INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

DISSERTATION

THE CYTOKINE RESPONSE TO MUCOSALLY TRANSMITTED FELINE IMMUNODEFICIENCY VIRUS INFECTION

Submitted by
Paul R. Avery
Department of Pathology

In partial fulfillment of the requirements
for the degree of Doctor of Philosophy
Colorado State University
Fort Collins, Colorado
Summer 2002

COLORADO STATE UNIVERSITY

March 22, 2002

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY PAUL R. AVERY ENTITLED THE CYTOKINE RESPONSE TO MUCOSALLY TRANSMITTED FELINE IMMUNODEFICIENCY VIRUS INFECTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Joseph E. Smith

Richard G. Titus

Edward A. Hoover

Advisor

Department Head

ABSTRACT OF DISSERTATION

THE CYTOKINE RESPONSE TO MUCOSALLY TRANSMITTED FELINE IMMUNODEFICIENCY VIRUS INFECTION

Understanding the immune response to retroviral infections is critical to the rational design of prevention and intervention strategies. The studies reported herein were designed to help understand the interaction of feline immunodeficiency virus (FIV) and the initial host immune response. In addition, we have begun to explore the use of adenoviral-mediated expression of murine interleukin 12 (mIL12) as a means to shift the immune response to favor host control of FIV.

We were able to document a significant increase in the T lymphocyte production of interleukin 10 (IL10) in both the spleen and colic lymph nodes of infected cats at four weeks post rectal infection (PI). At ten weeks PI, the high levels of IL10 were maintained yet the level of IFNγ in CD8+ T cells had increased to an even greater extent thereby normalizing the T cell IFNγ:IL10 ratio. The normalization of this ratio corresponded with a significant reduction in viral RNA levels in both the spleen and the colic lymph node T cells. Macrophage production of interleukin 6 (IL6) was increased by 10 weeks PI and this was correlated with the rising macrophage viral RNA levels noted at this timepoint.

To explore the significance of the early rise in interlukin 10, we utilized murine IL12 as a means to preferentially induce a strong cell-mediated immune response. Recombinant mIL12 augmented mitogen-induced proliferation of feline PBMC and induced the production of feline IFNγ *in vitro*. Cats treated with varying doses of mIL12 intraperitoneally developed dose-related hematotoxicities typical of those seen in mice. A replication defective adenoviral vector expressing mIL12 was shown to productively

infect feline cells and produce biologically active mIL12. Intraperitoneal adenovirus-mediated mIL12 delivery resulted in the systemic induction of feline IFNγ and, in a preliminary experiment, appears to have delayed the rise in plasma FIV RNA levels in mucosally infected cats. Future experiments are planned to expand upon these findings and to further define the significance of the early IL10-dominated cytokine response to FIV infection.

Paul R. Avery
Pathology Department
Colorado State University
Fort Collins, Colorado, 80523
Summer 2002

ACKNOWLEDGMENTS

I would like to recognize several people for their contributions to my training and to the studies included in this dissertation. Dr. Edward Hoover has been a mentor, a friend and will continue to serve as a role model for my future endeavors. His reputation within the academic community for providing an excellent training environment is, in my opinion, well deserved.

I would also like to acknowledge my committee members for their guidance and assistance throughout this work; Drs. Christine Wilcox, Richard Titus, Joseph Smith and Gary Cockerell.

All the members of the Hoover Laboratory have played a large role in shaping this thesis and providing a great work environment. I'd like to specifically mention Sharon Robinson, Candace Mathiason, Andrea Lauerman and Shelly Robertson for their contributions.

This research was funded by grants R01 A1 33773 and K08 A1 01421 from the Division of AIDS, NIAID, NIH.

DEDICATION

I dedicate this work to Anne for the incredible amount of support, both personally and professionally, that she has provided over the years.

TABLE OF CONTENTS

INTRODUCTION

Human immunodeficiency virus (HIV)

In the twenty years since it was first identified, HIV has become the fourth largest killer in the world (1). An estimated 60 million people have been infected with the virus since it was identified and 40 million people are currently infected (1). Despite the global impact of HIV and the intensive investigation of the virus and its lifecycle, our understanding of the immune response towards HIV is still incomplete. There are numerous, often conflicting, reports attempting to document the cytokine response to HIV during the natural course of infection.

Mucosal HIV Infection

The vast majority of HIV infections are acquired through sexual contact (2) (3) making examination of the early mucosal immune response to the virus particularly important. It is likely that critical aspects of the early local immune response can determine whether or not the infection becomes productive or influence the rapidity with which the infection progresses to clinical disease. Because these early events are difficult to study in naturally occurring HIV, animal models such as feline immunodeficiency virus (FIV) and simian immunodeficiency virus (SIV) serve important roles (4) (5) (6).

Feline immunodeficiency virus

FIV was identified in the mid 1980s and the clinical disease syndrome induced by the virus was shown to be extremely similar to that caused by HIV (7) (8). The acute phase in both viral infections is marked by high viral loads, lymphadenopathy, flu-like symptoms and early declines in the circulating CD4+ T cells (9) (10) (11). Analogous to HIV infection, the majority of FIV-infected cats enter a clinically asymptomatic period spanning several years, which is then followed by a period of progressive immunodeficiency ultimately culminating in resurgence of viremia and opportunistic infections (11) (12) (13) (14). The association between FIV and HIV has been further strengthened by recent evidence that FIV utilizes the co-receptor CXCR4 shown to be critical for infection with T cell tropic strains of HIV (15).

Mucosal FIV infection

The importance of the rectal and vaginal mucosal routes in the transmission of HIV prompted the search for similar modeling systems in FIV infection. FIV has been isolated from the saliva and semen of infected cats (16) (17) and has been transmitted via artificial insemination(18), suggesting mucosal routes may play a role in natural horizontal transmission. Work in our laboratory has established the oral, vaginal and rectal routes as viable means to transmit both cell-free and cell-associated FIV (19) (20) (6). These transmission routes allow us to study the immune response to the virus during and immediately following transit through mucosal barriers as models for sexually transmitted HIV infection.

Type 1 vs. type 2 cytokine patterns

The dichotomy of Th1 and Th2 cytokine responses within the CD4+ T cells of mice laid the groundwork for understanding how cytokines can shape the initial immune response to pathogens (21, 22). The initial focus on CD4+ T cells has expanded to include the cytokine contributions of all cells involved in the immune response and has given rise to the terms type 1 and type 2 (23). A type 1 immune response includes the cytokines interferon-gamma (IFNγ), interleukin 2 (IL2), tumor necrosis factor alpha (TNFα) and interleukin 12 (IL12) and is generally considered to be responsible for a strong cell-mediated immune response, whereas a type 2 response includes interleukin 4 (IL4), interleukin 5 (IL5), interleukin 6 (IL6), interleukin 10 (IL10) and interleukin 13 (IL13) and drives a dominant humoral immune response. This distinction is not absolute as certain immunoglobulin subclasses such as IgG2a are potently produced during a type 1 response (24). In general, IL4 and IL12 are thought to be the early initiators of type 2 and type 1 responses respectively (25). The cellular source of this initial IL4 production has remained somewhat elusive, whereas IL12 is produced by professional antigen presenting cells such as dendritic cells or macrophages. The initiation of one type of cytokine response tends to be self-propagating and inhibits the development of the other, thereby allowing one pathway to predominate in an immune response.

Blurring of the type 1 vs. type 2 dichotomy:

Despite the clear distinction between type 1 and type 2 cytokine responses in inbred strains of mice and the documented antagonism between each type of response, the dichotomy in naturally occurring diseases is less absolute. IL12 stimulates the potent production of the fellow type 1 cytokine IFNγ but it has also been shown to induce high levels of the type 2 cytokine IL10 (26). Individual clones of CD4+ T cells and CD8+ T cells have been shown to produce high levels of both IFNγ and IL10 indicating independent gene regulation (26). The simultaneous production of high levels of IL10 and IFNγ has also been reported in human patients with Lyme disease (*Borrelia burgdorferi*) (27) and tuberculosis (28). IL10, in its role of dampening type 1 responses, may be required to modulate the intensity of the IFNγ-driven cell-mediated immune response (29) (30) (31). It has therefore been suggested that it is the balance rather than the absolute levels of type 1 or type 2 cytokines that is critical in determining disease outcome.

The early cytokine response

Studies examining a wide array of pathogens have demonstrated how crucial the initial cytokine response to the organism is in determining disease outcome. A prominent example is the protozoal parasite *Leishmania major,* which causes self-limiting or progressive disease depending on the inbred mouse strain used. This divergence in pathogenesis has been linked to the natural tendency of each mouse strain to produce a type 1 or type 2 cytokine response (32). Recombinant IL12 administered to normally susceptible strains of mice can result in parasite clearance, but only if given during the first seven days post-infection (33). This early window of cytokine plasticity also exists in malarial infection with *Plasmodium chabaudi.* As with *Leishmania*, there are susceptible and resistant inbred strains of mice and resistance correlates with the early production of the type 1 cytokines IFNγ and IL12 (34). If IL12 is administered one day before or at the time of malaria challenge, increased levels of IFNγ and TNFα are induced and the typically susceptible mice are able to clear the parasite (35). This illustrates the critical role of cytokine balance in immune response priming.

The cytokine response in anti-viral immunity

Cytokines produced mainly by T lymphocytes and macrophages play a critical role in antiviral immune responses (36) (37) (38). A robust type 1 cytokine response early in a viral infection appears to be an important component in clearing virus infected cells from the body. The mechanisms of cytokine induced antiviral responses are varied and include alterations of MHC molecule and costimulatory molecule expression as well as direct activation or deactivation of immune cells (39). IFNγ stimulates the production of nitric oxide (NO) by activated macrophages, which has been shown to play a significant role in the response to ectromelia virus (40), herpes simplex virus 1 (41), Epstein Barr virus (42) and coxsackie virus (43). Type 1 cytokines, such as IFNγ and TNFα, can also directly inhibit viral replication (44).

Cytokine responses to HIV

The evidence for dichotomous cytokine responses in murine models of infectious disease spurred investigation into the cytokine response in HIV infection. There are several, often conflicting, reports about the existence of a type 1 to type 2 cytokine switch emerging during the progression of the chronic phase of HIV infection (45) (46) (47). Many studies have found the cytokine response during the chronic stages to be a mixture of type 1 and type 2 cytokines (48) (49) although an imbalance between the two cytokine responses appears to exist. Decreased production of IFNγ (50) or increased production of IL10 (51) has been associated with increased risk of disease progression and death. Recent data demonstrate that the relative balance of CD4+ T cell production of IL10 vs. IFNγ correlates with active replication and progression of disease and that highly active anti-retroviral treatment (HAART) decreases viral loads, shifting the balance towards IFNγ production (52). Adding IL12 or blocking IL10 restores the *in vitro* responsiveness of HIV+ cells to antigen, indicating that inhibition of type1 cytokines contributes to the immunologic defects of chronic HIV infection.

It is more difficult to study the acute phases of HIV-infection, as patients are often not identified immediately after exposure. The balance of cytokines during early HIV

infection has been described in a small number of papers. Individuals who had been HIV-infected for >3 but <12 months had increased PBMC-associated IL4 and IL10 with concurrent decreases in IFNγ and IL12 (53). Other studies examining peripheral blood during the acute phase of HIV infection have demonstrated early increases in TNFα (54) or IFNγ, IL10 and TNFα (55). Differences in methodologies and patient classification may be contributing to the lack of consensus in these studies but, to date, there has been no clear cut cytokine pattern identified during acute HIV infection.

Rare reports of cytokine production within lymphoid tissue of HIV+ people exist. A lymph node biopsy obtained during seroconversion revealed increases in IFNγ, IL10 and IL6 suggesting a mixed type1 and type 2 cytokine response (56). A larger study involving lymph node tissue from14 HIV+ people revealed an increase in both IFNγ and IL10 that persisted throughout all stages of established infection (49). A study in which IL10 was not examined, identified an increase in IFNγ in lymph node CD8+ T cells (57). These studies of lymph node cytokine production identify the simultaneous increases in IFNγ and IL10, a condition described as a type 0 or a mixed cytokine response.

Cytokine responses to SIV

The SIV model has been used to examine tissue cytokine responses that tend to be less accessible in HIV-infected people. All of the current data comes from infection systems in which the virus was administered via the intravenous route (IV). One study has documented the mild increase in PBMC-associated IL6 levels beginning 1 week PI which corresponded with peak viral replication (58). Other groups have compared *nef*-deleted or truncated viruses with their more pathogenic counterparts. One of these studies showed that the attenuated virus induced both IFNγ and IL10 while the pathogenic virus induced only IL10 in PBMC during the acute phase (59). Other groups have shown that there is less IL12 production and more IL10 and IL4 production in the lymphoid tissues of macaques infected with the pathogenic strain of virus than with the *nef*-deleted form (60). In addition, a *nef*-deleted virus engineered to produce IFNγ replicated to a much lower viral level when compared to the parent *nef*-deleted virus (61). A final study with

relevance to the expression of IFNγ showed that, with a low-dose IV challenge, there was an inverse relationship between lymph node levels of IFNγ and SIV antigenemia (62). When a higher dose of virus was administered, large increases in IFNγ were noted which showed no relationship with viral antigen levels. The authors speculated that some of the increase in IFNγ may have been coming from natural killer cells activated by the large viral challenge and therefore may not be reflective of acquired T cell responses (62). This hypothesis was supported by a concurrent increase in lymph node IFNα, which is a known NK cell activator. As a whole, the above SIV studies support the notion that the induction of type 1 cytokines is inhibited to some degree by pathogenic strains of SIV while type 2 cytokine production is augmented. This work also suggests that the IV route of infection may not be the most appropriate system to model naturally acquired HIV-infection.

Cytokine responses to FIV

Few studies examining the cytokine response in FIV infection have been conducted but these few studies provide some insight. Early work with FIV demonstrated increases in interleukin 6 (IL6) (63) (64), TNFα and interleukin 1 (IL1) (64) in chronically infected cats. Alveolar macrophages from acutely infected cats have been shown to produce increased levels of TNFα, IL6 and IL10 (65). IL2 levels have been shown to not differ significantly between naïve and FIV-infected cats (66) (64) (67). Two groups have documented simultaneously increased levels of IFNγ and IL10 in FIV-infected cats, one within weeks of viral challenge (67) and one during the chronic phase of infection (66). The overall pattern of cytokine responses to FIV infection has not fit neatly into a type1 or type 2 response, yet in a co-infection system with *Toxoplama gondii*, an elevated IL10/IL12 ratio was predictive of poor control of toxoplasmosis (68) suggesting that here may be a cytokine imbalance which inhibits type 1 cytokine responses.

Interleukin 12

Interleukin 12, initially identified as a factor secreted by Epstein-Barr virus transformed B cells, has subsequently been shown to be one of the most potent stimulators of the cell-mediated immune response (69-71). IL12 initiates a type1 cytokine response via the

induction of IFNγ from T cells and natural killer cells (NK) thereby augmenting cell-mediated cytotoxicity (72). Although it was first identified in B cells, the most biologically relevant producers of IL12 are monocyte/macrophages and dendritic cells (73, 74). IL12 is produced as a heterodimer in response to bacteria/bacterial products, endotoxins, intracellular parasites and CD40 ligation (71) (73) (75). Thus either direct contact with microbial products or activated T cells can lead antigen presenting cells such as macrophages and dendritic cells to release IL12.

IL12 is critical in the early host control/containment of many intracellular pathogens including *Toxoplasma gondii* (76), *Listeria monocytogenes* (77), *Leishmania major* (78) and *Mycobacterium tuberculosis* (79). Early intervention with IL12 has shown the pivotal role it plays in the control of some viral infections. Low doses of IL12 early in the infection of mice with lymphocytic choriomeningitis virus (LCMV) result in increases in splenic CD8+ T cell numbers and decreased viral levels (38). IL12-induced decreases in viral replication have also been demonstrated in murine models of cytomegalovirus virus (80), hepatitis B virus (81), encephalomyocarditis virus (82) and the murine acquired immunodeficiency (MAIDS) virus infection (83).

Preventing or decreasing IL12 production would seem an ideal way for a virus to circumvent the host immune response. The infection of human monocytes *in vitro* and rhesus macaques *in vivo* with measles virus leads to the ablation of IL12 production, which may be an important means of viral escape and the generalized immunosuppression seen in measles virus infection (84) (85). Hepatitis C virus, which frequently causes persistent infection and can lead to substantial liver disease, has been shown to specifically inhibit IL12 production from macrophages at what appears to be the transcriptional level (86). PBMC from HIV-infected patients have been shown to produce one fifth the IL12 of control PBMC when stimulated with *Staphylococcus aureus in vitro* (87). The mechanism for this IL12 inhibition remains unclear but may relate to the overproduction of IL10 (88).

Interleukin 10

IL10 was initially described as a factor secreted by murine Th2 cells that inhibited IFNγ production (89). IL10 has since been shown to have a variety of inhibitory effects on activated macrophages including the inhibition of nitric oxide production (90), the reduction in the surface expression of MHC class II molecules (91) and CD80/CD86 costimulatory molecules (92). In addition to blocking IFNγ production, IL10 has been shown to inhibit the production of IL12 and TNFα in monocytes (89, 93, 94). IL10 can also decrease IL12 production and costimulatory molecule expression on dendritic cells, the most potent initiators of an immune response (95, 96).

Adenoviral delivery of IL12

Replication-defective adenoviruses have been shown to be effective vehicles for the *in vivo* delivery of inserted DNA(97). Several adenoviral vectors that encode for both the p35 and p40 portion of the murine IL12 heterodimer have been developed and shown to produce biologically active IL12. (98) (99) (100). Much of the *in vivo* work with these vectors has focused on augmentation of cell-mediated immunity against murine tumors, where variable degrees of tumor reduction have been documented (101) (102) (103) (104). There are reports of the *in vivo* efficacy of adenovirus-IL12 vectors in decreasing mortality or infection rates in murine models of *Leishmania major* (105), *Klebsiella pneumoniae* (106) and *Toxoplasma gondii* (107) infection.

Dissertation research

Our understanding of the cytokine response to lentiviral infections remains far from complete. A detailed analysis of the early cytokine events that accompany mucosally transmitted FIV may help to explain some of the ways in which the virus escapes initial immune containment. This information could be crucial in the development of cytokine-based intervention or vaccine enhancement strategies for both FIV and HIV.

The first aim of this work was simply to develop an efficient and reliable means of detecting multiple cytokines in feline tissue samples. PCR-based methodologies exist

(108) (109) but are reagent and labor intensive when studying multiple cytokines from multiple tissues. Because the ribonuclease protection assay has proven to be a useful and efficient assay for RNA detection, we endeavored to develop this technology for the detection of feline cytokines.

A second major objective was to analyze the cytokines produced in the early stages after rectal mucosal transmission of FIVB2542 and to determine whether cellular or tissue specific responses were detectable. By examining two time points post-infection, we hoped to document the evolution of the acute cytokine response. We hypothesized that an early type 2 cytokine response would accompany the rapid rise in initial viral replication.

Our final goal was to attempt to influence the host's ability to control initial viral replication by administering IL12, thereby driving a more effective cell-mediated immune response. Adenoviral gene delivery systems were targeted as an efficient and self-limiting means to administer IL12.

1. Vass, A. 2001. AIDS now fourth biggest killer worldwide, report says. *British Journal of Medicine* 323, no. 7374:1271.
2. Royce, R.A., A. Sena, W. Cates, Jr., and M.S. Cohen. 1997. Sexual transmission of HIV. *N Engl J Med* 336, no. 15:1072.
3. Joint United Nations Programme on HIV/AIDS (UNAIDS)World Health Organization (WHO). 2000. *AIDS epidemic update.* December 2000.
4. Joag, S.V., I. Adany, Z. Li, L. Foresman, D.M. Pinson, C. Wang, E.B. Stephens, R. Raghavan, and O. Narayan. 1997. Animal model of mucosally transmitted human immunodeficiency virus type 1 disease: intravaginal and oral deposition of simian/human immunodeficiency virus in macaques results in systemic infection, elimination of CD4+ T cells, and AIDS. *J Virol* 71, no. 5:4016.
5. Elder, J.H., G.A. Dean, E.A. Hoover, J.A. Hoxie, M.H. Malim, L. Mathes, J.C. Neil, T.W. North, E. Sparger, M.B. Tompkins, W.A. Tompkins, J. Yamamoto, N. Yuhki, N.C. Pedersen, and R.H. Miller. 1998. Lessons from the cat: feline immunodeficiency virus as a tool to develop intervention strategies against human immunodeficiency virus type 1. *AIDS Res Hum Retroviruses* 14, no. 9:797.
6. Obert, L.A., Hoover, E. A. 2001. Early Target Cells in Mucosal FIV Infection. *Submitted, Journal of VIrology.*
7. Pedersen, N.C., E.W. Ho, M.L. Brown, and J.K. Yamamoto. 1987. Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. *Science* 235:790.
8. Pedersen, N.C. 1993. The feline immunodeficiency virus. *In* The Retroviridae, vol. 2. J.A. Levy, editor. 2 vols. Plenum Press, New York, NY. 181.
9. Bach, J.M., M. Hurtrel, L. Chrakrabarti, J.-P. Ganiere, L. Montagnier, and B. Hurtrel. 1994. Early stages of feline immunodeficiency virus infection in lymph nodes and spleen. *AIDS Research and Human Retroviruses* 10:1731.
10. Tompkins, M.B., P.D. Nelson, R.V. English, and C. Novotney. 1991. Early events in the immunopathogenesis of feline retrovirus. *Journal of the American Veterinary Medical Association* 199:1311.

11. English, R.V., P. Nelson, C.M. Johnson, M. Nasisse, W.A. Tompkins, and M.B. Tompkins. 1994. Development of clinical disease in cats experimentally infected with feline immunodeficiency virus. *Journal of Infectious Diseases* 170:543.

12. Pedersen, N.C., J.K. Yamamoto, T. Ishida, and H. Hansen. 1989. Feline immunodeficiency virus infection. *Veterinary Immunology and Immunopathology* 21:111.

13. Sparger, E.E., P.A. Luciw, J.H. Elder, J.K. Yamamoto, L.J. Lowenstine, and N.C. Pedersen. 1989. Feline immunodeficiency virus is a lentivirus associated with an AIDS-like disease in cats. *AIDS* 3:S43.

14. Yamamoto, J.K., E. Sparger, E.W. Ho, P.R. Andersen, T.P. O'Connor, C.P. Mandell, L. Lowenstine, R. Munn, and N.C. Pedersen. 1988. Pathogenesis of experimentally induced feline immunodeficiency virus infection in cats. *American Journal of Veterinary Research* 49:1246.

15. Willett, B., L. Picard, M. Hosie, J. Turner, K. Adema, and P. Clapham. 1997. Shared usage of the chemokine receptor CXCR4 by the feline and human immunodeficiency viruses. *Journal of Virology* 71, no. 9:6407.

16. Matteucci, D., F. Baldinotti, P. Mazzetti, M. Pistello, P. Bandecchi, R. Ghilarducci, A. Poli, F. Tozzini, and M. Bendinelli. 1993. Detection of feline immunodeficiency virus in saliva and plasma by cultivation and polymerase chain reaction. *Journal of Clinical Microbiology* 31:494.

17. Jordan, H.L., J. Howard, M.C. Barr, S. Kennedy-Stoskopf, J.K. Levy, and W.A. Tompkins. 1998. Feline immunodeficiency virus is shed in semen from experimentally and naturally infected cats. *AIDS Res Hum Retroviruses* 14, no. 12:1087.

18. Jordan, H.L., J.G. Howard, J.G. Bucci, J.L. Butterworth, R. English, S. Kennedy-Stoskopf, M.B. Tompkins, and W.A. Tompkins. 1998. Horizontal transmission of feline immunodeficiency virus with semen from seropositive cats. *J Reprod Immunol* 41, no. 1-2:341.

19. Burkhard, M.J., L.A. Obert, L.L. O'Neil, L.J. Diehl, and E.A. Hoover. 1997. Mucosal transmission of cell-associated and cell-free feline immunodeficiency virus. *AIDS Res Hum Retroviruses* 13, no. 4:347.

20. Burkhard, M.J., Mathiason, C.K., O'Halloran, K., Hoover, E.A. 2001. Kinetics of early FIV infection in cats exposed via the vaginal versus intravenous route. *Submitted, AIDS Research and Human Retrovirus.*

21. Mossman, T., and R. Coffman. 1989. TH1 and Th2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annual Review of Immunology* 7:145.

22. Mossman, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, and R.A. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of Immunology* 136:2348.

23. Clerici, M., and G.M. Shearer. 1994. The Th1-Th2 hypothesis of HIV infection: New insights. *Immunology Today* 15:575.

24. Stevens, T.L., A. Bossie, V.M. Sanders, R. Fernandez-Botran, R.L. Coffman, T. Mosmann, and E.S. Vitetta. 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* 334, no. 6179:255.

25. Mossman, T.R., and R.A. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual Review of Immunology* 7:145.

26. Gerosa F, P.C., Peritt D, Paiola F, Scupoli MT, Aste-Amezaga M, Frank I, Trinchieri G. 1996. Interleukin-12 primes human CD4 and CD8 T cell clones for high prodution of both interferon-gamma and interleukin-10. *Journal of Experimantal Medicine* 183, no. 6:2559.

27. Pohl-Koppe A, B.K., Steere AC, Logigian EL, Hafler DA. 1998. Identification of a T cell subset capable of both IFN-gamma and IL-10 secretion in patients with chronic *Borrelia burgdorferi* infection. *Journal of immunology* 160:1804.

28. Gerosa F, N.C., Righetti S, Micciolo R, Marchesini M, Cazzadori A, Trinchieri G. 1999. CD4+ T cell clones producing both interferon-gamma and interleukin 10 predominate in bronchoalveolar lavages of active pulmonary tuberculosis patients. *Clinical Immunology* 92:224.

29. Tumpey, T.M., V.M. Elner, S.H. Chen, J.E. Oakes, and R.N. Lausch. 1994. Interleukin-10 treatment can suppress stromal keratitis induced by herpes simplex virus type 1. *Journal of Immunology* 153:2258.

30. Gazinelli, R.T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kuhn, W. Muller, G. Trinchieri, and A. Sher. 1996. In the absence of IL10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent of CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. *Journal of Immunology* 157:798.

31. Hunter, C.A., L.A. Ellis-Neyes, T. Slifer, S. Kanaly, G. Grunig, M. Fort, D. Rennick, and F.G. Araujo. 1997. IL-10 is recquired to prevent immune hyperreactivity during infection with Trypanosoma cruzi. *Journal of Immunology* 158:3311.

32. Heinzel, F.P., M.D. Sadick, B.J. Holaday, R.L. Coffman, and R.M. Locksley. 1989. Reciprocal expression of interferon-gamma or interleukin 4 during resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *The Journal of Experimental Medicine* 169, no. 1:59.

33. Heinzel, F.P., D.S. Schoenhaut, R.M. Rerko, L.E. Rosser, and M.K. Gately. 1993. Recombinant interleukin 12 cures mice infected with Leishmania major. *The Journal of Experimental Medicine* 177, no. 5:1505.

34. Sam, H., Stevenson, M.M. 1999. In vivo IL-12 production and IL12 receptors β1 and β2 mRNA expression in the spleen are differentially up-regulated in resistant B6 and susceptible A/J mice during early blood-stage *Plasmodium chabaudi* AS malaria. *Journal of Immunology* 162:1582.

35. Stevenson, M.M., Tam, M.F., Wolf, S.F., Sher, A. 1995. IL-12-induced protection against blood-stage *Plasmodium chabaudi* AS requires IFNγ and TNFα and occurs via a nitric oxide-dependent mechanism. *Journal of Immunology* 155:2545.

36. Sher, A., T. Gazzinelli, I. Oswald, M. Clerici, M. Kullberg, J. Berzofsky, T. Mossman, and G. Shearer. 1993. Role of T-Cell derived cytokines in the

downregulation of immune responses in parasitic and retroviral infection. *Immunological Reviews*:183.

37. Berger, D.P., Homann.D., and M.B.A. Oldstone. 2000. Defining parameters for successful immunocytotherapy of persistent viral infection. *Virology* 266:257.
38. Orange, J.S., S.F. Wolf, and C.A. Biron. 1994. Effects of IL-12 on the response and susceptibility to experimental viral infections. *Journal of Immunology* 152:1253.
39. Belardelli, F. 1995. Role of interferons and other cytokines in the regulation of the immune response. *APMIS* 103:161.
40. Karupiah, G., Q.W. Xie, C. Buller, C. Nathan, C. Duarte, and J.D. MacMicking. 1993. Inhibition of viral replication by interferon-γ-induced nitric oxide synthase. *Science* 261:1445.
41. Adler, H., J.L. Beland, N.C. Del-Pan, L. Kabzik, J.P. Brewer, T.R. Martin, and I.J. Rimm. 1997. Suppression of herpes simplex virus 1-induced pneumonia in mice by inhibition of inducible nitric oxide synthase. *The Journal of Experimental Medicine* 185:1533.
42. Mannick, J.B., K. Asano, K. Izumi, E. Kieff, and J.S. Stamler. 1994. Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein-Barr reactivation. *Cell* 79:1137.
43. Lowenstein, C.J., S.L. Hill, A. Lafond-Walker, J. Wu, G. Allen, M. Landavere, N.R. Rose, and A. Herskowitz. 1996. Nitric oxide inhibits viral replication in murine myocarditis. *The Journal of Clinical Investigation* 97:1837.
44. Young, H.A., and K.J. Hardy. 1995. Role of interferon-γ in immune cell regulation. *Journal of Leukocyte Biology* 58:373.
45. Clerici, M., and G.M. Shearer. 1993. A TH1-->TH2 switch is a critical step in the etiology of HIV infection. *Immunology Today* 14:107.
46. Barcellini, W., G. Rizzardi, M. Borghi, C. Fain, A. Lazzarin, and P. Meroni. 1994. Th1 and TH2 cytokine production by peripheral blood mononuclear cells from HIV-infected patients. *AIDS* 8:757.

47. Romagnani, S., E. Maggi, and G. Del Prete. 1994. An alternative view of the Th1/Th2 switch hypothesis in HIV infection. *AIDS Research and Human Retroviruses* 10:iii.

48. Maggi, E., M. Mazzetti, A. Ravina, F. Annunziato, M. De Carli, M.P. Piccinni, R. Manetti, M. Carbonari, A.M. Pesce, G. Del Prete, and S. Romagnani. 1994. Ability of HIV to promote at T_H1 to T_H0 shift and to replicate preferentially in T_H2 and T_H0 cells. *Science* 265:244.

49. Graziosi, C., G. Pantaleo, K.R. Gantt, J.-P. Fortin, J.F. Demarest, O.J. Cohen, R.P. Sékaly, and A.S. Fauci. 1994. Lack of evidence for the dichotomy of T_H1 and T_H2 predominance in HIV-infected individuals. *Science* 265:248.

50. Ullum, H., A.C. Lepri, K. Bendtzen, J. Victor, P.C. Gotzsche, A.N. Phillips, P. Skinhoj, and B.K. Pedersen. 1997. Low production of inerferon γ is related to disease progression in HIV infection: evidence from a cohort of 347 HIV-infected individuals. *AIDS Research and Human Retroviruses* 13, no. 12:1039.

51. Stylianou, E., P. Aukrust, D. Kvale, F. Muller, and S.S. Froland. 1999. IL-10 in HIV infection: increasing serum levels with disease progression-down-regulatory effect of potent anti-retroviral therapy. *Clinical and Experimental Immunology* 116:115.

