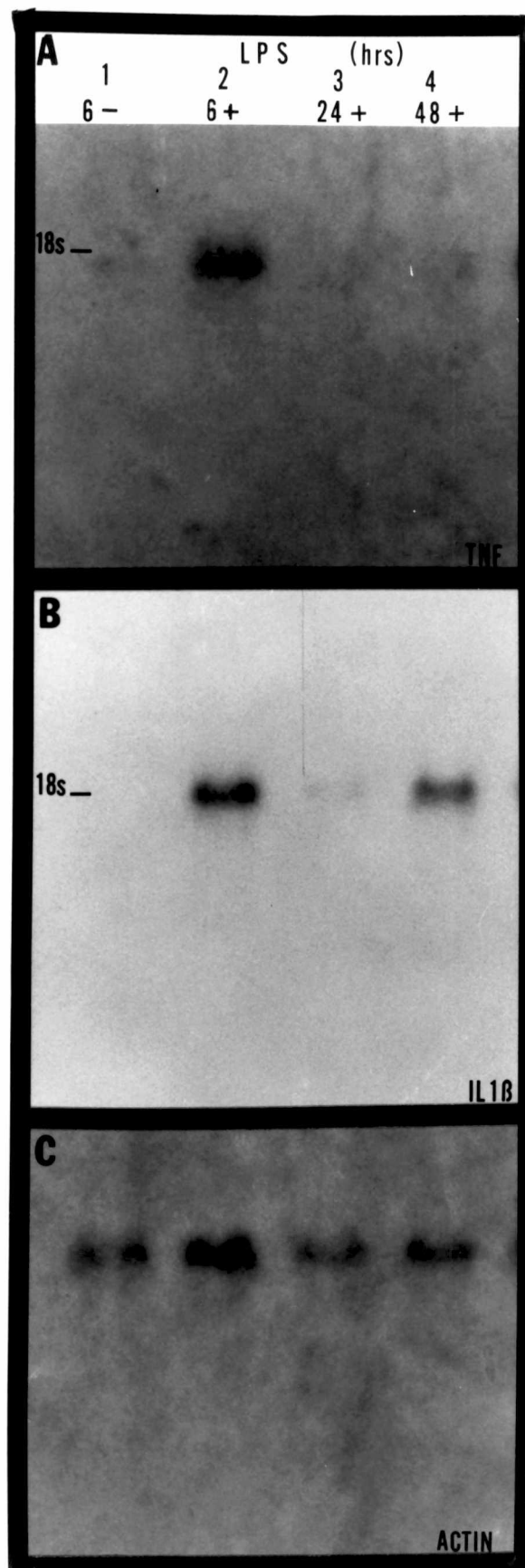
With the successful cloning of cDNAs for feline IL-1 β and TNF α , we further explored the kinetics of production of these two cytokines by bone marrow derived macrophages (3). Macrophages were cultured for 14 days *in vitro*, after which the medium were aspirat-

ed and cells re-cultured (without disturbing the adherent monolayer - Method 1, see Material and Methods) in the presence or absence of 10 ug/ml LPS for 6, 24 or 48 hrs. Total RNA was harvested from macrophage lysates and Northern blot analysis performed. Figure 2.3 is representative of our findings. In Panel A, a ^{32}P labeled cDNA probe for feline TNF α was hybridized to 10 ug of total RNA per lane. After 70 hour exposure, cytokine message in Lane 1, which contains RNA from cells not stimulated with LPS, is barely detectable indicating that the bulk of these cells were relatively quiescent or non-activated in *in vitro* culture. After LPS stimulation, the TNF α gene was activated with mRNA readily detectable at 6 hours followed by down-regulation of gene transcription with little or no mRNA detectable at 24 or 48 hours.

In Panel B of Figure 2.3, IL-1 β mRNA was readily detectable following 6 and 48 hour stimulations with LPS, but levels of mRNA for IL-1 β after 24 hours were greatly reduced. Actin mRNA levels visualized in Panel C show comparable amounts of total RNA were loaded per lane, it would appear that IL-1 β gene transcription was undergoing modulation.

These studies demonstrated bone marrow derived macrophages will produce TNF α and IL-1 β after appropriate stimulation, however, their gene transcription was tightly regulated with TNF α 's mRNA expression declining rapidly after 6 hours. IL-1 beta mRNA had a characteristic biphasic pattern with mRNA levels dropping significantly by 24 hours LPS stimulation with re-expression by 48 hours.

Figure 2.3 Kinetic Study: Northern blot analysis of LPS stimulated bone marrow derived macrophages - Method 1. After 14 days in culture, growth medium was changed and LPS stimulation of cultures begun without disturbing the adherent macrophage cell monolayer. Procedure fully described in Materials and Methods. Macrophages were stimulated for select periods of time at which point total RNA was harvested and Northern Blot analysis performed as outlined in Materials and Methods. Autoradiographic exposures were for 70 hours at -70°C in the presence of an intensifying screen. Filters were probed, stripped and reprobed in the following order, TNF α , IL-1 β , followed by actin. Ten μg of total RNA was added per lane for electrophoresis. The 18s ribosomal band marked by comparison with ethidium bromide stained agarose/formaldehyde gel prior to capillary transfer of RNA to nylon filter. Total RNA hybridized to ^{32}P -labeled probes for feline TNF α (Panel A), IL-1 β (Panel B), or actin (Panel C) for 18 hours followed by washing. Total RNA was derived from cells cultured in the presence of fresh growth medium without LPS for 6 hours (lane 1), with LPS for 6 hours (lane 2), with LPS for 24 hours (lane 3), or with LPS for 48 hours (lane 4).

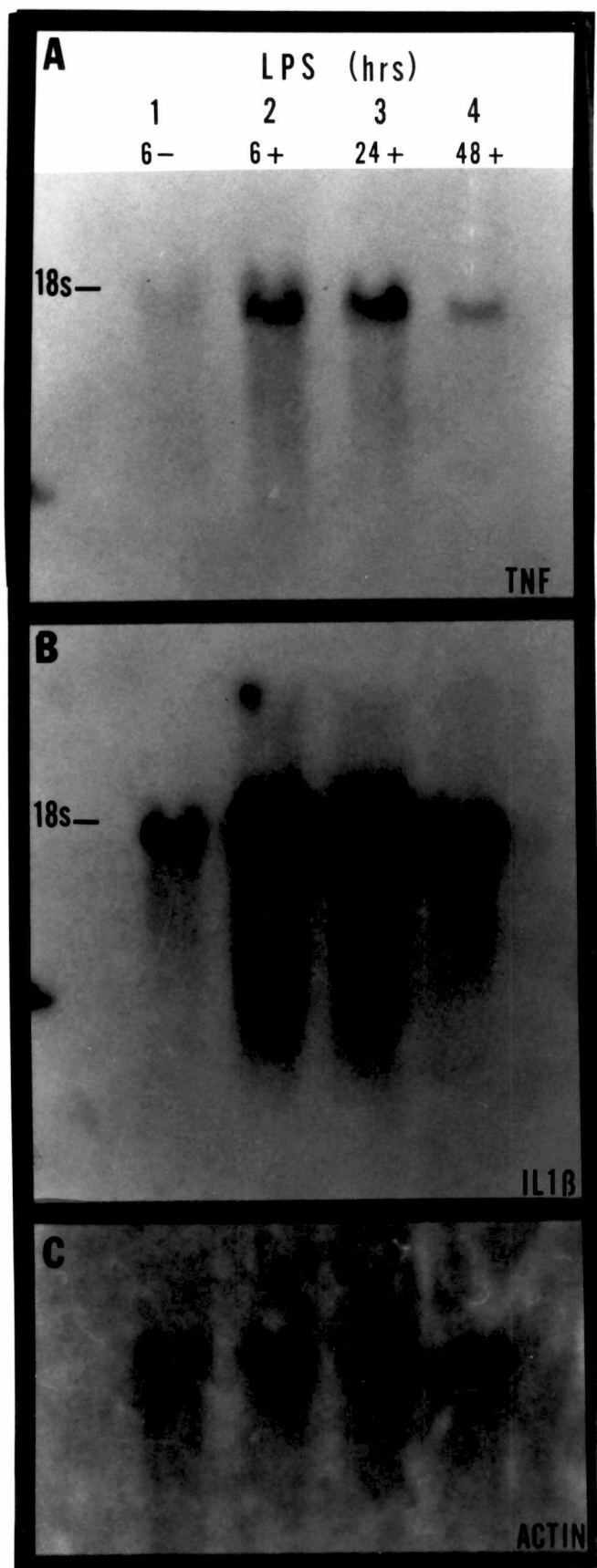


This pattern of TNF α and IL-1 β mRNA expression levels was reproducible as long as care was taken to prevent inadvertent activation of macrophages prior to LPS stimulation.

Interestingly, a separate pattern of cytokine mRNA induction was observed if cells were first harvested from tissue culture dishes by means of Hanks-EDTA (Method 2), washed, cell concentrations adjusted to 1×10^6 cells per ml and replated onto tissue culture plastic in the presence or absence of LPS (Compare Figures 2.3 and 2.4, Panels A and B). Removal of macrophages followed by re-adherence to plastic for even a short time caused cytokine gene activation with production of IL-1 β mRNA even in the absence of LPS stimulation (Figure 2.4, Panel B, Lane 1). Re-adherence may also have primed cells to produce increased amounts of TNF α mRNA following LPS stimulation since TNF α message is present in lane 1 and mRNA levels do not decrease until 48 hours (Figure 2.4, Panel A - lanes 2,3, and 4). The protracted presence of TNF α mRNA in Figure 2.4 (Panel A lanes 2,3, and 4) as compared to Figure 2.3 (Panel A lanes 2,3, and 4) may be due to LPS acting as a second signal for super-induction of TNF α production by previously "primed" macrophages. By 48 hours, TNF α gene transcription was undergoing down regulation.

In the absence of LPS stimulation (Figure 2.4 , Lane 1 - Panel B) macrophages were producing a significant amount of IL-1 β mRNA which increased greatly following LPS stimulation (Figure 2.4,

Figure 2.4 Kinetic Study: Northern blot analysis of LPS stimulated bone marrow derived macrophages - Method 2. After 14 days in culture, adherent macrophages were harvested from plastic, counted, replated in the presence or absence of 10 ug per ml of LPS in growth medium. Procedure described in Materials and Methods. Macrophages were stimulated for select periods of time at which point total RNA was harvested and Northern Blot analysis performed as outlined in Materials and Methods section. Exposure times were for 70 hours at -70°C in the presence of an intensifying screen, for all three probes. Filters were probed, stripped and reprobed in the following order, TNF α , IL-1 β , followed by actin. Ten ug of total RNA per lane added for electrophoresis. The 18s ribosomal band was marked by comparison with ethidium bromide stained agarose/formaldehyde gel prior to capillary transfer of RNA to nylon filter. Total RNA hybridized to ^{32} P-labeled probes for feline TNF α (Panel A), IL-1 β (Panel B), and actin (Panel C) for 18 hours followed by washing. RNA was derived from cells cultured in the presence of fresh growth medium without LPS for 6 hours (lane 1), with LPS for 6 hours (lane 2), with LPS for 24 hours (lane 3), or with LPS for 48 hours (lane 4).



Panel B - Lanes 2,3, and 4). Since comparable amounts of total RNA were loaded per lane (Panel C, actin probe, Figure 2.4), LPS activation of an already "primed" macrophage population (Lane 1, Panel B) resulted in superinduction of gene transcription with enhanced IL-1 β mRNA production or normal degradation of IL-1 β mRNA was blocked extending mRNA half life with consequent increased expression of IL-1 β mRNA at a given point in time. From these studies, the exact mechanism responsible for of increased mRNA could not be determined. However, it was apparent that even a slight modification in protocol (removal of cells prior to stimulation with LPS for determination of cell concentration versus a gentle medium change with no disturbance of the monolayer prior to LPS stimulation) may drastically alter cytokine induction patterns.

5. DISCUSSION

Large numbers of functional feline macrophages may be successfully obtained from bone marrow by *in vitro* culturing for 14 days. From an average input of 1.5×10^8 nucleated cells, an average of 2.3×10^7 cells was obtained at the end of 14 days of which approximately 90% were of typical mature macrophage morphology, 93% were positive for esterase activity and 89% were phagocytic for latex beads. It is imperative that the cultures be maintained for a minimum of 14 days, as the population of cells present at 7 days consists of PMN's and immature progenitor cells of varying lineage specificity. Whereas, 14 days was chosen as the routine harvest time, some cultures were allowed to go for 21 days without undue changes in the cell population (data not shown). No exogenous source of feline colony stimulating factors, such as the addition of Con A stimulated T cell supernates, was necessary in this system, thereby eliminating the need to euthanize a cat for preparation of Con A stimulated splenic cell supernates. Additionally, three separate fetal calf serum sources (Gibco vs Hyclone vs Cell Culture Laboratories) were compared and no difference was seen in ability of the complete culture medium prepared with any of these three sera to support the out-growth of macrophages over a 14 day period (data not shown). The probable source of macrophage colony stimulating factor or granulocyte-macrophage colony stimulating factor which is driving cellular development are the adherent bone marrow stromal cells (5). These cells are known to be major pro-

ducers of colony stimulating factors and to play a significant role in hematopoietic cell development (5,23).

Since only a small sample of bone marrow is needed for *in vitro* culturing, this technique has advantage over other methods in that euthanasia is not required (12,17). After a short rest period, we have utilized the same cat for bone marrow donation so large colonies of cats do not have to be maintained. Additionally, if cells are properly cultured, macrophages may be maintained in a quiescent or non-activated state as measured by a lack of detectable mRNA for TNF α or IL-1 β (Figure 2.3, Lane 1, Panels A and B). When properly activated with LPS, biologically active IL 1 was detectable in cell culture supernatants by an IL 1 conversion assay (Figure 2.2, Table 2.3).

The isolation of large numbers of macrophages by peritoneal lavage with isotonic saline has been reported (26). This technique also has the advantage that large numbers of cells may be obtained from one cat and animals are not euthanized. However, there are several disadvantages, including the need to subject cats to two peritoneal lavages separated by 9 -11 days for good macrophage recovery and the cell population which is obtained has gone through several potential activation steps, including elicited migration of cells into the peritoneal cavity and percoll separation. Attempts were made to prevent activation by adherence through the use of polypropylene tubes; however, prior to viral macrophage interaction studies, macrophages were purified by

adherence for 5 hours, washed free of non-adherent cells and utilized for studies (26). The presence of mRNA for IL-1 β and potentially TNF α in Lane 1 of Figure 2.4, Panels A and B, suggested very strongly that adherence for even a short period of time (5 or 6 hours) activated macrophages to produce monokines, thereby altering the macrophage's functional state. A similar observation has been reported for human monocytes (15). In comparison, (Figure 2.3, Lane 1 of Panels A and B) when macrophages were left undisturbed except for medium change 6 hours prior to total RNA harvest there was an absence of mRNA for IL-1 β and very little for TNF α , suggesting that these cells were in a non-activated state. Though LPS activated gene transcription for both TNF α and IL 1 β , as with other species, their transcription was independently regulated accounting for the differences observed between levels of mRNA for TNF α and IL 1 β following time courses of LPS stimulation (6,15).

