Université d'Ottawa • University of Ottawa

PERMISSION TO REPRODUCE AND DISTRIBUTE THE THESIS

PERMISSION DE REPRODUIRE ET DE DISTRIBUER LA THÈSE

NOM DE L'AUTEUR / NAME OF AUTHOR:	SINGH, Rekha
ADRESSE POSTALE / MAILING ADDRESS:	5610 Salvia Common Fremont, CA 94538, USA
GRADE / DEGREE:	**ANNÉE D'OBTENTION / YEAR GRANTED**
Ph.D.(Microbiology and Immunology)	2003
TITRE DE LA THÈSE / TITLE OF THESIS:	
Cellular Immune Responses in HSV and CMV Infections	

L'auteur permet, par la présente, la consultation et le prêt de cette thèse en conformité avec les règlements établis par le bibliothécaire en chef de l'Université d'Ottawa. L'auteur autorise aussi l'Université d'Ottawa, ses successeurs et cessionnaires, à reproduire cet exemplaire par photograhie ou photocopie pour fins de prêt ou de vente au prix coûtant aux bibliothèques ou aux chercheurs qui en feront la demande.

Les droits de publication par tout autre moyen et pour vente au public demeureront la propriété de l'auteur de la thèse sous réserve des règlements de l'Université d'Ottawa en matière de publication de thèses.

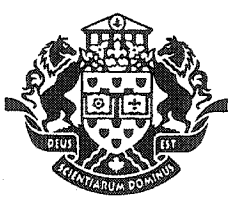
The author hereby permits the consultation and the lending of this thesis pursuant to the regulations established by the Chief Librarian of the University of Ottawa. The author also authorizes the University of Ottawa, its successors and assignees, to make reproductions of this copy by photographic means or by photocopying and to lend or sell such reproductions at cost to libraries and to scholars requesting them.

The right to publish the thesis by other means and to sell it to the public is reserved to the author, subject to the regulations of the University of Ottawa governing the publication of theses.

N.B. LE MASCULIN COMPREND ÉGALEMENT LE FÉMININ

April 14, 03
DATE

Rekha Singh
(AUTEUR) SIGNATURE (AUTHOR)



Université d'Ottawa · University of Ottawa

Université d'Ottawa · University of Ottawa

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

SINGH, Rekha

AUTEUR DE LA THÈSE - AUTHOR OF THESIS

Ph. D. (Microbiology and Immunology)

GRADE - DEGREE

Dept. of Biochemitry, Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT - FACULTY, SCHOOL, DEPARTMENT

TITRE DE LA THÈSE - TITLE OF THE THESIS

Cellular Immune Responses in HSV and CMV Infections

F. Diaz-Mitoma

DIRECTEUR DE LA THÈSE - THESIS SUPERVISOR

EXAMINATEURS DE LA THÈSE - THESIS EXAMINERS

J. Angel

M. Kozlowski

S. Sad

B. M. Sahai

J.-M. De Koninck, Ph.D.

LE DOYEN DE LA FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

SIGNATURE

DEAN OF THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

Cellular Immune Responses in HSV and CMV Infections

Rekha Singh

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in

Biochemistry, Microbiology and Immunology

Department of Biochemistry, Microbiology and Immunology

University of Ottawa

Ottawa, Ontario, Canada

May 2003

Abstract

Recurrent serious medical problems associated with the herpesvirus infections have provided impetus for investigating complex interactions between the virus and the host immune system leading to herpes pathogenesis. In spite of numerous immunological studies on herpesvirus infection, the exact mechanism of immune modulation by these viruses remains unclear. The herpes simplex virus (HSV) and cytomegalovirus (CMV) are the prominent members of the Herpesviridae family collectively responsible for the majority of herpes-related morbidity and mortality in humans. These viruses are therefore the subjects of this study that was undertaken to improve our understanding of the nature of immune response and the mechanism of viral modulation leading to suppression of viral replication or evasion of the host immune response *in vivo*.

HSV and CMV belong to two distinct subfamilies of the Herpesviridae family but share extensive similarities in their genetic organization, replication cycle, and the ability to persist lifelong in immunocompetent hosts capable of eliciting effective antiviral immune responses. The mechanisms by which these viruses overcome the immunological barriers are poorly understood. The present study has examined the T helper responses to HSV-2 and murine CMV (MCMV) and provides new insights into the nature and the modulation of the T helper responses by these viruses *in vivo*.