52. Ostrowski, M.A., J.X. Gu, C. Kovacs, J. Freedman, M.A. Luscher, and K.S. MacDonald. 2001. Quantitative and qualitiative assessment of human immunodeficiency virus type 1-specific CD4+ T cell immunity to gag in HIV-1-infected individuals with differential disease progression: reciprocal IFNg and interleukin-10 responses. *The Journal of Infectious Diseases* 184:1268.

53. Meroni, L., D. Trabbattoni, C. Balotta, C. Riva, A. Gori, M. Moroni, L. Villa, M. Clerici, and M. Galli. 1996. Evidence for type 2 cytokine production and lymphocyte activation in the early phases of HIV-1 infection. *AIDS* 10, no. 1:23.

54. Barcellini, W., G. Rizzardi, A. Poli, G. Tambussi, C. Velati, L. Meroni, A.G. Dalgleish, and A. Lazzarin. 1996. Cytokines and soluble receptor changes in the transition from primary to early chronic HIV type 1 infection. *AIDS Research and Human Retroviruses* 12, no. 4:325.

55. Graziosi, C., K.R. Gantt, M. Vaccarezza, J.F. Demarest, M. Daucher, M.S. Saag, G.M. Shaw, T.C. Quinn, O.J. Cohen, C.C. Welbon, G. Panteleo, and A.S. Fauci. 1996. Kinetics of cytokine expression during primary human immunodeficiency virus type 1 infection. *Proceedings of the National Academy of Sciences* 93, no. 9:4386.

56. Gray, C.M., L. Morris, J. Murray, J. Keeton, S. Shalekoff, S.F. Lyons, P. Sonnenberg, and D.J. Martin. 1996. Identification of cell subsets expressing intracytoplasmic cytokines within HIV-1 infected lymph nodes. *AIDS* 10:1467.

57. Emilie, D., M. Peuchmaur, M.C. Maillot, M.C. Crevon, N. Brousse, J.F. Delfraissy, J. Dormont, and P. Galanaud. 1990. Production of interleukins in human immunodeficiency virus-1-replicating lymph nodes. *Journal of Clinical Investigation* 86, no. 1:148.

58. Cheret, A., R. Le Grand, P. Caufour, N. Dereuddre-Bosquet, F. Matheux, O. Neildez, F. Theodoro, N. Maestrali, O. Benveniste, B. Vaslin, and D. Dormont. 1996. Cytokine mRNA expression in mononuclear cells from different tissues during acute SIVmac251 infection of macaques. *AIDS Research and Human Retroviruses* 12, no. 13:1263.

59. Beneviste, O., B. Vaslin, R. Le Grand, A. Cheret, F. Matheux, F. Theodoro, M.P. Cranage, and D. Dormont. 1996. Comparative IL2/IFNg and IL4/IL10 responses during acute infection of macaques inoculated with attenuated nef-truncated or pathogenic SIVmac251 virus. *The Proceedings of the National Academy of Sciences* 93:3658.

60. Zou, W., A.A. Lackner, M. Simon, I. Durand-Gasselin, P. Galanaud, R.C. Desrosiers, and D. Emilie. 1997. Early cytokine and chemokine gene expression in the lymph nodes of macaques infected with simian immunodeficiency virus is predictive of disease outcome and vaccine efficacy. *Journal of Virology* 71, no. 2:1227.

61. Giavedoni, L., S. Ahmad, L. Jones, and T. Yilma. 1997. Expression of gamma interferon by simian immunodeficiency virus increases the attenuation and reduces postchallenge virus load in vaccinated rhesus macaques. *Journal of Virology* 71, no. 2:866.

62. Khatissian, E., V. Monceaux, M. Cumont, L. Montagnier, B. Hurtrel, and L. Chakrabarti. 1996. Interferon-gamma expression in macaque lymph nodes during primary infection with simian immunodeficiency virus. *Cytokine* 8, no. 11:844.

63. Ohashi, T., R. Goitsuka, T. Watari, H. Tsujimoto, and A. Hasegawa. 1992. Elevation of feline interleukin 6-like activity in frline immunodeficiency virus infection. *Clinical Immunology and Immunopathology* 65, no. 3:207.

64. Lawrence, C.E., J.J. Callanan, B.J. Willett, and O. Jarrett. 1995. Cytokine production by cats infected with feline immunodeficiency virus:a longitudinal study. *Immunology* 85:568.

65. Ritchey, J.W., J.K. Levy, S.K. Bliss, W.A. Tompkins, and M.B. Tompkins. 2001. Constitutive expression of types 1 and 2 cytokines by alveolar macrophages from feline immunodeficiency virus-infected cats. *Veterinary Immunology and Immunopathology* 79, no. 1-2:83.

66. Liang, Y., L.C. Hudson, J.K. Levy, J.W. Ritchey, W.A. Tompkins, and M.B. Tompkins. 2000. T cells overexpressing interferon-gamma and interleukin-10 are found in both the thymus and secondary lymphoid tissues of feline immunodeficiency virus-infected cats. *The Journal of Infectious Diseases* 181, no. 2:564.

67. Dean, G.A., and N.C. Pedersen. 1998. Cytokine response in multiple lymphoid tissues during the primary phase of feline immunodeficiency virus infection. *Journal of Virology* 72, no. 12:9436.

68. Levy, J.K., J.W. Ritchey, J.B. Rottman, M.G. Davidson, Y.H. Liang, H.L. Jordan, W.A. Tompkins, and M.B. Tompkins. 1998. Elevated interleukin-10-to-interleukin-12 ratio in feline immunodeficiency virus-infected cats predicts a loss of type 1 immunity to Toxoplasma gondii. *The Journal of Infectious Diseases* 178, no. 2:503.

69. Chehimi, J., S. Starr, I. Frank, M. Rengaraju, S. Jackson, C. Llanes, M. Kobayashi, B. Perussia, D. Young, E. Nickbarg, S. Wolf, and G. Trinichieri. 1992. Natural killer cell stimulatory factor (NKSF) increases the cytotoxic activity of NK cells from both healthy donors and HIV-infected patients. *Journal of Experimental Medicine* 175:789.

70. Kobayashi, M., Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perussia B, Trinchieri G. 1989. Identification and purification of Natural Killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *Journal of Experimental Medicine* 170:827.

71. Trinchieri, G. 1994. Interleukin 12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood* 84, no. 12:4008.

72. Trinchieri G, G.F. 1996. Immunoregulation by interleukin 12. *Journal of Leukocyte Biology* 59:505.

73. D'Andrea A, R.M., Valiante NM, Chemini J, Kubin M, Aste M, Chan SH, Kobayashi M, Young D, Nickbarg E, Chizzonite R, Wolf SF, Trinchieri G. 1992. Production of natural killer cell stimulatory factor (NKSF/IL-12) by peripheral blood mononuclear cells. *Journal of Experimental Medicine* 176:1387.

74. Macatonia SE, H.N., Litton M, Vieira P, Hsieh CS, Culpepper JA, Wysocka M, Trinchieri G, Murphy KM, O'Garra A. 1995. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *Journal of Immunology* 154:5071.

75. Cella, M., D. Scheidegger, K. Palmer-Lehmann, P. Lane, A. Lanzavecchia, and G. Alber. 1996. Ligation of CD40 on dendritic cells triggers the production of high levels of interleukin-12 and enhances T cell stimulatory capacity. *The Journal of Experimental Medicine* 184:747.

76. Gazzinelli RT, W.M., Hayasi S, Denkers EY, Hieny S, Caspar P, Trinchieri G, Sher A. 1994. Parasite-induced IL-12 stimulates early IFNγ synthesis and resistance during acute infection with *Toxoplasma gondii*. *Journal of Immunology* 153:2533.

77. Hsieh CS, M.S., Tripp CS, Wolf SF, O'Garra A, Murphy KM. 1993. Development of Th1 CD4+ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* 260:547.

78. Heinzel, F.P., R.M. Rerko, F. Ahmed, and E. Pearlman. 1995. Endogenous IL-12 is required for control of Th2 cytokine responses capable of exacerbating leishmaniasis in normally resistant mice. *Journal of Immunology* 155:730.

79. Cooper AM, R.A., Rhoades ER, Callahan JE, Getzy DM, Orme IM. 1995. The role of interleukin-12 in acquired immunity to *Mycobacterium tuberculosis* infection. *Immunology* 84:423.

80. Orange, J.S., B. Wang, C. Terhorst, and C.A. Biron. 1995. Requirement for natural killer cell-produced interferon γ in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. *The Journal of Experimental Medicine* 182:1045.

81. Cavanaugh, V.J., L.G. Guidotti, and F.V. Chisari. 1997. Interleukin-12 inhibits hepatitis B virus replication in transgenic mice. *Journal of Virology* 71:3236.

82. Ozmen, L., M. Aguet, G. Trinchieri, and G. Garotta. 1995. The *in vivo* antiviral activity of interleukin-12 is mediated by γ interferon. *Journal of Virology* 69:8147.

83. Gazzinelli, R.T., N.A. Giese, and H.C.R. Morse. 1994. *In vivo* treatment with interleukin-12 protects mice from immune abnormalities observed during murine acquired immunodeficiency syndrome (MAIDS). *The Journal of Experimental Medicine* 180:2199.

84. Karp, C.L. 1999. Measles:immunosuppression, interleukin-12, and complement receptors. *Immunological Reviews* 168:91.

85. Polack, F.P., S.J. Hoffman, W.J. Moss, and D.E. Griffin. 2002. Altered synthesis of IL-12 and type 1 and type 2 cytokines in rhesus macaques during measles and atypical measles. *The Journal of Infectious Diseases* 185, no. 1:13.

86. Lee, C.H., Y.H. Choi, S.H. Yang, C.W. Lee, S.J. Ha, and Y.C. Sung. 2001. Hepatitis C virus core protein inhibits interleukin 12 and nitric oxide production from activated macrophages. *Virology* 279, no. 1:271.

87. Chehimi, J., S. Starr, I. Frank, A. D'Andrea, X. Ma, R. MacGregor, J. Sennelier, and G. Trinchieri. 1994. Impaired interleukin 12 production in human immunodeficiency virus-infected patients. *Journal of Experimental Medicine* 179:1361.

88. Chougnet, C., T.A. Wynn, M. Clerici, A.L. Landay, H.A. Kessler, J. Rusnak, G.P. Melcher, A. Sher, and G.M. Shearer. 1996. Molecular analysis of decreased

interleukin-12 production in persons infected with human immunodeficency virus. *The Journal of Infectious Diseases* 174:46.

89. Fiorentino, D., A. Zlotnik, P. Vieira, T. Mosmann, M. Howard, K. Moore, and A. O'Garra. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *Journal of Immunology* 146:3444.

90. Gazzinelli, R., M. Makino, S. Chattopadhyay, C. Snapper, A. Sher, A. Hugin, and H. Morse. 1992. CD4+ subset regulation in viral infection. *Journal of Immunology* 148:182.

91. de Waal Malefyt, R., J. Haanen, H. Spits, M. Roncarlo, A. te Velde, C. Figdor, K. Johnson, R. Kastelein, H. Yssel, and J.E. de Vries. 1991. Interleukin 10 and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of moncytes via downregulation of class II major histocompatibility complex expression. *The Journal of Experimental Medicine* 174:915.

92. Ding, L., P.S. Linsley, L. Huang, R.N. Germain, and E.M. Shevach. 1993. Il-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. *Journal of Immunology* 151:1224.

93. D'Andrea, A., M. Aste-Amezaga, N.M. Valiente, X. Ma, M. Kubin, and G. Trinchieri. 1993. Interleukin-10 inhibits human lymphocyte IFN-γ production by suppressing interleukin-12 synthesis in accessory cells. *The Journal of Experimental Medicine* 178:1041.

94. Ralph, P., I. Nakoinz, A. Sampson-Johannes, S. Fong, D. Lowe, H.Y. Min, and L. Lin. 1992. IL10; T lymphocyte inhibitor of human blood cell production of IL-1 and tumor necrosis factor. *Journal of Immunology* 148:808.

95. Buelens, C., F. Willems, G. Pierard, A. Delvaux, T. Velu, and M. Goldman. 1995. IL-10 inhibits the primary allogeneic T cell response to human peripheral blood dendritic cells. *Advances in Experimental Medicine and Biology* 378:363.

96. Mitra, R.S., T.A. Judge, F.O. Nestle, L.A. Turka, and B.J. Nickoloff. 1995. Psoriatic skin-derived dendritic cell function is inhibited by exogenous IL-10. Differential modulation of B7-1 and B7-2. *Journal of Immunology* 154:2668.

97. Graham, F.L. 2000. Adenovirus vectors for high-efficiency gene transfer into mammalian cells. *Immunology Today* 21, no. 9:426.

98. Bramson, J., M. Hitt, W.S. Gallichan, K.L. Rosenthal, J. Gauldie, and F.L. Graham. 1996. Construction of a double recombinant adenovirus vector expressing a heterodimeric cytokine: *in vitro* and *in vivo* production of biologically active interleukin-12. *Human Gene Therapy* 7:333.

99. Caruso, M., K. Pham-Nguyen, Y. Kwong, B. Xu, K. Kosai, M. Finegold, S.L.C. Woo, and S. Chen. 1996. Adenovirus-mediated interleukin-12 gene therapy for metastatic colon carcinoma. *The Proceedings of the National Academy of Sciences* 93:11302.

100. Lasarte, J.J., F.J. Corrales, N. Casares, A. Diaz de Cerio, C. Qian, X. Xie, F. Borras-Cuesta, and J. Prieto. 1999. Different doses of adenoviral vector expressing IL-12 enhance or depress the immune response to a coadministered antigen: the role of nitric oxide. *The Journal of Immunology* 162:5270.

101. Tanaka, M., Y. Saijo, G. Sato, T. Suzuki, R. Tazawa, K. Satoh, and T. Nukiwa. 2000. induction of antitumor immunity by combined immunogene therapy using IL-2 and IL-12 in low antigenic Lewis lung carcinoma. *Cancer Gene Therapy* 7, no. 11:1481.

102. Barajas, M., G. Mazzolini, G. Genove, R. Bilbao, I. Narvaiza, V. Schmitz, B. Sangro, I. Melero, C. Qian, and J. Prieto. 2001. Gene therapy of orthotopic hepatocellular carcinoma in rats using adenovirus coding for interleukin 12. *Hepatology* 33, no. 1:52.

103. Hull, G.W., M.A. McCurdy, Y. Nasu, C.H. Bangma, G. Yang, S. Shimura, H.M. Lee, J. Wang, J. Albani, S. Ebara, T.L. Timme, and T.C. Thompson. 2000. Prostate cancer gene therapy: comparison of adenovirus-mediated expression of interleukin 12 with interleukin 12 plus B7-1 for in situ gene therapy and gene-modified cell-based vaccines. *Clinical Cancer Research* 6, no. 10:4101.

104. Lohr, F., K. Hu, Z. Haroon, T.V. Samulski, Q. Huang, J. Beaty, M.W. Dewhirst, and C.Y. Li. 2000. Combination treatment of murine tumors by adenovirus-mediated local B7/IL12 immunotherapy and radiotherapy. *Molecular Therapy* 2, no. 3:195.

105. Gabaglia, C.R., B. Pedersen, M. Hitt, N. Burdin, E.E. Sercarz, F.L. Graham, J. Gauldie, and T.A. Braciak. 1999. A single intramuscular injection with an adenovirus-expressing IL-12 protects BALB/c mice against Leishmania major infection, while treatment with an IL-4-expressing vector increases disease susceptibility in B10.D2 mice. *Journal of Immunology* 162, no. 2:753.

106. Greenberger, M.J., S.L. Kunkel, R.M. Strieter, N.W. Lukacs, J. Bramson, J. Gauldie, F.L. Graham, M. Hitt, J.M. Danforth, and T.J. Standiford. 1996. IL-12 gene therapy protects mice in lethal Klebsiella pneumonia. *Journal of Immunology* 157, no. 7:3006.

107. Mack, D.G., and H.A. Jaffe. 2001. Effect of administration of a recombinant adenovirus expressing the genes ofr IFN-gamma and interferon-12 on acute murine toxoplasmosis. *Journal of Interferon and Cytokine Research* 21, no. 10:77.

108. Rottman, J., E. Freeman, S. Tonkonogy, and M. Tompkins. 1995. A reverse transcription-polymerase chain reaction technique to detect feline cytokine genes. *Veterinary Immunology and Pathology* 45:1.

109. Dean, G.A., J.A. Bernales, and N.C. Pedersen. 1998. Effect of feline immunodeficiency virus on cytokine response to Listeria monocytogenes *in vivo*. *Vet Immunol Immunopathol* 65, no. 2-4:125.

CHAPTER ONE

DEVELOPMENT OF A RIBONUCLEASE PROTECTION ASSAY TO DETECT FELINE CYTOKINES

INTRODUCTION

Current methods to quantify feline cytokines

Although feline immunodeficiency virus infection (FIV) in cats is a potentially valuable animal model of human lentiviral infection, the reagents and methods necessary to explore the interactions of FIV with the feline immune system are deficient. The standard ELISA-based techniques to measure feline cytokines do not exist and would require the generation and purification of antibodies to the individual cytokines. However, a quantitative competitive reverse transcription polymerase chain reaction (QC-RT-PCR) assay has been developed to measure feline interferon gamma (IFNγ), tumor necrosis alpha (TNFα), interleukin 2 (IL2), interleukin 4 (IL4), interleukin 6 (IL6), interleukin 10 (IL10) and interleukin 12 (IL12) (1). This assay has been shown to be a sensitive and quantifiable method to measure cytokine messages in feline infectious diseases. (2) (3).

The QC-RT-PCR assay relies on the addition of known concentrations of a fragment of DNA that encodes an internally truncated version of the cytokine to be assayed. The native PCR product can then be distinguished from the concurrently amplified competitive fragment based on size differential on an agarose gel. The concentration at which the competitive fragment and the native product are amplified to an equivalent amount can be used to determine the amount of cytokine message in the sample (Figure 1.1). To normalize the amount of cytokine message between samples, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used. The result is then expressed as a ratio of cytokine message to GAPDH message.

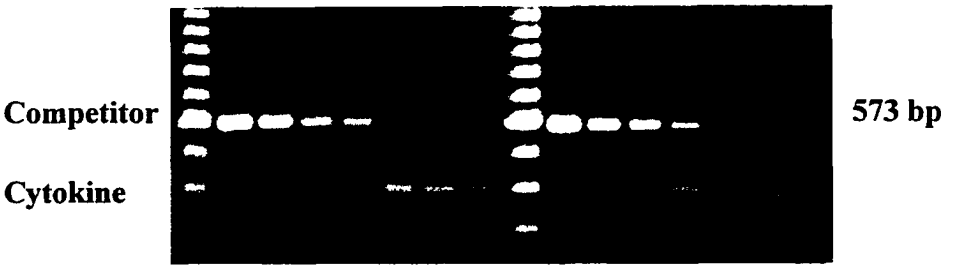

Figure 1.1: Two representative QC-RT-PCR reactions showing dilutions of input competitive DNA (upper bands) and increasing quantities of native DNA (lower bands). The point of equivalence of the two products can be calculated based on relative band intensities.

The fragment of DNA encoding all of the competitive sequences was provided to our laboratory by Dr. Mary Tompkins (Raleigh, NC) and was used in the initial pilot experiments. It soon became apparent that, while reliable, the use of this technique would require extremely large numbers of individual PCR reactions to quantify all the cytokines in the many samples. It was because of the potential time and expense involved with RT-PCR that we decided to develop a multiplex ribonuclease protection assay (RPA) for the measurement of feline cytokines. An additional attractive feature of the RPA is that, because no amplification steps are involved, more reliable quantification of products is possible (4).

Ribonuclease Protection Assay

The ribonuclease protection assay has been shown to be a very sensitive and specific method of detecting messenger RNAs (5) (6) (4). The assay involves the hybridization of a ^{32}P-labeled antisense RNA probe to the target RNA. This double stranded RNA, unlike the excess probe and unhybridized RNA, is then protected from RNase degradation and can be resolved by denaturing polyacrylamide gel electrophoresis. The amount of protected mRNA can be quantified using a phosphoimager (Amersham Biosciences, Piscataway, NJ) and related ImageQuant™ software. This technique has been applied in myriad human and murine diseases to measure cytokines, chemokines and other parameters of immune function (7) (8).

Cytokines

The sequences for most of the feline cytokines that we were interested in quantifying were part of the GenBank database, thereby allowing for the rapid generation of sequence-specific primers. We were fortunate to obtain sequence data directly from other investigators to complete the panel of needed cytokines. IFNγ, IL2 and TNFα were chosen as prototypical type 1 cytokines produced by T lymphocytes that have been shown to play an important role in viral infections (9) (10). IL4 and IL10 were chosen as what are generally considered to be prominent members of the T cell type 2 cytokine family (11) (12). Macrophages have been shown to constitutively express IL10, TNFα and IL6, all of which can influence viral replication (13) (14). Additionally, we chose IL12 because of the demonstrated ability of it to orchestrate a type 1 cytokine response to many infectious agents (15). Both the p40 and p35 subunits were included because the production of non-functional p40 homodimers occurs (16) (17) and the regulation for each subunit has been shown to differ (18).

MATERIALS AND METHODS

Cytokine Sequence Data and Primer Design

The sequences for feline IFNγ (accession #X86972), TNFα (X54000), IL2 (L19402), IL10 (U39569), IL6 (D13227), IFNβ (AB021707) were all available on the GenBank database. The partial sequences for feline IL12 and IL4 were kindly provided by Dr. Gregg Dean (NC State, Raleigh, NC). A partial sequence for feline GAPDH was generated by amplifying GAPDH with species consensus primers (1) and then by sequencing the product (Macromolecular Resources, Fort Collins, CO).

Primers pairs were generated using MacVector 7.0 Software (Genetics Computer Group, Madison, WI) with the following basic restrictions; 45-55% G+C content, Tm 55-80° C, 3 or less contiguous bonds between each primer and 4 or less contiguous bonds between either primer and the product. Primer pairs were chosen so that the various cytokine products would differ enough in size to be resolved on a polyacrylamide gel (Table 1.1).

Multiple primer pairs were tested before an IL12 product could be generated. The ultimately successful pair were generated based on a Gen Bank sequence (U83185, U83184) which was posted by Dr. Hans Lutz, Zurich, Switzerland during the period of assay development.

Table 1.1: Feline cytokine RPA probe sets and protected product sizes.

T Cell Probes	Product size (BPs)	Macrophage Probes	Product size (BPs)
IL10	369	IL6	422
IFNγ	302	IL10	369
TNFα	253	IL12 p35	315
IL4	230	TNFα	253
IL2	191	IFNβ	204
GAPDH	93	IL12 p40	163
		GAPDH	93

The selected primer pairs were as follows:

IFNγ; 5'-TTCGCTTTCCAGCTTTGCAT-3', 5'-CTGGAGCTGGTATTTAACAA-3'

TNFα; 5'-TGGCCTGCAACTAATCAACC-3', 5'-GTGTGGAAGGACATCCTTGG-3'

IFNβ;5'-TCTCGAAGTCTTTGCTTCAGCAC-3', 5'-GAGGTTCTGTTCAAGTTCACCAGG-3'

IL2; 5'-ACTGACTCTTATACTCGTCAC-3', 5'-GTCAATTCTGTGGCCTTCTTG-3'

IL4; 5'-GGTCTGCTTACTAGCATTTACCA-3', 5'-GGTGGAGCAGTTGTGATGTG-3'

IL6; 5'-GCAGAAAACAACCTGAATCTTCCG-3', 5'-GAGAAAGGAATGCCCGTGAAC-3'

IL10; 5'-GAGGACCCAGACATCAAAC-3', 5'-AGAGGTATGACCGGGTTCTCCAA-3'

IL12p35;5'-AGGAATGTTCCAGTGCCTCAAC-3',5'CACCTGGTACATCTTCAAGTCCTC-3'

IL12p40; 5'-ACCAGCAGCTTCTTCATCAGGG-3',5'-GGACCTGTACGCCAAATGTTAA-3'

Sample collection and cell isolation

Peripheral blood was collected from specific pathogen free (SPF) cats via jugular venipuncture under ketamine anesthesia. Peripheral blood mononuclear cells (PBMC) were isolated via density gradient centrifugation (Histopaque 1077; Sigma, St. Louis, MO). The cells were cultured at a concentration of 4 x 10^6 cells/ml in lymphocyte culture medium (LBT) composed of RPMI 1640 supplemented with 10% fetal calf serum

(Hyclone Laboratories, Logan, UT), 2% glutamine, 1% penicillin/streptomycin and 5 ug/ml concanavillin A (ConA) or Phorbol 12-myristate 13-acetate (PMA) (Sigma, St. Louis,MO) and ionomycin (Sigma). PBMC were cultured for 2 hrs at 37° C with 5% CO_2. This incubation protocol had been shown to maximally induce the cytokines of interest. In order to produce adequate quantities of IL6, IFNβ and IL12, the ConA was replaced by 1 μg/ml of lipopolysaccharide (LPS) and the cells were incubated for 4 hrs at 37° C.

Macrophage cultures were set up so that, after 1 hr at 37° C, the non-adherent cells were washed off the plates with PBS. The adherent cells were cultured for an additional 5 days in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The five day incubation period allowed for daily washes to remove lymphocytes. Purity was assessed by staining cytospin preparations (Shandon, Cheshire, England) with the macrophage marker Mac387 (Serotec, Raleigh, NC).

Splenic tissue and mesenteric and colonic lymph node samples were obtained from naïve cats immediately after euthanasia and snap frozen in liquid nitrogen. The samples were stored at –70° C until use.

cDNA Synthesis

RNA was extracted from the cells and tissues using Trizol™ (Invitrogen, Carlsbad, CA) according to the manufacturers instructions. One microgram of total RNA was then reverse transcribed using the cDNA Cycle Kit (Invitrogen) and the DNA product was brought up to a volume of 100 μL in distilled water.

Polymerase Chain Reaction

Hot start PCR was performed using Ampliwax PCR Gems (Perkin Elmer Corp., Norwalk, CT) in a total reaction volume of 50 μL. Thirty five cycles of 94° C for 15

seconds, 55° C for 20 seconds and 72° C for 30 seconds were carried out in a reaction mixture containing 3 mM $MgCl_2$, 200 μM each dNTP, 1X Gene Amp PCR buffer (Perkin Elmer Corp), 1 μM of each primer and 2.5 units of Pfu polymerase(Stratagene, Cedar Creek, TX). PCR products were run on 1.5% Seakem LE agarose gels (BMA, Rockland, ME) which were subsequently stained with ethidium bromide. The PCR products were excised from the agarose gels and purified using GenElute spin columns (Supelco, Bellafonte, PA).

Cloning of PCR Products

The gel purified PCR products were cloned into the pCR-Script Amp SK+ vector (Stratagene, La Jolla, CA) and streaked out onto Luria Broth (LB) plates containing 50 μg/ml ampicillin. White-appearing colonies were amplified in 10 mls of LB media and the resultant broths were pelleted for sequencing from both sides of the plasmid cloning sites (Macromolecular Resources, Fort Collins, CO). The sequencing allowed the selection of plasmids containing high fidelity inserts oriented in the anti-sense direction from the T7 promoter. High scale purification of the selected clones was then performed using MIDI Prep kits (Qiagen, Valencia, CA).

Ribonuclease Protection Assay

Plasmids encoding the appropriate cytokines were linearized using Not1 (New England Biolabs, Beverly. MA) and gel purified using spin columns (Supelco, Bellafonte, PA). Probe cocktails were prepared for the analysis of T cell cytokines and macrophage cytokines as outlined above with each probe present at a concentration of 5 ng/uL.

The probe sets were labeled with ^{32}P UTP using the Riboquant™ multi-probe RNase protection system (PharMingen, San Diego, CA). Briefly, the RNA probes were synthesized using T7 RNA polymerase at 37° C for 1 hr. The reaction was terminated by the addition of 2 units of DNase for an additional 30 minutes. The probes were then subjected to phenol/chloroform extraction followed by precipitation with 4 M ammonium

acetate and 100% ethanol. After an additional wash with 90% ethanol, the probes were air dried for 30 minutes. The probe mixture was resuspended in 50 μL of hybridization buffer and the activity was quantified in a scintillation counter. The probe mixture was used at a final concentration of 3 $X10^5$ Cherenkov counts/μL.

RNA samples were derived from Histopaque 1077 (Sigma) separated PBMC or snap frozen lymph nodes and spleens from healthy cats. The PBMC were incubated with ConA as described above and RNA was extracted with Trizol™ reagent (Invitrogen). One, 2 and 4 μg aliquots of total PBMC RNA were lyophilized in a Speed Vac concentrator (Savant, Farmingdale, NY). The tissue samples (50 μg) were pulverized in 1 ml of Trizol™ solution (Invitrogen) and 2, 5 and 10 μg of RNA was lyophilized.

Eight microliters of hybridization buffer was added to the samples and they were gently vortexed for 3 minutes. Two microliters of labeled probe was added to each sample and a drop of mineral oil was placed over each sample. The sample tubes were placed in a heat block set to 90° C and allowed to slowly ramp down to a temperature of 56° C where they remained for 12-18 hrs. At the end of the incubation, the temperature of the heat block was further reduced to 37° C and the samples remained at this temperature for 15 minutes.

The samples were then incubated with 1 μL of RNase A + T1 mixture for 45 minutes at 30 C to digest any unhybridized RNA. After an additional 15 minute incubation at 37° C with Proteinase K, the samples were extracted with Tris-saturated phenol and chloroform:isoamyl alcohol. The samples were then precipitated with 4 M ammonium acetate and ice-cold ethanol. The samples were allowed to air dry completely for approximately 45 minutes at which point they were re-suspended in 5 μL of 1X loading buffer. The samples were heated at 90° C for 3 minutes and then immediately placed on ice.

A denaturing 8% polyacrylamide sequencing gel was prepared and prerun at 40 volts for 45 minutes. The samples were loaded onto the gel along with a 1:150 dilution of the

probe mixture and run at 50 volts for 2 hours. An aliquot of a commercially prepared murine chemokine probe mixture (Pharmingen) was included to provide a molecular size marker. The gel was dried onto filter paper using the Speed Vac system and loaded into a radiograph film cassette (Dupont, Wilmington, DE) or a Phosphor screen (Amersham Biosciences, Piscataway, NJ). The film cassettes were incubated for 48-72 hrs at -70^{o} C whereas the Phosphor screens were incubated at room temperature for 72-96 hrs. Individual band intensity was determined using ImageQuant software (Amersham Biosciences, Piscataway, NJ) and all results were expressed as a ratio of cytokine to GAPDH.

Five PBMC samples and 5 macrophage samples were randomly chosen and analyzed two times using different preparations of the T cell probe set or the macrophage probe set respectively. Correlations between the two sample runs were calculated and Fisher's r to z transformation used to determine significance at a level of $p<0.05$ (StatView, Adept Scientific, Bethesda, MD).

RESULTS

Preparation of RPA Probe Sets

Each pair of selected primers produced clear, individual bands of the appropriate size (data not shown). Stimulating PBMC with LPS was necessary in order to amplify IL12, IL6 and IFNβ.

Because there were difficulties consistently amplifying IL12 and IL6 using Pfu polymerase, Taq polymerase was used. Despite the theoretical reduction in amplification fidelity, the Taq polymerase produced PCR products of the appropriate sequence. These amplimers were cloned into the TOPO-TA vector (Invitrogen) following the manufacturer's instructions.