When macrophages were left undisturbed prior to activation with LPS (Method 1), TNF α mRNA was present at 6 hours but gene transcription was rapidly down regulated so mRNA essentially disappears by 24 hours. This is in agreement with similar studies on murine and human LPS stimulated monocyte/macrophages, where TNF α gene expression and mRNA levels are rapidly down-regulated (14,25,27). Monocytes/macrophages tightly regulate TNF α production and chronic exposure to LPS results in a well known down modulation of TNF α mRNA (14). In contrast, gene transcription for

IL 1 β underwent a different type of regulation with mRNA present at 6 hours, decreased significantly by 24 hours, but recovered by 48 hours. Since actin mRNA levels in Panel C showed comparable amounts of total RNA were loaded per lane, it would appear that IL-1 β gene transcription was undergoing modulation and the downmodulation of mRNA at 24 hours was not due to unequal loading of total RNA at time of electrophoresis. This type of mRNA modulation may occur because macrophages develop a state of LPS refractoriness or down-modulation may be the result of endogenous cytokine production and/or mimicking of normal regulatory mechanisms (8,15). These studies demonstrated that bone marrow derived macrophages produced both TNF α and IL-1 β mRNA as well as biologically active IL-1 β (Table 2.3 and Figure 2.2) after LPS stimulation, however, gene transcription was tightly regulated. This pattern of TNF α and IL-1 β mRNA expression levels was reproducible as long as care was taken to prevent activation of the macrophages prior to their stimulation with LPS.

Interestingly, a separate pattern of cytokine mRNA induction was observed if cells were activated by plastic adherence prior to LPS induction of gene transcription (Method 2). Washing of cells followed by adherence to plastic for even a short time (6 hours) caused cytokine gene activation with production of IL-1 β mRNA in the absence of LPS stimulation (Figure 2.4, Panel B, Lane 1). Adherence activation may also have primed macrophages to produce increased amounts of TNF α mRNA following LPS stimulation since

mRNA levels did not drop until 48 hours post start of LPS stimulation (Figure 2.4, Panel A - lanes 2,3,and 4)(15). In examining the role of adherence in activation of human monocytes, Haskill et al (15) reported that plastic adherence induced transient expression of TNF α mRNA which was rapidly down-regulated by 4 hours. Following adherence, monocyte TNF α production was greatly increased if LPS was provided as a second signal for cytokine production (15). The earliest time point examined for TNF α mRNA was 6 hours, therefore, feline TNF α mRNA in LPS non-stimulated cultures may have already declined following transient activation by macrophage adherence to plastic. If feline macrophages react in a similar fashion to human monocytes, at 6 hours post adherence one would expect to see what is present in Lane 1 (Figure 2.4), only a small amount of mRNA when cells received no second signal in the form of LPS stimulation. The protracted presence of TNF α mRNA in Figure 2.4 (Panel A lanes 2,3, and 4) as compared to Figure 2.3 (Panel A lanes 2,3, and 4) was probably due to LPS acting as a second signal for super-induction of TNF α production by previously "primed" macrophages (15,25).

Following adherence, macrophages produced significant amounts of IL-1 β mRNA which increased greatly following LPS stimulation (Figure 2.4, Panel B - Lanes 2,3, and 4). Since comparable amounts of total RNA were loaded per lane (Panel C, actin probe, Figure 2.4), it would appear that LPS activation of an adherence "primed" macrophage population (Figure 2.4, Lane 1, Panel B) resulted in

enhanced IL-1 β mRNA by either superinduction of gene transcription or blockage of degradation of IL-1 β mRNA which resulted in extended mRNA half life with consequent accumulation of IL-1 β mRNA at set time points. The exact mechanism responsible for increased mRNA could not be determined from these experiments. However, from these studies, it may be appreciated that even a slight modification in protocol (removal of cells prior to stimulation with LPS for determination of cell concentration versus a gentle medium change with no disturbance of the monolayer prior to LPS stimulation) may drastically alter cytokine induction patterns.

The importance of the macrophage in first line host defense as well as its pivotal role in immunoregulation underscores the need for a system by which feline macrophages may be reproducibly generated for *in vitro* study. The system described here in has been used for 3 years in our laboratory and has proven to be highly reproducible. The interaction of these macrophages with viral agents such as Feline Immunodeficiency virus, Feline Leukemia virus and Feline Infectious Peritonitis virus may be studied *in vitro*. Additionally, since these macrophages can be maintained in a quiescent state, macrophages cultured in this manner maybe examined for modulation of monokine production following induction by specific activating agents.

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PART III:

**FELINE MONOKINES TNF α and IL 1 β : cDNA CLONING BY POLYMERASE
CHAIN REACTION TECHNOLOGY AND DETERMINATION OF NUCLEOTIDE
SEQUENCES**

1. ABSTRACT

Using degenerate oligodeoxyribonucleotides and polymerase chain reaction (PCR), cDNAs for feline TNF α and IL 1 β were amplified, cloned, and sequenced. To accomplish this, first strand cDNA synthesis was done on mRNA generated from LPS stimulated feline bone marrow derived macrophages. Using sets of degenerate primers, PCR was used to amplify cDNAs for IL 1 β and TNF α which were cloned into pGEM 3ZF(-) or pCR 1000 vectors and consensus nucleotide sequences generated.

The cDNA for feline TNF α cloned in this laboratory has a 98.6% match with coding regions of a genomic clone for feline TNF α which was recently published (22). The two feline TNF α clones differ by 8 nucleotide base pair (bp) changes which result in 5 amino acid changes in the predicted protein sequence. A search of Genbank and EMBL revealed feline TNF α cDNA has an 90%, 86%, 85%, 82% and 83% overall match with human, porcine, ovine, mouse and goat TNF α , respectively. The full length feline cDNA for TNF α from start to stop codon is 702 bp and encodes a predicted protein of 233 amino acids with a molecular weight of approximately 25,446 daltons (precursor form of mature TNF α).

The full length cDNA for feline IL 1 β is 804 bp long and encodes a predicted protein of 267 amino acids with a molecular weight of 31,892 daltons (precursor form). Feline IL 1 β has an overall match of 79%, 76%, 77.5% and 77% with IL 1 β cDNAs for human, bovine, rabbit and murine species, respectively.

2. INTRODUCTION

TNF α and IL 1 β are important cytokines involved in both inflammatory and immunoregulatory processes (7,11,19). To date, studies investigating the modulation of cytokines in feline infectious disease processes has been greatly hampered by a lack of appropriate molecular probes. Thus the use of heterologous cDNA probes for Northern analysis of feline mRNA results in poor quality blots with non-specific probe binding due to the low stringency washing conditions which must be utilized during cross species hybridization studies.

In an attempt to study feline viral interactions with macrophages, we developed a technique for growing large numbers of feline bone marrow derived macrophages *in vitro* (Part II of Thesis) but cross hybridization with murine or human IL 1 β ³²p labeled cDNA probes resulted in high backgrounds with low stringency washes and poor signal detection following higher wash stringencies during Northern blot analysis (6). To further our studies on the role of monokines in feline infectious disease processes, we cloned cDNAs for the precursor forms of feline IL 1 β and TNF α by using PCR technology. Northern blot analysis of LPS stimulated macrophages showed mRNA present in cell lysates for both TNF α and IL 1 β , of approximately 1.6 kb and 1.7 kb, respectively.

3. MATERIALS AND METHODS

3.1 Animals

In all experiments, 8 FeLV and FIV sero-negative random source adult cats (5 females and 3 males) were utilized as bone marrow donors. Animals were housed separately in cages at the animal facilities of the University of Tennessee School of Veterinary Medicine, Knoxville, Tennessee (a fully accredited AALAC facility) and their treatment, housing and care were in compliance with standards set forth in the "Guide for the Care and Use of Laboratory Animals" (NIH publication no. 85-23). Cats were part of a colony maintained by Dr. Al Legendre.

3.2 Feline bone marrow isolation technique and *in vitro* culturing method

Feline bone marrow isolation technique was previously described in Part II section 3.3 (6). Briefly, after clipping and surgical preparation, bone marrow was obtained from the femur of cats under general ketamine HCl (Ketalar^R, Parke Davis) anesthesia (1 mg/lb IV or 10 mg/lb IM). Approximately 0.5 ml of bone marrow was aspirated by a bone marrow needle directly into a 10 ml syringe containing 3 ml of Iscove's Modified Dulbecco's Medium (GIBCO) plus 100 units per ml of heparin sulfate (SIGMA Chemical Co., St. Louis, MO) (H-IMDMEM). Samples were mixed thoroughly, transferred to a sterile 15 ml conical centrifuge tube (Sarstedt, Inc, Newton, NC) containing 2 ml of H-IMDMEM and placed on ice until processed.

Cats were recovered from anesthesia and monitored for any adverse reactions. Bone marrow samples from each cat were kept separate with no pooling.

For cell separation, samples were brought up to 10 ml final volume with H-IMDMEM, vortexed 20 secs, and kept undisturbed at room temperature for 5 minutes. The supernate was carefully removed leaving a pellet containing bone chips and cartilage fragments. Five mls of bone marrow - H-IMDMEM mixture was layered over 3 ml of Ficoll-Sodium diatrizoate (Histopaque 1.077 - SIGMA, St. Louis, MO) in a 15 ml conical centrifuge tube and centrifuged at 400 xg for 25 minutes at 25°C. The interface band containing cells was removed, washed three times, and counted. Cell concentrations were adjusted to 1.0×10^6 to 2.0×10^6 cells per ml in complete medium consisting of IMDMEM containing 15% HI- FBS (HyClone), 2mM L-glutamine, 1mM sodium pyruvate, 100 units/ml penicillin, 100 ug/ml streptomycin, 10 mM Hepes and sodium bicarbonate to a pH of 7.2 to 7.3.

Ten mls of cell suspension were cultured in polystyrene 100 mm X 20 mm tissue culture dishes (FALCON, BAXTER, Atlanta,GA) at 37°C, in a humid 6% CO₂ chamber. At the end of one week, 10 ml of fresh complete medium was added to each plate and incubation continued for an additional 7 days. At the end of 14 days culture time, cells were used in the study.

Macrophages harvested for interleukin 1 β (IL 1 β) and tumor necrosis factor α (TNF α) mRNA and Northern blot analysis were

pulsed with 10 ug per ml lipopolysaccharide (LPS - *E. coli* serotype 0127:B8, SIGMA) in the following manner: For each culture dish containing 14 day old macrophages, complete medium was gently aspirated from the dishes and any non-adherent cells pelleted by centrifugation. Pelleted cells were resuspended in bone marrow growth medium containing 10 ug per ml of LPS. Twenty mls of medium plus LPS was gently layered over the undisturbed macrophage monolayer and incubated at 37°C, in a humid 6% CO₂ chamber for 6 hours at which point cells were collected and processed for total RNA isolation.

3.3 Total RNA isolation procedure and Northern Blot analysis

Cells were lysed in a 4M guanidine isothiocyanate (SIGMA) containing 0.5% sodium lauryl sarcosine (SIGMA), 25mM sodium citrate pH 7.0 (SIGMA), and 47 mM 2-mercaptoethanol. The lysate was then centrifuged at 36,000 rpm for 18 hours in a Beckman ultracentrifuge (rotor - Sorval AH650 swinging bucket rotor) through a 5.7 M Cesium chloride cushion (5).

The RNA pellet was resuspended in DEPC treated water, phenol/chloroform extracted, and ethanol precipitated. The total RNA was quantified by spectrophotometry and aliquoted for cDNA preparation or Northern Blot analysis. For Northern Blot analysis, 10 ug of total RNA for mRNA size determination or 5 ug of a RNA molecular weight marker (0.16 - 1.77 kb RNA Ladder, GIBCO BRL, Grand Island, N.Y.) was denatured for fractionation in a 1.0%

agarose/ 1.1 M formaldehyde gel containing 500 ugs of ethidium bromide for RNA visualization. The formaldehyde-agarose gel RNA fractionation method has been described in Part II section 3.7 of the thesis (6). Gels were electrophoresed at a constant current of 95 volts until the bromophenol blue dye front traveled approximately 14 cm. At this point, the gel was visualized with UV for size confirmation and quality of RNA loading, soaked briefly in water to remove gel fragments, and RNA transferred overnight to nylon filters (Nytran, Schleicher and Schuell, Inc, Keene, NH) by a sponge modification of the capillary transfer method (10).

Post transfer, nylon filters were UV-crosslinked to stabilize RNA (17) (Stratalinker UV Crosslinker, Stratagene) and filters were prehybridized at 60°C for a minimum of 1 hour in 5X SCC, 0.05M sodium phosphate buffer pH 6.5, 5X Denhardt's solution, 1% SDS, 200 microgram per ml of denatured salmon sperm DNA as a blocker. Following prehybridization, filters were hybridized overnight (18 hours) at 60°C in the same prehybridization mix plus the appropriate ³²P nick translated (Amersham kit N5500) cDNA probe. Nick translated probes had an activity of at least 1 to 2 x 10⁷ cpm/μg of DNA specific activity. Post hybridization, filters were washed in the following order: (1) Two times at room temperature for 15 minutes each in 2X SCC plus 0.1% SDS. (2) Two times at room temperature for 15 minutes each in 0.2X SCC plus 0.1% SDS. (3) Once for 20 minutes at 55°C with 0.2X SCC plus 0.1% SDS. (4) Highest stringency wash, once for 30 minutes at 60°C with 0.1X SCC

plus 0.1% SDS.

Following washes, blots were exposed for 70 hours to Kodak XAR 5 film in the presence of Dupont Cronex Lightning plus intensifying screens at -70°C. Following exposure, radioactive cDNA probes were stripped from nylon filters by washing filters in 50% formamide/6X SSPE at 65° C for 30 minutes followed by rinsing briefly in 2X SSPE. Filters were then reprobed with a second radiolabeled probes. In these studies, the following pattern was maintained: Filters were first probed with TNF α specific probes, blot stripped followed by probing with an IL-1 β specific probe.

The ^{32}P labeled TNF α probe was prepared from the plasmid, pTNF α B9, which contains a 570 bp Eco R1, Hind III fragment of feline TNF α cDNA. The ^{32}P labeled IL-1 β probe was prepared from the plasmid, pIL1#2, which contains a 750 bp, Eco R1, Hind III fragment of feline IL-1 β cDNA.