Of the total 61 samples examined in this study, a majority (~89 %) of them were from individuals who exhibited at least one episode of active disease (AD) and were therefore symptomatic. The results presented in this thesis provide evidence for two successive immunological stages of HSV-2 disease in symptomatic individuals. The first stage is characterized by a T cell proliferative response to HSV-2 antigen that is

accompanied by Th1 type T helper response; the samples in this stage are referred to as responders. The second stage is marked by a selective absence of both T cell proliferative and Th1 helper responses to HSV-2, as indicated by diminished T cell proliferation and Th1 cytokine production by peripheral blood mononuclear cells (PBMC) cultures after stimulation with HSV-2 antigen, despite normal responsiveness to anti-CD3 antibody. The samples in this stage are referred to as non-responders. Among the 61 samples from 43 HSV-2 seropositive participants included in this study, 47 samples were responders (~77 %) and 14 non-responders (~23 %), and samples from 3 individuals progressed from responders to non-responders during the course of this study. Such loss of T cell responsiveness to HSV-2 antigen in PBMC samples from symptomatic patients represents HSV-2-specific immunodeficiency that may lead to mortality.

Infection with HSV-2 triggers a Th1 type immune response characterized by high levels of interferon-γ (IFN-γ) and low interleukin (IL)-4 production by PBMC cultures in response to HSV-2 antigen, regardless of whether PBMC were derived from symptomatic or asymptomatic responders, or from responders with AD or non-active disease (NAD). The PBMC from symptomatic responders also produce high levels of IL-10 presumably from non-T cell sources. Although IFN-γ is known to suppress HSV-2 replication, its levels in symptomatic responders are insufficient to prevent the onset of recurrence. The PBMC from asymptomatic responders produce markedly higher levels of IFN-γ (~3 fold) and much lower (undetectable) levels of IL-10 than PBMC from symptomatic responders, suggesting a protective effect of this cytokine combination. The precise trigger for AD in HSV-2 seropositive individuals remains unknown. Neither Th1 nor Th2 cytokine response by itself appears to constitute such a trigger.

B7-1/B7-2 costimulation of T cells by antigen-presenting cells is essential for T cell activation by antigen. HSV-2 infection affects the expression of both B7-1 and B7-2 on monocytes, the key antigen presenting cells *in vivo*, potentially in two ways with opposing outcomes. It abrogates or diminishes the IFN-γ-upregulation of B7-1 (in 8 out of 9 patients) and B7-2 (in 6 out of 9 patients) on monocytes. However, the infection also augments the expression of B7-1 and B7-2 on monocytes through an IFN-γ-independent mechanism (in 9 out of 9 patients). Although the clinical significance of these opposing effects is presently unclear, these may be related to the immunological mechanism or strategy leading to recurrent disease in immunocompetent hosts.

Like HSV-2, infections with MCMV in mice also led to a predominant Th1 type immune response characterized by high levels of IFN-γ and low IL-4 production. Studies with specific MCMV mutants, containing Tn3 transposon in the open reading frame of M25, M27, M43, or m09 gene, led to the identification of M43 gene that specifically suppresses IL-4 response. The Tn3 disruption of M43, but not M25, M27 or m09, gene of MCMV led to a strong IL-4 response ($p = 0.0002$) despite the presence of a dominant IFN-γ response. These results provide insight into the possible role of a herpesvirus gene that can profoundly modulate the nature of T helper response *in vivo*. The presence of a homologous genetic element in other herpesvirus genomes may explain the dominant Th1 immune response triggered by HSV-2 and possibly other herpesviruses. The obvious importance of this finding, beyond herpesvirus immunopathogenesis, lies in the ability of M43 and homologous genes to globally modulate the nature of cytokine response *in vivo* and suppress Th2 cytokine-mediated diseases. The assessment of immunological, clinical and pharmacological significance of M43 and related genes would require further studies.