Multiple clones containing the IFNγ and IL6 inserts were sequenced. All contained the insert in the opposite orientation necessary to generate an anti-sense product using the T7 promoter. There was no obvious explanation for the directional insertion of a blunt ended, Pfu-generated, PCR product. It is possible that, although they are truncated sequences, that the plasmid product in the sense orientation was toxic to the bacteria preventing their growth. In order to proceed with assay development, NotI and BamHI (New England Biolabs, Beverly, MA) were used to excise the complete IFNγ and IL6 insert out of the pCR-script vector. The excised DNA band was then directionally inserted into the TOPO-TA vector (Invitrogen) so that an antisense product could be generated with the T7 promoter.

Ribonuclease Protection Assay

T cell Probe Set

The T cell probe set (IL10, IFNγ, TNFα, IL4, IL2 and GAPDH) consistently produced protected fragments of the appropriate size for IL10, IFNγ, TNFα, IL2 and GAPDH when ConA stimulated PBMC were used as the RNA source (Figure 1.2). The specificity of the protected RNA fragments was determined based on the size of the resolved band. The size of the product was determined by direct comparison to the known sizes of the included murine chemokine probe set as well as by generating a standard curve and plotting log nucleotide length against migration distance on semi-log paper (5) One to two micrograms of total RNA was sufficient to produce readily identifiable protected RNA cytokine bands. Dilutional series of input RNA resulted in proportional decreases in the signal strength of GAPDH, validating its use in sample quantification (Figure 1.3).

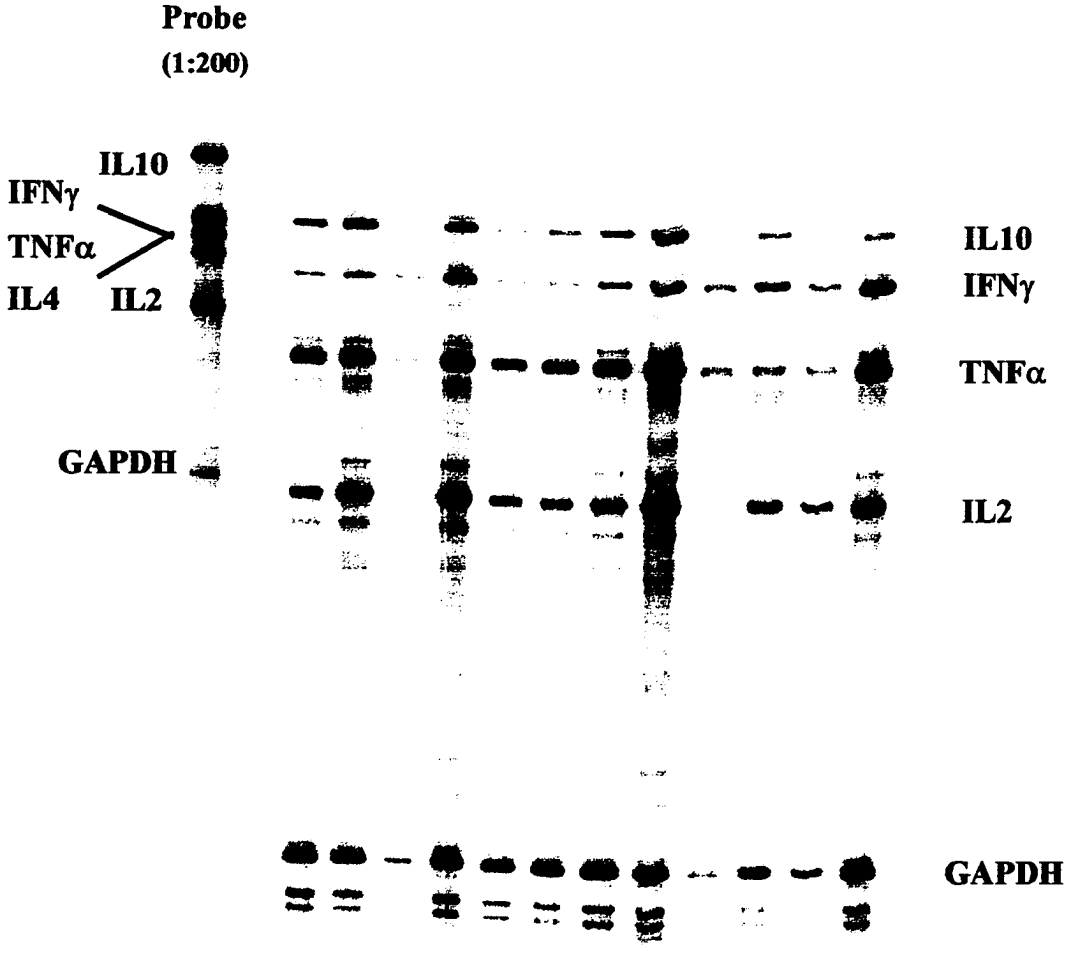

Figure 1.2: Representative Ribonuclease Protection Assay of mitogen-stimulated feline lymphocytes. Peripheral blood mononuclear cells were stimulated for 4 hrs with 5 μg/ml ConA and total RNA was extracted. Two micrograms of total RNA was used for each hybridization reaction.

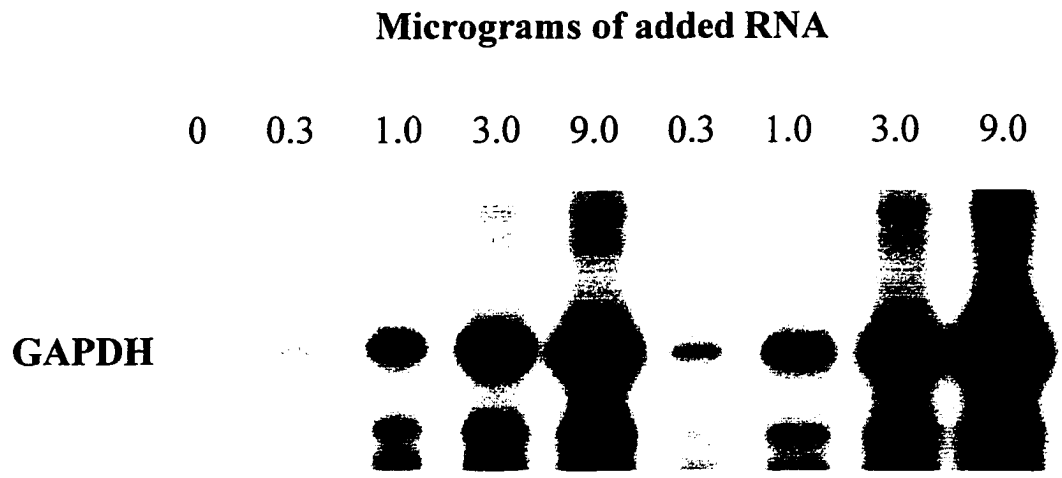

Figure 1.3: The ribonuclease protection assay (RPA) detection of the housekeeping gene GAPDH appears to be linear over the range of 0.3 to 9.0 μg of total RNA. Two samples from feline lymph nodes were diluted and assayed via RPA.

Snap frozen, unstimulated lymph node tissue from naïve cats consistently demonstrated a more restricted cytokine profile. Without mitogen stimulation, IL10 and TNFα bands could be resolved but a minimum of 6-9 μg of total RNA was generally required before bands were visible. When bulk lymph node cells were cultured with either ConA or PMA/ionomycin, all of the cytokines except IL4 could be readily resolved. Faint bands corresponding to IL4 could be seen in samples originating from the mesenteric or colonic lymph nodes.

interleukin 4

Because protected RNA corresponding to IL4 could not be demonstrated in stimulated PBMC and only weak bands were seen in lymph nodes draining the GI tract, we performed a brief experiment to confirm the identity of the weak bands. A separate probe set was synthesized that was identical to the T cell set except the plasmid encoding IL4 was excluded. When samples from the colonic lymph nodes were analyzed with both probe sets, the presumed IL4 bands were only present in the samples probed with the complete set (Figure 1.4). This experiment demonstrated that the faint bands were in fact specific for IL4.

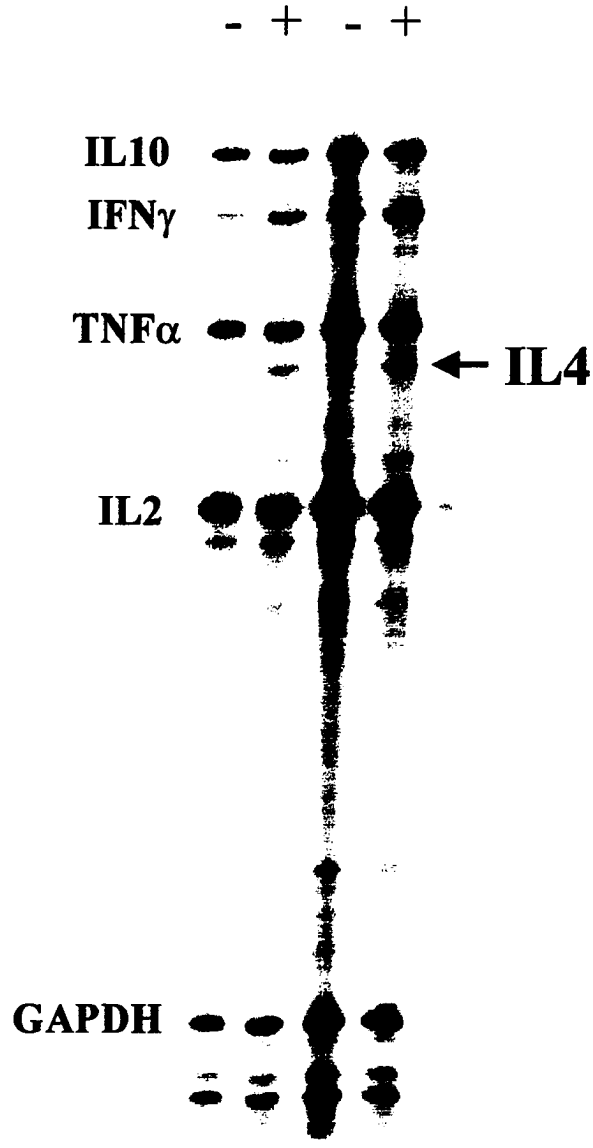

Figure 1.4: Interleukin 4 can be detected in the colonic lymph nodes of cats. Two samples were assayed with probe sets lacking (-) or including (+) the IL4 probe. Suspensions of lymph node cells were cultured with 5 μg/ml ConA for four hours and RNA was extracted. Two micrograms of RNA were added to each hybridization reaction. Intereukin 4 was not detected in similarly treated peripheral blood mononuclear cells.

Macrophage Probe Set

The macrophage probe set consistently produced protected fragments of the appropriate size for IL6, IL10, TNFα and GAPDH (Figure 1.5). IFNβ and both p40 and p35 subunits of IL12 were generally below the limits of reliable detection, although very faint bands of the appropriate size could occasionally be seen. The number of background bands was increased in the macrophage RPA samples but specific protected bands were identified by plotting log nucleotide length against migration distance on semi-log paper. In addition, T cell samples generated with the T cell probe set were run on the same gels for size comparison.

Phosporimager Analysis

Once the probe sets were established and the RPA methodology refined using exposure to plain radiographic film, we shifted to using Phosphor screens (Amersham Biosciences). Exposure times were comparable between the two capture systems although, extending the exposure time beyond that of the radiograph film, improved the clarity of the image. The optimum exposure time within the Phosphor cassette was determined to be 72 to 96 hrs.

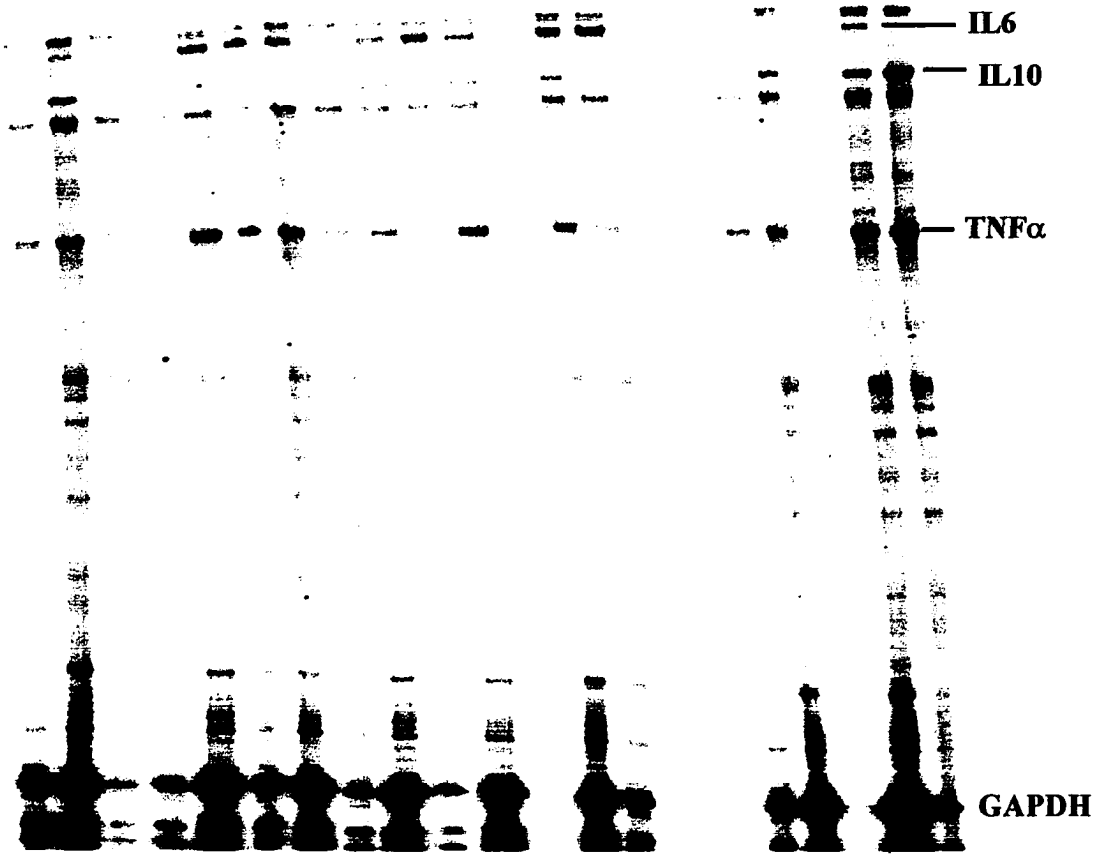

Figure 1.5: A representative Ribonuclease Protection Assay of adherence-purified feline macrophages. Peripheral blood mononuclear cells were allowed to adhere for 1 hr, plates were washed and the adherent cells were cultured for an additional 5 days. Total RNA was extracted and 4 μg was used for each hybridization reaction.

Reproducibility

Sample measurements repeated two times with different batches of either macrophage or T cell probe sets were highly correlated with an R value of 0.952 ($p<0.0001$) (Figure 1.6).

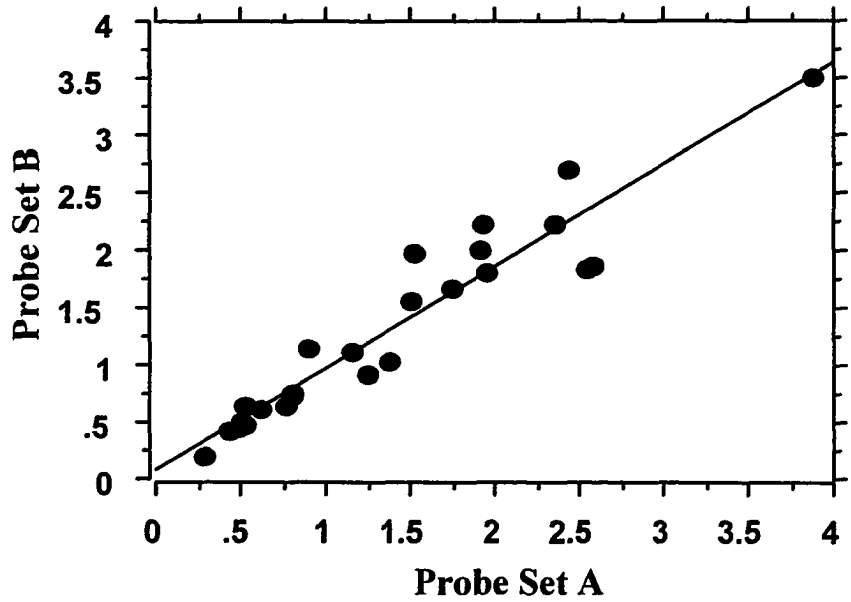

Figure 1.6: There is a high correlation between cytokine values obtained when the assay is repeated with two different generations of probe mixture. Values for both T cell cytokines and macrophage cytokines are included. $R=0.952$, $p<0.0001$.

DISCUSSION

We generated a ribonuclease protection assay that can simultaneously quantify 5-6 feline cytokines per 1-2 μg of RNA sample. As many as 24 RNA samples could be run per assay. The existing QC-RT-PCR assay for cytokines would require 1008 individual PCR reactions in order to quantify the same number of cytokines as one RPA. The RPA has the added advantage of not introducing the potential artifacts of multiple rounds of amplification which can be seen with PCR (4). The RPA produced repeatable results even when new probe mixtures were synthesized *de novo*, an important feature for long-term *in vivo* studies.

One limitation of the assay is the prolonged exposure time which extends the total assay time to 5-6 days, but this issue is less concerning when one calculates that the time necessary to quantify the same 144 cytokine samples using QC-RT-PCR is similar. The assay also appears to be less sensitive than RT-PCR which may necessitate mitogen stimulation when the total amount of sample RNA is limited. The inability to detect macrophage IL12 and INFβ may be a reflection of the culture conditions used to generate relatively pure populations of macrophages. The addition of LPS might increase message levels sufficiently to allow quantification.

Current applications of RPA cytokine quantification include toxicology (19), rheumatology (20), transplantation (21), oncology (22) in addition to infectious diseases (23) (8). Future directions with this assay may include conversion to a non-radioisotopic detection method which would decrease regulatory and safety issues as well as allow the storage of stably labeled probe sets. On balance, the ability of this RPA assay to specifically, reproducibly and efficiently quantify feline cytokines makes it a valuable tool to study the feline immune response.

REFERENCES

1. Rottman, J., E. Freeman, S. Tonkonogy, and M. Tompkins. 1995. A reverse transcription-polymerase chain reaction technique to detect feline cytokine genes. *Veterinary Immunology and Pathology* 45:1.
2. Levy, J.K., J.W. Ritchey, J.B. Rottman, M.G. Davidson, Y.H. Liang, H.L. Jordan, W.A. Tompkins, and M.B. Tompkins. 1998. Elevated interleukin-10-to-interleukin-12 ratio in feline immunodeficiency virus-infected cats predicts loss of type 1 immunity to Toxoplasma gondii. *J Infect Dis* 178, no. 2:503.
3. Liang, Y., L.C. Hudson, J.K. Levy, J.W. Ritchey, W.A. Tompkins, and M.B. Tompkins. 2000. T cells overexpressing interferon-gamma and interleukin-10 are found in both the thymus and secondary lymphoid tissues of feline immunodeficiency virus-infected cats. *J Infect Dis* 181, no. 2:564.
4. Rottman, J.B. 2002. The ribonuclease protection assay: a powerful tool for the veterinary pathologist. *Veterinary Pathology* 39, no. 1:2.
5. Gilman, M. 1993. Ribonuclease Protection Assay. *In* Current protocols in Molecular Biology, vol. 1. F.M. Ausubel, editor. John Wiley and Sons, Inc., New York. 4.7.1.
6. Plath, A., F. Peters, and R. Einspanier. 1996. Detection and quantitation of specific mRNAs by ribonuclease protection assay using denaturing horizontal polyacrylamide electrophoresis. *Electrophoresis* 17, no. 3:471.
7. Muller, K., S. Ehlers, W. Solbach, and T. Laskay. 2001. Novel multi-probe RNase protection assay sets for the detection of murine chemokine gene expression. *J Immunol Methods* 249, no. 1-2:155.
8. Stalder, A.K., and I.L. Campbell. 1994. Simultaneous analysis of multiple cytokine receptor mRNAs by RNase protection assay in LPS-induced endotoxemia. *Lymphokine Cytokine Res* 13, no. 2:107.
9. Berger, D.P., Homann.D., and M.B.A. Oldstone. 2000. Defining parameters for successful immunocytotherapy of persistent viral infection. *Virology* 266:257.

10. Khatissian, E., V. Monceaux, M. Cumont, L. Montagnier, B. Hurtrel, and L. Chakrabarti. 1996. Interferon-gamma expression in macaque lymph nodes during primary infection with simian immunodeficiency virus. *Cytokine* 8, no. 11:844.

11. Meroni, L., D. Trabattoni, C. Balotta, C. Riva, A. Gori, M. Moroni, M. Villa, M. Clerici, and M. Galli. 1995. Evidence for type 2 cytokine production and lymphocyte activation in the early pahses of HIV-1 infection. *AIDS* 10:23.

12. Ralph, P., I. Nakoinz, A. Sampson-Johannes, S. Fong, D. Lowe, H.Y. Min, and L. Lin. 1992. IL10; T lymphocyte inhibitor of human blood cell production of IL-1 and tumor necrosis factor. *Journal of Immunology* 148:808.

13. Takeshita, S., E.C. Breen, M. Ivashchenko, P.G. Nishanain, T. Kishimoto, D.L. Vredevoe, and O. Martinez-Maza. 1995. Induction of IL-6 and IL-10 production by recombinant HIV-1 envelope glycoprotein 41 in the THP-1 human monocytic cell line. *Cellular Immunology* 165, no. 2:234.

14. Rabbi, M.F., A. Finnegan, L. Al-Harthi, S. Song, and K.A. Roebuck. 1998. Interleukin-10 enhances tumor necrosis factor-alpha activation of HIV-1 transcription in latently infected T cells. *Journal of Acquired Immune Deficiency Syndrome and Human Retroviruses* 19, no. 4:321.

15. Trinchieri, G. 1994. Interleukin 12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood* 84, no. 12:4008.

16. Heinzel, F.P., A.M. Hujer, F.N. Ahmed, and R.M. Rerko. 1997. In vivo production and function of IL-12 p40 homodimers. *Journal of Immunology* 158, no. 9:4381.

17. Ling, P., M.K. Gately, U. Gubler, A.S. Stern, P. Lin, K. Hollfelder, C. Su, Y.C. Pan, and J. Hakimi. 1995. Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. *Journal of Immunology* 154, no. 1:116.

18. Bette, M., S.C. Jin, T. Germann, M.K. Schafer, E. Weihe, E. Rude, and B. Fleischer. 1994. Differential expression of mRNA encoding interleukin-12 p35 and p40 subunits in situ. *European Journal of Immunology* 24, no. 10:2435.

19. Bhandari, N., and R.P. Sharma. 2002. Fumonisin B(1)-induced alterations in cytokine expression and apoptosis signaling genes in mouse liver and kidney after acute exposure. *Toxicology* 172, no. 2:81.

20. Scola, M.P., S.D. Thompson, H.I. Brunner, M.K. Tsoras, D. Witte, D. Van, A.A. Grom, M.H. Passo, and D.N. Glass. 2002. Interferon-gamma:interleukin 4 ratios and associated type 1 cytokines expression in juvenile rheumatoid arthritis synovial tissue. *Journal of Rheumatology* 29, no. 2:369.

21. Mannon, R.B., K. Roberts, P. Ruiz, V. Laubach, and T.M. Coffman. 1999. Inducible nitric oxide synthase promotes cytokine expression in cardiac allografts but is not required for efficient rejection. *Journal of Heart and Lung Transplantation* 18, no. 9:819.

22. Takahashi, K., S. Tanaka, and A. Ichikawa. 2001. Effect of cimetidine on intratumoral cytokine expression in an experimental tumor. *Biochemical and Biophysical Research Communications* 281, no. 5:1113.

23. Sauder, C., and J.C. de la Torre. 1999. Cytokine expression in the rat central nervous system following perinatal Borna disease virus infection. *Journal of Neuroimmunology* 96, no. 1:29.

CHAPTER TWO

TISSUE CYTOKINE RESPONSES TO MUCOSAL INFECTION WITH FELINE IMMUNODEFICIENCY VIRUS SUBTYPE B

INTRODUCTION

FIV was recognized as a feline pathogen in the years immediately after HIV emerged in the first cohorts of homosexual men (1). While there are some differences between the two viruses in terms of the breadth of cellular targets and genomic complexity, the two lentiviruses produce a clinical disease course that is virtually identical. Both HIV and FIV induce an acute flu-like illness characterized by lymphadenopathy, high levels of circulating virus and declines in circulating CD4+ T cells which, after a period of weeks, gives way to a long clinically asymptomatic period (2) (3) (4) (5). After several years of clinical latency, both viruses induce a terminal period of clinical immunodeficiency with wasting and opportunistic infections (6) (7).

The cytokines elaborated early in a variety of infectious diseases are critical to the course of infection. A cytokine response dominated by interferon-gamma (IFNγ), tumor necrosis factor alpha (TNFα) and interleukin 12 (IL12) (type 1) has been shown to be important in the clearance of intracellular pathogens including viruses (8) (9) (10). These cytokines are instrumental in establishing cytotoxic T lymphocytes (CTL) and several studies have shown a correlation between the emergence of CTL and the reduction of circulating HIV and SIV (11) (12) (13). Type 2 cytokines (interleukin 4 (IL4), interleukin 10 (IL10), interleukin 13 (IL13)) have been shown to antagonize the production and effects of type 1 cytokines and it has been postulated that they play a role in viral escape from immune surveillance (8). Contradictory data has been generated to both establish and refute the notion that the cytokine response to HIV moves from a type 1 response to a type 2 response during the progression to immunologic deterioration and clinical AIDS (14) (15) (16) (17) (18).

Studying the early events in naturally occurring HIV infection can be quite difficult as information concerning the exact timing of infection and seroconversion is usually lacking. In addition, the majority of the viral replication occurs within the lymphoid tissues and sampling these sites in people requires invasive surgical procedures. It was our plan to use a rectal mucosal transmission model of FIV to simulate the early transmission of HIV and to necropsy cats at serial time points to determine the tissue cytokine response to the virus. In order to further explore the cellular source of the cytokines we purified CD4 and CD8-positive T cells as well as macrophages from each lymphoid organ.

MATERIALS AND METHODS

Animals and sample collection

Sixteen week old cats from a specific pathogen-free (SPF) breeding colony maintained at Colorado State University (Fort Collins, CO) were inoculated by atraumatic exposure of the rectal mucosa with 1 ml of either 200 $TCID_{50}$/ml (50% tissue culture infective dose; n=4) or 1 ml of 400 $TCID_{50}$/ml cell-free infectious FIV-B supernatant (n=10). Age-matched control cats received 1 ml of cell-free non-infectious cell culture medium (n=15). The inoculations were repeated 24 hrs after the first exposure. Blood collection was performed under ketamine anesthesia every 5 days until FIV proviral DNA was detected by PCR and then collections were performed every 14 days. Six FIV infected and 7 naïve controls were euthanized at four weeks post-inoculation (PI). The remaining 8 FIV-infected and 8 naïve controls were sacrificed at ten weeks PI.

Blood was collected immediately prior to euthanasia and then a necropsy was performed. The colic lymph nodes and a portion of the spleen were collected immediately following euthanasia and placed in ice cold Hanks' Balanced Salt Solution (GIBCO, Grand Island, NY) containing DNase.

Virus inocula

The first cell-free cell culture virus inoculum (200 $TCID_{50}$/ml) was generated by the coculture of naïve feline peripheral blood mononuclear cells (PBMC) with PBMC from a cat acutely infected with FIV-B via the intravenous route. The second cell culture inoculum (400 $TCID_{50}$/ml) was generated by the coculture of naïve PBMC with the PBMC from a pool of 3 cats acutely infected with FIV-B via the rectal mucosal route. Infectivity of the supernatants was determined via titration and aliquots were frozen at – 70° C.

Processing of PBMC and tissue

PBMC were separated by density gradient centrifugation (Histopaque 1077, Sigma, St. Louis, MO). Colic lymph nodes and spleen were mechanically dispersed by passage through a fine wire mesh and resuspended in cold Hanks' Balanced Salt Solution (GIBCO). The erythrocytes from the splenic samples were lysed by incubation in 5 mls of ACK lysis buffer for 10 minutes. The isolated cells were washed three times in sterile Dulbecco's phosphate buffered saline (PBS) (GIBCO) and placed in LBT media containing Phorbol 12-myristate 13-acetate (PMA) (Sigma, St. Louis, MO) and ionomycin (Sigma) at a concentration of 4 x 10^6 cells/ml. PMA/ionomycin replaced ConA as the mitogen in these experiments in order to avoid the cellular clumping induced by ConA prior to magnetic separation. After 2 hrs of incubation at 37° C, the non-adherent cells were washed three times in PBS. The adherent cells were cultured for an additional 5 days in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Magnetic purification of cells

The isolated cells were resuspended in 1 ml of PBS supplemented with 2% fetal bovine serum (FBS) (HyClone, Logan, UT) and 4 μg of murine IgG for 30 minutes at 4° C. The cells were washed two times with PBS and resuspended in 800 μL PBS 2% FBS. FITC-

conjugated antibodies to either feline CD4 (Hoover Laboratory) or CD8 (Southern Biotechnology, Birmingham, AL) were added and the cells were incubated at 4° C for an additional 30 minutes. After washing the cells three times they were resuspended in 800 μL of PBS 2% FBS with 40 μL of anti-FITC magnetic beads (Miltenyi Biotec, Auburn,CA) and incubated for 15 minutes at 4° C. The cells were washed one time in PBS and reusupended in 500 μL of degassed PBS supplemented with 2mM EDTA and 0.5% bovine serum albumin (separation buffer).

MACS LS separation columns were placed in a MidiMACS Separation Unit (Miltenyi Biotec) and pre-loaded with 3 ml of cold, degassed separation buffer. The cell suspensions were applied to individual LS columns and washed three times with 3 ml of degassed separation buffer. The columns were removed from the MidiMACS unit and 5 mls of separation buffer was applied and pushed through the column with the supplied plunger. The eluted cells were pelleted and resuspended in 500 μL of separation buffer and the above procedure was repeated with a new LS separation column. Cells were quantified in the final eluants and 1 x 10^5 cells were stained with the same FITC-labeled antibody used to sort and a PE-labeled antibody for either CD4 or CD8 and a tricolor-labeled antibody for B220 (BD Pharmingen, San Diego, CA). Flow cytometry with a EPICS XL-MCL (Coulter, Hialeah, FL) was performed to assess the final purity of the sorted cells. The remaining cells were lysed in 1 ml of Trizol Reagent (GIBCO BRL, Grand Island, NY) and stored at –70° C until further processing according to the manufacturers instructions. The adherence-purified macrophages were similarly lysed with Trizol directly in the culture flasks. The RNA was lyophilized in 2 μg aliquots using a Speed Vac concentrator (Savant, Farmingdale, NY) immediately prior to performing the ribonuclease protection assay (RPA).

Ribonuclease Protection Assay

Two probes sets were developed for feline cytokines. The set used with RNA from purified CD4+ and CD8+ T cells consisted of probes for IL10, IFNγ, TNFα, IL4, IL2 and the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The set

used with RNA from macrophages consisted of IL6, IL10, TNFα, IL12 p40 and p35, interferon-beta (IFNβ) and GAPDH.