3.4 Preparation of first strand cDNA, oligonucleotides utilized, and conditions for polymerase chain reactions

Following total RNA isolation, first strand cDNA was synthesized with reverse transcriptase (RT) from 1 - 2 μg of total RNA by manufacturer's protocols using either Boehringer Mannheim (cDNA Synthesis Kit, cat. no. 1013 882) or Promega Reverse Transcription Systems (cat. no. A3500, Promega, Madison, WI) with oligo (dT) 15 as the RT primer for IL 1 β . For TNF α RT, a specific 47 nucleotide antisense primer (designated primer # 3) with the

following sequence 5'-CC-AAG-CTT-TCA-CAG-GGC-AAT-GAT-CCC-AAA-GTA-GAC-CTG-CCC-GGA-CTC-3' was utilized for both RT and as the 3' primer during PCR. This sequence contains a Hind III cloning site with a CC cap on the 5' end and is antisense for a short segment of the 3' end of TNF α cDNA including the stop codon. This sequence was utilized successfully for cloning of porcine TNF α cDNA (24). Prior to RT assay, RNA samples were heated at 65°C for 5 min then rapidly cooled on ice to decrease any potential interference in DNA synthesis due to RNA secondary structure. Additionally, reactions were carried out at 42°C for 1 hour minimum as opposed to 15 minutes recommended in manufacturer's RT assay conditions. After RT assay and first strand cDNA formation, RT activity was destroyed by boiling RNA/cDNA hybrids for 5 minutes with quenching on ice for 5 minutes. Samples were spun and the final 20 μ l volume was aliquoted into three 6 μ l samples or two 10 μ l samples for polymerase chain reaction (PCR) amplification.

For TNF α PCR, the following two 5' primers were utilized to generate cDNAs of TNF α , each 5' primer is sense and starts with a GG cap followed by an Eco RI restriction site: 1. primer #1: a 29 mer previously used for cloning porcine TNF α (24) consisting of 5'-GG-GAA-TTC-GCC-ACC-ACG-CTC-TTC-TGC-CTG-3' or 2. primer # 2: a 29 mer derived from the ATG start codon of a reported human TNF α cDNA sequence 5'-GG-GAA-TTC-ATG-AGC-ACT-GAA-AGC-ATG-ATC-3'. Upon PCR amplification, utilization of primer # 2 and primer #3 resulted in a full length cDNA copy of approximately 700 bp while primer

1 and primer # 3 yielded an approximate 570 bp cDNA fragment lacking the first 130 bp of the 5' end of TNF α cDNA.

For cloning of IL-1 β , several sets of oligonucleotides were designed by comparing areas with high degrees of homology between human, murine, and bovine IL 1 β cDNAs. Several sets of degenerate oligonucleotides were tried before success was achieved. The successful 5' sense primers utilized, include the following: 1: Primer # 1 - 5'-GGGAATTC-GAA-ATG-ATG-ATG-GCT-TAC-TAC-AGT-3', a 29 mer beginning approximately 30 bp 3' of the anticipated ATG start codon, Primer # 2 - 5'-GGGAATTC-ATC-CTC-TCC-AGC-CA-TCT-TCA-TTG-3', a 32 mer designed from similarities in sequences present in human, murine and bovine reported cDNAs and located some 40 bp upstream of the anticipated ATG start codon. These primers had a GG cap followed by an Eco RI restriction site for cloning. The 3' antisense primers had the following sequences: 1. Primer # 3 a 29 mer 5'-CCAAGCTT-GAA-GTC-AGT-TAT-ATC-CTG-GCC-3' designed from similarities just upstream (5') of the predicted termination codon with a 5' CC cap followed by a Hind III restriction site for cloning, and 2. Primer # 4 an Oligo (dT) 17 adaptor with an Xho I, Sal I, and Cla I restriction sites, 5'-GAC-TCG-AGT-CGA-CAT-CGA-TTT-TTT-TTT-TTT-TTT-TT-3'. Primers # 1 and 3 were designed to yield a 750 bp cDNA fragment and Primers # 2 and 3 a 800 bp fragment upon PCR amplification. It was necessary to use an Oligo (dT) adaptor (Primer # 4) to get the 3' end including the stop codon of cDNA for feline IL 1 β as other primers designed with "nucleotide wobbles" to account

for species diversity of the nucleotides reported for this area failed to generate PCR fragments. As an internal positive control for RNA integrity and RT efficiency, every sample of RNA that was RT to cDNA for amplification by PCR was tested for PCR amplification of actin at the time of PCR amplification of cytokine cDNA. The actin primers utilized were derived from human sequences as reported (2). The sequences of these primers are as follows: 1. 5' sense Primer 5'-GTG-GGG-CGC-CCC-AGG-CAC-CA-3', a 20 mer and 2. 3' antisense Primer 5'-CTC-CTT-AAT-GTC-ACG-CAC-GAT-TTC-3', a 24 mer. Every test sample that underwent PCR amplification for cytokine cDNA concurrently underwent PCR amplification for actin expression to test the integrity of the beginning RNA sample and the efficiency of RT. Samples found negative by PCR for actin expression were invariably negative for cytokine expression indicating poor quality RNA or inefficient RNA to cDNA production.

The PCR reaction was performed directly on first strand cDNA synthesized using a GeneAmp DNA reagent kit (Perkin Elmer Cetus, Norwalk, CT). PCRs were performed in a final volume of 100 μ l which contained 6-10 μ l of first-strand cDNA prepared from total RNA, 10 μ l of 10x PCR reaction buffer, 16 μ l of 1.25mM dNTP mix, 1.0 μ M appropriate 5' sense primer, 1.0 μ M appropriate 3' antisense primer, and 2.5 units of *Taq* DNA polymerase. The reactions typically were performed for 30 - 35 cycles with 1 minute ramp times and each cycle consisting of 1 minute denaturing at 94°C, 1 minute annealing at 55°C, and 2 minute extensions at 72°C. All reactions

were carried out in a Perkin Elmer Cetus, DNA thermal cycler. A 10ul sample of PCR product was analyzed by electrophoresis in a 1.0% native agarose submarine gel with a 1 kb DNA molecular weight marker (Gibco BRL, Grand Island, NY) for presence of amplified cDNA fragments.

3.5 Cloning of PCR fragments and sequencing conditions

PCR amplified fragments for cloning were separated from oligonucleotide primers by electrophoresis in 2% native agarose gels containing ethidium bromide (0.5 ug/ml). PCR amplified bands were separated by electrophoresis, then isolated onto NA 45 DEAE paper (Schleicher and Schuell, Keene, NH) inserted into the gel in an incision just ahead of the band of interest. Amplified DNA stacks up on the NA 45 paper during electrophoresis and is eluted off by incubating at 65°C for 1 hour in a high salt buffer (1M NaCl in 0.05M arginine free base). After elution, DNA was extracted with phenol/chloroform, and ethanol precipitated. Precipitated fragments were either directly cloned into pCR 1000 vectors (see below) or restriction enzyme digested in a final 100ul volume with 1x restriction enzyme buffer (usually Eco RI and Hind III) for 2-3 hours at 37°C, phenol-chloroform extracted, ethanol precipitated and resuspended in water for ligation. Digested fragments were directionally cloned into restriction enzyme (Eco RI and Hind III) digested pGem 3Zf(-) vector (Promega) using a Stratagene DNA Ligation Kit (Stratagene, La Jolla, CA) and the manufacturer's

protocol except, that ligations were carried out for 24 to 48 hours at 4°C. Ligated DNA (10 μ l) was transformed into JM 101 competent cells using standard transformation procedures (16). Ampicillin resistant, β -galactosidase negative bacterial colonies were screened for the presence of p-Gem plasmids with appropriately sized DNA inserts. Representative colonies with inserts were grown up overnight in Terrific Broth and miniprep plasmid DNA preparations prepared by a modified alkaline lysis protocol, which prepared DNA of such purity that it was usable for enzyme restriction studies to determine the presence of plasmid inserts as well as for sequencing (23).

Double stranded DNA sequencing was performed using the Sequenase version 2.0 kit (United States Biochemical Corporation, Cleveland, OH) with ^{35} S dATP as the label (Amersham, >1000 Ci/mmol) and Sp6 or T7 universal sequencing primers (United States Biochemical). To facilitate sequencing, internal primers were also utilized during the sequencing of IL 1 β . Sequencing products were electrophoresed in a 6%(8M urea) denaturing 45-cm acrylamide sequencing gel (Base Runner Nucleic Acid Sequencer, International Biotechnologies, Inc, New Haven, CT), gels fixed in 5% methanol and acetic acid for 15 minutes, dried on a gel dryer and exposed overnight at room temperature to Kodak XAR-5 film. Films were developed in an automatic film processor.

For cloning of PCR fragments not digested, fragments from PCR reactions were directly cloned into pCR 1000 TA vectors utilizing

the TA Cloning System (Invitrogen, San Diego, CA) and the manufacturers recommendations with the following exceptions in the transformation protocol. For transformation of INV1 α F' competent cells, 2 μ l of the ligation mixture was utilized for transformation (not 1 μ l) and 200 or 300 μ l (not 25 μ l) of transformed cells were plated onto kanamycin plates with top agar containing 2% BluGal and 50 μ g/ml kanamycin. Plates were incubated for 48 hours prior to screening of white colonies for cDNA plasmid inserts. All subsequent bacterial growth, DNA separation and sequencing performed as previously described for pGEM 3Zf(-) plasmid.

The cDNA sequences were analyzed and protein structure predicted by the DNASTAR sequence analysis program (DNASTAR INC, Madison, WI.).

4. RESULTS

4.1 PCR amplification of IL 1 β and TNF α cDNA

From total RNA, single stranded cDNA was prepared by first strand cDNA synthesis using reverse transcriptase. From single stranded cDNA, cDNA for TNF α and IL 1 β were amplified using PCR and appropriate primer sets for TNF α or IL 1 β . The PCR products were separated by electrophoresis in a 1% native agarose gel, and visualized by ethidium bromide staining and UV light. With TNF α , single PCR products with expected base pair sizes were visualized, a 570 bp product for primer sets #1 and #3 and a 700 bp product for primer sets #2 and #3. Primer sets #2 and #3 were designed to span the expected coding region of cDNA for TNF α , therefore, clones derived from PCR reactions using primer sets #2 and #3 represented complete cDNA clones of feline TNF α .

For IL 1 β , single bp products of 750 bp or 800 bp was visualized for primer sets #1 and #3, and #2 and #3, respectively. However, neither of these clones represented a complete cDNA copy of IL 1 β mRNA. Primer sets #1 and #3 yielded clones lacking both the 5' and 3' ends of IL 1 β , whereas, primer sets #2 and #3 covered the 5' end of the molecule containing the expected ATG start codon but lacked the 3' end of the cDNA molecule. Since a degenerate primer designed to span the termination codon and the 3' end of the cDNA failed to amplify a PCR fragment, an oligo(dT) 17 non-specific primer adaptor (primer # 4) was utilized. For primer set #1 and #4, two PCR products were visualized, a strong

band at approximately 1500 bp and a weaker band around 800 bp. Since the 1500 bp had the greater potential for encoding a more complete cDNA for IL 1 β , these bands were isolated, cloned, and sequenced. In all cases, PCR products were separated from primers by electrophoresis in 2% native agarose gels and DNA for cloning captured on DEAE Dextran paper. Cloning and sequencing methods were previously described in Materials and Methods section.

4.2 Sequencing of feline TNF α and IL 1 β

Three separate PCR reactions utilizing cDNA generated from three separate RT reactions were performed for every primer set of both TNF α and IL 1 β . Additionally, a minimum of two PCR clones (usually more) from each primer set PCR product ligation and transformation were sequenced in their entirety. Therefore, a minimum of 6 separate clones derived from 3 different PCR reactions were sequenced for each primer set and a consensus sequence generated to minimize potential aberrations due to reverse transcriptase or Taq polymerase infidelity (8,9,28). In many cases, clones were sequenced in both the sense and antisense direction, though sequencing problems due to compressions and other sequencing artifacts were minimal. In this manner, numerous overlapping clones were sequenced for both monokines. The only clones not sequenced in their entirety were the IL 1 β clones generated by primers #1 and #4 (oligo (dT) 17). In this case, internal primers for IL 1 β were used for sequencing to prevent the necessity of

sequencing extensive areas of the non-coding poly A tail region.

The entire nucleotide consensus sequence and predicted protein sequence of feline TNF α cDNA is displayed in Figure 3.1 and feline IL 1 β cDNA in Figure 3.2. The cDNA for feline TNF α cloned in this laboratory has a 98.6% overall match with coding regions of a genomic clone of feline TNF α published recently (22). These two feline TNF α clones differ by 8 nucleotide bp changes which result in 5 amino acid changes in the predicted amino acid protein sequence. The 5 amino acid changes are underlined in Figure 3.1. A search of Genbank and EMBL revealed that feline TNF α cDNA has a 90%, 86%, 85%, 82% and 83% overall match with human, porcine, ovine, mouse and goat TNF α , respectively (Table 3.1). The full length feline cDNA for TNF α from start to stop codon is 702 bp and encodes a predicted protein of 233 amino (aa) acids with a molecular weight of approximately 25,446 daltons. This represents the probable precursor form of mature TNF α (19,27). The Leu at amino acid position 77 is starred and based upon homology comparisons with the NH₂-terminal of human, murine, and porcine TNF α cDNA sequences (Val/Leu/Leu-Arg-Ser-Ser), represents amino acid number 1 for the mature form of feline TNF α . There are 76 amino acids preceeding this leucine residue, and based upon comparisons with human and murine TNF α cDNAs (25,26) probably represents a sequence which is involved in the secretion or processing of mature feline TNF α . It is also interesting to note the presence

Figure 3.1 The predicted protein sequence and cDNA nucleotide consensus sequence of feline TNF α clones. The sequence is translated from the first ATG encountered. The 5' primer sequences are identical to nucleotides encoding amino acids 1 through 7. The 3' primer sequences are antisense to amino acids 222 through the termination codon except for the serine residue at 223 which in the primer is encoded by TCT. Primer sequences are enclosed in parentheses. The 5 amino acid changes in our reported protein sequence from the recently reported genomic clone of feline TNF α are underlined. Leu at amino acid 77 is starred and is the probable start of the mature processed protein for feline TNF α (aa 77 through 234). The 76 amino acids preceding this residue are probably involved in processing and secretion of the mature feline TNF α . Note the presence of Arg-Arg (aa 28-29) and Lys-Lys (aa 19-20) dipeptides in the first 30 amino acids of feline TNF α , these are potential enzymatic cleavage sites. There are two cysteine residues (aa positions 145 and 177) present in the mature TNF α peptide which could be involved in a single intramolecular disulphide bond formation.