Acknowledgments

I would like to express my sincere gratitude and thanks to my doctoral degree supervisors, Dr. Fransisco Diaz-Mitoma and Dr. Fenyong Liu, for their support during this study. I am very much thankful to Dr. Ashok Kumar and Dr. Alexander Mackenzie, the members of my thesis advisory committee, for their thoughtful discussions and encouragement, and for reviewing the thesis and offering numerous valuable comments. I gratefully acknowledge my thesis examiners for critically reviewing the thesis and providing insightful constructive criticism, which resulted in significant improvement of this thesis. Special thanks are due to Louise Larocque, Peihua Cheng, Claudia Galvis, and Erik Haghjoo for their help during experimentation, to Dr. Gerry Abenes for many useful discussions, and to Manfred Lee and Dr. Xiaoyan Zhan for providing the recombinant murine Cytomegaloviruses. I would like to thank all the members of Dr. Diaz-Mitoma's, and Dr. Liu's Labs for being good friends and making my stay in the lab memorable. I also wish to thank the office staff of the Children Hospital of Eastern Ontario, Virology Division Ottawa, Ontario, Canada, and the School of Public Health, University of California, Berkeley, USA, for their helpful cooperation. Finally, I wish to express my gratitude and indebtedness to my parents and all family members and friends, for their inspiration, encouragement, and support. Most of all, I extend my affectionate appreciation to my husband Rakesh, for his constant motivation and encouragement, and to Aparna and Ashish for being wonderful children. Their unfailing support, devoted understanding, and cooperation were a continuous source of inspiration in my pursuit.

This thesis is dedicated to

my parents

Table of Contents

List of Tables

List of Figures and Illustrations

Page

Abbreviations

Ab	antibody
AD	active disease
Ag	antigen
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cells
AS	asymptomatic HSV-2 seropositive
bp	base pairs
CMI	cell-mediated immunity
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
°C	degree Celsius
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dpi	days post infection
ds	double stranded
EBV	Epstein Barr Virus
ELISA	enzyme linked immunosorbant assay
gp	glycoprotein
HCMV	human cytomegalovirus
HLA	human leukocyte antigen
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HHV-6	human herpes virus type 6
HHV-7	human herpes virus type 7
HHV-8	human herpes virus type 8
ICTV	International Committee on the Taxonomy of Viruses
IFN-γ	Interferon-gamma
IL	interleukin
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-10	Interleukin-10
kDa	kilodaltons
LTR	long terminal repeat

MFI	median fluorescence intensity
MHC	major histocompatibility complex
M/mM	molar/millimolar
µM	micro molar
µl	microliter
MCMV	murine cytomegalovirus
NAD	non active disease
ng	nanogram
NK cell	natural killer cell
NO	nitric oxide
NR	non responder
OD	optical density
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
pfu	plaque forming unit
pg	picogram
R	responder
Rv	recombinant virus
RvM25	recombinant MCMV with disrupted M25 gene
RvM27	recombinant MCMV with disrupted M27 gene
RvM43	recombinant MCMV with disrupted M43 gene
Rvm09	recombinant MCMV with disrupted m09 gene
SCID	sever combined immunodeficiency
sd	standard deviation
s.e.m.	standard error of the mean
SI	stimulation index
SV40	simian virus 40
TAP	transporter associated with antigen processing
TCR	T cell receptor
Th	T helper
Th1	T helper type1
Th2	T helper type2
Tn	transposon
TNF	tumor necrosis factor
VZV	Varicilla-Zoster Virus
WT	wild type MCMV

Chapter 1

INTRODUCTION

HSV and human CMV (HCMV), members of Herpesvirdae family, cause acute and persistent infections which involves latency and recurrent disease. These viruses inflict severe opportunistic infections in immunocompromised individuals such as AIDS and transplant patients, and neonates (1-9). Both HSV and HCMV employ mechanisms to overcome the antiviral immune responses of the host (10-25). To prevent complete evasion there are counter balance mechanisms (26-37), making it difficult to understand the immunopathogenesis of these infections. Previous studies have shown that $CD4^+$ T cells play a major role in the pathogenesis of these infections (37-44). Further, these infections alter T helper type-1 and –2 (Th1 and Th2) responses leading to predominant Th1 response (45-49) which play a vital role in viral clearance (38-43). In spite of numerous extensive studies related to HSV and CMV diseases, the underlying cause of alteration in Th responses during these infections are still unclear. Therefore, the main objective of this study was to investigate the factors leading to modulation of Th responses during infection with these herpesviruses. The results of present study provide useful insight into potential mechanisms underlying the alteration in Th responses during such infections. This chapter describes genomic organization, and biological and immunological characteristics of Herpesvirdae family in general, and of HSV and CMV in particular, followed by the hypothesis and objectives of the present study.

1.1 *Herpesvirdae* Family

1.1.1 General Description

Herpes viruses are highly disseminated in nature, and over 100 different types have been identified. Among those that have been at least partially characterized, eight herpesviruses have been isolated from humans. These are herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), human cytomegalovirus (HCMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), and human herpesvirus-6, -7, and -8 (50,51).