The probe sets were labeled with ^{32}P UTP using the Riboquant™ multi-probe RNase protection system (BD PharMingen). Briefly, the RNA probes were synthesized using T7 RNA polymerase at 37° C for 1hr. The reaction was terminated by the addition of 2 units of DNase for an additional 30 minutes. The probes were then subjected to phenol/chloroform extraction followed by precipitation with 4 M ammonium acetate and 100% ethanol. After an additional wash with 90% ethanol, the probes were air dried for 30 minutes. The probe mixture was resuspended in 50 μL of hybridization buffer and the activity was quantified in a scintillation counter. The probe mixture was used at a final concentration of 3 $X10^5$ Cherenkov counts/μL. Eight microliters of hybridization buffer was added to the lyophilized samples and they were gently vortexed for 3 minutes. Two microliters of labeled probe was added to each sample and a drop of mineral oil was placed over each sample. The sample tubes were placed in a heat block set to 90° C and allowed to slowly ramp down to a temperature of 56° C where they remained for 12-18 hrs. At the end of the incubation, the temperature of the heat block was further reduced to 37° C and the samples remained at this temperature for 15 minutes.

The samples were then incubated with 1 μL of RNase A + T1 mixture for 45 minutes at 30° C to digest any unhybridized RNA. After an additional 15 minute incubation at 37° C with Proteinase K, the samples were extracted with Tris-saturated phenol and chloroform:isoamyl alcohol. The samples were then precipitated with 4 M ammonium acetate and ice-cold ethanol. The samples were allowed to air dry completely for approximately 45 minutes at which point they were re-suspended in 5 μL of 1X loading buffer. The samples were heated at 90° C for 3 minutes and then immediately placed on ice.

A denaturing 8% polyacrylamide sequencing gel was prepared and run at 40 volts for 45 minutes. The samples were loaded onto the gel along with a 1:150 dilution of the probe mixture and run at 50 volts for 2 hours. The gel was dried onto filter paper using the

Speed Vac system and loaded into a Phosphor screen (Amersham Biosciences, Piscataway, NJ) and incubated at room temperature for 72-96 hrs. Individual band intensity was determined using ImageQuant software (Amersham Biosciences) and all results expressed as a ratio of cytokine to GAPDH.

DNA PCR for provirus detection

DNA was extracted from PBMC using the QIAamp blood kit (Qiagen, Chatsworth, CA). One microgram of DNA was amplified by nested PCR using FIV gag primers. First round primers were Gag129: 5'-CGTAACTACAGGACGAGAACCTGG-3' and Gag802: 5'-CCAACTTTCCCAATGCTTCAAG-3' and second round primers were Gag3: 5'-TTGACCCAAAAATGGTGTCCA-3' and Gag4: 5'-TTCTGCTTGTTGTTCTTGAGT-3' resulting in a 293 bp product. For both first and second round reactions, hot start polymerase chain reaction was performed with Ampliwax PCR gems (Perkin Elmer, Norwalk, CT). Each round consisted of 35 cycles of 94° C for 15 seconds, 55° C (first round) or 60° C (second round) for 20 seconds and 72° C for 30 seconds. The first and second round reaction mixtures contained 3 mM MgCl2 200 μM of each dNTP, 1X Gene Amp 10X PCR buffer II (Perkin Elmer), 2.5 units AmpliTaq DNA polymerase (Perkin Elmer) and 0.1 μM of each first round primer or 0.05 μM of each second round primer. Product was visualized on a 1.2% agarose gel stained with ethidium bromide. Amplimer specificity has been previously shown via Southern blot analysis (19).

RT-PCR for viral RNA

Viral RNA levels in plasma and tissue were quantified using a quantitative competitive reverse transcriptase PCR (QC-RT-PCR) assay based on the method described by Diehl et al (20) (21). A single round, 40 cycle reaction was run as described above using the primers Gag3 and Gag4. Three fold serial dilutions of competitor RNA containing from 10^6-$10^{3.6}$ copies were added to viral RNA samples. Ethidium bromide stained amplimers were quantified using AlphaImager™ software (Alpha Innotech, San Leandro, CA) and

the point of equivalence of competitor and virus was determined. Input amounts of RNA consisted of 25 μL of Alsever's (Sigma) plasma and 25 ng of total tissue RNA per PCR reaction.

FIV antigen ELISA

Productive *in vitro* infection was assessed by capsid antigen (p26) capture ELISA described by Dreitz et al (22) performed on macrophage supernatants. Optical densities (OD), measured by absorbance at A450, were recorded using a Dynatech 5000MRTM microplate reader (Dynatech Corp., Chantilly, VA). Positive reactions were defined as those with a minimum OD of 0.1 and at least twice that of negative control supernatants run in parallel.

Statistical Analysis

Data were analysed with StatView software (Adept Scientific, Bethesda, MD). Samples were determined to be normally distributed using the Kolmogorov-Smirnov test. Comparisons were made using a two-tailed Student's t test with significance defined as $p<0.05$. Correlations were calculated and p values determined using Fisher's r to z transformation with significance defined as a $p<0.05$.

RESULTS

Rectal mucosal infection

Thirty cats were inoculated with 2 doses of FIVB2542 via the rectal mucosal route. Overall, PBMC from 14 of the 30 cats became DNA PCR positive (47% rate of infection) beginning 14-22 days PI. The pool of virus generated from cats acutely infected via the rectal route did not demonstrate any augmented ability to subsequently cross the rectal mucosa i.e. 4 of 9 cats (44%) became PCR positive. Five exposed, PCR-negative cats were euthanized to search for potential virus in tissues and virus was not detectable via DNA or RT-PCR in any tissue sampled (not shown).

Purity of sorted cell populations

The purity of the magnetically sorted CD8+ lymphocytes was consistently >94%, with some samples reaching 98% purity. The sorted CD4+ lymphocytes ranged from 91-97% pure with the majority falling in the 94-96% range. Adherence purified macrophages were 89-92% pure based on cytochemical staining for the marker MAC387 (Serotec Inc, Raleigh, NC).

Cytokine patterns in naïve cats

PMA/ionomycin-stimulated CD4+ lymphocytes produce significantly higher levels of IL2 and IL10 RNA when compared to stimulated CD8+ lymphocytes in both the colonic lymph node and spleen (Figure 2.1a and 2.1b). TNFα levels were consistently higher in the CD4+ lymphocytes than the CD8+ lymphocytes but only in the colonic lymph node (Figure 2.1c). Using the RPA detection method, IL4 was only detectable in the CD4+ lymphocytes of the colonic lymph node (data not shown).

Cytokines at four weeks post infection

Lymphocytes

Four weeks PI, IL10 was the only cytokine message in infected cats that differed significantly from that in controls. This was true for IL10 in CD8+ T cells of the colonic lymph node and for both CD4+ and CD8+ T cells of the spleen (Figure 2.2). IFNγ, TNFα, IL2 and IL4 levels did not differ between FIV+ and control cats (data not shown).

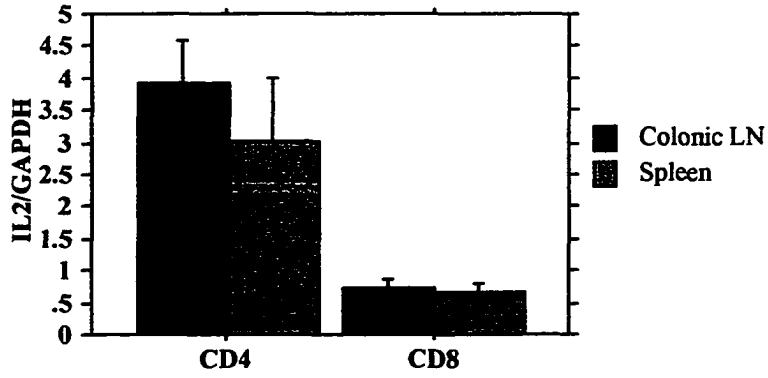

Figure 2.1a: Mitogen-stimulated CD4+ T cells produce more IL2 message than CD8+ T cells in the spleen and colonic lymph node of naïve cats. Cytokine RNA was measured via a ribonuclease protection assay on cells that had been stimulated for 2 hrs with PMA/ionomycin. Significant differences exist between CD4 and CD8+ T cells in both colonic lymph node and spleen $p<0.01$, two-tailed Student's T test.

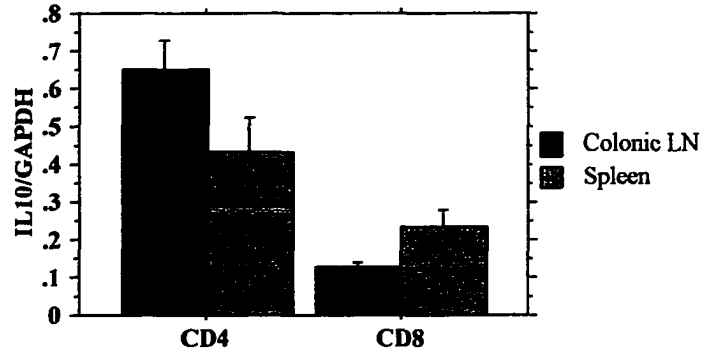

Figure 2.1b: Mitogen-stimulated CD4+ T cells produce more IL10 message than CD8+ T cells in the spleen and colonic lymph node of naïve cats. Cytokine RNA was measured via a ribonuclease protection assay on cells that had been stimulated for 2 hrs with PMA/ionomycin. Significant differences exist between CD4 and CD8+ T cells in the colonic lymph node $p<0.001$ and in the spleen $p<0.05$, two-tailed Student's T test.

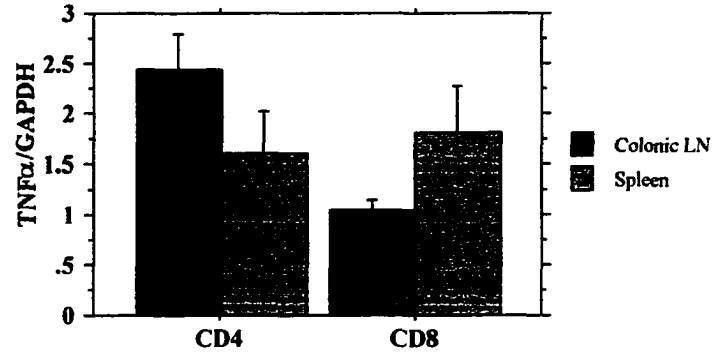

Figure 2.1c: Colonic CD4+ T cells produce more TNFα message than CD8+ T cells in naïve cats while no difference is noted in the spleen. Cytokine RNA was measured via a ribonuclease protection assay on cells that had been stimulated for 2 hrs with PMA/ionomycin. Differences between CD4 and CD8+ T cells in the colonic lymph node $p<0.001$, two-tailed Student's T test.

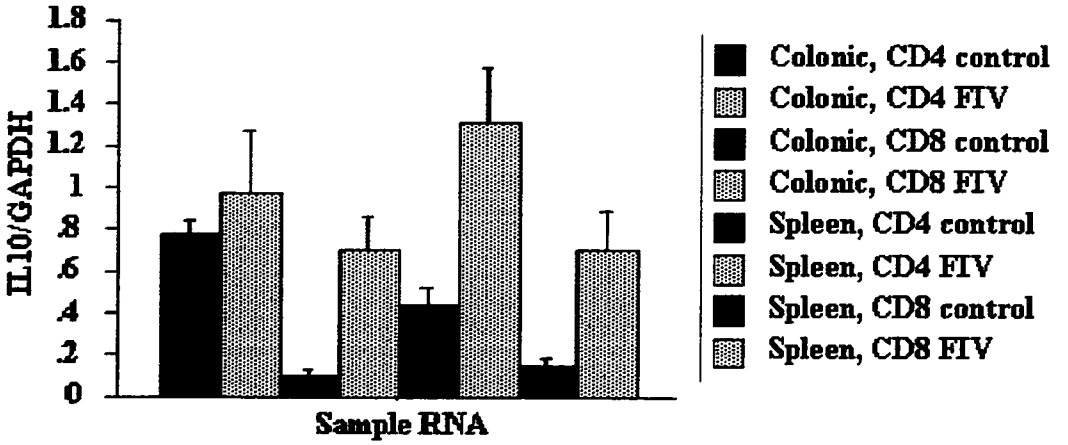

Figure 2.2: IL10 RNA levels are increased in CD4+ T cells of the spleen and in CD8+ T cells of both spleen and colonic lymph node. Cytokine RNA was analyzed via RPA from CD4 and CD8+ T cells in the colonic lymph node and spleen 4 weeks post-infection with FIVB or sham inoculation. Differences were significant in colonic LN and splenic CD8+ T cells ($p<0.01$) and splenic CD4+ T cells ($p<0.05$), two-tailed Student's T test.

Macrophages

The number of macrophages present in colonic lymph nodes was sufficiently low to preclude the evaluation of cytokines in the vast majority of cats. Two of the week 10 control cats did have sufficient numbers macrophages in their colonic lymph nodes but comparisons between infected and naïve cats were not possible.

The spleen yielded sufficient numbers of macrophages in 24 of the 29 cats. Interleukin 6, IL10 and TNFα were the only cytokines detectable in the adherence-purified macrophages. Interleukin 12 and IFNβ were consistently below the limits of detection (not shown). There were no statistically significant differences in the cytokine RNA levels of FIV+ macrophages when compared to those of controls (not shown).

Cytokines at ten weeks post infection

Lymphocytes

IL10 levels in FIV-infected cats remained significantly elevated over controls in both CD4+ T cells and CD8+ T cells in the colonic lymph node and spleen at 10 weeks PI

(Figure 2.3). By 10 weeks PI, a highly significant difference in the levels of IFNγ in the CD8+ lymphocyte population was detected between FIV+ and control cats (Figure 2.4). This rise in IFNγ was not seen in the CD4+ T cells of infected cats. There were no differences in TNFα or IL2 levels in either lymphocyte population in the spleen or colonic lymph node (not shown).

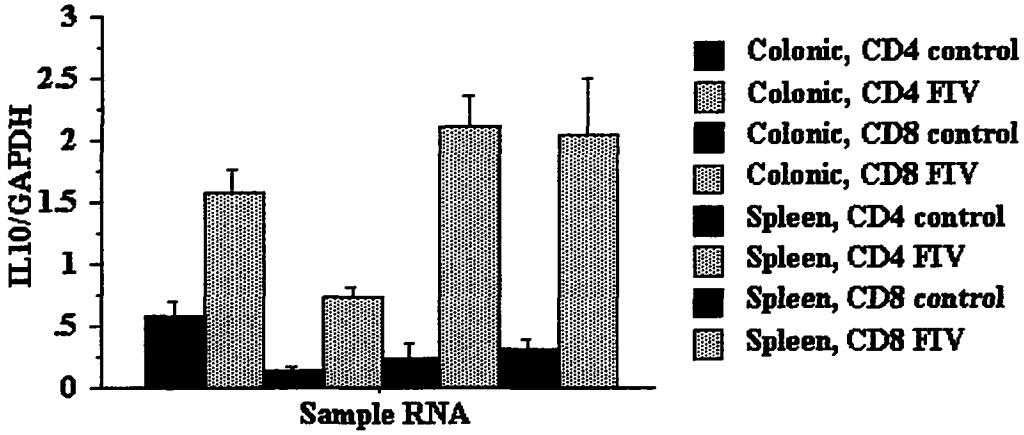

Figure 2.3: IL10 RNA levels are increased in CD4 and CD8+ T cells in both the colonic lymph node and spleen taken at 10 weeks post rectal inoculation with FIVB or media. Cytokine RNA was analyzed via ribonuclease protection assay. Differences were significant in colonic LN and splenic CD4 and CD8+ T cells $p<0.01$, two-tailed Student's T test.

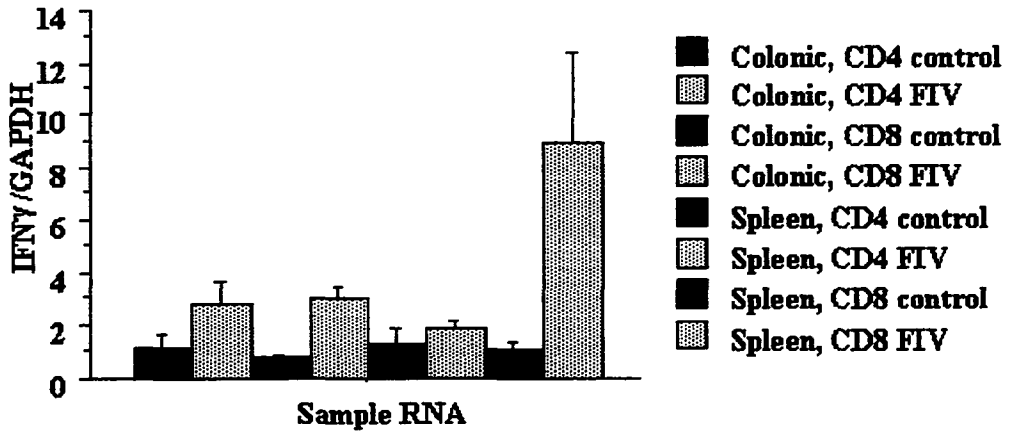

Figure 2.4: IFNγ RNA levels are increased in CD8+ T cells in the colonic lymph node and spleen taken at 10 weeks post rectal inoculation with FIVB or media. Cytokine RNA was analyzed via ribonuclease protection assay. Differences were significant in colonic LN and splenic CD8+ T cells $p<0.01$,two-tailed Student's T test. There was no difference in the CD4+ T cells.

The early and persistent rise of IL10 followed by an even larger increase in IFNγ at 10 weeks PI resulted in a significantly decreased IFNγ/IL10 ratio within the CD8+ T cells at 4 weeks PI which returned to normal by 10 weeks PI (Figure 2.5a). This is in contrast to the pattern of viral tissue RNA levels seen in Figure 2.5b.

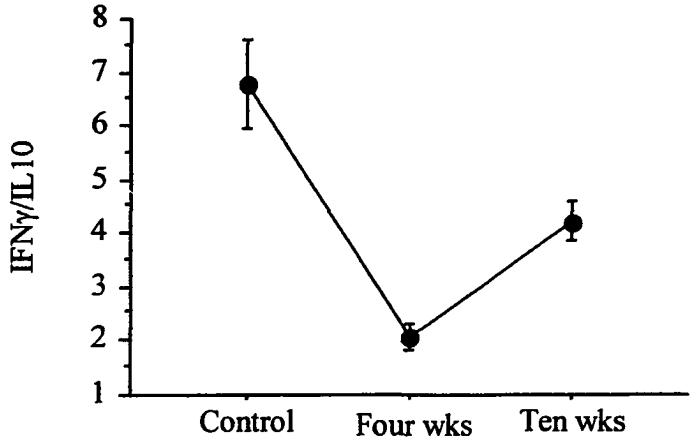

Figure 2.5a: The IFNγ/IL10 ratio decreases between 4 and 10 weeks post rectal inoculation with FIV in both colonic and splenic CD8+ T cells. Cytokine RNA was measured using a ribonuclease protection assay. There is a significant difference between Control and Four wks $p<0.01$ and between Four and Ten wks $p<0.001$, two-tailed Student's T test.

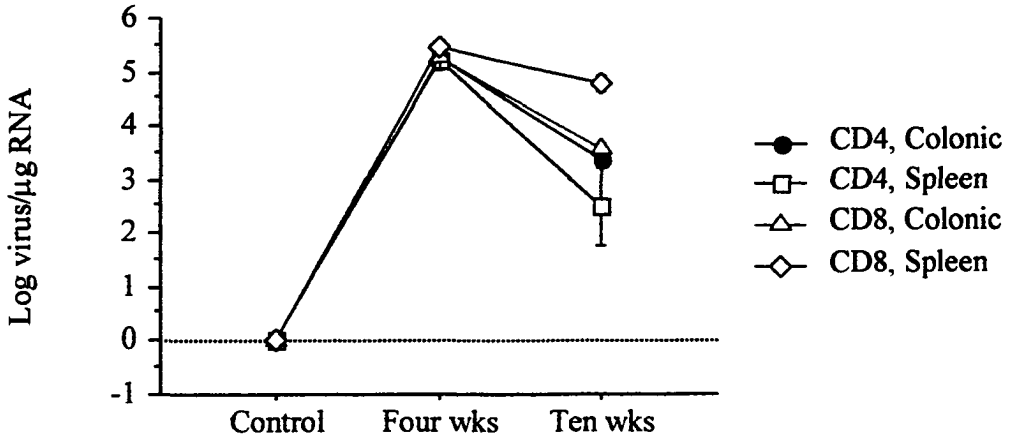

Figure 2.5b: Viral RNA levels decrease between 4 and 10 weeks post rectal inoculation with FIV in CD4 and CD8+ T cells in both the spleen and colonic lymph node. Virus was measured using QC-RT-PCR. There is a significant difference between Four and Ten wks in all populations. CD4 Colonic, CD4 Spleen, CD8 Colonic $p<0.001$, CD8 Spleen $p<0.05$, two-tailed Student's T test

Macrophages

Interleukin 6 levels were significantly increased in the splenic macrophages of FIV+ cats at week 10 PI (Figure 2.6a). Both TNFα and IL10 remained unchanged (not shown).

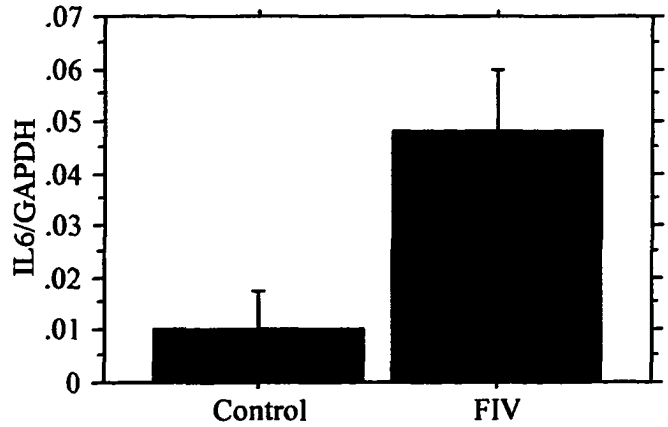

Figure 2.6a: IL6 RNA levels are increased in macrophages 10 weeks after rectal inoculation. Cytokine RNA was analyzed via RPA from adherence-purified splenic macrophages taken 10 weeks post infection with FIVB or sham inoculation. $p<0.05$, two-tailed Student's T test.

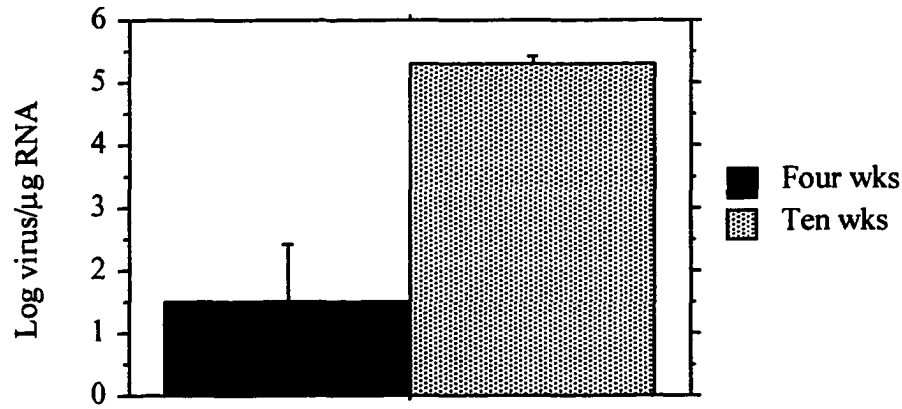

Figure 2.6b: Viral RNA levels increase in adherence-purified splenic macrophages between 4 and 10 weeks post FIV infection. Macrophage viral levels were measured by QC-RT-PCR. There is a significant difference between 4 and 10 weeks PI, $p<0.01$ two-tailed Student's T test.

Virus Levels

Viral RNA levels within the plasma of infected cats were quite high (7.7×10^5 to 4.7×10^6 viral copies/ml) at 4 weeks PI but significantly decreased by 10 weeks PI (Figure 2.7). This reduction over time was mirrored in the tissue CD4 and CD8+ T cell viral levels in both colonic lymph node and spleen (Figure 2.5b). At 10 weeks PI there was more viral RNA in the spleen than in the colonic lymph node (Figure 2.5b). Within the spleen, there was significantly more viral RNA in the CD8+ than in CD4+ T cell population (Figure 2.5b).

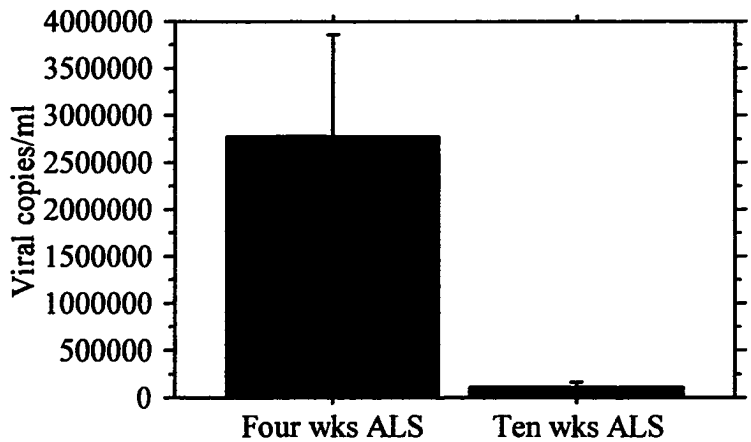

Figure 2.7: Plasma viral RNA levels decrease between 4 and 10 weeks post FIV infection. Plasma RNA levels were determined by quantitative competitive reverse transcriptase PCR (QC-RT-PCR) at four and ten weeks post rectal inoculation with FIVB. $p<0.05$, two-tailed Student's T test.

The pattern of viral RNA in splenic macrophages was opposite that in lymphocytes and plasma. Macrophage viral RNA levels significantly increased to very high levels between weeks 4 and 10 PI (Figure 2.6b). Interestingly, no viral antigen could be detected in the supernatants from any of the infected macrophage cultures (not shown).

Cytokines and viral levels

There were no significant correlations between cytokine levels and virus levels in infected CD4+ or CD8+ T cells. Macrophage IL6 levels were correlated with the amount of viral RNA within macrophages ($r=0.889$, $p<0.05$) (Figure 2.8).

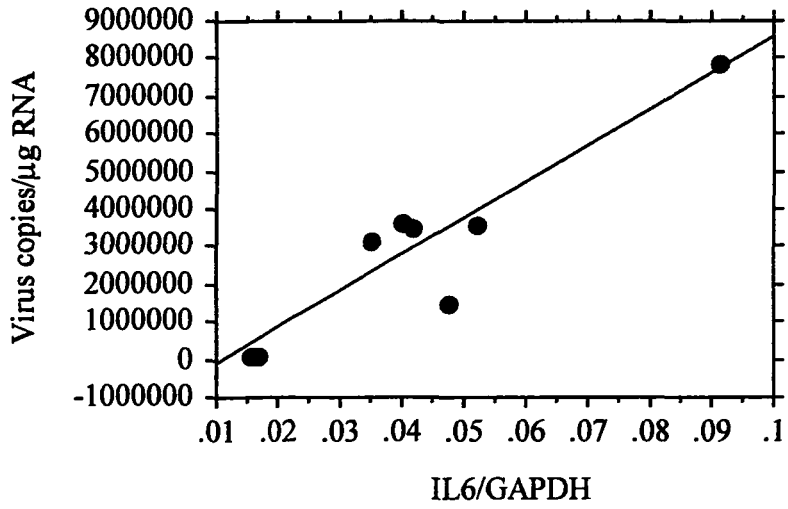

Figure 2.8: IL6 RNA levels correlate with viral RNA levels within the macrophages of the spleen at 4 and 10 weeks post rectal inoculation of FIVB. Macrophage viral levels were measured by QC-RT-PCR, cytokine message was analyzed via RPA. $R=0.889$ $p<0.05$

DISCUSSION

The rate of rectal FIVB2542 transmission was lower than would have been expected based on initial studies in our laboratory(23). The present study, along with concurrent work in this laboratory (24), have indicated that the true transmission rate is approximately 50%. This rate appeared to be independent of the tissue culture infectious dose (TCID) calculated with each supernatant pool. In addition, *in vivo* passage of the virus via the rectal route did not appear to increase the ability of the virus pool to cross the rectal mucosa. This implies that crossing the rectal mucosa does not in itself select for virus subtypes with altered tropism, as has been postulated for HIV-transmission (25) (26). The early viral stocks used to infect cats had been stored at -70° C for a period of 2

years. We generated two new viral stocks to guard against declining infectivity with storage yet saw no difference in rate of infection. Low-level or transient viremia has been seen in macaques rectally inoculated with SIV (27). We euthanized several exposed, PCR-negative cats and were unable to detect any evidence of a tissue virus reservoir. The intact rectal mucosa appears to provide a relatively effective barrier against retroviral transmission and the transmission rates seen in sexually exposed people likely reflect repeated exposures and/or minor mechanical trauma to the mucosa.

The drop in tissue viral levels within the lymphocyte subsets between weeks 4 and 10 PI mirrored the plasma viral pattern that we saw in this experiment and in previous work in this laboratory using the rectal route of infection (23). This would lead to the assumption that viral production within the CD4 and CD8+ T cells in the lymphoid tissue compartments directly contributes to the circulating pool of virus. While HIV can infect CD8+ T cells in some situations (28), CD8+ T cells are not considered one of the major cellular targets. FIV has been described as infecting CD8+ T cells yet this study documents for the first time that CD8+ T cell associated virus can actually exceed that of CD4+ T cell virus at certain times PI. Although FIV is known to infect B cells (29), the contribution of B lymphocytes to tissue virus levels was not examined in this study. The pattern of viral RNA levels in the macrophages was opposite to that seen in the lymphocytes. An earlier study of FIV infection utilizing RNA *in situ* hybridization has documented the progressive increase in macrophage-associated virus during the early weeks to months post-infection (30). Monocyte/macrophages have been implicated as a major source of persistent HIV infection particularly in those patients being treated with highly active anti-retroviral treatment (HAART) (31). The ability of the virus to maintain low level replication in the long-lived population of monocytes is thought to contribute to the escape of immune clearance (32). Despite the high levels of viral RNA that we documented in the adherence-purified macrophages, we were unable to detect viral antigen in the supernatants. There is precedent for the adherence-stimulated up-regulation of FIV viral RNA synthesis in macrophages without concurrent release of virus (33), suggesting that these cells may be a persistent source of low level virus infection. Chronically HIV-infected human promonocytic cell lines have also shown increased viral

replication with activation and differentiation to macrophages (34) (35). The ability of FIV to shift from the predominant infection of lymphocytes to macrophages may represent an *in vivo* evolution of tropism in order to avoid the apparent immune-mediated decrease in virus levels seen in the lymphocytes.

The general cytokine induction patterns seen in mitogen-stimulated naïve lymphoid cells mirrors that seen in other species. That feline CD4+ lymphocytes produce more IL2, IL4 and IL10 than CD8+ lymphocytes is consistent with data from human studies (36) (37). Detectable IL4 only in the colonic lymph node CD4 + T cells likely reflects the cytokine environment of the gastrointestinal mucosa. It has been shown that the intraepithelial lymphocytes of the murine large intestine express 6-10 fold more IL4 than those found in the small intestine and that IL4 is the predominant cytokine secreted in the large intestine(38).