Protein Translation of Feline Tumor Necrosis Factor alpha cDNA

	v3		v6		v9		v12		v15							
(Met	Ser	Thr	Glu	Ser	Met	Ile)	Arg	Asp	Val	Glu	Leu	Ala	Glu	Glu	Ala	Leu
(atg	agc	act	gaa	agc	atg	atc)	cgg	gac	gtg	gag	ctg	gcc	gag	gag	gca	ctc
v18		v21		v24		v27		v30		v33						
Pro	Lys	Lys	Ala	Gly	Gly	Pro	Gln	Gly	Ser	Arg	Arg	Cys	Leu	Cys	Leu	Ser
ccc	aag	aag	gca	ggg	ggc	ccc	cag	ggc	tcc	<u>aga</u>	agg	tgc	ttg	tgc	ctc	agc
	v36		v39		v42		v45		v48		v51					
Leu	Phe	Ser	Phe	Leu	Leu	Val	Ala	Gly	Ala	Thr	Thr	Leu	Phe	Cys	Leu	Leu
ctc	ttc	tcc	ttc	ctc	ctc	gtc	gca	gga	gcc	acc	acg	ctc	ttc	tgc	ctg	ctg
	v54		v57		v60		v63		v66							
His	Phe	Gly	Val	Ile	Gly	Pro	Gln	Arg	Glu	Glu	Leu	Pro	His	Gly	Leu	Gln
cac	ttt	gga	gtg	atc	ggc	ccc	cag	agg	gaa	gag	ctc	cca	cat	ggc	ctg	caa
v69		v72		v75		v78		v81		v84						
Leu	Ile	Asn	Pro	Leu	Pro	Gln	Thr	* Leu	Arg	Ser	Ser	Arg	Thr	Pro	Ser	Ser
cta	atc	aac	cct	ctg	ccc	cag	aca	ctc	aga	tca	tct	tct	cga	act	ccg	agt
	v87		v90		v93		v96		v99		v102					
Asp	Lys	Pro	Val	Ala	His	Val	Val	Ala	Asn	Pro	Glu	Ala	Glu	Gly	Gln	Leu
gac	aag	cca	gta	gcc	cat	gta	gta	gca	aac	ccc	gaa	gct	gaa	ggg	caa	ctc
	v105		v108		v111		v114		v117							
Gln	Trp	Leu	Ser	Arg	Arg	Ala	Asn	Ala	Leu	Leu	Ala	Asn	Gly	Val	Glu	Leu
cag	<u>tg</u>	ctg	agc	cgg	cgt	gcc	aat	gcc	ctc	ctg	gcc	aat	ggc	gtg	gag	ctg
v120		v123		v126		v129		v132		v135						
Thr	Asp	Asn	Gln	Leu	Lys	Val	Pro	Ser	Asp	Gly	Leu	Tyr	Leu	Ile	Tyr	Ser
aca	gac	aac	caa	ctg	aag	gtg	ccc	tca	gac	ggg	ctg	tac	ctc	atc	tac	tcc
	v138		v141		v144		v147		v150		v153					
Gln	Val	Leu	Phe	Thr	Gly	Gln	Gly	Cys	Pro	Ser	Thr	His	Val	His	Leu	Thr
cag	gtc	ctc	ttc	acg	ggc	caa	gga	tgt	cct	tcc	aca	cat	gtg	<u>cac</u>	ctc	acc
	v156		v159		v162		v165		v168							
His	Thr	Ile	Ser	Arg	Phe	Ala	Val	Ser	Tyr	Gln	Thr	Lys	Val	Asn	Leu	Leu
cac	<u>acc</u>	atc	agc	cgc	ttt	gcc	ggt	tcc	tac	cag	acc	aag	gtc	aac	ctc	ctc
v171		v174		v177		v180		v183		v186						
Ser	Ala	Ile	Lys	Ser	Pro	Cys	Gln	Arg	Glu	Thr	Pro	Glu	Gly	Ala	Glu	Ala
tct	gcc	atc	aag	agc	cct	tgc	cag	agg	gag	acc	cca	gag	ggg	gct	gag	gcc
	v189		v192		v195		v198		v201		v204					
Lys	Pro	Trp	Tyr	Glu	Pro	Ile	Tyr	Leu	Gly	Gly	Val	Phe	Gln	Leu	Glu	Lys
aag	ccc	tg	tat	gag	ccc	atc	tac	ctg	gga	ggg	gtc	ttc	caa	ctg	gag	aag
	v207		v210		v213		v216		v219							
Gly	Asp	Arg	Leu	Ser	Ala	Glu	Ile	Asn	Leu	Pro	Ala	Tyr	Leu	Asp	Phe	Ala
ggt	gat	cga	ctc	agc	<u>gct</u>	gag	atc	aat	ctg	cct	gcc	tat	ctt	gac	ttt	gcc
v222		v225		v228		v231		v234								
(Glu	Ser	Gly	Gln	Val	Tyr	Phe	Gly	Ile	Ile	Ala	Leu	ter)				
(gag	<u>tcc</u>	ggg	cag	gtc	tac	ttt	ggg	atc	att	gcc	ctg	tga)				

of Arg-Arg (aa 28-29) and Lys-Lys (aa 19-20) dipeptides in the first 30 amino acids of feline TNF α . Pairs of basic amino acids are reported to serve as cleavage sites for the release of physiologically important peptides from precursor molecules. Similar pairs basic amino acids have been reported in human TNF α 's precursor sequence (26). The predicted mature form of feline TNF α is 158 aa in length beginning at amino acid number 77 (Figure 3.4) and encodes a protein with a predicted molecular weight of 19,175.

There are 6 asparagine (Asn) residues present in the predicted amino acid sequence of mature feline TNF α (aa residues 77 through 234) for possible glycosylation sites. However, the high mannose precursor is reported to be transferred only to Asn residues that have Ser or Thr residues located 2 residues to their carboxy-terminal side with Asn-X-(Thr or Ser) tripeptide (where X is any amino acid) as the universal acceptor (25). Based on the above criteria, the deduced amino acid sequence reported here for feline TNF α or for genomic feline TNF α previously reported (22), like human TNF α , have no Asn residues that fulfill this criteria. This would suggest that mature feline TNF α is not a glycoprotein. There are two cysteine residues (aa positions 145 and 177) present in the mature TNF α peptide which could be involved in a single intramolecular disulfide bond formation. The two cysteine residues present in the mature form of human TNF α are linked by a disulfide bridge which appears to be essential for TNF α cytotoxicity (1).

Figure 3.2 The predicted protein translation and cDNA nucleotide consensus sequence of feline IL 1 β clones. The sequence is translated from the first ATG codon. The predicted amino terminus of mature IL 1 β protein is marked at position 116. The 115 amino acid residues prior to this are probably involved in proteolytic processing of the precursor molecule to the mature form. Note the basic dipeptide Lys-Lys at positions 75-76. Potential N-glycosylation sites are marked at amino acid positions 37, 58, and 217.

Protein Translation of Feline Interleukin 1 beta cDNA

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                                v3          v6
atcctctcca gccaatcttc attgctcagg tttctgaagc cgcc atg gca cca gta cct gaa ctc
                                Met Ala Pro Val Pro Glu Leu
                                v9          v12          v15          v18          v21          v24
Thr Ser Glu Met Met Ala Tyr Tyr Ser Asp Glu Asn Asp Leu Phe Phe Glu Ala Asp
acc agt gaa atg atg gct tac tac agt gat gag aat gac ctg ttc ttt gag gct gat

v27          v30          v33          v36          v39          v42          v45
Gly Pro Glu Lys Met Lys Gly Ser Leu Gln Asn Leu Ser His Ser Phe Leu Gly Asp
ggc ccc gaa aag atg aag ggc agc ctc caa aac ctg agc cac agt ttt ctg gga gat

                                v48          v51          v54          v57          v60          v63
Glu Gly Ile Gln Leu Gln Ile Ser His Gln Pro Asp Asn Lys Ser Leu Arg His Ala
gag ggc atc cag ttg caa atc tcc cac cag ccc gac aac aag agt ctt agg cat gcc

                                v66          v69          v72          v75          v78          v81
Val Ser Val Ile Val Ala Met Glu Lys Leu Lys Lys Ile Ser Phe Ala Cys Ser Gln
gtg tcg gtc att gtg gct atg gag aaa ctg aag aag ata tct ttt gcc tgc tca caa

v84          v87          v90          v93          v96          v99          v102
Pro Leu Gln Asp Glu Asp Leu Lys Ser Leu Phe Cys Cys Ile Phe Glu Glu Glu Pro
ccc ctc cag gat gag gac ctg aag agc ctc ttt tgc tgc atc ttt gaa gaa gaa ccc

                                v105          v108          v111          v114          *          v117          v120
Ile Ile Cys Asp Thr Trp Asp Asp Gly Phe Val Cys Asp Ala Ala Ile Gln Ser Gln
atc atc tgt gac acg tgg gat gac ggt ttt gtg tgt gat gca gcc ata cag tca cag

                                v123          v126          v129          v132          v135          v138
Asp Tyr Thr Phe Arg Asp Ile Ser Gln Lys Ser Leu Val Leu Ser Gly Ser Tyr Glu
gac tac acg ttc cga gac ata agc caa aag agc ctg gtg ctg tct ggc tca tac gag

v141          v144          v147          v150          v153          v156          v159
Leu Arg Ala Leu His Leu Asn Gly Gln Asn Met Asn Gln Gln Val Val Phe Arg Met
ctt cgg gct ctc cac ctc aat gga cag aat atg aac caa caa gtg gtg ttc cgc atg

                                v162          v165          v168          v171          v174          v177
Ser Phe Val His Gly Glu Asn Ser Lys Lys Ile Pro Val Val Leu Cys Ile Lys
agc ttt gtg cac ggg gag gaa aat agt aag aag ata cca gta gtg ttg tgc atc aag

                                v180          v183          v186          v189          v192          v195
Lys Asn Asn Leu Tyr Leu Ser Cys Val Met Lys Asp Gly Lys Pro Thr Leu Gln Leu
aaa aat aac ctg tac ctg tcc tgt gtg atg aaa gac ggg aaa ccc acc cta cag ctg

v198          v201          v204          v207          v210          v213          v216
Glu Met Leu Asp Pro Lys Val Tyr Pro Lys Lys Lys Met Glu Lys Arg Phe Val Phe
gag atg tta gac ccc aaa gtt tac cca aag aag aag atg gaa aag cga ttt gtc ttc

                                v219          v222          v225          v228          v231          v234
Asn Lys Thr Glu Ile Lys Gly Asn Val Glu Phe Glu Ser Ser Gln Phe Pro Asn Trp
aac aag aca gaa atc aag ggc aat gtg gaa ttt gag tct tcc cag ttc ccc aac tgg

                                v237          v240          v243          v246          v249          v252
Tyr Ile Ser Thr Ser Gln Ala Glu Glu Met Pro Val Phe Leu Gly Asn Thr Lys Gly
tac atc agc acc tct caa gca gaa gaa atg cct gtc ttc cta gga aat acc aaa ggt

v255          v258          v261          v264          v267
Gly Gln Asp Ile Thr Asp Phe Ile Met Glu Ser Ala Ser ter
ggc cag gat ata act gac ttc atc atg gaa agc gct tcc taaagaga cctgtaccct

aaagagagtc caaatgtg

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The full length cDNA for feline IL 1 β (Figure 3.2) is 804 bp long and encodes a predicted protein of 267 amino acids with a molecular weight of 31,892 daltons (precursor form of IL 1 β). This molecular weight is in close agreement with the molecular weight of proteins predicted from the cDNA for the precursor forms of murine (30,935 daltons) and human (30,749 daltons) IL 1 β (13,21). Feline IL 1 β has an overall match of 79%, 76%, 77.5% and 77% with IL 1 β cDNAs for human, bovine, rabbit and murine species, respectively (Table 3.1). The sequence context surrounding the ATG methionine start codon of feline IL 1 β conforms to the CC(A/G)CC ATGG sequence which has been proposed as a consensus sequence for eukaryotic initiator sites (18).

Due to incomplete homology with human, murine, and bovine IL 1 β , it is difficult to predict the exact location at which the precursor protein is cleaved to yield the mature form of IL 1 β but comparisons between amino acid sequences suggest that amino acid residue 116 is the probable amino terminal for the mature form of feline IL 1 β . This prediction is based upon the following amino acid motif Val-(His/Cys)-Asp (cleavage site) Ala/Val that was derived for cleavage of rabbit, human, murine, bovine, and rat IL 1 β precursor molecule, with Aspartic acid as a critical residue (4). However, N-terminal sequencing of isolated feline IL 1 β is necessary in order to confirm this cleavage site.

If cleavage occurs at this site, it would result in a mature feline IL 1 β protein of 152 amino acids with a predicted molecu-

Table 3.1 Comparison of Feline TNF α and IL 1 β cDNA consensus sequences with reported cDNA sequences from other species

Percent Overall Match of other species cDNA with Feline		
	TNF α	IL 1 β
Feline (genomic)	98.6%	N/A ^a
Human	90 %	79 %
Murine	82 %	77 %
Porcine	86 %	N/A
Ovine	85 %	N/A
Caprine	83 %	N/A
Bovine	N/A	76 %
Rabbit	N/A	77.5%

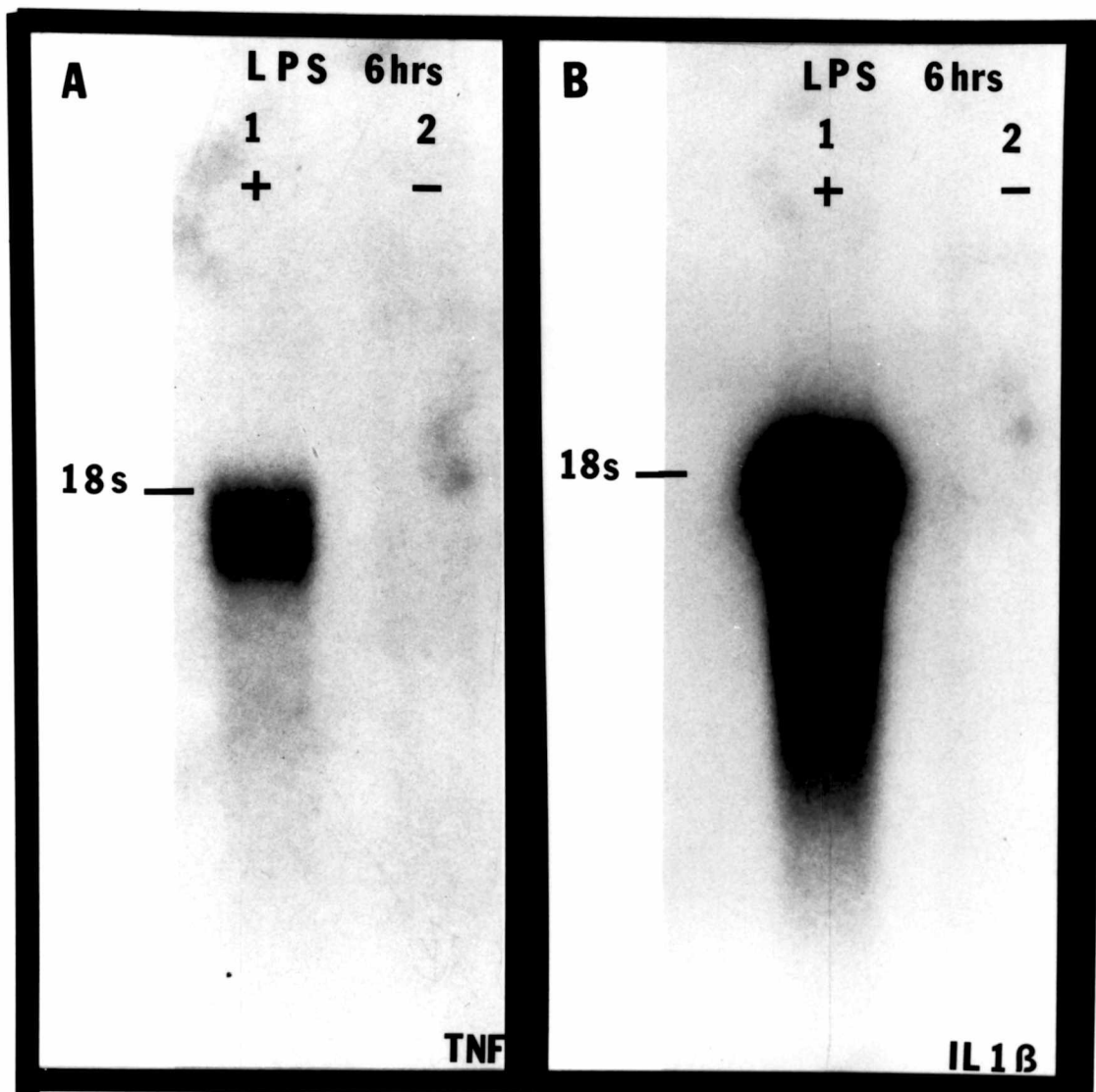
^aN/A indicates that these comparisons were not available when Genbank and EMBL data bases were accessed.

lar weight of 20,096 daltons. Note the presence of the basic amino acid dipeptide Lys-Lys at positions 75-76, which may be involved in proteolytic processing of the precursor IL 1 β to the mature form. There are 10 asparagine residues in the predicted protein structure of mature feline IL 1 β (aa residues 116 through 267) but only asparagine at position 217 has the potential to be glycosylated as well as additional asparagines at amino acid positions 37 and 58 in the precursor form of IL 1 β . In the predicted mature IL 1 β peptide, there are two cysteine residues at positions 176 and 186 which may serve to stabilize the protein by formation of intramolecular disulfide bonds.