1.1.2 Common Architectural Features

The viruses included in *Herpesviridae* family have distinctive virion architecture. A typical herpesvirus consists of an electron dense core containing a linear double stranded (ds) DNA in the form of a torus (52). The core is surrounded by an icosadeltahedral capsid 100-110 nm in diameter comprised of 162 capsomeres. An amorphous, sometimes asymmetric material called tegument surrounds the capsid (50-53). The outer most covering of the virus, the envelope, has a typical trilaminar appearance. The herpesvirus envelope contains numerous protrusions of spikes. The spikes consist of glycoproteins. The number and relative amounts of viral glycoproteins vary in different subfamilies. The most interesting feature of herpesvirus DNAs is their sequence arrangement with the presence and location of reiteration of terminal sequences greater than 100 bp (50,54,55).

1.1.3 Biological Characteristics

The herpesviruses are distinguished by their biological properties (50,54,55): i) All herpesviruses encode a large array of enzymes involved in nucleic acid metabolism (e.g., thymidine kinase, thymidylate synthetase, dUTPase, ribonucleotide reductase, etc.), DNA synthesis (e.g., DNA helicase and primase), and possibly, modifying proteins (e.g., protein kinase). The exact array of genes encoding enzymes may vary somewhat from one herpesvirus to another. ii) Their replication and assembly occur in the nucleus. iii) The cell is lysed as a result of virus infection. iv) They have the capacity to enter a state of latency in which only small subsets of the viral genes are expressed.

1.1.4 Nomenclature and Classification

The members of *Herpesviridae* family have been classified by the herpesvirus study group of the International Committee on the Taxonomy of Viruses (ICTV) into three subfamilies on the basis of biological properties. These subfamilies are the *alphaherpesvirinae*, the *betaherpesvirinae*, and the *gammaherpesvirinae*. The *alphaherpesvirinae* are characterized by a broad host range, and are highly lytic in culture. The *betaherpesvirinae* have a restricted host range, grow more slowly in culture, and cells infected with these often show enlargement. The *gammaherpesvirinae* mainly infect lymphoblastoid cells (50,56).

1.2 Herpes Simplex Viruses (HSV)

1.2.1 General Description

HSV, a member of *alphaherpesvirinae* subfamily, were the first of the human herpesviruses to be discovered and are among the most intensively investigated of all viruses. HSV has been isolated from nearly all visceral and mucocutaneous sites. Their important features are their biological properties, particularly their ability to cause a variety of infections, neurovirulence, to remain latent in their host for life, and to be reactivated to cause lesions at or near the site of initial infection (1,57).

1.2.2 Sub-Types

More than 40 years after their isolation it was suggested that there were two serotypes of HSV, HSV-1 and HSV-2 (58). Not until 1961 were practical plaque assays published, and only much later was the genome size and the extent of homology between these two viruses reported (59). In 1968, Nahmias and Dowdle (60) demonstrated antigenic and biologic differences between HSV-1 and HSV-2. HSV-1 was shown to be more frequently associated with nongenital infection (infection above the belt), whereas HSV-2 was associated with genital disease (infection below the belt) (61). Other differences in the restriction digest pattern suggesting different organization of genes between the two strains of HSV were demonstrated by restriction endonuclease technology (62).

1.2.3 Pathology

The changes that occur due to replication of HSV are similar for both primary and recurrent infection but vary in the quantitative extent of cytopathology. The histopathologic changes represent a combination of virally mediated cellular death and associated inflammatory response. Changes induced by viral infection include ballooning of the infected cells and the appearance of condensed chromatin within the nuclei of cells, followed by subsequent degeneration of the cellular nuclei, generally within parabasal and intermediate cells of the epithelium. Cells lose intact plasma membranes and form multinucleated giant cells. With cell lysis, a clear fluid containing large quantities of virus appears between the epidermis and dermal layer (61,63). The vesicular fluid contains cell debris, inflammatory cells, and often times, multinucleated giant cells. In dermal substructures there is an intense inflammatory response. With healing, the vesicular fluid becomes pustular with the recruitment of inflammatory cells and, then, scabs. Vascular changes in the area of infection include perivascular cuffing and areas of hemorrhagic necrosis. These findings become particularly prominent when organs of the body other than skin are involved (61).