The cytokine pattern at four weeks post FIV infection is one dominated by IL10 produced by lymphocytes in both the colonic lymph node and the spleen. IL10 was initially described as a factor secreted by murine Th2 cells that inhibited the production of IFNγ (39). It has since been shown to have a variety of inhibitory effects on activated macrophages including the inhibition of nitric oxide production (40), the reduction in the surface expression of MHC class II molecules (41) and CD80/CD86 costimulatory molecules (42). In addition to blocking IFNγ production, IL10 has also been shown to inhibit the production of IL12 and TNFα in monocytes (43) (44) (45). Dendritic cells have been shown to be the most potent initiators of an immune response and IL10 can decrease IL12 production and costimulatory molecule expression on these cells as well (46) (47).

IL10 has also been shown to inhibit the production of many chemokines including MIP-1α, MIP-1β and RANTES all of which have been shown to have anti-viral activity in HIV infection (48) (49). IL10 also upregulates the expression of CCR1, CCR2 and CCR5 on monocytes which have been shown to be co-receptors for macrophage tropic strains of HIV (50). There is evidence that FIV may be able to use CCR3 to enter cells

(51). The effect of IL10 on levels of feline CCR3 has not yet been investigated and how these chemokines play a role in controlling FIV infection remains to be elucidated.

Elevations of IL10 have been reported in HIV-infected patients in all stages of infection (52) (53). Many HIV+ people have demonstrable defects in proliferative recall responses to HIV as well as non-HIV proteins such as tetanus toxoid and influenza (54). The *in vitro* neutralization of IL10 restores the defective recall responses in patients who have between 200-500 CD4 cells/μl (55) indicating that IL10 is playing a role in the generalized dampening of the immune response in HIV.

In addition to Th1 and Th2 CD4+ T cells, other T cells with distinct cytokine secretion patterns have been described. A subset of lymphocytes that produce high levels of IL10 have been termed Tr1 cells (56) (57). Recent work provided the first evidence of pathogen-specific Tr1 cells that express large quantities of IL10 in a murine model of *Bordetella pertussis* infection (58). The pathogen-derived molecule was shown to specifically inhibit IL12 and augment IL10 production from dendritic cells, which then directed naïve T cells into the Tr1 subtype. Type 1 cytokine responses were inhibited by the increased levels of IL10 in this system. Circulating Tr1 cells have been demonstrated in HIV-infected people and the numbers of Tr1 cells were significantly higher in those patients with active replication or progressive disease (59). Although dendritic cells were not examined in the current work, this model for pathogen-specific skewing of dendritic cell cytokine production is an attractive explanation for the early IL10-dominated response in FIV infection.

The sum total of the above described effects of IL10 on monocyte/macrophages and dendritic cells would be to decrease their ability to direct a viral-specific cell-mediated immune response while potentially increasing their susceptibility to lentiviral infection. Any impaired ability to mount a cell-mediated immune response, shown to be critical for control of HIV and SIV replication (11), would be advantageous to the virus. The fact that we have detected a selective increase in IL10 during peak FIV replication provides

indirect evidence that FIV may exploit the immunosuppressive effects of IL10 to establish a persistent infection.

The cytokine response at 10 weeks post FIV infection evolves into what has been described as a type 0 response with elevations in both IL10 and IFNγ. A significant elevation in IFNγ was only documented in the CD8 positive population in both the colonic lymph node and spleen while both CD4 and CD8+ T cells maintained the elevated production of IL10. With the RPA methodology that we employed, it is not possible to discern whether individual CD8+ T cells produced both IL10 and IFNγ or whether the increase in IFNγ is a result of a separate, expanding population of viral effector cells. A technique such as intracellular staining for IFNγ and IL10 and subsequent flow cytometry for cell phenotype would be required to answer this question. A recent study examining IFNγ production in PBMC from HIV+ patients demonstrated that the increased IFNγ levels were found only in the CD8+ lymphocytes and, more specifically, in CD8+CD28- cells (60). Cells with this phenotype have been shown to be effective cytolytic cells in HIV (61) and cytomegalovirus infections (62). Increasing levels of CD8+ T cell IFNγ as FIV levels are dropping raises this possibility that viral-specific CTL are emerging between 4 and 10 weeks. This would be consistent with observations in SIV and HIV infections in which the initial control of viremia is correlated with the emergence of viral peptide specific CTL (11) (63) (12) (13).

At first glance, the concept of a single population of CD8+ T cells capable of producing both IFNγ and IL10 seems contradictory. Nevertheless, CD8+ T cells producing both cytokines have emerged as a distinct phenomenon in several infectious diseases. *Leishmania major* infection in mice provides a dramatic example of how disease outcome can be altered by the type of cytokine response generated (64). Even though Leishmania-resistant strains of mice generate a strong type cytokine response dominated by IFNγ and IL12, they are generally unable to completely clear the parasite (65) (66). In these persistent dermal lesions, up to 25% of the T cells secreting IFNγ are also secreting IL10 (67). The administration of anti-IL-10 receptor antibody to mice resulted in the sterile

cure of the parasite indicating that IL10 plays a significant role in preventing cure despite the concurrent presence of IFNγ (67). T cell clones producing both IFNγ and IL10 have also been documented in human infections with *Mycobacterium tuberculosis*(68) and *Borrelia burgdorferi* (69). The dual-producing clones are seen in the bronchoalveolar lavage fluid of pulmonary tuberculosis patients yet are not present in the peripheral blood suggesting that they are important at the site of active infection/inflammation (68). The production of both cytokines was shown to be IL12-dependent in the *B.burgdorferi* model system, consistent with the data showing that IL12 can stimulate CD4 and CD8+ T cells to produce both IFNγ and IL10 (70). IL10 has been shown to be a potent inhibitor of inflammatory responses and the concurrent presence of IL10 and IFNγ is thought to be one means by which the host prevents uncontrolled inflammation (71). This may be a cytokine pattern that intracellular pathogens such as FIV have evolved to exploit as a means of maintaining persistent, chronic infection.

While we cannot determine from our current data whether the delayed rise in CD8+ T cell production of IFNγ is due to an emerging population of virus effector cells or from a subset of T cells capable of producing both IL10 and IFNγ, the rise in IFNγ and subsequent restoration of the IFNγ:IL10 ratio is temporally associated with a decreasing viral load. It is becoming more apparent that it is the relative proportions of cytokines rather than the absolute production of type 1 or type 2 cytokines that influence disease progression. Recent work in HIV infected people has confirmed this principle by showing that the relative balance of CD4+ T cell production of IL10 vs. IFNγ correlates with active replication and progression of disease and that highly active anti-retroviral treatment (HAART) can decrease viral loads and shift the balance towards IFNγ production(59). Our findings in the FIV model corroborate these findings in that the balance of IL10 versus IFNγ shifted back to a more normal ratio in CD8+ T cells as viral levels came under initial host control.

The elevated IL6 seen at 10 weeks PI in the macrophages is a phenomenon described in many viral infections. IL6 has been shown to be elevated in HIV infection, (72) SIV (73) (74) and FIV (75) (76) infection. The *in vitro* infection of monocytes with HIV induces

IL6 production (77) which has been shown to be mediated by binding of the envelope glycoprotein 41 (gp41) (78). In addition, HIV infection has been shown to have a priming effect whereby increased amounts of IL6 are produced by subsequent ligation of the CD40 receptor (79). The intravenous infection of macaques with SIV has been shown to lead to a rapid rise in PBMC and peripheral lymph node levels of IL6 (73) (74) whereas people naturally infected with HIV via the mucosal route tend to have a more delayed increase associated with the transition into the chronic phase of infection (17).

IL6 has the ability to increase the production of HIV in chronically infected *ex vivo* monocytes and in macrophages infected *in vitro*(80). Despite the ability of IL6 to increase viral replication in monocytes, lymphocyte HIV replication appears to be unaffected (80). The increase in macrophage HIV production occurs at the post-transcriptional level, increasing HIV proteins and RT activity without the accumulation of viral RNA (80). The feline macrophage cultures that we examined at 10 weeks PI had very high levels of intracellular viral RNA but undetectable levels of viral antigen in the supernatants. The correlation between macrophage IL6 levels and macrophage viral RNA levels in our cats supports the notion that the two may be linked but, either IL6 acts to stimulate viral replication via a different mechanism in feline macrophages, or it is the infection of the macrophages that has induced high levels of IL6.

In summary, we have documented an early IL10-dominated cytokine response in both CD4 and CD8+ T cells during mucosal FIV infection. The IL10-dominated phase correlated with the period of peak viral replication. This cytokine response evolved to include concurrent increases in CD8+ T cell production of IFNγ, which was temporally related to decreasing lymphocyte and plasma virus levels. A delayed increase in macrophage IL6 production was associated with a shift to increased macrophage infectivity. Determining the functional significance of these cytokine changes on the documented downregulation of viral replication is a subject for further study. The apparent shift in cellular infection pattern from lymphocytes to macrophages introduces the possibility that FIV cellular tropism may be evolving during the acute immune response, a possibility that will be explored in future experiments.

REFERENCES

1. Pedersen, N.C., E.W. Ho, M.L. Brown, and J.K. Yamamoto. 1987. Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. *Science* 235:790.
2. Beebe, A.M., T.G. Faith, E.E. Sparger, M. Torten, N.C. Pedersen, and S. Dandekar. 1994. Evaluation of *in vivo* and *in vitro* interactions of feline immunodeficiency virus and feline leukemia virus. *AIDS* 8:873.
3. Beebe, A.M., N. Dua, T.G. Faith, P.F. Moore, N.C. Pedersen, and S. Dandekar. 1994. Primary stage of feline immunodeficiency virus infection: viral dissemination and cellular targets. *J Virol* 68, no. 5:3080.
4. Barlough, J.E., C.D. Ackley, J.W. George, N. Levy, R. Acevedo, P.F. Moore, B.A. Rideout, M.D. Cooper, and N.C. Pedersen. 1991. Acquired immune dysfunction in cats with experimentally induced feline immunodeficiency virus infection: Comparison of short-term and long-term infections. *Journal of Acquired Immune Deficiency Syndromes* 4:219.
5. Bach, J.M., M. Hurtrel, L. Chrakrabarti, J.-P. Ganiere, L. Montagnier, and B. Hurtrel. 1994. Early stages of feline immunodeficiency virus infection in lymph nodes and spleen. *AIDS Research and Human Retroviruses* 10:1731.
6. English, R.V., P. Nelson, C.M. Johnson, M. Nasisse, W.A. Tompkins, and M.B. Tompkins. 1994. Development of clinical disease in cats experimentally infected with feline immunodeficiency virus. *Journal of Infectious Diseases* 170:543.
7. Sparger, E.E., P.A. Luciw, J.H. Elder, J.K. Yamamoto, L.J. Lowenstine, and N.C. Pedersen. 1989. Feline immunodeficiency virus is a lentivirus associated with an AIDS-like disease in cats. *AIDS* 3:S43.
8. Sher, A., T. Gazzinelli, I. Oswald, M. Clerici, M. Kullberg, J. Berzofsky, T. Mossman, and G. Shearer. 1993. Role of T-Cell derived cytokines in the

downregulation of immune responses in parasitic and retroviral infection. *Immunological Reviews*:183.

9. Orange, J.S., S.F. Wolf, and C.A. Biron. 1994. Effects of IL-12 on the response and susceptibility to experimental viral infections. *Journal of Immunology* 152:1253.
10. Berger, D.P., Homann.D., and M.B.A. Oldstone. 2000. Defining parameters for successful immunocytotherapy of persistent viral infection. *Virology* 266:257.
11. Kuroda, M.J., J.E. Schmitz, W.A. Charini, C.E. Nickerson, M.A. Lifton, C.I. Lord, M.A. Forman, and N.L. Letvin. 1999. Emergence of CTL coincides with clearance of virus during primary simian immunodeficiency virus infection in rhesus monkeys. *Journal of Immunology* 162:5127.
12. Ogg, G.S., S. Kostense, M.R. Klein, S. Jurriaans, D. Hamann, A.J. McMichael, and F. Miedema. 1999. Longitudinal phenotypic analysis of human immunodeficiency virus type 1-specific cytotoxic T lymphocytes: correlation with disease progression. *Journal of Virology* 73, no. 11:9153.
13. Wilson, J.D., G.S. Ogg, R.L. Allen, C. Davis, S. Shaunak, J. Downie, W. Dyer, C. Workman, S. Sullivan, A.J. McMichael, and S.L. Rowland-Jones. 2000. Direct visualization of HIV-1-specific cytotoxic T lymphocytes during primary infection. *AIDS* 14, no. 3:225.
14. Clerici, M., and G.M. Shearer. 1993. A TH1-->TH2 switch is a critical step in the etiology of HIV infection. *Immunology Today* 14:107.
15. Barcellini, W., G. Rizzardi, M. Borghi, C. Fain, A. Lazzarin, and P. Meroni. 1994. Th1 and TH2 cytokine production by peripheral blood mononuclear cells from HIV-infected patients. *AIDS* 8:757.
16. Meyaard, L., S. Otto, I. Keet, R. van Lier, and F. Miedema. 1994. Changes in cytokine secretion patterns of CD4+ T-cell clones in human immunodeficiency virus infection. *Blood* 84:4262.
17. Graziosi, C., G. Pantaleo, K.R. Gantt, J.-P. Fortin, J.F. Demarest, O.J. Cohen, R.P. Sékaly, and A.S. Fauci. 1994. Lack of evidence for the dichotomy of T_H1 and T_H2 predominance in HIV-infected individuals. *Science* 265:248.

18. Romagnani, S., E. Maggi, and G. Del Prete. 1994. An alternative view of the Th1/Th2 switch hypothesis in HIV infection. *AIDS Research and Human Retroviruses* 10:iii.

19. O'Neil, L.L., M.J. Burkhard, L.J. Diehl, and E.A. Hoover. 1995. Vertical transmission of feline immunodeficiency virus. *Semin Vet Med Surg (Small Anim)* 10, no. 4:266.

20. Diehl, L.J., C.K. Mathiason-DuBard, L.L. O'Neil, and E.A. Hoover. 1996. Plasma viral RNA load predicts disease progression in an accelerated feline immunodeficiency virus model. *J Virol* 70:2503.

21. Diehl, L.J., C.K. Mathiason-DuBard, L.L. O'Neil, and E.A. Hoover. 1995. Longitudinal assessment of feline immunodeficiency virus kinetics in plasma by use of a quantitative competitive reverse transcriptase PCR. *J Virol* 69:2328.

22. Dreitz, M.J., S.W. Dow, S.A. Fiscus, and E.A. Hoover. 1995. Development of monoclonal antibodies and capture immunoassays for feline immunodeficiency virus. *Am J Vet Res* 56, no. 6:764.

23. Burkhard, M.J., L.A. Obert, L.L. O'Neil, L.J. Diehl, and E.A. Hoover. 1997. Mucosal transmission of cell-associated and cell-free feline immunodeficiency virus. *AIDS Res Hum Retroviruses* 13, no. 4:347.

24. Obert, L.A., and E.A. Hoover. 2000. Relationship of lymphoid lesions to disease course in mucosal feline immunodeficiency virus type C infection [In Process Citation]. *Vet Pathol* 37, no. 5:386.

25. Berger, E.A., P.M. Murphy, and J.M. Farber. 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annual Reviews of Immunology* 17:657.

26. Miller, C.J. 1998. Host and viral factors influencing heterosexual HIV transmission. *Reviews in Reproduction* 3, no. 1:42.

27. Trivedi, P., D. Horejsh, S.B. Hinds, P.W. Hinds, M.S. Wu, M.S. Salvato, and C.D. Pauza. 1994. Intrarectal transmission od simian immunodeficiency virus in macaques:selective amplification and host responses to transient or persistent viremia. *Journal of Virology* 70, no. 10:6876.

28. Imlach, S., S. McBreen, T. Shirafuji, C. Leen, J.E. Bell, and P. Simmonds. 2001. Activated peripheral CD8 lymphocytes express CD4 *in vivo* and are targets for infection with human immunodeficiency virus type 1. *Journal of Virology* 75, no. 23:11555.

29. English, R.V., C.M. Johnson, D.H. Gebhard, and M.B. Tompkins. 1993. *In vivo* lymphocyte tropism of feline immunodeficiency virus. *Journal of Virology* 67:5175.

30. Beebe, A.M., N. Dua, T.G. Faith, P.F. Moore, N.C. Pedersen, and S. Dandekar. 1994. Primary stage of feline immunodeficiency virus infection: Viral dissemination and cellular targets. *Journal of Virology* 68:3080.

31. Perelson, A., S., P. Essenger, Y. Cao, M. Vesanen, A. Hurley, K. Sakseal, M. Markowitz, and D.D. Ho. 1997. Decay characteristics of HIV-1 infected compartments during combination therapy. *Nature* 387, no. 188-191.

32. Schutten, M., C.A. van Baalen, C. Guillon, R.C. Huisman, P.H. Boers, K. Sintnicolaas, R.A. Gruters, and A.D. Osterhaus. 2001. Macrophage tropism of human immunodeficiency virus type 1 facilitates *in vivo* escape from cytotoxic T-lymphocyte pressure. *Journal of Virology* 75, no. 6:2706.

33. Dow, S.W., C.K. Mathiason, and E.A. Hoover. 1999. *In vivo* monocyte tropism of pathogenic feline immunodeficiency viruses. *J Virol* 73, no. 8:6852.

34. Goletti, D., A.L. Kinter, P. Biswas, S.M. Bende, G. Ploi, and A.S. Fauci. 1995. Effect of cellular differentiation on cytokine-induced expression of human immunodeficiency virus in chronically infected promonocytic cells; dissociation of cellular differentiation and viral expression. *Journal of Virology* 69:2540.

35. Folks, T.M., J. Justement, A. Kinter, C.A. Dinarello, and A.S. Fauci. 1987. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. *Science* 238:800.

36. Hagiwara, E., F. Abbasi, G. Mor, Y. Ishigatsubo, and D.M. Klinman. 1995. Phenotype and frequency of cells secreting IL-2,IL-4,IL-6,IL1-,IFN and TNF-alpha in human peripheral blood. *Cytokine* 7, no. 8:815.

37. Garcia Vela, J.A., I. Delgado, R. Bornstein, B. Ivarez, M.C. Auray, I. Martin, F. Ona, and F. Gilsanz. 2000. Comparative intracellular cytokine production by *in*

vitro stimulated T lymphocytes from human umbilical cord blood (HUCB) and adult peripheral blood (APB). *Analytical Cellular Pathology* 20, no. 2-3:93.

38. Beagley, K.W., K. fujihashi, A.S. Lagoo, S. Lagoo-Deenadaylan, C.A. Black, A.M. Murray, A.T. Sharmanov, M. Yamamoto, J.R. McGhee, C.O. Elson, and H. Kiyono. 1995. Differences in intraepithelial lymphocyte T cell subsets isolated from murine small versus large intestine. *Journal of Immunology* 154, no. 11:5611.
39. Fiorentino, D., A. Zlotnik, P. Vieira, T. Mosmann, M. Howard, K. Moore, and A. O'Garra. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *Journal of Immunology* 146:3444.
40. Gazzinelli, R.T., I.P. Oswald, S.L. James, and A. Sher. 1992. IL-10 inhibits parasite killing and nitric oxide production by IFNγ-activated macrophages. *Journal of Immunology* 148:1792.
41. de Waal Malefyt, R., J. Haanen, H. Spits, M. Roncarlo, A. te Velde, C. Figdor, K. Johnson, R. Kastelein, H. Yssel, and J.E. de Vries. 1991. Interleukin 10 and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of moncytes via downregulation of class II major histocompatibility complex expression. *The Journal of Experimental Medicine* 174:915.
42. Ding, L., P.S. Linsley, L. Huang, R.N. Germain, and E.M. Shevach. 1993. Il-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. *Journal of Immunology* 151:1224.
43. Ralph, P., I. Nakoinz, A. Sampson-Johannes, S. Fong, D. Lowe, H.Y. Min, and L. Lin. 1992. IL10; T lymphocyte inhibitor of human blood cell production of IL-1 and tumor necrosis factor. *Journal of Immunology* 148:808.
44. Fiorentino, D., A. Zlotnick, T. Mosmann, M.H. Howard, and A. O'Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. *Journal of Immunology* 147:3815.
45. D'Andrea, A., M. Aste-Amezaga, N.M. Valiente, X. Ma, M. Kubin, and G. Trinchieri. 1993. Interleukin-10 inhibits human lymphocyte IFN-γ production by

suppressing interleukin-12 synthesis in accessory cells. *The Journal of Experimental Medicine* 178:1041.

46. Mitra, R.S., T.A. Judge, F.O. Nestle, L.A. Turka, and B.J. Nickoloff. 1995. Psoriatic skin-derived dendritic cell function is inhibited by exogenous IL-10. Differential modulation of B7-1 and B7-2. *Journal of Immunology* 154:2668.

47. Buelens, C., F. Willems, G. Pierard, A. Delvaux, T. Velu, and M. Goldman. 1995. IL-10 inhibits the primary allogeneic T cell response to human peripheral blood dendritic cells. *Advances in Experimental Medicine and Biology* 378:363.

48. Berkman, N., M. John, G. Roesems, P.J. Jose, P.J. Barnes, and K.F. Chung. 1995. Inhibition of macrophage inflammatory protein-1 alpha expression by IL-10. Differential sensitivities in human blood monocytes and alveolar macrophages. *Journal of Immunology* 155:4412.

49. Marfaing-Koka, A., M. Maravic, M. Humbert, P. Galanaud, and D. Emilie. 1996. Contrasting effects of IL4, IL10 and corticosteroids on RANTES production by human monocytes. *International Immunology* 8:1587.

50. Sozzani, S., S. Ghezzi, G. Iannolo, W. Luini, A. Borsatti, N. Polentarutti, A. Sica, M. Locati, C. Mackay, T.N. Wells, P. Biswas, E. Vicenzi, G. Poli, and A. Mantovani. 1998. Interleukin 10 increases CCR5 expression and HIV infection in human monocytes. *The Journal of Experimental Medicine* 187:439.

51. Johnston, J., and C. Power. 1999. Productive infection of human peripheral blood mononuclear cells by feline immunodeficiency virus: implications for vector development. *Journal of Virology* 73, no. 3:2491.

52. Meroni, L., D. Trabattoni, C. Balotta, C. Riva, A. Gori, M. Moroni, M. Villa, M. Clerici, and M. Galli. 1995. Evidence for type 2 cytokine production and lymphocyte activation in the early pahses of HIV-1 infection. *AIDS* 10:23.

53. Stylianou, E., P. Aukrust, D. Kvale, F. Muller, and S.S. Froland. 1999. IL-10 in HIV infection: increasing serum IL-10 levels with disease progression-down-regulatory effect of potent anti-retroviral therapy. *Clinical and Experimental Immunology* 116:115.

54. Clerici, M., N.I. Stocks, R.A. Zajec, R.N. Boswell, D.R. Lucey, C.S. Via, and G.M. Shearer. 1989. detection of three distinct patterns of T helper cell

dysfunction in asymptomatic human immunodeficiency virus-seropositive patients. *Journal of Clinical Investigation* 84:1892.

55. Landay, A.L., M. Clerici, F. Hashemi, H. Kessler, J.A. Berzofsky, and G.M. Shearer. 1996. *In vitro* restoration of T cell immune function in human immunodeficiency virus-positive persons: effects of interleukin-12 and anti-IL10. *The Journal of Infectious Diseases* 173:1085.

56. Roncarlo, M., R. Bacchetta, C. Bordignon, S. Narula, and M.K. Levings. 2001. Type 1 T regulatory cells. *Immunological Reviews* 182:68.

57. Groux, H. 2001. An overview of regulatory T cells. *Microbes and Infection* 3, no. 11:883.

58. McGuirk, P., C. McCann, and K.H.G. Mills. 2002. Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates IL10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by Bordetella pertussis. *The Journal of Experimental Medicine* 195, no. 2:221.

59. Ostrowski, M.A., J.X. Gu, C. Kovacs, J. Freedman, M.A. Luscher, and K.S. MacDonald. 2001. Quantitative and qualitiative assessment of human immunodeficiency virus type 1-specific CD4+ T cell immunity to gag in HIV-1-infected individuals with differential disease progression: reciprocal $I\square N\gamma$ and interleukin-10 responses. *The Journal of Infectious Diseases* 184:1268.

60. Caruso, A., S. Licenziati, A.D. Canaris, A. Cantalamessa, M. Corulli, B. Benzoni, L. Peroni, A. Balsari, and A. Turano. 1996. Characterization of t cell subsets involved in the production of IFN-γ in asymptomatic HIV-infected patients. *AIDS Research and Human Reteroviruses* 12, no. 2:135.

61. Ho, H.N., L.E. Hultin, R.T. Mitsuyasu, J.L. Matud, M.A. Hausner, D. Bockstoce, C.C. Chou, S. O'Rourke, J.M.G. Taylor, and J.V. Giorgi. 1993. Circulating HIV-specific CD8+ cytotoxic T cells express CD38 and HLA-DR antigens. *Journal of Immunology* 150:3070.

62. McFarland, H.I., S.R. Nahill, J.W. Maciaszek, and R.M. Welsh. 1992. CD11b (Mac-1): A marker for CD8+ cytotoxic T cell activation and memory in virus infection. *Journal of Immunology* 149:1326.

63. Borrow, P., H. Lewicki, B.H. Hahn, G.M. Shaw, and M.B.A. Oldstone. 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *Journal of Virology* 68:6103.

64. Heinzel, F.P., M.D. Sadick, B.J. Holaday, R.L. Coffman, and R.M. Locksley. 1989. Reciprocal expression of interferon-gamma or interleukin 4 during resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *The Journal of Experimental Medicine* 169, no. 1:59.

65. Aebischer, T., S.F. Moody, and E. Handman. 1993. Persistence of virulent Leishmania major in murine cutaneous leishmaniasis: a possible hazard for the host. *Infection and Immunity* 61:220.

66. Bogdan, C., N. Donhauser, R. Doring, M. Rollighoff, A. Diefenbach, and M.G. Rittig. 2000. Fibroblasts as host cells in latent leishmaniosis. *The Journal of Experimental Medicine* 191:2121.

67. Belkaid, Y., K.F. Hoffman, S. Mendez, S. Kamhavi, T.A. Wynn, and D. Sacks. 2001. The role of intereukin-10 in the persistence of Leishmania major in the skin after healing and the therapeutic potential of anti-IL-10 recpetor antibody. *The Journal of Experimental Medicine* 194:1497.

68. Gerosa, F., S. Nisii, S. Righetti, R. Micciolo, A. Marchesini, A. Cazzadori, and G. Trinchieri. 1999. CD4+ T cell clones producing both interferon-gamma and interleukin-10 predominate in the bronchoalveolar lavages of active pulmonary tuberculosis patients. *Clinical Immunology* 92:224.

69. Pohl-Koppe, A., K.E. Balashov, A.C. Steere, E.L. Logigian, and D.A. Hafler. 1998. Identification of a T cell subset capable of both IFN-gamma and IL10 secretion in patients with chronic *Borrelia burgdorferi* infection. *Journal of Immunology* 160:1804.

70. Gerosa F, P.C., Peritt D, Paiola F, Scupoli MT, Aste-Amezaga M, Frank I, Trinchieri G. 1996. Interleukin-12 primes human CD4 and CD8 T cell clones for high prodution of both interferon-gamma and interleukin-10. *Journal of Experimantal Medicine* 183, no. 6:2559.

71. Trinchieri, G. 2001. regulatory role of T cells producing both interferon γ and interleukin 10 in persistent infection. *The Journal of Experimental Medicine* 194, no. 10:F53.

72. Breen, E.C., A.R. Rezai, K. Nakajima, G.N. Beall, R.T. Mitsuyasu, T. Hirano, T. Kishimoto, and O. Martinez-Maza. 1990. Infection with HIV is associated with elevated IL-6 levels and production. *Journal of Immunology* 144:480.

73. Cheret, A., R. LeGrand, P. Caufour, N. Deeuddre-Bosquet, F. Matheux, O. Neildez, F. Theodoro, N. Maestrali, O. Beneviste, B. Vaslin, and D. Dormont. 1996. Cytokine mRNA expression in mononuclear cells from different tissues during acute SIVmac251 infection of macaques. *AIDS Research and Human Retroviruses* 12, no. 13:1263.

74. Khatissian, E., L. Chakrabati, and B. Hurtrel. 1996. Cytokine patterns and viral load in lymph nodes during the early stages of SIV infection. *Research in Virology* 147, no. 2-3:181.

75. Ohashi, T., R. Goitsuka, T. Watari, H. Tsujimoto, and A. Hasegawa. 1992. Elevation of feline interleukin 6-like activity in frline immunodeficiency virus infection. *Clinical Immunology and Immunopathology* 65, no. 3:207.

76. Lawrence, C.E., J.J. Callanan, B.J. Willett, and O. Jarrett. 1995. Cytokine production by cats infected with feline immunodeficiency virus:a longitudinal study. *Immunology* 85:568.

77. Nakajima, K., O. Martinez-Maza, T. Hirano, E.C. Breen, P.G. Nishanain, J.F. Salazar-Gonzalez, J.L. Fahey, and T. Kishimoto. 1989. Induction of IL-6 production by HIV. *Journal of Immunology* 142:531.

78. Takeshita, S., E.C. Breen, M. Ivashchenko, P.G. Nishanain, T. Kishimoto, D.L. Vredevoe, and O. Martinez-Maza. 1995. Induction of IL-6 and IL-10 production by recombinant HIV-1 envelope glycoprotein 41 in the THP-1 human monocytic cell line. *Cellular Immunology* 165, no. 2:234.

79. Bergamini, A., F. Bolacchi, B. Bongiovanni, V. Colizzi, G. Cappelli, I. Uccella, M. Cepparulo, M. Capozzi, G. Mancino, and G. Rocchi. 2000. Human immunodeficiency virus type 1 infection modulates the interleukin-1beta and IL-6

responses of human macrophages to CD40 ligand stimulation. *The Journal of Infectious Diseases* 182, no. 3:776.

80. Poli, G., P. Bressler, A. Kinter, W.C. Timmer, A. Rabson, J.S. Justernet, S. Stanley, and A.S. Fauci. 1990. Interleukin 6 induces human immunodeficiency virus expression in infected monocytic cells alons and in synergy with tumor necrosis factor a by transcriptional and post-translational mechanisms. *The Journal of Experimental Medicine* 172, no. 151-158.

CHAPTER THREE

THE EFFECT OF RECOMBINANT INTERLEUKIN 12 ON INITIAL CONTROL OF FIV INFECTION

INTRODUCTION

Interleukin 12 (IL12), initially identified as a factor secreted by Epstein-Barr virus transformed B cells, has subsequently been shown to be one of the most potent stimulators of the cell-mediated immune response (1) (2) (3). IL12 initiates a type 1 cytokine response via the induction of interferon gamma (IFNγ) from T cells and natural killer cells (NK) thereby augmenting cell-mediated cytotoxicity (4). IL12 plays a crucial role in the development of specific immunity to many intracellular pathogens, including *Toxoplasma gondii* (5), *Listeria monocytogenes* (6), *Leishmania major* (7) and *Mycobacterium tuberculosis* (8).

The role of IL12 in lentiviral infections is less well defined but there is accumulating evidence that defective production of IL12 may play a role in establishing and maintaining long term infection. Impaired production of IL12 has been documented in HIV-infected people and the *in vitro* addition of IL12 can restore HIV-specific cell-mediated immune responses in these patients (9) (10) (11). In addition, the co-administration of IL12 with various vaccine candidates, has been shown to enhance the cell-mediated immune response to HIV antigens (12). These data suggest that the early addition of IL12 may be beneficial in host control of lentiviral replication.