4.3 Northern Blot analysis of LPS stimulated macrophages for IL 1 β and TNF α mRNA

Total RNA (10 ug) from bone marrow derived macrophages pulsed 6 hours with LPS was denatured, electrophoresed through a formaldehyde/urea denaturing agarose gel and blotted to nylon filters as described in Materials and Methods. Filters were prehybridized, and then hybridized with ^{32}P labeled TNF α or IL 1 β specific probes, washed and exposed for 70 hours at -70°C with an intensifying screen. The filter was first probed with the TNF α specific probe, then stripped and reprobed with the IL 1 β specific probe. Figure 3.3, Panel A, Lane 1.0 shows a TNF α specific message of

Figure 3.3 Expression of mRNA for TNF α and IL β in LPS stimulated bone marrow derived macrophages. Lane 1 in each panel was loaded with 10ug of total RNA from LPS stimulated (6 hours) macrophages while Lane 2 contains 10ug of total RNA from non-LPS stimulated macrophages. Size of mRNA was determined by comparison with a 0.16 to 1.77 kb RNA ladder run on the same gel. Panel A RNA was probed with a TNF α specific ^{32}P labeled cDNA probe while in Panel B RNA was probed with a IL 1 β specific ^{32} labeled cDNA probe.



approximately 1.6 kb present in LPS stimulated macrophages. In Panel B, Lane 1.0 of Figure 3.3, IL 1 β specific mRNA of approximately a 1.7 kb size is present in LPS stimulated macrophages. Lane 2.0 in both panels contains 10 ug of total RNA derived from non-LPS stimulated macrophage cultures. No message is present in Lane 2.0 of either Panel A or B, indicating that some activating stimuli (LPS in this case) is necessary to activate gene expression of TNF α and IL β in quiescent feline macrophages.

5. Discussion

Using PCR technology, we have cloned and sequenced cDNAs for feline TNF α and IL 1 β . The nucleotide sequences presented are "consensus" sequences of multiple cDNA clones generated by multiple PCR reactions. This minimized potential problems due to misincorporation of nucleotides by *Taq* polymerase, especially during early cycles where subsequent amplification steps would lead to a significant portion of the cDNA population generated possessing the misincorporated base pair (9). By sequencing multiple clones from multiple PCR reactions, a true consensus sequence of the population is formed, significantly decreasing the risk of reporting a misincorporated base pair (8,9). However, we are aware that for TNF α only, the sequences which overlap the most 5' and 3' primer sites may have a mismatch present, since primer design may have lead to a skewing of the sequence and forced a mismatch to occur. In cases of mismatch, the sequences will match those reported for human TNF α since human sequences were utilized in the design of primers where mismatching of bases between species occurred. However, at the serine amino acid residue 223, the codon present in feline TNF α consensus sequences is TCC, but the primer sequence at this location was designed from the human sequence, TCT (which also codes for serine) so in this case the primer sequence did not force a mismatch of bases. Since the most 5' and 3' primers used for cloning IL 1 β were external to the coding region, this type of primer induced mismatching was not a problem

for the reported feline IL 1 β sequence.

The nucleotide consensus sequence and predicted protein sequence of feline TNF α cDNA is displayed in Figure 3.1 and feline IL 1 β cDNA in Figure 3.2. The consensus sequence of feline TNF α cDNA cloned has a 98.6% overall match with the coding regions of the recently reported genomic clone of feline TNF α (22). The two clones differ by 8 nucleotide changes which result in 5 amino acid differences in the predicted protein sequence. A search of Genbank and EMBL revealed our consensus feline TNF α cDNA has a 90%, 86%, 85%, 82% and 83% overall match with human, porcine, ovine, mouse and goat TNF α , respectively (Table 4.1) (24-26,30). The full length feline cDNA for TNF α is 702 bp and encodes a predicted protein of 233 amino acids with a molecular weight of 25,446 daltons. This represents the probable precursor form of mature TNF α (19,27). Based upon homology comparisons with the NH₂-terminal of human, murine, and porcine TNF α cDNA sequences (Val/Leu/Leu-Arg-Ser-Ser), Leu at position 77 is probably the first amino acid for the mature, processed form of feline TNF α . There are 76 amino acids preceeding this leucine residue, and based upon comparisons with human and murine TNF α cDNAs (25,26) probably represent a sequence which is involved in the secretion or processing of mature feline TNF α . It is also interesting to note the presence of Arg-Arg (aa 28-29) and Lys-Lys (aa 19-20) dipeptides in the first 30 amino acids of feline TNF α . Pairs of basic amino acids are reported to serve as cleavage sites for the release of physio-

logically important peptides from precursor molecules. Similar pairs of basic amino acids have been reported in human TNF α precursor sequence (26). The predicted mature, processed form of feline TNF α is 158 aa in length and begins at amino acid residue number 77 and encodes a protein with a predicted molecular weight of 19,175. Northern blot analysis (Figure 3.3) of LPS stimulated macrophages, detected one species of mRNA of approximately 1.6 kb for TNF α .

There are 6 asparagine (Asn) residues present in the predicted amino acid sequence of mature feline TNF α (aa residues 77 through 234) but none appear to fulfill the criteria reported necessary to be available as potential N-glycosylation sites (14). Therefore, it appears from the deduced amino acid sequence reported here for feline TNF α , as well as that reported for the genomic feline TNF α clone (22), that mature feline TNF α , like human TNF α , is not a glycoprotein. There are two cysteine residues (aa positions 145 and 177) present in the mature feline TNF α peptide which could be involved in a single intramolecular disulfide bond formation. Two cysteine residues present in the mature form of human TNF α are linked by a disulfide bridge, which appears to be essential for TNF α cytotoxicity (1).

The full length cDNA for feline IL 1 β (Figure 3.2) is 804 bp long and encodes a predicted protein of 267 amino acids with a molecular weight of 31,892 daltons (the probable precursor form of feline IL 1 β). This molecular weight is in close agreement with

the molecular weight of proteins predicted from the cDNA for the precursor forms of murine (30,935 daltons) and human (30,749 daltons) IL 1 β (13,21). Feline IL 1 β has an overall match of 79%, 76%, 77.5% and 77% with IL 1 β cDNAs for human, bovine, rabbit and murine species, respectively (Table 3.1) (4,13,20,21). Sequences surrounding the ATG methionine start codon of feline IL 1 β conform to the CC(A/G)CC ATGG sequence which has been proposed as a consensus sequence for eukaryotic initiator sites (18).

Due to incomplete homology with human, murine, and bovine IL 1 β , it is difficult to predict the exact location at which the precursor protein is cleaved to yield the mature form of IL 1 β but comparisons between amino acid sequences suggest that amino acid residue 116 is the probable amino terminal for the mature form of feline IL 1 β . However, N-terminal sequencing of the mature form of isolated feline IL 1 β protein is necessary in order to confirm this cleavage site.

If cleavage occurs at this site, it would result in a mature feline IL 1 β protein of 152 amino acids with a predicted molecular weight of 20,096 daltons. There has been a report of the isolation of biologically active feline IL 1 from LPS stimulated alveolar macrophages (12). This IL 1 activity had a molecular weight that ranged from 15000 to 20000 by Sepharacyl S-200 gel filtration. Though the authors believed they had isolated feline IL 1 α due to chromatofocusing analysis, it is possible that their samples contained both IL 1 α and IL 1 β which could possibly

account for the wide spread in molecular weight. Northern blot analysis (Figure 3.3) of LPS stimulated bone marrow derived macrophages detected the presence of one species of mRNA for IL 1 β of 1.7 to 1.8 kb in size.

The predicted protein structure of mature feline IL 1 β (aa residues 116 through 267) has one asparagine residue at position 217 which has the potential to be N-glycosylated. Additionally, in the precursor form of feline IL 1 β , additional asparagine residues at amino acid positions 37 and 58 also fulfill the criteria for potential sites of N-glycosylation. In the predicted mature IL 1 β peptide, there are two cysteine residues at positions 176 and 186 which may serve to stabilize the protein by formation of intramolecular disulfide bonds.

The cloning and sequencing of these important feline cytokines not only provides information concerning cytokine homology across species at both the nucleic acid and protein level, in addition, two valuable reagents are now available for use in exploration of the importance of these two important cytokines during pathogenesis of feline infectious diseases. Feline leukemia virus (15), feline immunodeficiency virus (3), and feline infectious peritonitis virus (29) are all viral agents known to infect feline macrophages but their influence upon macrophage gene expression has yet to be thoroughly investigated. These cDNA clones will be of benefit in evaluating whether selected viral agents modulate feline macrophage gene expression and may help determine the

possible role of these cytokines in the pathogenesis of these viral agents *in vivo*.

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PART IV:

**DIFFERENTIAL EXPRESSION OF LPS INDUCED $\text{TNF}\alpha$ AND IL-1 β mRNA
IN FELINE MACROPHAGES DERIVED FROM THE BONE MARROW OF NORMAL
AND FELV INFECTED CATS**

1. ABSTRACT

Macrophages were cultured *in vitro* from the bone marrow of clinically normal cats and cats persistently infected with Feline Leukemia Virus (FeLV). Macrophages were examined for phagocytic capacity of latex beads, tumor necrosis factor alpha (TNF α) and interleukin 1 beta (IL-1 β) mRNA expression, esterase activity and the presence of FeLV gp 70 antigen by immunohistochemistry. A comparison of macrophages derived from FeLV negative and positive cats (population mean 90% versus 96% macrophages, respectively), no significant difference was detected between the percent of macrophages in the final cell population ($p=0.1490$), nor in their ability to phagocytize latex beads (mean 85% vs 88%, respectively, $p=0.9551$) or in expression of nonspecific esterase (a marker of macrophage maturity) activity (mean 95% vs 95%, respectively, $p=1.0$). However, macrophages derived from the bone marrow of FeLV positive cats, when stimulated with lipopolysaccharide (LPS), differentially expressed TNF α and IL-1 β mRNA depending upon the replication status of FeLV. Mature macrophages derived from FeLV infected but clinically normal cats did not appear to be replicating FeLV (negative for FeLV gp 70 antigen by immunohistochemistry) and Northern Blot analysis of total RNA, demonstrated no significant difference for TNF α or IL-1 β mRNA levels from FeLV negative macrophage control cultures. Interestingly, the gp 70 antigen positive cells in these cultures consisted exclusively of residual lymphocytes. In contrast, bone marrow derived macrophages

from one FeLV positive cat, which was exhibiting clinical signs of infection, were positive for FeLV gp 70 antigen by immunohistochemistry (replicating virus) and Northern Blot analysis demonstrated no TNF α mRNA and greatly decreased IL-1 β mRNA levels following LPS stimulation of macrophages when compared to LPS stimulated FeLV negative macrophages. Equivalent amounts of total RNA, as determined by actin mRNA levels, were loaded for each sample, therefore the difference in cytokine mRNA levels could not be attributed to improper loading of total RNA in one lane during electrophoresis. These findings indicate that FeLV may modulate TNF α and IL-1 β gene expression, but only when macrophages are actively replicating FeLV. However, one must be cautious in interpretation of these results, since macrophages were derived from bone marrow of random source cats (known to be FeLV positive and FIV negative), other undetected factors may be contributing to mRNA modulation and by mere happenstance FeLV is replicating in these macrophages. Additional investigations using infectious FeLV cDNA clones and transfection studies into mature macrophages followed by FeLV expression, LPS stimulation and Northern Blot analysis are necessary to properly address this issue.

2. INTRODUCTION

As a first line of defense, macrophages play a major role in host surveillance and immunoregulation by secretion of a wide variety of immunoregulatory factors such as cytokines (1,4,25). As an antigen processing and presenting cell, the macrophage plays a critical role in determining whether cellular immune response to invading pathogens will occur (27). The title for a recent review most aptly conveys the overall importance of the macrophage during immune responses, "Lymphocytes play the music but the macrophage calls the tune" (25). If an infectious agent has the ability to infect and replicate within monocyte/macrophages, thereby changing the cells functional capacity, it would not only be an important pathogenic factor for the infectious agent, but could also result in widespread dysfunction of the host's immune system (12,15,16,18).

Previous work by Hoover et al (12) established the pivotal role that macrophages play in restricting replication of FeLV in acute and persistently infected cats. These studies demonstrated that age related resistance for FeLV infection (older cats are less susceptible to infection), correlated with age related acquisition of macrophage mediated immune functions. Additionally, FeLV was found to resume replication whenever macrophage function was disturbed by glucocorticoid therapy or other *in vivo* macrophage suppressive treatments. While it is clear that myelomonocytic

precursors in bone marrow and mature macrophages replicate virus, few studies have addressed the issue of FeLV influence on macrophage gene expression. These experiments were designed to determine if macrophages derived from the bone marrow of FeLV infected cats were actively replicating FeLV and if viral replication altered expression of TNF α and/or IL 1 β mRNAs, two important macrophage immunoregulatory and inflammatory cytokines.

3. MATERIALS AND METHODS

3.1 Animals

Seven FeLV negative and 5 FeLV positive random source cats of varying adult ages were utilized as bone marrow donors. Animals were housed separately in cages at the animal facilities of the University of Tennessee School of Veterinary Medicine, Knoxville, Tennessee (a fully accredited AALAC facility) and their treatment, housing and care were in compliance with standards set forth in the "Guide for the Care and Use of Laboratory Animals" (NIH publication no. 85-23). These animals were part of a cat colony maintained by Dr. Al Legendre with protocols on file with the Animal's Cares and Concerns Committee for removal of bone marrow. The FeLV status of cats were determined by the Feline Leukemia Virus Test Kit, Leukassay*F II (Pitman-Moore, Inc., Washington Crossing, N.J.).