1.2.4 Pathogenesis in Human

The pathogenesis of human disease is dependent on intimate, personal contact of a susceptible individual with someone excreting HSV. The clinical manifestation and course of HSV infections depend on the anatomic site of the infection, the age and immune status of the host, and the antigenic type of the virus. First episode of HSV

disease, especially primary infections with either HSV-1 or HSV-2 in which the host lacks HSV antibodies in acute phase serum are frequently accompanied by systemic signs and symptoms, involve both mucosal and extra mucosal sites, are characterized by a longer duration of symptoms and a longer time during which virus can be isolated from lesions, and have a higher rate of complications than recurrent episodes of the disease. Immunosuppressed patients, especially those with defects in cell-mediated immunity, have both, more frequent and more severe disease (1,57,61,63-66). Neurovirulence and latency are the two important properties of HSV that influence human disease. The propensity of HSV to replicate in nervous system tissue can result in profound disease with severe neurologic consequences. At least three HSV glycoproteins gB, gD, and gH are involved in virus binding and penetration of cells. The fundamental principle of disease pathogenesis, then, is the propensity of virus to replicate at mucosal surfaces, be transported to dorsal root ganglia, and become latent (57,58,61).

After latency is established, a proper stimulus can cause reactivation to occur causing virus to become evident at mucocutaneous sites and appear as skin vesicles or mucosal ulcers. Transport of the virion is by retrograde axonal flow. Though unusual, the primary infection can spread beyond the dorsal root ganglia, thereby becoming systemic. Such circumstances include disseminated neonatal HSV infection with multiorgan involvement, multiorgan disease of pregnancy, and rarely, dissemination in patients who require significant immunosuppressive therapy. Thus, the systemic disease is the consequence of viremia in a host incapable of limiting replication to mucosal surfaces (57,61). The general sites of infection and clinical syndromes associated with HSV-1 and HSV-2 are gingivostomatitis, herpes labialisrhinitis, pharyngotonsilitis, pneumonitis,

herpetic keratitis, herpes gladiatorum, herpes paronychia, herpes whitlow, meningoencephalitis, and urogenital disease. HSV-2 is one of the most common sexually transmitted diseases (58,61,64).

1.2.5 Animal Models for HSV

The commonly used animal models for HSV disease are mouse, guinea pig, and rabbit (57,67,68). However, due to the advantage of size, cost, availability of defined inbred strains and well-characterized immunologic reagents, the mouse has become the most popular animal model for studying the immunobiology and pathogenesis of herpes diseases (57).

1.2.6 Epidemiology

Herpes simplex viruses are distributed worldwide. HSV infections occur throughout the year. Human are the sole reservoirs for transmission of HSV. HSV disease ranges from the usual case of mild illness to sporadic severe and life-threatening disease in immunocompromised individuals such as neonates, AIDS patients, and patients undergoing transplantation. Although HSV-1 and HSV-2 are usually transmitted by different routes and involve different areas of the body, there is a great deal of overlap between the epidemiology and clinical manifestations of infections. However, several serological assays, such as neutralization, indirect immunofluorescence, passive hemagglutination, radioimmunoassays (RIA), enzyme-linked immunosorbant assay

(ELISA), can measure the relative selectivity of serum antibodies to the two subtypes (58,61,68,69).

Primary HSV-1 infections usually occur in young children, less than 5 years of age, and are most often asymptomatic. The mouth and lips are the most common sites of HSV-1 infections; however, any organ can become infected with this virus. With clinical illness, gingivostomatitis usually is the manifestation of the disease (58,61). Antibodies to HSV-2 are not routinely detected until puberty, and antibodies prevalence rates are correlated with past sexual activity. Many people who have Ab to HSV-1 or HSV-2 are asymptomatic. Corneal infection with HSV is a major cause of corneal blindness in the Western Hemisphere. The increased frequency of genital herpes in Western industrialized countries coincides with increasing numbers of seronegative persons entering their sexually active years; this finding suggests that prior HSV-1 infections may offer some protection against the acquisition of clinically symptomatic genital HSV-2 disease (69-71).