The goals in this study were to determine whether the currently available recombinant IL12s (murine and human) have cross-reactive activity in feline cells and whether the early administration of IL12 could alter the replication kinetics of FIV infection. Our previous documentation of a strong IL10-dominated immune response during peak replication of FIV (P. Avery, unpublished data) provided us a relevant system to explore the effects of early type 1 cytokine intervention.

MATERIALS AND METHODS

Recombinant Interleukin 12

Recombinant murine and human IL12 were kindly provided by the Dr. J. Sypek (The Genetics Institute, Andover, Mass). A total of 20 μg of murine and 40 μg of human IL12 were stored at –70° C until use.

Adenoviruses

Replication-defective adenoviruses were kindly provided by Dr. Frank Graham (AdMEM12R and AdCA35, McMaster University, Ontario, Canada), Dr. Savio Woo (Adv.mIL12, Mount Sinai School of Medicine, New York, NY) and Dr. Jesus Prieto (RAdIL12 and AdLacZ, University of Navarra, Pamplona, Spain). The plaque-purified viral stocks were stored at –70° C until use. All three vectors have deletions of the E1 and E3 region of the adenovirus where the p35 and p40 subunits of murine IL12 have been inserted. Both of the vectors AdMEM12R and RAdIL12 are under the influence of the murine cytomegalovirus (CMV) promoter while Adv.mIL12 utilizes the Rous sarcoma virus (RSV) long terminal repeat promoter. AdCA35 and AdLacZ are identical to the IL12-expressing vectors from their respective laboratories except the LacZ gene is inserted in place of the IL12 p35 and p40 genes.

FIV inoculum

Cell-free cell culture virus inoculum (200 $TCID_{50}$/ml) was generated by the co-culture of naïve feline PBMC with PBMC from a cat acutely infected with FIV-B 2542 via the intravenous route. Infectivity of the supernatants was determined via titration and aliquots were stored in liquid nitrogen. This stock of virus was shown to infect 4/4 cats when 1 ml was administered oral-nasally.

Cells and cell lines

Feline peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation (Histopaque 1077, Sigma, St. Louis, MO). The cells were cultured at a concentration of 4 x 10^6 cells/ml in lymphocyte culture medium (LBT) composed of RPMI 1640 supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT), 2% glutamine, 1% penicillin/streptomycin and 5 μg/ml concanavillin A (ConA). Crandell feline kidney cell line (CrFK) cells (13) were cultured in 6 well multiwell plates (Becton Dickinson, Franklin Lakes, NJ) in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Feline lymphocyte endothelial cell line (FLE) was derived in this laboratory from adherent cells within a feline lymph node. These cells were also cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. 293 cells (transformed human embryonic kidney cells) (ATCC, Manassas, VA) were cultured at 3 x 10^6 cells/ml in Joklik-modified minimum essential medium (Gibco BRL, Grand island, NY) with 10% fetal bovine serum and 1% penicillin/streptomycin.

FIV antigen ELISA

Productive *in vitro* infection was assessed by capsid antigen (p26) capture ELISA described by Dreitz et al (14) performed on macrophage/PBMC supernatants. Optical densities (OD), measured by absorbance at A_{450}, were recorded using a Dynatech 5000MRTM microplate reader (Dynatech Corp., Chantilly, VA). Positive reactions were defined as those with a minimum OD of 0.1 and at least twice that of negative control supernatants run in parallel.

Reverse Transcriptase Activity

Reverse transcriptase (RT) activity was measured using the protocol of Goldstein et al (15) in 96 well plates. Fifteen microliters of clarified supernatant were used in each reaction and incorporated ^{32}P was measured with a Microbeta plate reader (Wallac).

Nitric oxide detection

Tissue culture supernatants or serum samples were evaluated for the presence of NO_2- as previously described (16). Briefly, samples were mixed with an equal volume of Griess reagent (Sigma, St. Louis, MO) and incubated at room temperature for 15 minutes. Plates were read at A_{550} in an automatic plate reader (Molecular Devices). $NaNO_2$ was used to generate a standard curve.

IL-12 induced proliferation

Feline PBMC were cultured in 96 well plates at a concentration of 2 x 10^6 cells/ml in 200 µL of LBT media supplemented with 5 µg/ml ConA for 48 hrs. Recombinant mIL12 or hIL12 was added at a concentration of 0, 0.5, 2,5, 10 or 20 U/ml for an additional 12 hrs. Each well was pulsed with 10 µCi of tritiated thymidine (NEN), and incubated for an additional 12 hrs. The plates were harvested onto membranes using a Microbeta Harvestor (Wallac) and incorporated thymidine was detected using Microbeta Microsoft Workstation 2.6 software (Wallac) and expressed as counts per minute (CPM).

***In vitro* induction of cytokines**

Feline PBMC were cultured in six well plates at a concentration of 4 x 10^6 cells/ml in LBT supplemented with 5 µg/ml ConA for 48 hrs. Recombinant murine IL12 was added at concentrations of 0, 5 or 10 U/ml and the cells were cultured for an additional 12-24 hrs. The non-adherent cells were removed and washed once with 10 mls sterile phosphate-buffered saline (PBS). One ml of Trizol™ (Invitrogen, Carlsbad, CA) was

added to the culture wells and pipetted up and down several times. The same Trizol was then used to lyse the pelleted cells. The cellular lysates were stored at -70^{o} C until further processing according to the manufacturer's instructions. RNA was quantified via spectrophotometer and 2 μg aliquots were lyophilized in a Speed Vac concentrator (Savant, Farmingdale, NY).

In vivo administration of recombinant mIL12

Four 6 month old female cats were inoculated intraperitoneally with 0, 1.0, 2.5 or 5 μg of recombinant mIL12 in a total volume of 200 μL of sterile PBS. The cats were anesthetized with subcutaneous ketamine/acepromazine and received the injections every other day for a total of 4 doses. Blood was collected via jugular venipuncture every 2 days for the first 8 days and every 5 days thereafter. The cats receiving 0 and 2.5 μg of mIL12 were euthanized at day 8 post initiation whereas the cats receiving 1 and 5 μg of mIL12 were euthanized at day 30 post initiation. Blood, spleen, colonic and mesenteric lymph nodes were collected at necropsy.

In vitro adenoviral infection

Cell lines were cultured as described above. When cells were approximately 80% confluent, the culture media was taken off and adenovirus at a multiplicity of infection of 30 (MOI) was added in 500 μL of PBS. The plates were incubated at room temperature with periodic rocking for 30 minutes at which point fresh media was added and the plates were returned to 37^{o} C. Cell culture supernatants were collected at 24 and 48 hours post inoculation.

ELISA for murine IL12

MaxiSorp^{TM} 96 well plates (Nalge Nunc International, Denmark) were incubated for 12 hrs at 4 C with 8 μg/ml of the anti-mouse IL12 antibody G297-289 (BD PharMingen, Torrey Pines, CA) in 100 μL of 0.1 M sodium bicarbonate. The plates were then washed

8 times in an Auto-strip plate washer (Bio-Tek Instruments, Winooski, VT). 200 μL of PBS with 10% goat serum was added and the plates were incubated at room temperature (RT) for 4 hrs. The contents of the plates were emptied over a sink and 100 μL of the samples was added. The plates were then incubated at 4° C for 12 hrs after which they were washed again 8 times. The secondary biotinylated anti-IL12 antibody C17.8 (BD PharMingen) was added at a concentration of 0.5 μg/ml and incubated at RT for 45 minutes. The plates were washed an additional 8 times and streptavidin-horse radish peroxidase (BD PharMingen) was added at a concentration of 0.5 μg/ml for an additional 30 minutes at RT. Plates were washed for a final 8 times and equal portions of TMB peroxidase substrate and peroxidase solution (Kirkegaard and Perry, Gaithersburg, MD) were added for 10 minutes at which point the reaction was stopped by adding 100 μL of 0.1 M phosphoric acid. Optical densities were determined on a plate reader at a wavelength of 450 nm (DynaTech, Chantilly, VA). A standard curve was generated using 2 fold dilutions of recombinant murine IL12 (BD Pharmingen) starting with 5 ng/ml.

Adenoviral supernatant stimulation of feline PBMC

Feline PBMC were cultured at a concentration of 4 x 10^6 cells/ml in LBT media supplemented with 5 μg/ml ConA for 48 hrs. 10, 50 or 100 μL of supernatants from adenovirally infected CrFK cells was added to the PBMC and they were cultured for an additional 24 hrs. Both adherent and non-adherent cells were lysed with Trizol™ and the lysates were stored at –70° C until further processing via the manufacturers instructions.

***In vitro* effects of adenoviral IL12 on FIV replication**

Feline macrophages were purified from 4 x 10^6 cells/ml of PBMC by adherence for 1 hr at 37° C. The adherent cells were cultured for an additional 2 days in DMEM supplemented with 10% fetal bovine serum, penicillin/streptomycin and 100 U/ml human granulocyte-monocyte colony stimulating factor (GMCSF). The plates were washed two times with PBS and infected with either AdMEMR12 or AdLacZ at a MOI of 10 and 30.

The macrophages were incubated for an additional 12 hrs at which point, fresh, autologous, non-adherent PBMC were added at a concentration of 2 x 10^6 cells/ml and the cells were subsequently cultured in LBT media without GMCSF. 24 hrs later, FIVB2452 cell-free supernatants were added to the cultures at a dilution of 1:64 or 1:512 based on previous titration assays. Wells cultured with virus-free media served as negative controls. After 24 hrs, cells were washed 1x with PBS to remove any remaining virus and supernatants were monitored every two days for levels of mIL12, p26 antigen (14), reverse transcriptase (RT) activity and cell viability was monitored via trypan blue exclusion.

Adenoviral infection of cats

One cat each was inoculated oral-nasally with 1 x 10^{10} plaque forming units (pfus), 1 x 10^9 pfus or 1 x 10^8 pfus of RAdIL12 in 500 μL PBS. An additional cat was inoculated with 1 x 10^9 pfus AdLacZ. Blood was collected at 24 and 48 hrs PI and spleen and retropharyngeal lymph nodes were collected at necropsy at 48 hrs PI.

Three cats were inoculated intraperitoneally with 1 x 10^9 pfus of AdMEM12R in 100 μL of PBS while one cat was inoculated with 1 x 10^9 pfus AdLacZ. Blood was collected at 24, 48 and 72 hrs PI for serum determination of mIL12 as well as complete blood counts (CBC).

Three cats were inoculated with 1 x 10^9 pfus AdMEM12R via the intraperitoneal route and two cats were inoculated with 1 x 10^9 pfus AdMEME12R via the oral-nasal route. An additional three control cats received 1 x 10^9 pfus Ad LacZ intraperitoneally and two cats received 1 x 10^9 pfus oral-nasally. All cats were bled at 6 and 12 hrs PI and one cat each from the AdIL12 group and AdLacZ group was bled and necropsied at 12 and 24 hrs PI. Two additional cats from each intraperitoneal group were bled and necropsied at 48 hrs PI. Tonsil, spleen and colonic lymph nodes were collected from all cats and snap frozen in liquid nitrogen.

Three cats were inoculated with 1 x 10^9 pfus AdMEM12R and three control cats were inoculated with 1 x 10^9 pfus AdLacZ via the intraperitoneal route. Twenty-four hrs later, all cats were challenged oral-nasally with 0.8 mls of FIVB2542 cell-free supernatant (300 TCID). The cats were bled every 4 days for the first 4 weeks of the study to examine serum levels of mIL12 and to determine the presence of pro-viral DNA. Plasma was frozen at –70 C from each time-point for viral RNA quantification. Complete blood counts were performed every 8 days to monitor hematologic parameters.

RESULTS

In vitro effects of recombinant IL12

Proliferation

Both recombinant murine and human IL12 augmented ConA-induced proliferation in both feline PBMC and lymph node cells (Figure 3.1). There was a dose-related increase in proliferation up to 2.5 U of IL12/ml.

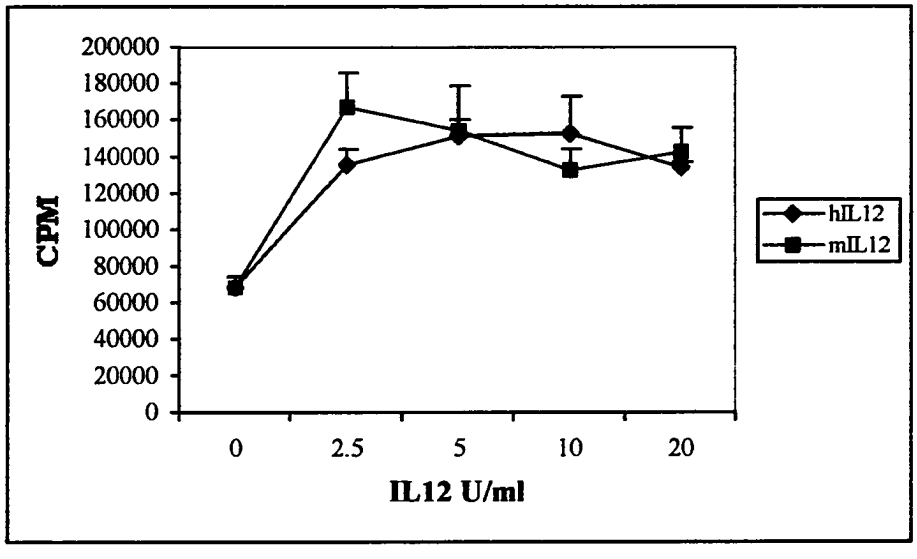

Figure 3.1: Recombinant human and murine IL12 augment proliferation in ConA-primed feline PBMC. Cells were stimulated for 48 hrs with 5 μg/ml ConA followed by 12 hrs with rIL12.

Cytokine production

There was a dose responsive induction of feline IFNγ in cultures treated with either recombinant murine or human IL12. Interleukin 10 levels were unchanged over the doses of IL12 used (Figure 3.2).

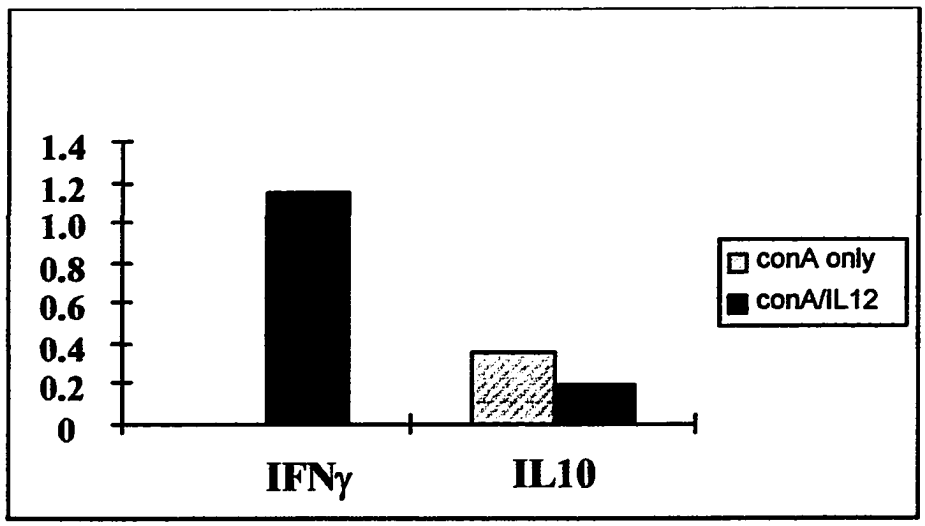

Figure 3.2: Recombinant murine IL12 induces *in vitro* IFNγ production in feline PBMC without inducing IL10. Cells were stimulated with conA for 48 hrs followed by 20 U of mIL12 for an additional 12 hrs. IFNγ was measured using QC-RT-PCR. Results representative of 4 individual experiments.

***In vivo* effects of recombinant IL12**

Toxicity

Cats tolerated all three doses of recombinant murine IL12 without evidence of illness. There was no evidence of pyrexia, anorexia and all cats continued to gain weight. There were no abnormalities including induction of liver enzymes seen on blood biochemical panels submitted throughout the study. All of the cats receiving IL12 had a marked drop in both neutrophil and red blood cell (RBC) numbers within 2 days of the initial dose (Figures 3.3a and 3.3b). Both parameters continued to decline throughout the treatment period reaching a nadir at the time of the last injection. There was a gradual recovery of both parameters in the cats that were followed for an additional 3 weeks post treatment.

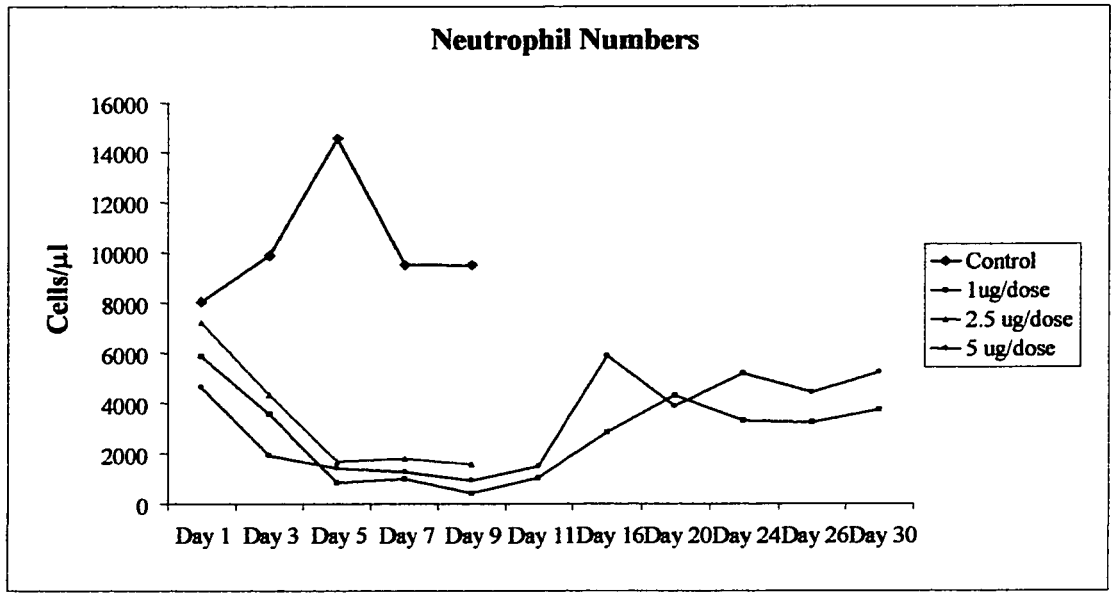

Figure 3.3a: *In vivo* inoculation of recombinant IL12 induces transient neutropenia in cats. Cats were inoculated with one of three doses of rIL12 intraperitoneally every other day starting on Day 1 for a total of five doses. All cats remained clinically asymptomatic.

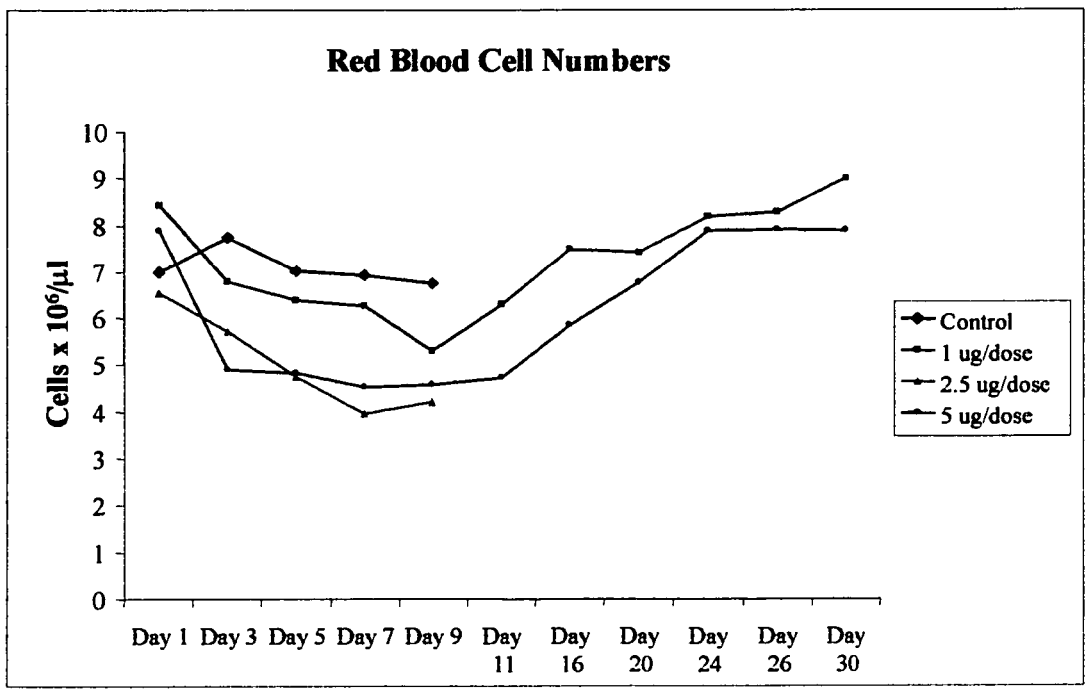

Figure 3.3b: Red blood cell numbers decrease transiently in cats treated with rIL12. Cats were treated as described in Figure 3a and developed a rapid decrease in circulating red blood cell numbers.

Cytokine induction

The intraperitoneal administration of mIL12 induced transient increases in IFNγ production in ConA-stimulated PBMC (Figure 3.4). Levels of IFNγ declined to pretreatment levels within 48 hrs of discontinuing IL12 administration. Analysis of mesenteric, colonic and splenic lymphoid tissue obtained 72 hrs post treatment failed to reveal significant differences in IFNγ levels between the control cat and the cat receiving 2.5 μg of mIL12 (data not shown).

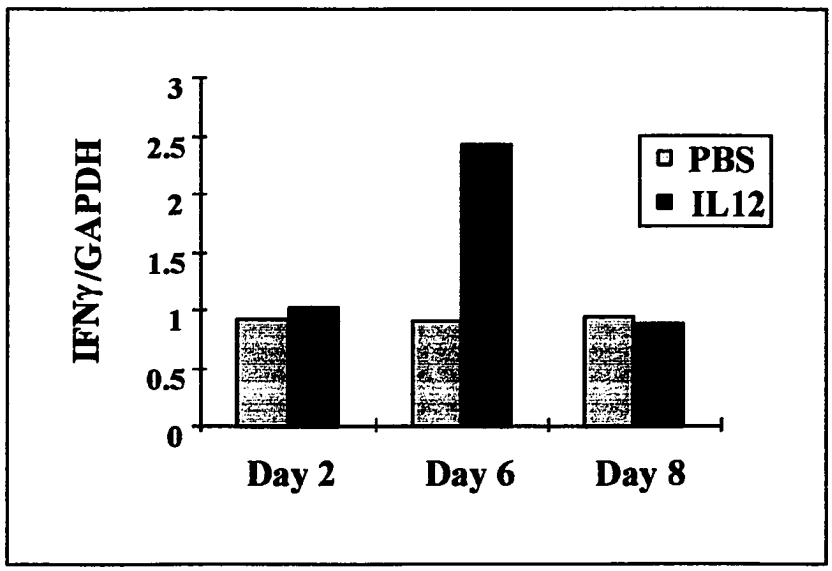

Figure 3.4: *in vivo* mIL12 primes feline PBMC to produce IFNγ. One cat received PBS and one cat received 2.5 μg/ml recombinant mIL12 via the intraperitoneal route. Injections were administered every other day beginning on Day 0 and ending on Day 6. PBMC were purified and stimulated for 2 hrs with PMA/ionomycin and RNA was extracted for cytokine analysis via RPA.

In vitro adenoviral infection of feline cells

Murine IL12 production

In vitro infection of CrFK, FLE and human 293 cells at a MOI of 30 with AdMEM12R resulted in the production and release of murine IL12 (Figure 3.5). AdMEM12R-infected

CrFK cell supernatants contained substantially more mIL12 than CrFK cells infected with Adv.mIL12 or RAdIL12 (Figure 3.6).

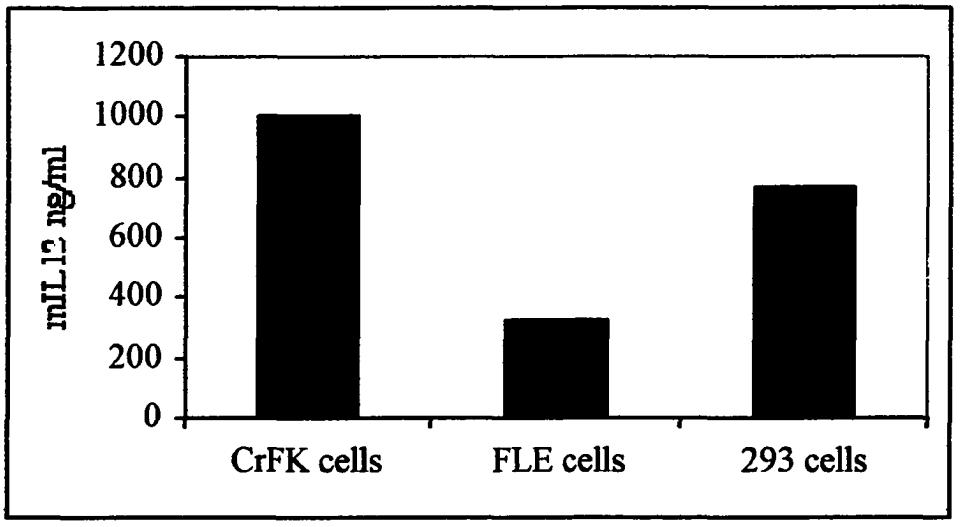

Figure 3.5: AdMEM12R infection of feline cells results in the production of mIL12. Crandell feline kidney (CrFK) cells, feline lymphocyte endothelial (FLE) cells and human 293 cells were infected with AdMEM12R at a MOI of 30. Twenty-four hours later supernatant levels of murine IL12 were determined by sandwich ELISA.

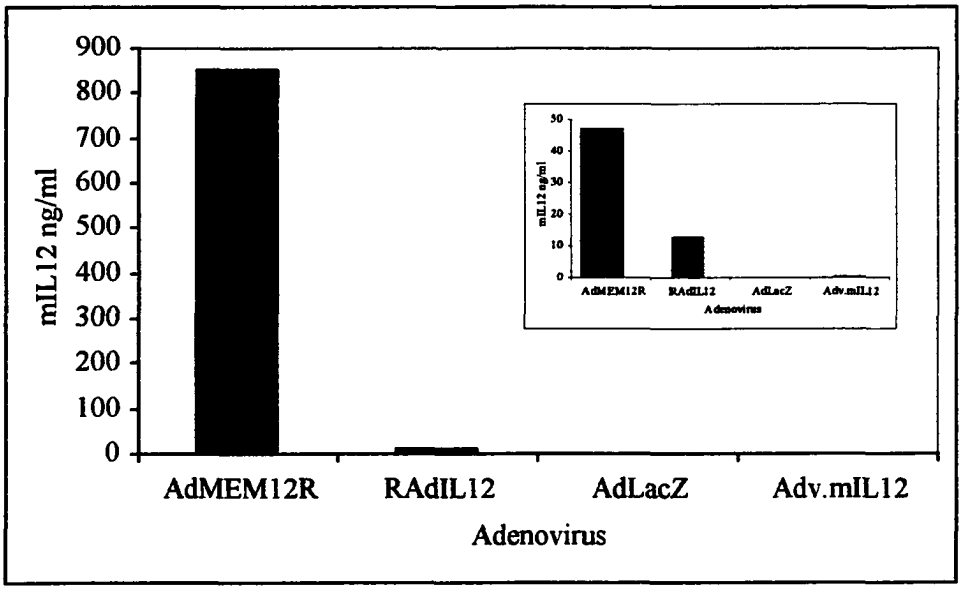

Figure 3.6: AdMEM12R produces more murine IL12 in feline cells than other adenoviral vectors. Crandell feline kidney cells were infected at a MOI of 30 with various adenoviruses expressing mIL12. Supernatant mIL12 levels were measured 24 hrs later by ELISA. Inset demonstrates low, yet detectable, mIL12 in all IL12 vectors.

Supernatant-induction of feline IFNγ

Supernatants from AdMEM12R-infected CrFK cells induced the production of IFNγ from mitogen-activated feline PBMC whereas AdLacZ-infected CrFK supernatants had no effect (Figure 3.7). There were no differences in the RNA levels of IL10 and TNFα between the two groups.

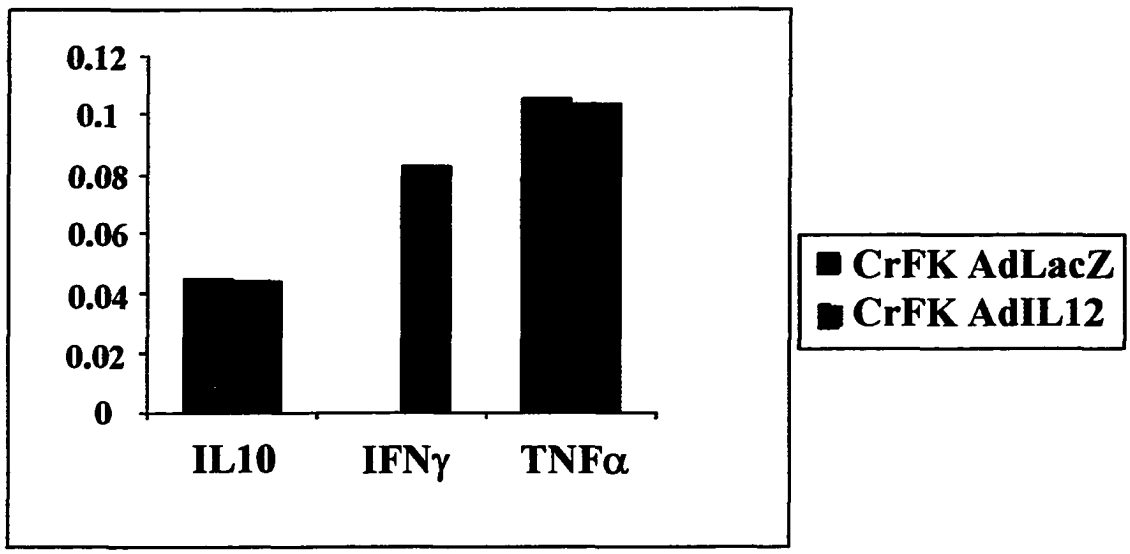

Figure 3.7: Supernatants from AdMEM12R-infected feline cells induce IFNγ production in feline PBMC. Feline CrFK cells were infected with AdMEM12R or AdLacZ (MOI 30) for 24 hrs at which point supernatant was collected. ConA stimulated feline PBMC were cultured with 10 μL of AdMEMIL12 or AdLacZ infected supernatant for 12 hours. Cytokines were measured by ribonuclease protection assay.

***In vitro* effects of adenoviral IL12 on FIV replication**

Feline macrophages infected with AdMEMR12 at a MOI of either 10 or 30 produced detectable levels of mIL12 for the first 7 days or 15 days of culture, respectively. Feline macrophages expressing mIL12 had the effect of delaying and diminishing the degree of FIV replication in PBMC *in vitro*. In the AdMEM12R-treated cultures there was an increase in the time necessary to detect viral antigen in the supernatant (Figure 3.8) and detectable RT activity was delayed and decreased (Figure 3.9). Cell viability assessed by trypan blue exclusion was the same between groups (not shown). NO_2- was not detected

in the supernatants at days 2, 4, 6 or 12 post AdMEM12R infection (data not shown). Thus, macrophages primed to produce mIL12 are able to alter the kinetics of subsequent FIV infection of PBMC.