3.2 Feline bone marrow isolation technique and *in vitro* culturing method

Bone marrow isolation and culturing technique has been previously described in Part II of this Thesis (6). Briefly, cats were anesthetized with ketamine HCl (Ketalar^R, Parke Davis, Morris Plains, NJ), the lumbosacral area clipped, surgically prepped, and 0.5 ml of bone marrow aspirated by bone marrow needle from the femur directly into a 10 ml syringe containing 3 ml of Iscove's

Modified Dulbecco's Medium (GIBCO) plus 100 units per ml of heparin sulfate (SIGMA Chemical Co., St. Louis, MO) (H-IMDMEM).

For separation, samples were brought to 10 ml final volume with H-IMDMEM, and 5 mls layered over 3 ml of Ficoll-Sodium diatrizoate (Histopaque 1.077 - SIGMA, St. Louis, MO) in a 15 ml conical centrifuge tube. Tubes were centrifuged at 400 xg for 25 minutes at 25°C. The cellular interface band was removed, washed, and viability assessed by 0.2% trypan blue dye exclusion (SIGMA). Viability was usually greater than 96% and never dropped below 94%. Cell concentrations were adjusted to 2.0×10^6 cells per ml in complete media consisting of IMDMEM containing 15% HI-FBS (HyClone), 2mM L-glutamine, 1mM sodium pyruvate, 100 units/ml penicillin, 100 ug/ml streptomycin, 10 mM Hepes and sodium bicarbonate to a pH of 7.2 to 7.3.

Ten mls of cell suspension were cultured in polystyrene 100 mm X 20 mm tissue culture dishes (FALCON, BAXTER, Atlanta, GA) at 37°C, in a humid 6% CO₂ chamber. At the end of one week, 10 ml of complete IMDMEM was added to each plate and incubation continued for an additional 7 days. At the end of 14 days, macrophages were harvested, tested for the presence of FeLV antigen in tissue culture supernates by ELISA (Feline Leukemia Virus Test Kit, Leukassay*F II), tested for esterase activity, phagocytic ability, and Northern blot analysis of lipopolysaccharide (LPS -*E. coli* serotype 0127:B8, SIGMA) induction of cytokine mRNA.

At 14 days, macrophages were pulsed with 10 ug per ml LPS by

one of two methods. Method 1 - For each culture dish, media was aspirated, non-adherent cells pelleted by centrifugation, and cells resuspended in complete growth media containing 10 ug per ml of LPS. For each dish undergoing LPS stimulation, 20 ml of media plus LPS was gently layered over the undisturbed macrophage monolayer and incubated at 37°C, in a humid 6% CO₂ chamber for 6 hours at which point cells were lysed for RNA isolation. Method 2 - Loosely adherent macrophages were gently washed from culture dishes, while adherent cells were removed by incubating at room temperature with Hanks balanced salt solution (Ca²⁺ and Mg²⁺ free) (GIBCO) plus 2.6 mM EDTA (SIGMA) followed by gentle pipetting. Cells were resuspended in complete media at 1 x 10⁶ cells per ml plus 10 ug per ml LPS. Cultures were incubated in a 37°C, humid 6% CO₂ incubator for 6 hours at which point cells were lysed in guanidium isothiocyanate lysing solution and stored at -70°C until RNA isolation.

3.3 Cytochemical and phagocytic characterization of cell population

Non adherent cells were removed from dishes by gentle pipetting. Adherent cells were removed by incubating with 5 ml of Hank's balanced salt solution (Ca²⁺ and Mg²⁺ free) plus 2.6 mM EDTA. Adherent and non adherent cells were mixed, concentrations determined, and cytopins prepared (Cytospin, Shandon Inc., Pittsburgh, PA) for differential, immunoperoxidase and nonspecific

esterase stains by spinning 100 μ l of 1×10^6 cells/ml onto glass slides for 5 minutes at 700 RPM. Cytospins were allowed to dry at room temperature and stained with Diff-Quik stain (Difco, Detroit, MI) for differentials, nonspecific esterase stains were done by the method of Li et al (14), and immunoperoxidase stains done as detailed below. A minimum of 200 cells were counted for differential analysis and esterase determination, entire slide scanned for immunoperoxidase studies.

For phagocytosis assays, bone marrow derived macrophages were harvested, counted and plated at 2.0×10^5 cells per ml in complete IMDMEM plus 10 μ g per ml of LPS in 8 chamber Lab-Tek slides. Ten microliters of a 1:100 dilution of 0.8 micron Latex beads (SIGMA) was added per well. Activated macrophages plus beads were incubated at 37°C in a humid 6% CO₂ atmosphere for 24 hours. After incubation, cells were fixed, and stained with Diff-Quick and permanently mounted. A cell was deemed phagocytic if it had phagocytized a minimum of 10 beads, however, phagocytic cells frequently contained so many beads that they were not countable within the cell.

3.4 Immunoperoxidase staining for FeLV envelope antigen, gp 70

C11D8 monoclonal antibody was used to recognize FeLV gp 70 antigen (11). C11D8 is an IgG2b isotype and was kindly donated by Dr. Chris Grant (Pacific Northwest Research Foundation).

The immunoperoxidase technique was modified from that of Gen-

delman et al (10). Due to the presence of Fc receptors on the macrophage, purified feline IgG Fc fragment (Jackson Immuno-research Labs, West Grove, PA.) was used in the initial blocking steps to saturate macrophage Fc receptors by the method of Pullen et al (21). Briefly, cytopins were prepared from non-stimulated macrophages. Cells were fixed with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature (RT), then covered with the following solutions (all prepared in PBS pH 7.2): (1) 20 minute wash in PBS at RT, (2) 20 minute wash in PBS plus 20% fetal calf serum (FBS), (3) 20 minute incubation in PBS + 20% FBS + 20ug/ml purified feline IgG Fc fragment at RT, (4) C11D8 monoclonal antibody or a murine IgG2b monoclonal antibody standard (Southern Biotechnology), 10 ug per slide in PBS + 20% FBS for 1 hour at 37°C, (5) Wash 10 minutes in PBS + 20% FBS at RT, (6) 30 minutes at 37°C with biotinylated rabbit anti-mouse IgG F(ab')₂ (Zymed, San Francisco, CA), 1:100 dilution in PBS, (7) Wash 10 minutes RT in PBS, (8) Incubate 30 minutes at RT with peroxidase-conjugated streptavidin (Zymed, San Francisco, CA), 1:100 dilution in PBS, (9) Wash PBS at RT for 10 minutes, (10) develop label in 3,3'-diaminobenzidine tetrahydrochloride (SIGMA), 0.5 mg/ml in 0.05M Tris Base with 0.01% hydrogen peroxide for 7 minutes at RT, (11) Wash in water for 5 minutes at RT, (12) Stain for 5 minutes at RT in 0.5% copper sulfate to enhance color, (13) Wash in water, 5 minutes at RT, (14) Counterstained in 0.5% methyl green for 20 to 30 minutes at RT, (15) Slides were rinsed in water, (16) Dehydrat-

ed through alcohol gradients, (17) Slides were air dried and permanently mounted with Permount.

3.5 Total RNA isolation procedure and Northern Blot analysis

RNA was isolated by the method of Chirgwin et al (3). Briefly, cells for RNA isolation were lysed in a 4M guanidinium isothiocyanate (SIGMA) lysing solution containing 0.5% sodium lauryl sarcosine (SIGMA) , 25mM sodium citrate pH 7.0 (SIGMA), and 47 mM 2-mercaptoethanol (SIGMA). The lysate was centrifuged at 36,000 rpm for 18 hours in a Beckman ultracentrifuge (rotor - Sorval AH650 swinging bucket rotor) through a 5.7 M cesium chloride cushion (3).

After phenol/chloroform extraction followed by ethanol precipitation, total RNA was quantitated by spectrophotometry and 10 to 15ug per lane denatured for fractionation in a 1.0% agarose/1.1 M formaldehyde gel containing 500 ugs of ethidium bromide for RNA visualization. The 10X running buffer consisted of 0.5M boric acid, 50mM sodium borate, 100 mM sodium sulfate and 10 mM EDTA. RNA was denatured in 20 ul of the following buffer mix: 0.63 ml denatured formamide (BRL), 0.20 ml 37% formaldehyde, 0.063 ml of 10X running buffer, 0.10 ml of 50% glycerol in DEPC treated water, and 0.030 ml of a 0.6% solution of bromophenol blue/ xylene cyanol, boiled for 10 minutes, and placed on ice for 10 minutes prior to gel loading. Gels were electrophoresed at a constant current of 95 volts until the bromophenol blue dye front traveled approximate-

ly 14 cm. At this point, gels were visualized with UV for comparisons of equality of RNA loading, soaked briefly to remove gel fragments, then transferred to nylon filters (Nytran, Schleicher and Schuell, Inc, Keene, NH) overnight by a sponge modification of the capillary transfer method (9).

After transfer, nylon filters were UV-crosslinked to stabilize RNA (13) (Stratalinker UV Crosslinker, Stratagene) and filters were prehybridized at 60°C for a minimum of 1 hour in 5X SCC, 0.05M sodium phosphate buffer pH 6.5, 5X Denhardt's solution, 1% SDS, 200 ug per ml of denatured salmon sperm DNA as a blocker. Following prehybridization, filters were hybridized overnight (18 hours) at 60°C in the same prehybridization mix plus the appropriate ^{32}P labeled nick translated (Amersham kit N5500) cDNA probe. Nick translated probes had a specific activity of 1 to 2 x 10^7 cpm/ug of DNA specific activity. Post hybridization, filters were washed in the following order: (1) Two times at room temperature for 15 minutes each in 2X SCC plus 0.1% SDS. (2) Two times at room temperature for 15 minutes each in 0.2X SCC plus 0.1% SDS. (3) Once for 20 minutes at 55°C with 0.2X SCC plus 0.1% SDS. (4) Final wash, once for 30 minutes at 60°C with 0.1X SCC plus 0.1% SDS.

Following washes, blots were exposed 70 hours to Kodak XAR 5 film in the presence of Dupont Cronex Lightning plus intensifying screens at -70°C. Autoradiographs were scanned using a LKB Ultros-can XL Laser Densitometer (LKB, Bromma, Sweden) and quantitated

using the LKB 2400 Gelscan XL Software Package (LKB, Bromma, Sweden). Following exposure, radioactive cDNA probes were stripped from nylon filters by washing filters in 50% formamide/6X SSPE at 65° C for 30 minutes followed by rinsing briefly in 2X SSPE. Filters were then reprobed with additional radiolabeled cDNA probes. In all studies, the following pattern was maintained: Filters were first probed with TNF α specific probes, blot stripped followed by probing with an IL-1 β specific probe, blot stripped then probed with an actin specific cDNA probe.

DNA probes for feline TNF α and IL-1 β were constructed in this laboratory and the paper characterizing their preparation and sequencing is in progress (5) (see Part III of Thesis). The ³²P labeled TNF α probe was prepared from the plasmid, pTNF α B9, which contains a 570 bp Eco R1, Hind III fragment of feline TNF α cDNA. The ³²P labeled IL-1 β probe was prepared from the plasmid, pIL1#2, which contains a 750 bp, Eco R1, Hind III fragment of feline IL-1 β cDNA. The actin cDNA plasmid (pA1) was a gift from Dr. Janet Lathey (Scripps Institute) and contains a 750 bp Eco R1, Hind III actin cDNA fragment.

3.6 Statistics

Statistics were done using the Instat Statistical Software Package (GraphPAD Software, San Diego, CA.). The Mann-Whitney two sample unpaired nonparametric test was used for analysis.

4. RESULTS

4.1 Comparison of macrophages derived from FeLV positive and negative cats: Differential, phagocytosis and esterase

As can be seen in Table 4.1, there was no significant difference in the number of mature macrophages derived from the bone marrow of FeLV negative and positive cats (population mean 90% versus 96% macrophages, respectively), $p=0.1490$, nor in the ability of macrophages from FeLV positive cats to phagocytize latex beads (mean 85% and 88%, negative versus positive, respectively, $p=0.9551$) nor in the percent of mature macrophages staining positive for nonspecific esterase activity (mean 93% vs 94%, negative versus positive, respectively, $p=1.0$).

4.2 Detection of FeLV gp70 antigen in bone marrow derived cell populations by immunoperoxidase staining

Due to the isotype of the C11D8 monoclonal antibody, IgG2b, F(ab')₂ fragments could not be generated by enzyme digestion (2). Therefore, purified feline IgG Fc fragments were used as specific blockers for the macrophage's Fc receptor. The following standards were included for each immunoperoxidase stained set of slides, macrophages from a FeLV negative cat treated with C11D8 monoclonal antibody, FeLV negative and positive cells treated with a murine IgG2b MOPC standard. If any of these slides were positive for antibody binding, that particular set of slides were considered non valid due to non-specific binding of antibody to improp-

Table 4.1 Comparison of differential, esterase stains and phagocytosis of latex beads between macrophages derived from FeLV positive and negative cats.

	FeLV Status ^a			
	<u>FeLV</u>	<u>Negative</u>	<u>FeLV</u>	<u>Positive</u>
	Differential^d			
PMN ^b	(0 -17%)	4%	(0 - 3%)	1%
Lymphocytes	(3 - 9%)	4%	(1 - 5%)	3%
Macrophages	(80-90%)	90%	(95-98%)	96% p= 0.1490 ^e
Other ^c	(0 - 2%)	2%	(0 - 1%)	<1%
	Esterase^d			
Positive	(79-99%)	93%	(81-99%)	94% p= 1.0
Negative	(1 -12%)	7%	(2 -25%)	6%
	Phagocytosis^d			
Positive	(78-97%)	85%	(75-98%)	88% p= 0.9551
Negative	(3 -22%)	11%	(2 -25%)	13%