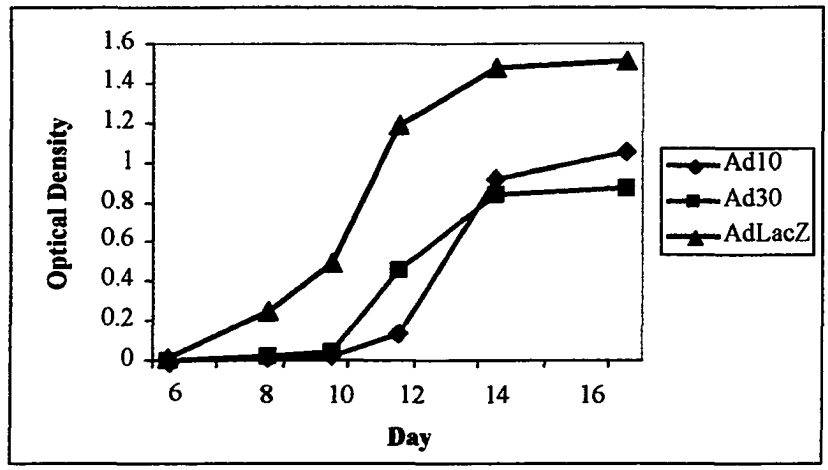

Figure 3.8: Macrophages producing mIL12 alter the kinetics of p26 antigen release during *in vitro* FIV infection. Feline macrophages were pre-infected with 10 or 30 MOI of AdMEM12R or 30 MOI of AdLacZ and 24 hours later autologous PBMC were added. The cells were infected with FIVB2542 cell-free supernatants 24 hrs later. FIV p26 ELISA was performed on supernatants beginning 6 days after FIV infection.

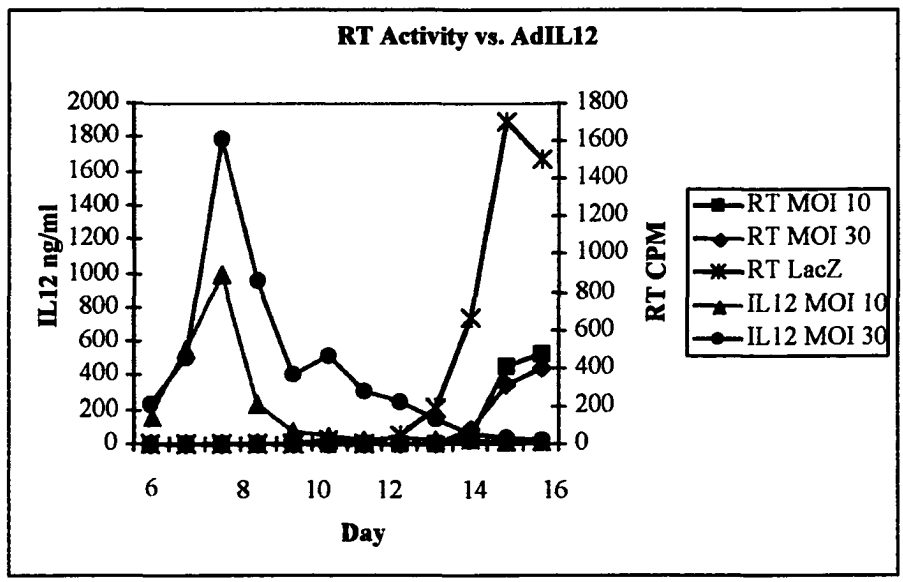

Figure 3.9: Macrophages producing mIL12 decrease reverse transcriptase activity during *in vitro* FIV infection. Feline macrophages were infected with Adenovirus and FIV as described in Figure 8. IL12 levels were monitored by ELISA and reverse transcriptase activity (RT) was monitored by the incorporation of radioactive dTTP.

In vivo effects of adenoviral IL12

Serum mIL12 levels

In all cats treated with AdMEM12R via the intraperitoneal route, acute elevations in serum murine IL12 were detected. In the initial study, the peak appeared to be 24 hrs post inoculation with a dramatic decrease by 48 hrs PI and a return to baseline by 72 hrs PI (Figure 3.10a). Subsequent studies examining earlier time points demonstrated that serum mIL12 could be detected as early as 6 hrs PI and levels were still rising up to 24 hrs PI (Figure 3.10b). Cats treated with AdMEM12R via the oral-nasal route had undetectable levels of mIL12 up to 48 hrs PI (data not shown). These studies demonstrate the rapid dissemination of mIL12 after IP infection, yet suggest a more limited expression after oral-nasal exposure.

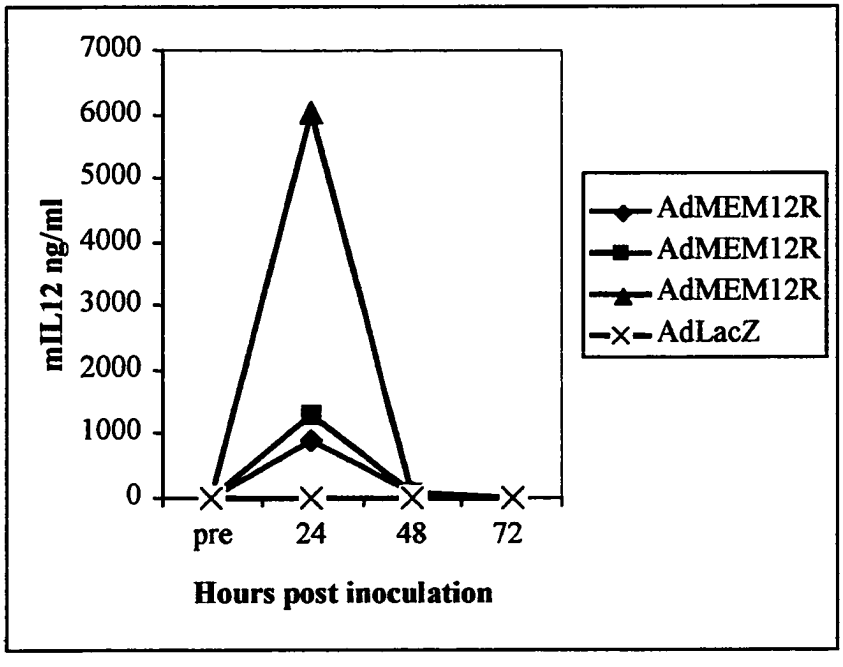

Figure 3.10a: AdMEM12R infection results in the *in vivo* production of mIL12. Three cats were inoculated with 1 x 10^9 pfus of AdMEM12 or AdLacZ via the intraperitoneal route. Blood was collected every 24 hrs and serum mIL12 levels were assayed by ELISA.

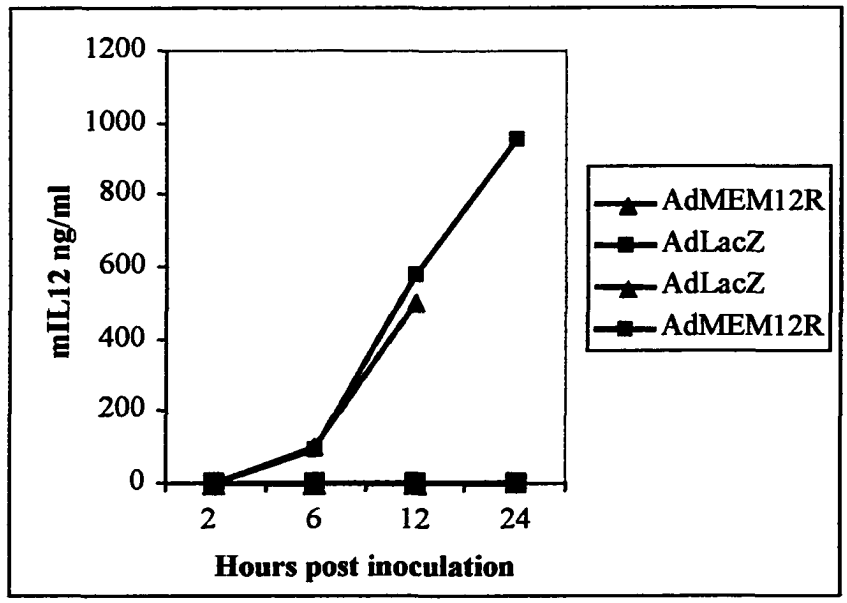

Figure 3.10b: AdMEM12R infection leads to the rapid release of mIL12. Cats were infected with either 1 x 109 pfus of AdMEM12R or AdLacZ via the intraperitoneal route and serum mIL12 levels monitored with ELISA 2, 6, 12 and 24 hrs later.

Toxicity

All cats treated with either AdMEM12R or AdLacZ remained asymptomatic without any clinical evidence of adverse effects. All cats treated with AdMEM12R via the intraperitoneal route developed the same acute decrease in neutrophil and red blood cell numbers as seen in the recombinant IL12 treated cats (Figure 3.11a, 3.11b). In addition, platelet numbers showed a similar acute reduction (Figure 3.11c). All three hematologic parameters returned to normal during the three week study period. By contrast, cats treated with AdMEM12R via the oral-nasal route had no demonstrable hematologic abnormalities. These findings again demonstrate the differences in systemic expression of AdenoIL12 given by the two routes.

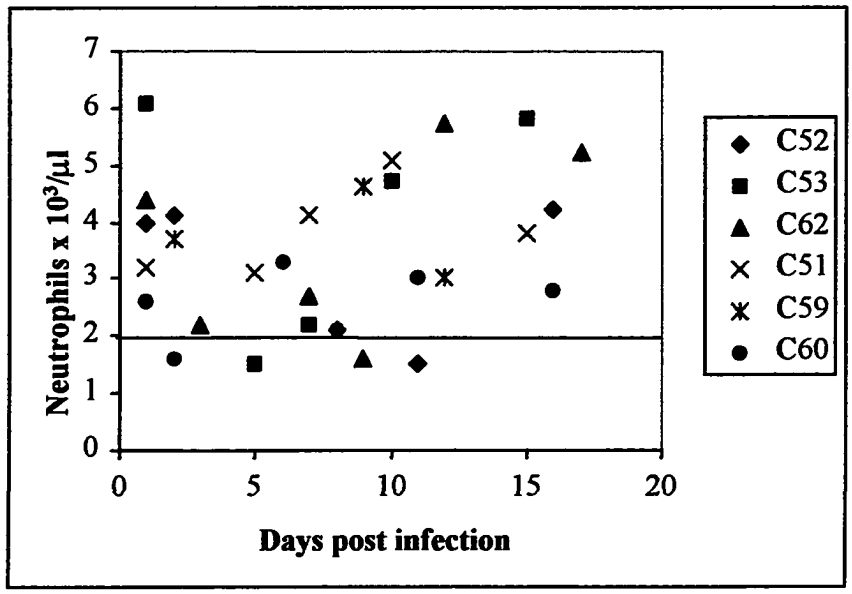

Figure 3.11a: Adenoviral delivery of mIL12 *in vivo* results in rapid, transient neutropenia. Three cats (C52,53,62) were infected with 1 x 109 pfus of AdMEM12R IP while 3 cats (C51,59,60) were infected with 1 x 109 pfus AdLacZ. Neutrophils were quantified using an automated counter and manual differential. The lower end of the normal reference range is delineated.

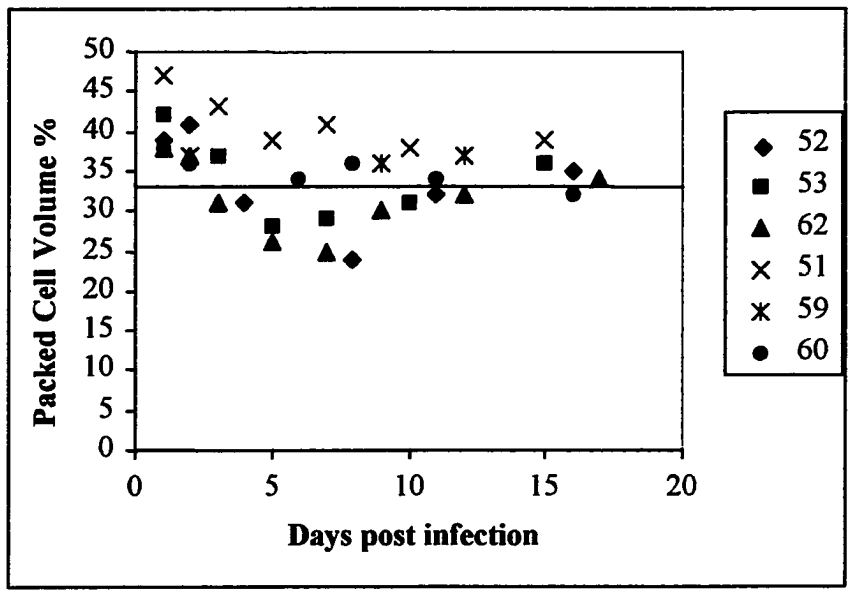

Figure 3.11b: Adenoviral delivery of mIL12 *in vivo* results in the rapid induction of transient anemia. Three cats (52,53,62) were infected with 1 x 109 pfus of AdMEM12R IP while 3 cats (51,59,60) were infected with 1 x 109 pfus AdLacZ. The packed cell volume of red blood cells was measured on an automated counter. The lower end of the normal reference range is delineated.

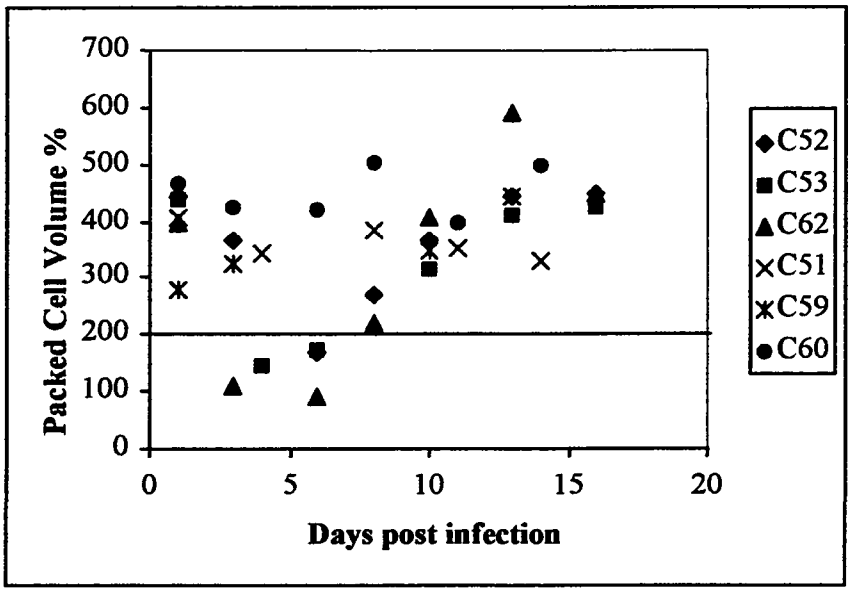

Figure 3.11c: Adenoviral delivery of mIL12 *in vivo* results in the rapid induction of transient thrombocytopenia. Three cats (C52,53,62) were infected with 1 x 109 pfus of AdMEM12R IP while 3 cats (C51,59,60) were infected with 1 x 109 pfus AdLacZ IP. Platelets were quantified using an automated counter and platelet clumping was ruled with a manual inspection of a peripheral blood smear. The lower end of the normal reference range is delineated.

Serum NO_2- concentrations

There was no detectable NO_2- in any of the sera tested (data not shown).

Cytokine induction

Tumor necrosis factor alpha

Cats treated with AdMEM12R or AdLacZ via the oral-nasal route had increased levels of TNFα in the tonsil at 24 hrs PI but not at 12 hrs PI (Figure 3.12a). This was true in the tonsil of the IP treated cats as well although the cat treated with AdLacZ appears to have had an earlier rise at 12 hrs (Figure 3.12b). The levels of TNFα within the spleen and colonic lymph nodes of cats treated with AdMEM12R and AdLAcZ by either route were more variable without any apparent trends (not shown). There appears to be a non-specific rise in TNFα that is secondary to the administration of the adenovirus.

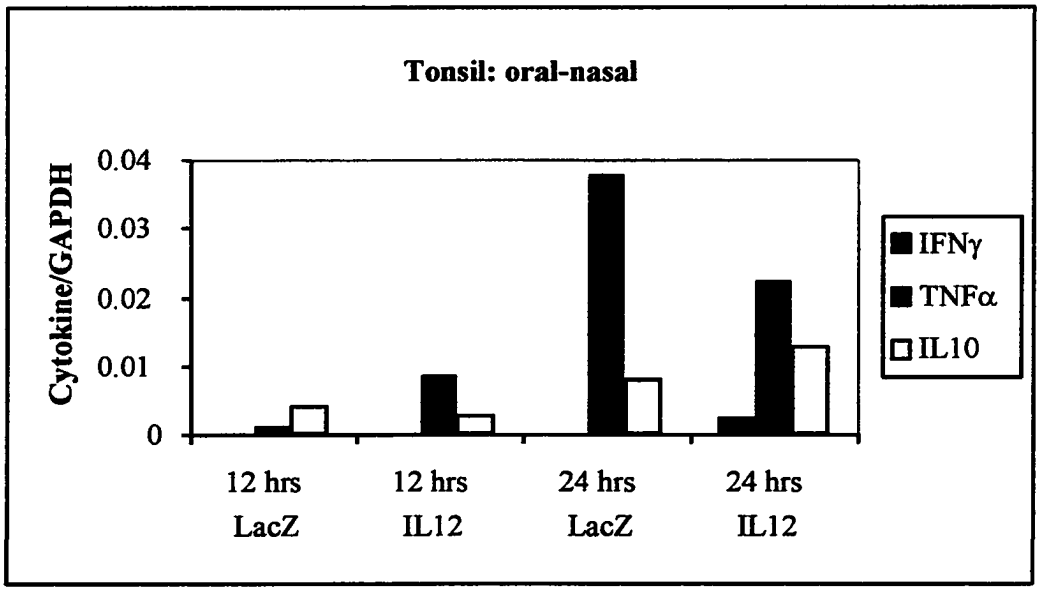

Figure 3.12a: Oral-nasal administration of AdMEM12 results in detectable levels of IFNg in the tonsil. Two cats were inoculated with 1 x 109 pfus AdMEM12R and two cats were inoculated with 1 x 109 pfus of AdLacZ via the oral-nasal route. One cat from each group was euthanized at 12 and 24 hrs PI and cytokine RNA levels were measured in the tonsil using a ribonuclease protection assay.

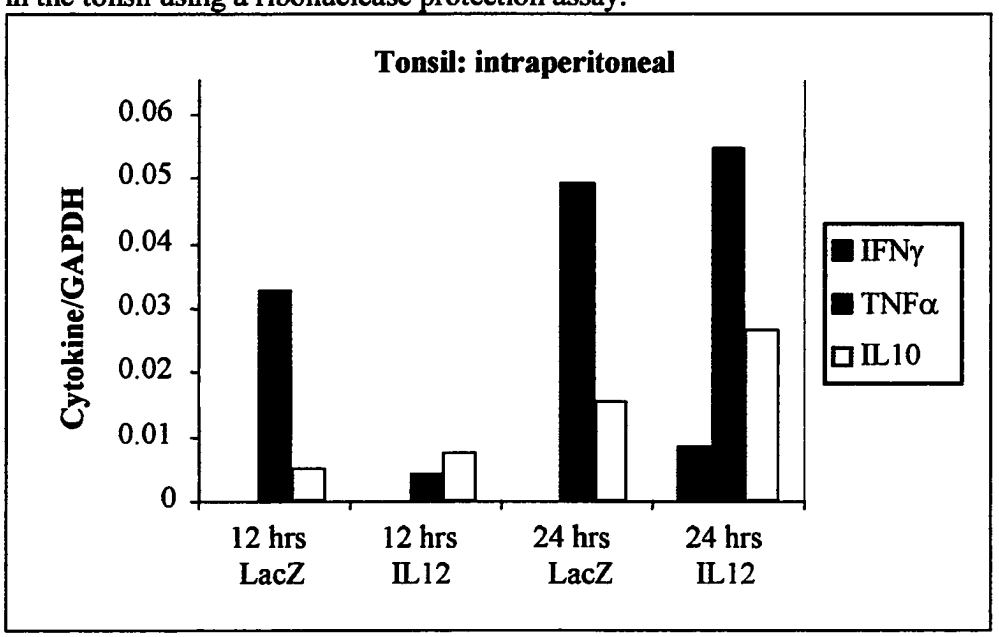

Figure 3.12b: Intraperitoneal administration ofAdMEM12R results in detectable IFNγ and mild increases in IL10 in the tonsil. Two cats were inoculated with 1 x 109 pfus AdMEM12R and two cats were inoculated with 1 x 109 pfus of AdLacZ via the intraperitoneal route. One cat from each group was euthanized at 12 and 24 hrs PI and cytokine RNA levels were measured in the tonsil using a ribonuclease protection assay.

Interleukin10

IL10 levels were moderately increased in the spleen of AdMEM12R treated cats as compared with AdLacZ treated cats. This was particularly evident in the IP-treated cats and appeared to occur as early as 12 hrs PI (Figure 3.13a). The increases in the oral-nasal-treated cats were more modest and did not occur until 24 hrs PI (Figure 3.13b). There were mild increases in IL10 in the tonsils of AdMEM12R cats treated by either IP or oral-nasal routes. The AdMEM12R cats treated via the IP route had increased IL10 as compared with the AdLacZ cats at 12 and 24 hrs PI in the colonic lymph node whereas there were no differences seen in the oral-nasal treated cats (Figures 3.14a and 3.14b). Both routes of administration of adenovirus IL12 appear to have produced increases in local and systemic IL10 production.

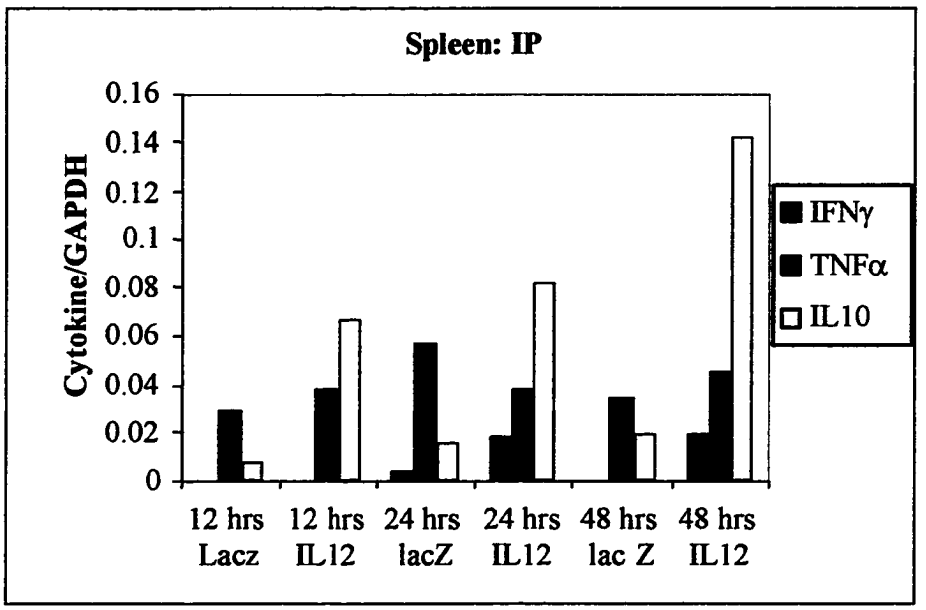

Figure 3.13a: Intraperitoneal administration of AdMEM12R results in detectable IFNγ and increases in IL10 in the spleen. Three cats were inoculated with 1 x 10^9 pfus AdMEM12R and three cats were inoculated with 1 x 10^9 pfus of AdLacZ via the intraperitoneal route. One cat from each groupswas euthanized at 12, 24 and 48 hrs PI and cytokine RNA levels were measured in the spleen using a ribonuclease protection assay.

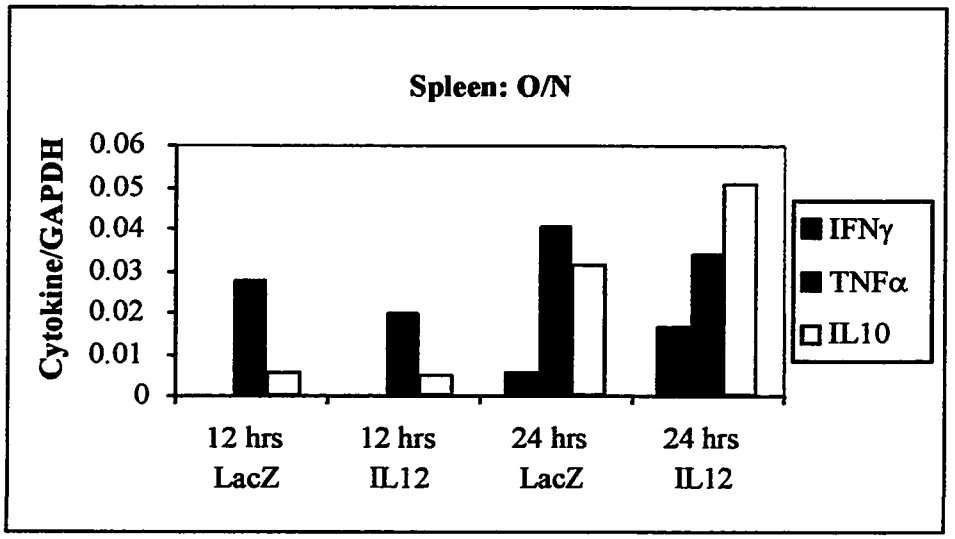

Figure 3.13 b: Oral-nasal administration of AdMEM12R results in detectable IFNγ and mild increases in IL10 in the spleen. Two cats were inoculated with 1 x 109 pfus AdMEM12R and two cats were inoculated with 1 x 109 pfus of AdLacZ via the oral-nasal route. One cat from each group was euthanized at 12 and 24 hrs PI and cytokine RNA levels were measured in the spleen using a ribonuclease protection assay.

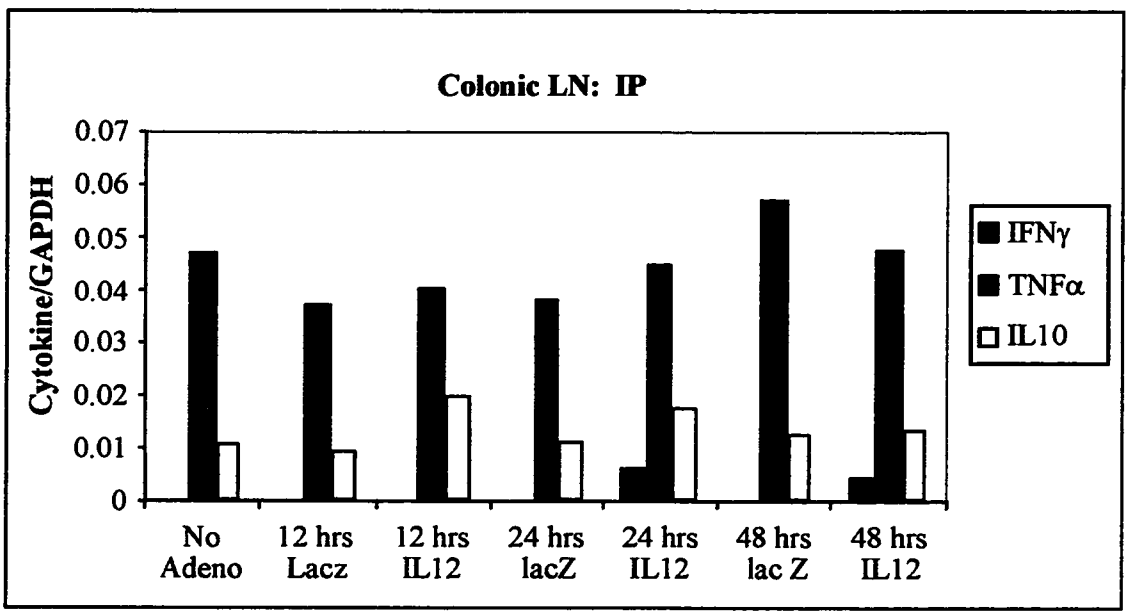

Figure 3.14a: Intraperitoneal administration of AdMEM12R results in detectable IFNγ in the colonic lymph node. Three cats were inoculated with 1 x 109 pfus AdMEM12R and three cats were inoculated with 1 x 109 pfus of AdLacZ via the intraperitoneal route. One cat from each group was euthanized at 12, 24 and 48 hrs PI and cytokine RNA levels were measured in the spleen using a ribonuclease protection assay. The colonic lymph node from a naïve cat served as a negative control.

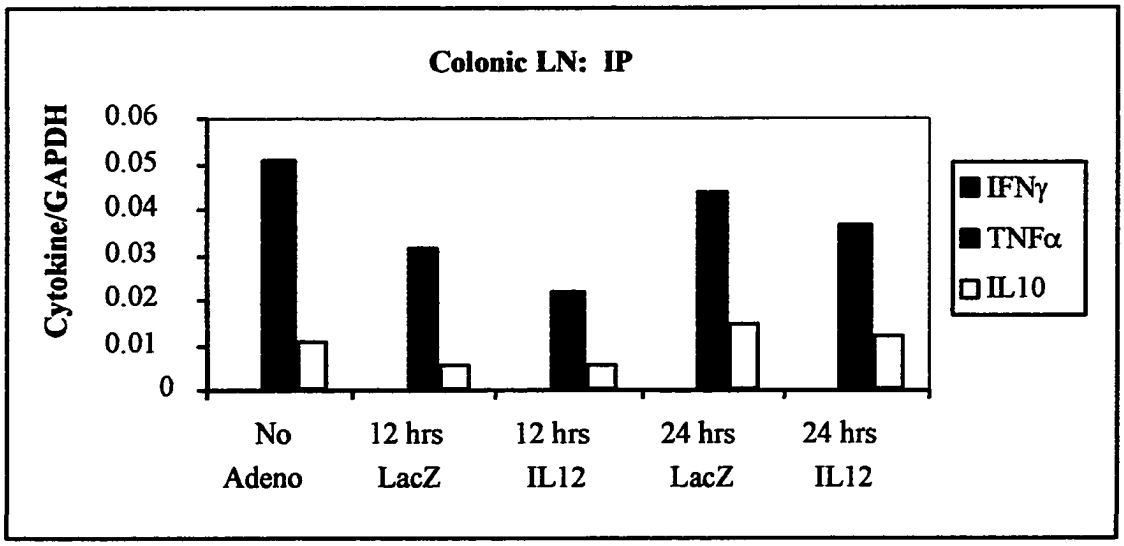

Figure 3.14b: Oral-nasal administration of AdMEM12R appears to have no effect on cytokine production in the colonic lymph node. Two cats were inoculated with 1 x 109 pfus AdMEM12R and two cats were inoculated with 1 x 109 pfus of AdLacZ via the oral-nasal route. One cat from each group was euthanized at 12 and 24 hrs PI and cytokine RNA levels were measured in the spleen using a ribonuclease protection assay. The colonic lymph node from a naïve cat served as a negative control.

Interferon-gamma

IFNγ was detected in the tonsils of AdMEM12R cats treated via either route at 24 hrs PI but not before (Figures 3.12a and 3.12b). There was no IFNγ detected in the colonic lymph node of the oral-nasal-treated cats whereas IP treatment resulted in the detection of IFNγ in the AdMEM12R treated cats beginning at 24 hrs PI (Figures 3.14a and 3.14b). IFNγ was detectable at low levels in the spleens of AdLacZ treated cats at 24 hrs PI but was present at higher levels in the AdMEM12R treated cats and persisted through 48 hrs in the IP AdMEM12R-treated cats (Figures 3.13a and 3.13b). The induction of IFNγ was either restricted to AdMEM12R-treated cats or augmented in cats treated with AdMEM12R.