^aDetermined by ELISA on 14th day of culture

^bPMN= polymorphonuclear leukocytes

^cOther= precursors for granulocytes, macrophages and erythrocytes

^dStaining methodology described in materials and methods

^ep value as determined by Mann-Whitney nonparametric test

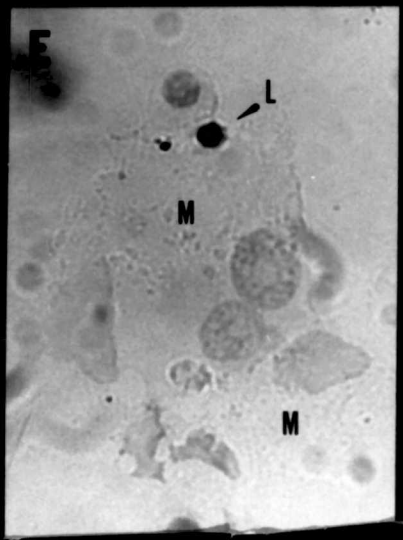
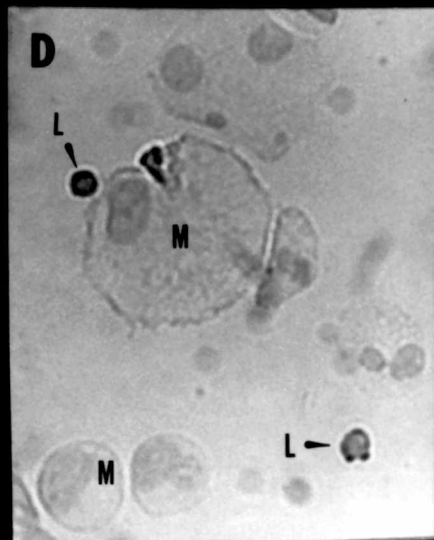
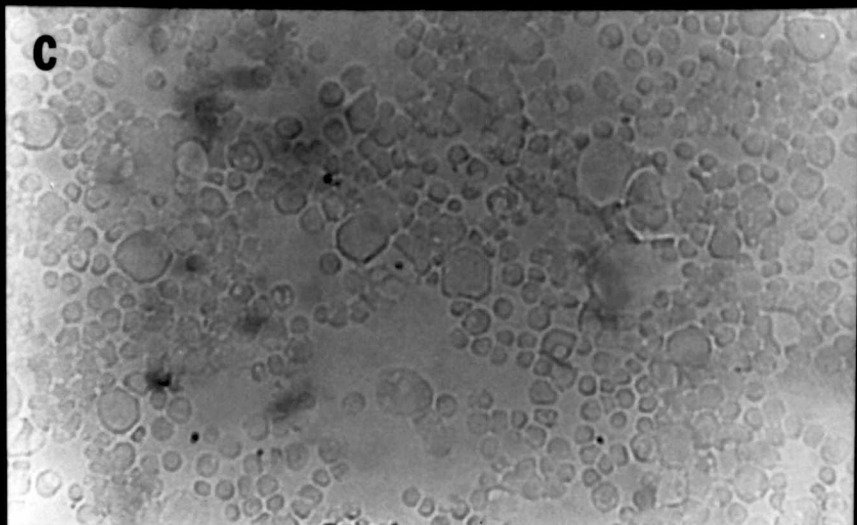
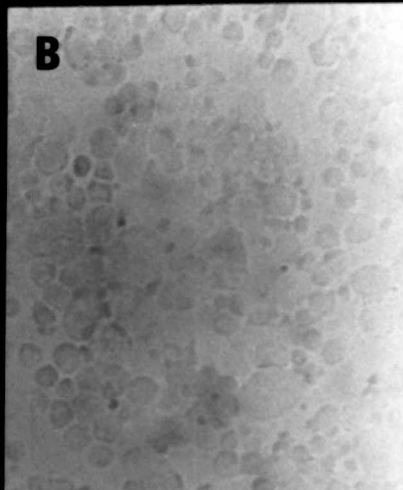
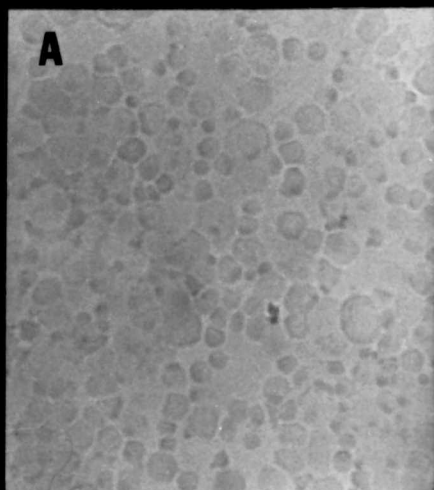
erly blocked Fc receptors and a new set of slides were stained.

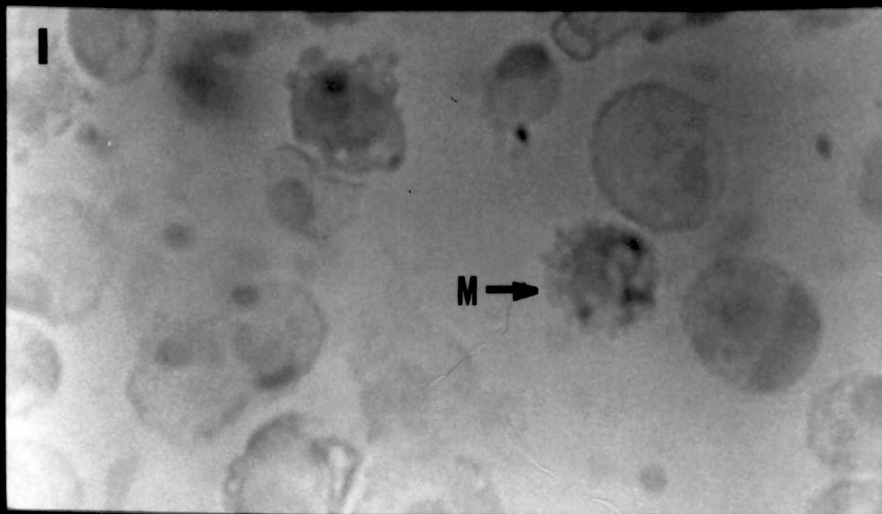
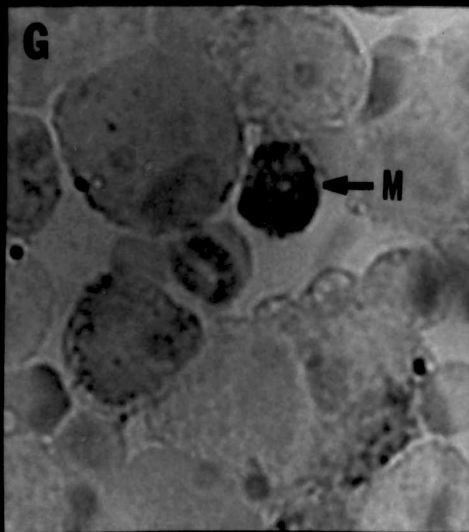
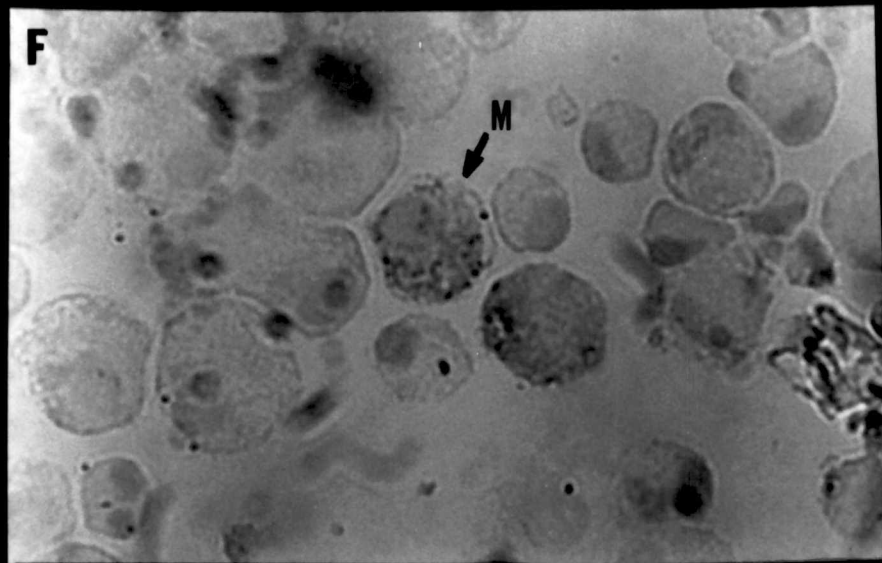
Figure 4.1 is representative of the results of immunoperoxidase stains for detection of FeLV gp70 antigen in the macrophage cell populations. In Panels A and B, no specific immunoglobulin staining was seen when macrophages were derived from FeLV negative cats and stained with a murine standard IgG2b (Panel A) or with the C11D8 monoclonal specific for FeLV gp70 antigen (Panel B). The use of methyl green as a counterstain imparts very little color to cells not positive for immunoglobulin staining, and for this reason, unstained cells are faint and appear to fade into the background. Panel C is representative of macrophages derived from FeLV positive cats stained with the murine control MOPC IgG2b monoclonal antibody. Once again no antigen specific or Fc receptor staining is present so the cells are stained only with methyl green counterstain. When examined for the presence of viral antigen, the staining pattern seen in Panels D (Cat #1) and E (Cat #2) was common for all macrophage populations derived from FeLV positive cats with one exception. In these micrographs, cells were stained with the C11D8 monoclonal antibody but only residual lymphoid cells (L) stained darkly indicating the presence of gp70 antigen and replicating FeLV. The macrophages (M) were uniformly negative for the presence of FeLV antigen. Note the obvious size differential between the FeLV gp70 positive lymphocytes and adjacent antigen negative macrophages. Interestingly, the cells derived from one FeLV positive cat (Cat #3) failed to show this

Figure 4.1 Micrographs of FeLV gp70 antigen detection by immunoperoxidase methodology on cells derived from bone marrow cultures. Panels A and B are cells derived from FeLV negative cats that have been treated with a nonspecific murine standard MOPC IgG2b antibody and with the C11D8 monoclonal antibody, respectively. Panels A and B are both 100x magnification. Panels C, D, and E are representative of cells derived from FeLV positive cats (Cats 1 for C and D, and Cat 2 for E) not replicating FeLV in their macrophages. In Panel C, cells were treated with a nonspecific murine standard MOPC IgG2b antibody while Panels D and E were treated with C11D8, FeLV specific monoclonal antibody. In Panels D and E, two darkly staining lymphocytes, positive for FeLV gp70 antigen have been marked. Panel C is at 100x magnification and Panels D and E are at 400x magnification. Panels F through I represent cells from Cat #3, a FeLV positive cat replicating FeLV in its macrophages. All of these cells were treated with the C11D8 monoclonal antibody. In each case, a monocyte/macrophage positive for FeLV antigen has been arrowed. Panels F,G,H, and I are all at 400x magnification.

L = lymphocyte

M = Monocyte/Macrophage





staining pattern. The micrographs in Panels F through I are representative of the staining pattern seen for Cat #3. Interestingly, an occasional lymphocyte as well as monocytes and macrophages were positive for FeLV gp70 antigen. In Panel F, a macrophage (M) is clearly visible in the the center of the micrograph with dark staining clusters of FeLV antigen present. This is also true for the micrographs in Panels G, H, and I. In Panel I, the nucleus configuration clearly indicates that this cell as a monocyte with a small amount of FeLV antigen detectable.

4.3 Northern blot analysis of TNF α and IL-1 β in LPS stimulated macrophages derived from FeLV negative versus FeLV positive cats

What triggered the replication and expression of FeLV antigen in macrophages and monocytes derived from FeLV latently infected Cat #3 is unknown. However, it was interesting to note that northern blot analysis for cytokine mRNA was also unique for Cat #3 when compared to FeLV negative cats and FeLV positive cats not replicating virus in their macrophages. In Figures 4.2 and 4.3, results are presented for Northern blot analysis of total RNA from LPS stimulated and non stimulated macrophages derived from separate FeLV negative cats as well as previously mentioned FeLV positive Cats #2 and #3. In Table 4.2, the densitometry results of the corresponding northern blots in Figures 4.2 and 4.3 are presented. In Figure 4.2, Lanes 1 and 2 contained RNA derived from unstimulated and 6 hour LPS stimulated (Method 1) macrophages,

Figure 4.2 Northern blot analysis of 6 hour LPS stimulated (Method 1) and non-stimulated bone marrow derived macrophages from a FeLV negative cat and FeLV positive Cat #2. Lane 1 in each panel was loaded with 15ug of total RNA from non stimulated macrophages while Lanes 2 and 3 were loaded with 15ug of total RNA from LPS stimulated macrophages. RNA in Lanes 1 and 2 were derived from a FeLV negative cat while Lane 3 was derived from FeLV positive Cat #2. Panel A was probed with a TNF α specific ^{32}P labeled cDNA probe, Panel B was probed with a IL-1 β specific ^{32}P labeled cDNA probe and Panel C was probed with an actin specific ^{32}P labeled cDNA probe. Autoradiographs were exposed for 70 hours at -70°C . The 18s ribosomal band is marked.

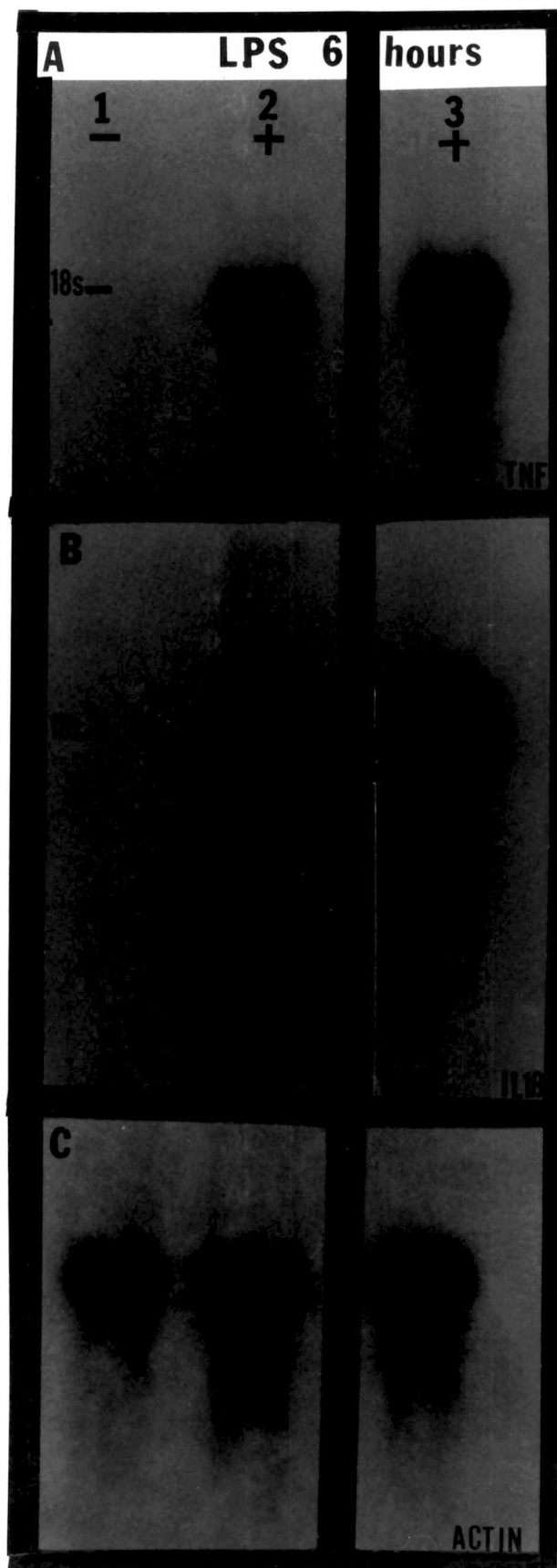


Figure 4.3 Northern blot analysis of LPS stimulated and non-stimulated macrophages (Method 2) derived from a FeLV negative cat and FeLV positive Cat #3. Lanes 1 and 3 in each panel were loaded with 10ug of total RNA from non LPS stimulated but adherent activated macrophages while Lanes 2 and 4 were loaded with 10ug of total RNA from LPS stimulated macrophages. RNA in Lanes 1 and 2 were derived from a FeLV negative cat while Lanes 3 and 4 were derived from FeLV positive Cat #3. Panel A was probed with a TNF α specific ^{32}P labeled cDNA probe, Panel B was probed with a IL-1 β specific ^{32}P labeled cDNA probe and Panel C was probed with an Actin specific ^{32}P labeled cDNA probe. Autoradiographs were exposed for 70 hours at -70°C . The 18s ribosomal band is marked.