Statistical analysis was not feasible given the low number of animals used in this pilot study.

Effects of concurrent FIVB infection

All three AdMEM12R treated cats had detectable levels of mIL12 in the serum by 24 hrs PI with a decrease to undetectable by 72 hrs PI (Figure 3.15). After oral-nasal exposure to FIVB, one of the three AdLacZ treated cats became DNA PCR positive in PBMC by 10 days post FIVB infection while two of three AdMEM12R treated cats became DNA PCR positive by 20 days PI. The AdLAcZ treated cat had plasma viral RNA levels of 1000 copies/ml at 16 days PI and 1.46 x 10^6 copies/ml 21 days PI. The two AdMEM12R cats both had undetectable plasma viral RNA levels at days 16 and 21 PI (Figure 3.16). All three cats had undetectable levels of viral RNA yet remained viral DNA PCR positive when assayed 8 weeks later. Thus, despite small numbers of infected animals and incomplete follow-up, adenoviral delivery of mIL12 may have altered the kinetics of *in vivo* FIV replication.

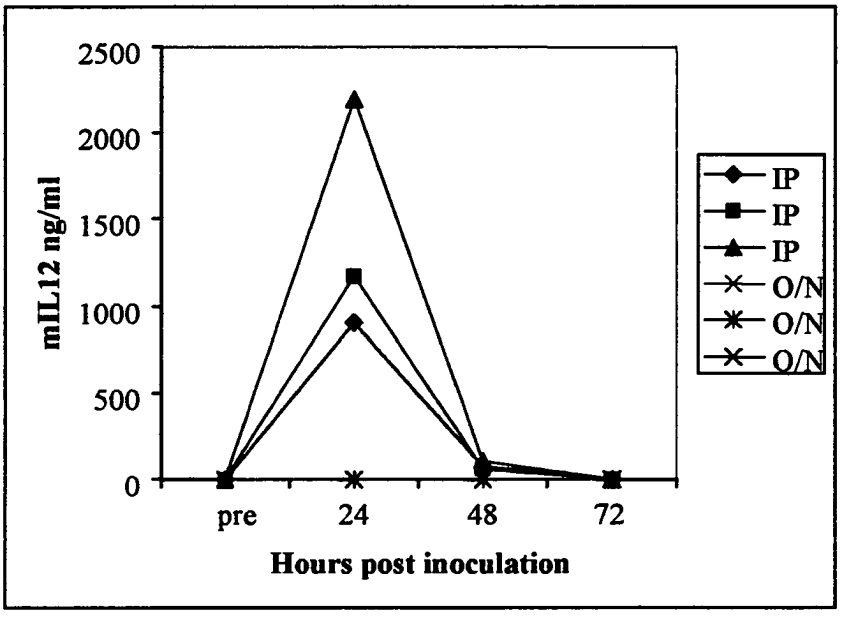

Figure 3.15: Intraperitoneal, but not oral-nasal, inoculation of AdMEM12R results in detectable serum mIL12 levels. Cats were inoculated with 1 x 109 pfus AdMEM12R and serum levels of mIL12 were monitored by ELISA.

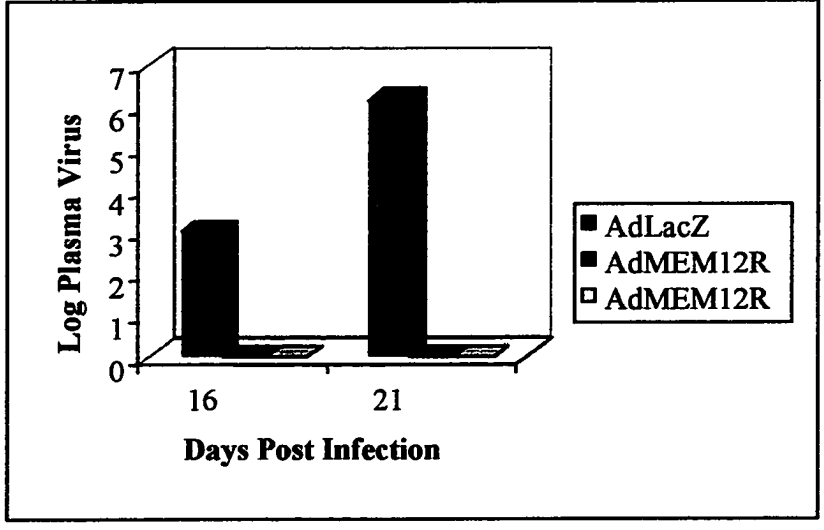

Figure 3.16: AdMEM12R pre-treatment delays the detection of plasma viral RNA after FIV infection. One cat was inoculated with AdLAcZ and two cats were inoculated with AdMEM12R via the intraperitoneal route. Twenty-four hours later they were challenged with cell-free FIVB2542 supernatants via the oral-nasal route. All three cats were positive for FIV provirus at the time points shown. Viral RNA was quantified using QC-RT-PCR.

DISCUSSION

Data from our laboratory (P. Avery, manuscript in preparation) have shown that interleukin 10 (IL10) dominates the early response to mucosally acquired FIV infection. Although IL10 has recently been shown to have more pleotropic effects than initially thought, it has several well-described inhibitory effects on the cell-mediated immune response. This fact, coupled with the notable lack of antiviral IFNγ or TNFα induction, lead us to speculate that FIV was exploiting cytokines to inhibit the cell-mediated immune response during the critical early stage of viral infection.

The role of interleukin 12 (IL12) in driving a strong type 1, cell-mediated immune response has been demonstrated in many infectious diseases in murine systems. Supplying or augmenting the production of IL12 in the early phases of protozoal, bacterial or viral infections can tip the balance of the immune response allowing the clearance of the infectious agent. Our goals in this study were to investigate the cross-species efficacy of murine IL12 in cats and begin to investigate the role of early IL12 intervention in the control of mucosal FIV infection.

We have provided evidence that murine IL12 can induce the production of feline IFNγ *in vitro* and both IFNγ and IL10 *in vivo*. This data is consistent with published work in the both murine and human systems. Stimulation of isolated PBMC results in the production of IFNγ only (17) whereas, stimulation with IL12 during antigen specific expansion, results in the production of both IFNγ and IL10 (18) (19). Mice treated with mIL12 *in vivo* produce increased amounts of both IFNγ and IL10 (20). The simultaneous induction of both cytokines has been documented within individual T cell clones (18). Given that IL10 can inhibit the production of IFNγ (21), this work shows that these cytokines can also be independently regulated in the same cell. It is thought that the dual induction of IL10 and IFNγ may allow fine control over the magnitude of the cell-mediated immune response to a given pathogen.

We have demonstrated that adenoviral vectors will replicate in feline cells despite the fact that there are no known natural adenoviral infections of cats. Several replication defective adenoviral vectors produced mIL12 in feline cell lines although AdMEM12R produced far more than the others tested. The levels of mIL12 released into the supernatants 24 PI of CrFK and FLE cell lines are within the range seen with ADMEM12R infection of some murine cell lines (22). The explanation for decreased IL12 production in the RSV driven construct Adv.mIL12 is likely related to promoter efficiency. Adenoviral constructs using the CMV promoter have been shown in several systems to produce more product than RSV driven constructs (23) (24) (25). Other investigators have documented cell-specific differences in the strength of CMV versus RSV driven transcription (26). Either mechanism may be playing a role in the *in vitro* CrFK comparisons performed in this study. AdMEM12R and RAdIL12 both utilize the murine CMV promoter and have both IL12 subunits in the E1 region separated by the encephalomyocarditis virus internal ribosome entry site promoter but the differences in mIL12 production may be related to differences in the virus vectors themselves (27).

The mIL12 released by AdMEM12R-infected cells has biologic activity in feline PBMC as evidenced by the induction of IFNγ. When controlling for the amount of mIL12 added to PBMC, it appears that the mIL12 released by the adenovirus may have a lower

biological activity than the recombinant mIL12. A stimulus of 6.5 ng/ml recombinant mIL12 induced 0.775 of IFNγ/GAPDH in feline PBMC whereas 12.5 ng/ml of AdMEM12R-produced mIL12 induced 0.045 of IFNγ/GAPDH in feline PBMC. These comparisons were not made using PBMC from the same cat due to the time elapsed between the use of recombinant mIL12 and the AdMEM12R vector so some individual response variation cannot be completely ruled out. Nevertheless, the IFNγ responses to AdMEM12R supernatants have generally been in this lower range. It is also possible that the adenoviral-packaged mIL12 may consist of some non-functional p40 homodimers but this would not explain the discrepancy as the sandwich ELISA used to quantify the adenoviral-derived mIL12 requires that both p35 and p40 subunits be present.

The kinetics of the release of mIL12 into the serum of IP-treated cats was similar to that seen in adenovirus IL12-infected mice. The peak in mice has been reported to occur approximately 24 hrs after inoculation with a rapid drop off to very low levels by 72 hrs PI(28). IL12 levels were very low by 48 hrs PI and undetectable by 72 hrs PI in our cats. The slightly increased rate of clearance may be due to the fact that AdMEM12R contains the murine CMV promoter whereas most of the work done in mice has utilized human CMV-driven constructs. Murine CMV driven adenoviruses exhibit an earlier peak of expression than human CMV driven constructs (29).

The magnitude of serum mIL12 induction after IP injection in cats is difficult to compare to that in mice as data are not published with this particular vector. The AdMEM12R vector has been shown to produce up to a six-fold increase in mIL12 production *in vitro* when compared with a human CMV driven construct (AdmIL-12.1) produced by the same laboratory (29). The AdmIL12.1 construct produces 128 ng/ml mIL12 in mice when given IP at a dose of 1×10^9 pfus/ml whereas we saw 1-6 μg/ml of mIL12 using AdMEM12R at the same dose in our cats (28). If the increased replication efficiency seen *in vitro* is recapitulated *in vivo*, the levels of mIL12 that we have documented in cats may be similar to those seen in similarly treated mice. The fact that we only saw mild hematologic toxicities in cats with such high levels of IL12 expression adds indirect evidence to the suspicion of decreased biologic activity.

The hematologic toxicities that we observed in mIL12-treated cats have been characterized in both murine and non-human primates systems (30) (31) (32). The changes that we observed are most consistent with those seen in mIL12 treated mice, which develop neutropenia, anemia and thrombocytopenia (31). The treated mice also developed a transient lymphopenia, which we did not observe in the treated cats. Treatment of non-human primates with recombinant human IL12 also induces a transient thrombocytopenia, anemia and lymphopenia but neutrophil numbers tend to increase (33). While the mechanism behind the hematotoxicity is not completely clear, it appears to be a combination of bone marrow suppression and peripheral destruction of cells. It has been clearly shown to be IFNγ-dependent by examining the response in IFNγ knock-out mice (30). This suggests that we are inducing increased levels of feline IFNγ within the bone marrow in mIL12-treated cats.

Interleukin-12 given at high doses can actually impair the immune response as has been seen in lymphocytic choriomeningitis virus (LCMV) infection where high levels of IL-12 lead to higher viral loads and decreased cytotoxic T lymphocyte (CTL) responses(34). These inhibitory effects have been shown to be dependent of the induction of nitric oxide (NO_2-) (27, 35). We examined NO_2- levels in the serum from cats treated with AdMEM12R and, despite the induction of high circulating levels of mIL12, we were unable to detect any. Although we were unable to demonstrate any induction of nitric oxide, the measurement of inducible nitric oxide synthase (iNOS) may provide a more sensitive means of detecting increased nitric oxide in serum samples.

The brief increase in mitogen-stimulated IFNγ production seen in the PBMC of recombinant mIL12 treated cats is typical of that seen in mice. Mice treated for 5 consecutive days with mIL12 had elevations in PBMC-derived IFNγ that returned to baseline within 2 days of discontinuing treatment (36). We were unable to document any increase in tissue IFNγ RNA but the samples were taken 72 hrs after discontinuing the mIL12 injections so any potential increase may have resolved by that time-point. When we examined AdMEM12R treated cats during the acute phase of the adenoviral infection,

we were able to demonstrate increases in IFNγ and IL10 levels as compared to AdLacZ treated cats. The induction of IL10 and IFNγ was the most notable in the spleen of IP infected mice where there was a sustained increase in IL10 beginning at 12 hrs and induction of IFNγ beginning at 24 hrs. In a similar fashion, IFNγ was detectable and IL10 appeared increased in the more distant lymphoid tissue, the tonsil, in IP infected cats suggesting a disseminated response. There were also increases in TNFα but there was no difference between the two groups of adenovirus and adenoviral infections alone can induce TNFα release (37). There appear to be mild increases of IFNγ and IL10 in the tonsil and spleen of cats inoculated with AdMEM12R oral-nasally suggesting that this may be a viable means to deliver IL12 to mucosal tissues. The effect was not seen in the colonic lymph nodes. The small overall animal number and individual cats at each time-point warrant caution in interpreting the data but the results are consistent with a systemic effect of IL12 within 24 hrs after IP infection and a more modest and focal response after oral-nasal administration.

The *in vitro* production of mIL12 by feline macrophages led to a delay and decrease in subsequent reverse transcriptase (RT) levels produced by *in vitro* FIV-infected PBMC. There is precedent for this antiviral effect in similar experiments performed with HIV. Human macrophage cultures infected with HIV were co-cultured with autologous PBMC with and without recombinant human IL12. The addition of hIL12 resulted in a 75-90% reduction in RT activity which was IFNγ-dependent (38). We did not measure IFNγ in this study but our previous work with adenoviral-expressed mIL12 would support the notion that IFNγ was induced in these cultures. Interferon-γ has been shown to be a potent activator of macrophages and can stimulate the release of H_2O_2, proteases and inducible nitric oxide synthase, all of which are important in the clearance of intracellular pathogens (39). We were unable to detect increased nitric oxide in the supernatants of AdMEM12R-infected cultures despite the fact that the Greiss reaction has been validated in feline culture systems(40).

The dose of FIVB pool chosen to inoculate cats via the oral-nasal route had previously been shown to infect 4/4 cats (C. Mathiason, unpublished results). The 50% infectivity

seen in the 6 inoculated cats in this study was disappointing but not surprising given the variability we have seen when attempting to infect cats across a mucosal barrier. The intact rectal and oral mucosa appear to present a formidable mechanical barrier to FIV infection.

The early proviral data was prematurely interpreted as a failure of early IL12 intervention to alter the course of infection. That 1/3 control cats and 2/3 IL12-treated cats became infected within the time period typical for this route of infection suggested no effect. It was not until stored plasma samples were analyzed for viral RNA levels that a potential difference between the two groups was identified. There was a delay in the ability to detect both viral DNA and RNA in the AdMEM12R pretreated cats. Unfortunately, the plasma was not processed until several weeks after the DNA samples and critical sample time-points were missed. We do not know if, in addition to a delay, there was an overall decrease in level of plasma viremia in the IL12 treated cats. Recent work giving twice weekly injections of rhesus IL12 in the SIV system showed that, while there was no delay in the induction of plasma viremia, the magnitude was significantly decreased in rIL12-treated macaques(41). The lack of any delay may be explained by the fact that these macaques were inoculated intravenously whereas our cats may have been able to control the more localized inoculum for some period of time before dissemination to the plasma. While no protection from infection was seen in the SIV study, survival was significantly prolonged in the IL12-treated monkeys and tissue pro-viral levels were significantly decreased out to 58 weeks PI (41). Tissue viral levels will be examined in our cats to further explore this phenomenon. Clearly, this experiment will have to be repeated with larger numbers of animals and closer monitoring during the acute phase in order to document any potential benefit of adenoviral-delivered IL12.

In summary, the present studies demonstrate that murine IL12 has relevant biologic activity in feline cells and cats *in vivo*. In addition, adenoviral delivery appears to be a viable means of administering murine IL12 to cats. Further work needs to be done to explore the potential down regulation of early FIV replication *in vivo* and to examine the long term consequences of such intervention on viral control and disease outcome.

1. Chehimi, J., S. Starr, I. Frank, M. Rengaraju, S. Jackson, C. Llanes, M. Kobayashi, B. Perussia, D. Young, E. Nickbarg, S. Wolf, and G. Trinichieri. 1992. Natural killer cell stimulatory factor (NKSF) increases the cytotoxic activity of NK cells from both healthy donors and HIV-infected patients. *Journal of Experimental Medicine* 175:789.
2. Trinchieri, G. 1994. Interleukin 12: A cytokine produced by antigen presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood* 84:4008.
3. Kobayashi, M., Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perussia B, Trinchieri G. 1989. Identification and purification of Natural Killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *Journal of Experimental Medicine* 170:827.
4. Trinchieri G, G.F. 1996. Immunoregulation by interleukin 12. *Journal of Leukocyte Biology* 59:505.
5. Gazzinelli RT, W.M., Hayasi S, Denkers EY, Hieny S, Caspar P, Trinchieri G, Sher A. 1994. Parasite-induced IL-12 stimulates early IFNγ synthesis and resistance during acute infection with *Toxoplasma gondii*. *Journal of Immunology* 153:2533.
6. Hsieh CS, M.S., Tripp CS, Wolf SF, O'Garra A, Murphy KM. 1993. Development of Th1 CD4+ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* 260:547.
7. Heinzel, F.P., R.M. Rerko, F. Ahmed, and E. Pearlman. 1995. Endogenous IL-12 is required for control of Th2 cytokine responses capable of exacerbating leishmaniasis in normally resistant mice. *Journal of Immunology* 155:730.
8. Cooper AM, R.A., Rhoades ER, Callahan JE, Getzy DM, Orme IM. 1995. The role of interleukin-12 in acquired immunity to *Mycobacterium tuberculosis* infection. *Immunology* 84:423.
9. Clerici, M., D.R. Lucey, J.A. Berzofsky, L.A. Pinto, T.A. Wynn, S.P. Blatt, M.J. Dolan, C.W. Hendrix, S.F. Wolf, and G.M. Shearer. 1993. Restoration of HIV-

specific cell-mediated immune responses by interleukin-12 *in vitro*. *Science* 262:1721.

10. Chehimi, J., S. Starr, I. Frank, A. D'Andrea, X. Ma, R. MacGregor, J. Sennelier, and G. Trinchieri. 1994. Impaired interleukin 12 production in human immunodeficiency virus-infected patients. *Journal of Experimental Medicine* 179:1361.
11. Marshall JD, C.J., Gri G, Kostman JR, Montaner LJ, Trinchieri G. 1999. The interleukin-12-mediated pathway of immune events is dysfunctional in human immunodeficiency virus-infected individuals. *Blood* 94, no. 3:1003.
12. Belyakov, I.M., J.D. Ahlers, J.D. Clements, W. Strober, and J.A. Berzofsky. 2000. Interplay of cytokines and adjuvants in the regulation of mucosal and systemic HIV-specific CTL. *Journal of Immunology* 165, no. 11:6454.
13. Crandell RA, F.C., Nelson-Rees WA. 1973. Development, characterization, and viral susceptibility of a feline (Felis catus) renal cell line (CRFK). *In Vitro* 9, no. 3:176.
14. Dreitz, M.J., S.W. Dow, S.A. Fiscus, and E.A. Hoover. 1995. Development of monoclonal antibodies and capture immunoassays for feline immunodeficiency virus. *Am J Vet Res* 56, no. 6:764.
15. Goldstein, S., R. Engle, R.A. Olmsted, V.M. Hirsch, and P.R. Johnson. 1990. Detection of SIV antigens by HIV-1 antigen capture immunoassays. *Journal of Acquired Immune Deficiency Syndromes* 3:98.
16. Green, S.J., D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnock, and S.R. Tannenbaum. 1982. Analysis of nitrate, nitrite and ^{15}N nitrate in biological fluids. *Annals of Biochemistry* 126:131.
17. Chan SH, P.B., Gupta JW, Kobayashi M, Pospisil M, Young HA, Wolf SF, Young D, Clarh SC, Trinchieri G. 1991. Induction of IFN-γ production by NK cell stimulatory factor (NKSF): characterization of the responder cells and synergy with other inducers. *Journal of Experimental Medicine* 173:869.
18. Gerosa, F., C. Paganin, D. Peritt, F. Paiola, M.T. Scupoli, M. Aste-Amezaga, I. Frank, and G. Trinchieri. 1996. Interleukin-12 primes human CD4 and CD8 T cell

clones for high production of both interferon-γ and interleukin-10. *The Journal of Experimental Medicine* 183:2559.

19. Windhagen, A., D.E. Anderson, A. Carrizosa, R.E. Williams, and D.A. Hafler. 1996. IL-12 induces human T cells secreting IL-10 with IFN-γ. *The Journal of Immunology* 157:1127.

20. Morris, S., K. Madden, J. Adamovicz, W. Gause, B. Hubbard, M. Gately, and F. Finkelman. 1994. Effects of IL-12 on *in vivo* cytokine gene expression and Ig isotype selection. *Journal of Immunology* 152:1047.

21. D'Andrea, A., M. Aste-Amezaga, N.M. Valiente, X. Ma, M. Kubin, and G. Trinchieri. 1993. Interleukin-10 inhibits human lymphocyte IFN-γ production by suppressing interleukin-12 synthesis in accessory cells. *The Journal of Experimental Medicine* 178:1041.

22. Putzer, B.M., M. Hitt, W.J. Muller, P. Emtage, J. Gauldie, and F.L. Graham. 1997. Interleukin 12 and B7-1 costimulatory molecule expressed by an adenovirus vector act synergistically to facilitate tumor regression. *The Proceedings of the National Academy of Sciences* 94:10889.

23. Tong, X., D.G. Engehausen, C.T. Freund, I. Agoulnik, Z. Guo, M.K. Oehler, T.E. Kim, A. Hasenburg, C.F. Contant, S.L. Woo, and D.G. Kieback. 1998. The efficacy of adenovirus-mediated gene therapy of ovarian cancer is enhanced by using the cytomegalovirus promoter. *Anticancer research* 18, no. 2A:719.

24. Elshami, A.A., J.W. Cook, K.M. Amin, H. Choi, J.Y. Park, L. Coonrod, J. Sun, K. Molnar-Kimber, J.M. Wilson, L.R. Kaiser, and S.M. Albelda. 1997. The effect of promoter strength in adenoviral vectors containing herpes simplex virus thymidine kinase on cancer gene therapy *in vitro* and *in vivo*. *Cancer Gene Therapy* 4, no. 4:213.

25. Johnson, L.G., R.J. Pickles, S.E. Boyles, J.C. Morris, H. Ye, Z. Zhou, J.C. Olsen, and R.C. Boucher. 1996. *In vitro* assessment of variables affecting the efficiency and efficacy of adenovirus-mediated gene transfer to cystic fibrosis airway epithelia. *Human Gene Therapy* 7, no. 1:51.

26. Smith, R.L., D.L. Traul, J. Schaack, G.H. Clayton, K.J. Staley, and C.L. Wilcox. 2000. Characterization of promoter function and cell-typr specific expression

from viral vectors in the nervous system. *The Journal of Virology* 74, no. 23:11254.

27. Lasarte, J.J., F.J. Corrales, N. Casares, A. Diaz de Cerio, C. Qian, X. Xie, F. Borras-Cuesta, and J. Prieto. 1999. Different doses of adenoviral vector expressing IL-12 enhance or depress the immune response to a coadministered antigen: the role of nitric oxide. *The Journal of Immunology* 162:5270.

28. Bramson, J., M. Hitt, W.S. Gallichan, K.L. Rosenthal, J. Gauldie, and F.L. Graham. 1996. Construction of a double recombinant adenovirus vector expressing a heterodimeric cytokine: *in vitro* and *in vivo* production of biologically active interleukin-12. *Human Gene Therapy* 7:333.

29. Addison, C.L., M. Hitt, D. Kunsken, and F.L. Graham. 1997. Comparison of the human versus the murine cytomegalovirus immediate early gene promoters for transgene expression by adenoviral vectors. *Journal of General Virology* 78:1653.

30. Eng, V.M., B.D. Car, B. Schnyder, M. Lorenz, S. Lugli, M. Aguet, T.D. Anderson, B. Ryffel, and V.F.J. Quesniaux. 1995. The stimulatory effects of IL-12 on hematopoiesis are antagonized by IL-12-induced interferon-γ *in vivo*. *The Journal of Experimental Medicine* 181:1893.

31. Tare, N.S., S. Bowen, R.R. Warrier, D.M. Carvajal, W.R. Benjamin, J.H. Riley, T.D. Anderson, and M. Gately. 1995. Administration of recombinant interleukin-12 to mice suppresses hematopoiesis in the bone marrow but enhances hematopoiesis in the spleen. *Journal of Interferon and Cytokine Research* 15:377.

32. Bree, A.G., F.J. Schlerman, M.D. Kaviani, R.C. Hastings, S.L. Hitz, and S.J. Goldman. 1994. Multiple effects on peripheral hematology following administration of recombinant human interleukin 12 to nonhuman primates. *Biochemical and Biophysical Research Communications* 204, no. 3:1150.

33. Sarmiento, U.M., J.H. Riley, P.A. Knaack, J.M. Lipman, J.M. Becker, M. Gately, R. Chizzonite, and T.D. Anderson. 1994. Biologic effects of recombinant human interleukin-12 in squirrel monkeys. *Laboratory Investigation* 71, no. 6:862.

34. Orange, J.S., S.F. Wolf, and C.A. Biron. 1994. Effects of IL-12 on the response and susceptibility to experimental viral infections. *The Journal of Immunology* 152:1253.

35. Koblish, H.K., C.A. Hunter, M. Wysocka, G. Trinchieri, and W.M.F. Lee. 1998. Immune suppression by recombinant interleukin-12 involves interferon g induction of nitric oxide synthase (iNOS) activity: inhibitors of NO generation reveal the extent of rIL-12 viccine adjuvant effect. *The Journal of Experimental Medicine* 188, no. 9:1603.

36. Mazzolini, G., I. Narvvaiza, A. Perez-Diez, M. Rodriguez-Calvillo, C. Qian, B. Sangro, J. Ruiz, J. Prieto, and I. Melero. 2001. Genetic heterogeneity in the toxicity to systemic adenoviral gene transfer of interleukin-12. *Gene Therapy* 8, no. 4:259.

37. Zhang, Y., N. Chirmule, G. Gao, R. Qian, M. Croyle, B. Joshi, J. Tazelaar, and J.M. Wilson. 2001. Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. *Molecular Therapy* 3, no. 5:697.

38. Akridge, R.E., and S.G. Reed. 1996. Interleukin-12 decreases human immunodeficiency virus type 1 replication in human macrophage cultures reconstituted with autologous peripheral blood mononuclear cells. *The Journal of Infectious Diseases* 173:559.

39. Young, H.A., and K.J. Hardy. 1995. Role of interferon-γ in immune cell regulation. *Journal of Leukocyte Biology* 58:373.

40. Ponti, W., T. Rubino, M. Bardotti, G. Poli, and D. Parolaro. 2001. Cannabinoids inhibit nitric oxide production in bone marrow derived feline macrophages. *Veterinary Immunology and Immunopathology* 82:203.

41. Ansari, A.A., A.E. Mayne, J.B. Sundstrom, P. Bostik, B. Grimm, J.D. Altman, and F. Villinger. 2002. Administration of recombinant rhesus interleukin-12 during acute simian immunodeficiency virus infection leads to decreased viral loads associated with prolonged survival in SIVmac251-infected rhesus macaques. *Journal of Virology* 76, no. 4:1731.

CONCLUSIONS AND FUTURE DIRECTIONS

The data presented in this dissertation provide some interesting answers to the questions surrounding the cytokine response in the acute phase of FIV infection. Like most research however, these answers raise further questions. Instead of being definitive, this work provides a framework for future studies exploring the issues of cytokine induction from a more mechanistic angle.

The ribonuclease protection assay has proven to be a reliable and sensitive means of measuring feline cytokines. Areas of future exploration with this assay would include more optimization in an attempt to detect feline interleukin 12 (IL12). As the T cell cytokine assay benefited from mitogen stimulation, so too may the macrophage RPA. The ability of FIV-positive and negative macrophages to respond to lipopolysaccharide might offer insight into the basal levels of IL12 production during infection.

The early, selective rise in T cell IL10 production that we noted is somewhat unusual in the context of work published with SIV and HIV. To the best of my knowledge, all of the cytokine data available from SIV studies involves intravenous viral inoculation. It seems likely that this massive, systemic influx of virus could lead to altered cytokine kinetics. The length of a potential IL10-dominated phase may be dramatically shortened or bypassed altogether. Work with tissue samples during the acute phases of HIV infection will likely continue to be difficult and hampered by the imprecise nature of the exposure. If FIV is responsible for the induction of IL10, it provides one attractive explanation of the ability of the virus to escape immune containment.

The fundamental question raised by the IL10 data is at what level in the virus-host interaction is this skewing of cytokine profile occurring? Examination of tissue cytokine profiles in the days following infection would help determine how rapidly IL10 is induced. It seems likely that, as has been shown in other models of infectious disease, early viral interactions with dendritic cells are important in generating subsequent T cell

cytokine profiles. Are the CD4+ T cells that are producing high levels of IL10 equivalent to the Tr1 cells documented in people? Work is ongoing in this laboratory to isolate feline dendritic cells and it would be interesting to see what cytokines are induced by *in vitro* exposure to virus. Does FIV block or decrease dendritic cell IL12 production as a means of inducing high levels of IL10?

The data demonstrating simultaneous increases in IFNγ and IL10 at 10 weeks post infection is consistent with several of the studies in FIV, SIV and HIV mentioned throughout the thesis. This work identifies CD8+ T cells as the population with increased levels of both IFNγ and IL10. The studies documenting elevations in both cytokines have generally been conducted with lymphoid tissues samples, which would seem to have particular relevance given that viral replication is occurring in these sites. The notion of the simultaneous increases in cytokines that have been traditionally considered antagonistic is gaining more widespread acceptance in several models of infectious disease. Our data support the concept that cytokine responses to infectious diseases are generally more complex than the Th1/Th2 dichotomy would suggest. Relevant questions from this portion of the thesis center around the exact identity of the CD8+ T cells. Is it the same cell producing both IL10 and IFNγ or are we seeing a delayed rise in anti-viral, IFNγ-producing CD8+ T cells?

Antibodies to feline IFNγ have just become commercially available and could be adapted to intracellular cytokine staining. We have documented a restricted MHC class I repertoire in our closed breeding colony and have begun to develop class I/peptide tetramer complexes to label viral specific CD8+ T cells. In addition, ELISPOT technology has been refined for feline samples in another laboratory. Techniques such as these may allow us to answer some of the questions surrounding the evolution of this delayed production of IFNγ by CD8+ T cells.

The adenoviral delivery of IL12 obviously has a lot more room for exploration. The early pilot study data is encouraging and we have shown cross-species efficacy. A larger adenoviral pretreatment/FIV infection experiment is set to begin in the next few weeks.

Viral levels will be monitored more closely to document any potential effect of the IL12 on viral replication kinetics. Any documentation of IL12-induced decreases in viral replication would help substantiate our proposed role of IL10 in the early inhibition of the anti-viral immune response. Adenoviral gene delivery could be also be exploited in peptide vaccine approaches. As mentioned previously, peptide/tetramer work has begun in this laboratory and the identification of immunodominant peptides could be used to construct FIV peptide/IL12 expressing adenoviruses for vaccine studies.