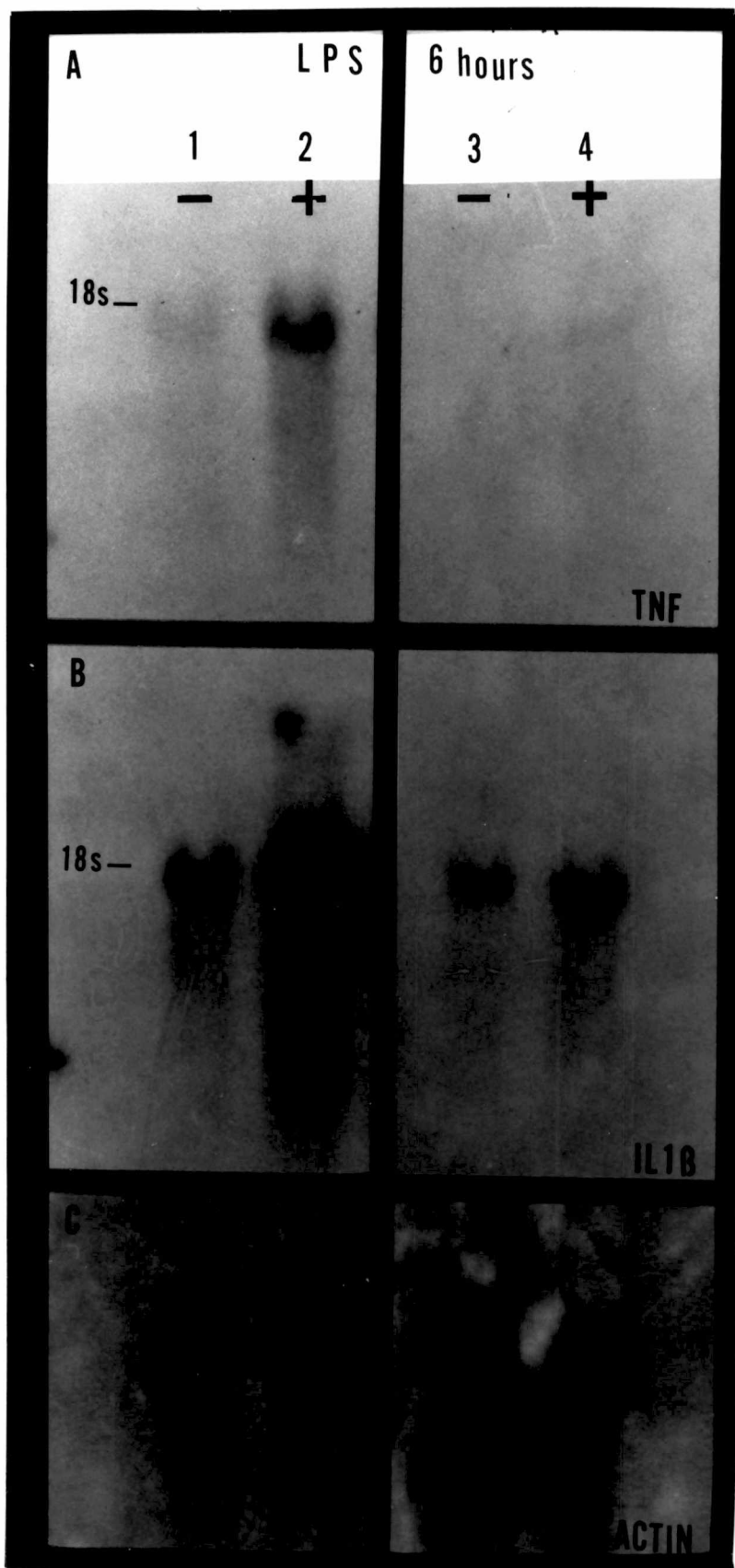


Table 4.2 Compilation of densitometry results of Figure 4.2 and Figure 4.3 Northern blot analysis.

Figure 4.2 and 4.3 Densitometry

Densitometry Results for Figure 4.2^a

cDNA Probe	FeLV Negative		Cat #2	
	No LPS	LPS	No LPS	LPS
TNF α	0	16.18	N/D ^b	31.2
IL-1 β	0	96.0	N/D	108.5
Actin	30.9	44.0	N/D	44.0

Densitometry Results for Figure 4.3^a

cDNA Probe	FeLV Negative		Cat #3	
	No LPS	LPS	No LPS	LPS
TNF α	1.1	9.1	0.3	0.3
IL-1 β	4.5	75.0	1.4	7.2
Actin	13.2	19.3	15.6	15.8

^aDensitometry values were normalized to area during 2 dimensional analysis. The values presented represent relative density, with larger numbers correlating with increased binding of radioactive cDNA probes and presence of increased mRNA levels

^bN/D - these samples were not tested

respectively, derived from a FeLV negative cat while lane 3 contained RNA from 6 hour LPS stimulated macrophages derived from Cat #2. Cat #2 is representative of FeLV positive cats that only had FeLV antigen present in their lymphocytes upon immunoperoxidase staining (see Figure 4.1, Panel E). In Lane 1, no cytokine message for either TNF α (Panel A) or IL-1 β (Panel B) was present demonstrating the necessity for LPS stimulation for activation of these cytokine genes when the macrophage monolayer is undisturbed during LPS stimulation. In Lanes 2 and 3, LPS stimulation rapidly activated TNF α (Panel A) and IL-1 β (Panel B) gene expression in macrophages derived from FeLV negative and positive cats (Cat #2), respectively. Densitometry readings are listed in Table 4.2. There is a 2 fold greater expression of IL-1 β mRNA by macrophages derived from the FeLV positive cat (Cat #2), but this is not considered significant. Densitometry readings for TNF α and actin (Figure 4.2, Panel C) mRNA levels also show no significant difference in the expression of these mRNAs by macrophages derived from FeLV negative or positive cats not replicating FeLV in their macrophages. The data indicate there is no modulation of mRNA levels for TNF α , IL-1 β , or actin in bone marrow derived macrophages from FeLV positive cats that are not replicating virus in their macrophages.

In contrast, Figure 4.3 contains RNA derived from a FeLV negative cat and Cat #3, which appeared to be replicating FeLV within

its monocyte/macrophage population (Figure 4.2, Panels F through I). Lanes 1 and 3 contain RNA derived from macrophages which have not been stimulated by LPS, but they were activated by removal and re-adherence onto plastic for 6 hours prior to total RNA harvest. Activation through adherence probably accounts for the low level of IL-1 β message in Lanes 1 (FeLV negative cat) and 3 (Cat #3) of Panel B, and of TNF α message in Lane 1 of Panel A. In Lane 2, a 6 hour stimulation with LPS resulted in significant upregulation of both TNF α and IL-1 β mRNA production (Panels A and B, respectively) for the FeLV negative cat but not for Cat #3 which appeared to be replicating FeLV within its macrophage population. Following a 6 hour stimulation with LPS, Cat #3's macrophages still failed to undergo activation of the TNF α gene (Lane 4, Panel A and Table 4.2) and production of IL-1 β mRNA was greatly decreased (Lane 4, Panel B and Table 4.2). Since actin mRNA levels are roughly equivalent (Figure 4.3, Lanes 1,2,3, and 4, Panel C and Table 4.2), Cat #3's macrophages must be undergoing some type of modulation of gene activation for these cytokines by some as yet undefined mechanism.

5. DISCUSSION

Earlier investigations by Hoover et al (12) have established the pivotal role that mature feline macrophages play in restricting the replication of FeLV in acute and latently infected cats. Additionally, when FeLV is present in the bone marrow as a latent infection, FeLV was found to resume replication when ever macrophage function was disturbed by some systemic treatment that interfered with macrophage function. What is not clear, is what role if any FeLV may play in modulation of macrophage cell function. As a key cell involved in both antigen processing and presentation (27) as well as immunoregulation (1,8,25), the ability to modulate this cell's function would be of significant advantage for an infectious agent such as FeLV.

Other members of the Retroviridae family such as the human immunodeficiency virus (15) and visna virus (17-19) are known to be intimately associated with macrophages, and may even cause dysregulation of macrophage cytokine production (17,24). This dysregulation may have important ramifications upon the pathogenesis of the disease. However, both visna virus and human immunodeficiency virus are members of the Lentivirus subfamily and as such are distantly related to FeLV, a member of the Oncornaviridae subfamily (17,23).

Due to a lack of suitable molecular probes, few studies have addressed the issue of FeLV modulation of macrophage function such as gene expression. Due to the recent cloning of feline IL-1 β and

TNF α by this laboratory (5), specific molecular reagents for conducting such a study became available. The experiments in this report were designed to determine if macrophages derived from the bone marrow of FeLV latently infected cats have altered macrophage function. Herein, we report that macrophages derived from the bone marrow of latently infected cats do not differ significantly in their ability to phagocytize latex beads ($p = 0.9551$) or in their production of mRNA for TNF α or IL-1 β following LPS stimulation, only if they are not actively replicating FeLV (positive for gp70 antigen by immunoperoxidase staining).

In comparison, one FeLV positive cat began to exhibit clinical signs of aggression and *in vitro* its bone marrow derived macrophages to replicated FeLV (as evidenced by the presence of gp70 antigen). These macrophages retained their capacity to phagocytize latex beads (85% phagocytosis, data not shown) but failed to produce TNF α mRNA and produced greatly decreased levels of IL-1 β mRNA when appropriately stimulated with LPS. These findings suggest that FeLV may modulate TNF α and IL-1 β gene expression since the presence of the virus correlated with downmodulation of cytokine gene expression, when macrophages were actively replicating FeLV. However, one must be cautious in interpretation of these results, since macrophages were derived from bone marrow of random source cats (known to be FeLV positive and FIV negative), other undetected factors may be contributing to the cytokine mRNA modulation and by mere happenstance FeLV is replicating in these

macrophages. Additionally, this may be an aberration of this particular FeLV positive cat, since this cat was undergoing a reacerbation of disease. Additional investigations using infectious cDNA clones of FeLV and transfection studies into mature feline monocyte/macrophages followed by FeLV expression, LPS stimulation and northern blot analysis are necessary to properly address this issue.

However, though the data in this paper is far from giving a conclusive cause and effect for virus and macrophage interaction, the data presented in this paper does offer a tantalizing glimpse at one possible mechanism by which FeLV could interfere with feline immune responses, by inhibiting the production of immunoregulatory cytokines such as TNF α and IL-1 β . Inhibition of cytokine production may be direct due to replication of FeLV within the cell or indirect in that p15E or some other viral protein may block signal transduction within the macrophage so that cells cannot respond to external stimuli, or indirect by causing production of a powerful inhibitory cytokine such as transforming growth factor beta. It is likely that the virus itself or a viral component such as p15E may be blocking signal transduction in the macrophage, since a similar mechanism has been evoked to explain inhibition of T cell (26) and PMN function (7,28) when exposed to FeLV. One fact is quite clear, the dysregulation of cytokines such as IL-1 β and TNF α would contribute significantly to the immunosuppression which is seen as such a key factor in

the pathogenesis of FeLV associated diseases (20,22,23).

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PART V:
CONCLUSIONS AND FUTURE PROSPECTS

CONCLUSIONS AND FUTURE PROSPECTS

Due to the diverse nature of this dissertation, several points should be addressed. In Part II, a procedure for the *in vitro* growth of large numbers of feline bone marrow derived macrophages was reported which does not necessitate euthanasia of the animal. In feline research, this technique will be very useful for the generation of large numbers of macrophages *in vitro* which maybe used to study feline pathogen-macrophage interactions with special emphasis upon viral agents such as feline infectious peritonitis virus, feline leukemia virus, and feline immunodeficiency virus. These three viral agents cause immunosuppression and significant mortality in the feline population of the United States, any information regarding their pathogenic mechanisms would be of obvious benefit to the population.

Perhaps the most significant portion of this dissertation is found in Part III, the cloning and sequencing of cDNA for the feline monokines, tumor necrosis factor α and interleukin 1 β . These cDNA clones are valuable reagents for individuals involved in all aspects of feline research, whether investigating immunological phenomena or the pathogenic mechanisms of specific feline infectious agents. With these cDNA clones, specific molecular questions concerning normal or aberrant regulation of macrophage gene expression may now be explored. Considering the importance of macrophages as a host's first line of defense, and their important roles in antigen processing and presentation, immunoregulation,

and inflammatory responses, it will not be surprising to find that certain feline pathogens may replicate in macrophages and hinder the production of specific monokines, possibly TNF α or IL-1 β which now may be easily detected at the molecular level.

The original intent of Part IV of this dissertation was to determine whether FeLV infection of macrophages altered macrophage function, with special emphasis upon the monokines TNF α and IL-1 β . However, no strong conclusions can be drawn concerning this point from the data present in Part IV of this dissertation. The finding that macrophages derived from the bone marrow of one FeLV positive cat appeared not only to be replicating FeLV but also had a detectable alteration in the production of TNF α and IL-1 β mRNA induction by LPS, would suggest that FeLV may be capable of modulating macrophage function but one must be cautious in their evaluation of these results. Since the population of cats from which bone marrow samples were harvested were of random source, a number of other infectious agents may also be present in the bone marrow cultures, their presence may have gone undetected, but they could possibly be contributing or modulating macrophage functions. It may have just been mere happenstance that FeLV was also replicating in the same cells. In order to more fully study the question of viral modulation of macrophage gene expression in felines, working *in vitro* model systems of infection or in the case of retroviruses of transfection of populations of normal macrophages which are grown in bulk and then split for experimentation or the

establishment of a continuous *in vitro* feline monocyte/macrophage cell line must occur. Only then could proper controls be done for each experiment so any true modulation of the macrophages gene expression could be detected. Unfortunately, my attempts at *in vitro* transfection of an infectious cDNA clone of FeLV into normal feline macrophages for viral expression and detection of possible modulation of monokine production failed. However, transfection studies must be successfully completed in order to adequately explore viral modulation of macrophage gene transcription.

VITA

Sandra Leigh (Edmonds) Daniel was born in Charlotte, North Carolina on July 24, 1955. She has attended a number of educational institutions throughout the south. In 1977, she graduated with Honors with a B.S. Degree in Zoology from North Carolina State University at Raleigh. In the fall of 1977 she entered The University of Georgia at Athens and graduated with a MS Degree in Medical Microbiology in 1979. At this time, she entered the School of Veterinary Medicine at Auburn University and graduated with Honors in 1983 with a Doctor of Veterinary Medicine and a husband, Dr. Gregory B. Daniel. After 4 1/2 years at University of Illinois in Champaign-Urbana, the Daniel's (including one year old Joseph) moved to Knoxville, Tennessee, where she re-entered a Ph.D. program in Comparative and Experimental Medicine in Spring of 1988. In May of 1992, she will receive a Ph.D. from the University of Tennessee at Knoxville in Comparative and Experimental Medicine. She is a member of the Gamma Sigma Delta Agriculture Honor Society, Phi Zeta Veterinary Honor Society and Phi Kappa Phi.

Following graduation, her current plans are to spend some quality time with her husband and 5 year old Joseph before seeking a career position in teaching or research.