1.2.7 Genital Herpes

Genital Herpes is a sexually transmitted disease mainly associated with HSV-2. Recurrent genital infection is the largest reservoir of HSV-2. Although most genital infections are caused by HSV-2, a recognizable but variable proportion is attributable to HSV-1. The distinction in virus type is significant, because genital HSV-1 infections are usually both less severe and less prone to recurrences than genital HSV-2 infections. From 1970 to 1985, annual incidence of HSV-2 infection in HSV-2-seronegative individuals has increased by 82 %. Incidence in 1985 was higher in women than in men

(9.9 vs. 6.9 per 1,000), higher in Blacks than in Caucasians (20.4 vs. 6.3 per 1,000), and highest in the group aged 20-29 years (14.6 and 22.5 per 1,000 in men and women, respectively). Thus, by 1985, approximately 1,640,000 +/- 150,000 people (730,000 men and 910,000 women) were being infected annually with HSV-2 in the United States (69,70,72).

In primary disease, the incubation period is about 6 days and severe local symptoms last for about 10 days. Genital HSV infections are characterized by symptomatic recurrences that may occur 4 to 15 times per year, with lesions lasting for periods of 3 to 12 days (65). Genital symptoms include pain, itching, erythema, swelling, discharge, dysuria, and inguinal lymphadenopathy. General symptoms include malaise, fever, headache, and root pains down the legs. Severe dysuria or neurologic dysfunction may lead to urinary retention. Vesicles may occur on the external genitalia, rectum, urethra, and the mucosa of the bladder.

Clinical lesions typically present with wavelike crops of vesicles that coalesce to form bullae or large tender ulcers involving the external genitalia. Genital infection with HSV-2 in adult women may be a risk factor for cancer of the cervix. In some cases, dissemination after primary genital infection lead to severe manifestations of disease, such as necrotizing hepatitis with or without thrombocytopenia, leukopenia, disseminated intravascular coagulopathy, and encephalitis (61,64-66). Genital HSV transmission is usually due to asymptomatic viral shedding by individuals who are unaware that they are infected and clinical screening fails to detect most infections. HSV-2 infection is also implicated in the transmission and acquisition of HIV (73).

1.2.8 Neonatal Herpes

The most serious complication of urogenital infections in a pregnant woman is transmission of HSV to the neonate. Maternal primary infection before 20 weeks gestation in some women has been associated with spontaneous abortions. HSV causes severe infections with congenital anomalies and a high mortality rate in human newborns. Some of the survivors have crops of cutaneous vesicles that recur at 3 to 5 week intervals for many years. The incidence of neonatal herpes is 1/2,500-1/5,000 births. Most infections of the newborn are caused by HSV-2, but there is no clinical difference if there is an infection with HSV-1. Skin lesions are the most common presenting manifestations in newborns with progression to more generalized disease occurring in 70 % of infants whose presenting sign is a skin vesicle (69-74).

1.2.9 HSV Immunology

In experimental animal models, polyspecific anti-HSV antiserums, monoclonal or monospecific anti-HSV antibodies, natural killer (NK) cells, macrophages, and $CD8^+$ CTLs, $CD4^+$ Th cells can, under appropriate conditions, confer protection against subsequent viral challenge (27-31,41-43,75-89).

1.2.10 Innate Immunity

In early studies, a vital role for NK cells in HSV clearance was suggested (75). In murine model for HSV eye infection, activation of NK cells appears to play a role in preventing direct anterior to posterior spread of the virus in the inoculated eye (90). Activated

macrophages also help in viral clearance. IFN-γ is also known to affect macrophage function, for example, upregulating potential antiviral activities such as NO and TNF-α production. It has been suggested that NO may act to inhibit HSV replication (31,83,91,92).

1.2.11 Adaptive Immunity

1.2.11.1 Humoral Immunity

Analysis of antibody mediated reactions have elucidated host responses to viral polypeptides and correlated these responses with the development of neutralizing antibodies (66,87). In persons with a primary infection, humoral antibodies can usually be detected at about 6 days, and reach a peak in about 2-3 weeks. In the newborn, IgM antibodies to HSV can be detected within 1-4 weeks after birth and are present for 6 months or more (66,87-89). The role of humoral antibodies in the pathogenesis of reactivation disease is less clear. The results of functional assays of humoral immunity appear to remain relatively constant in patients with recurrent herpetic infections, whereas the cellular immune responses to HSV Ag *in vitro* fluctuate throughout the course of the disease. Also, the passive transfer of antibodies after intradermal challenge with HSV does not offset paralysis or abrogates viral replication and establishment of latency. Immunization with live HSV before viral challenge also yields no strong correlation between neutralizing antibody titer and protection of local sensory ganglia from viral colonization (87,93).

1.2.11.2 Cell Mediated Immunity

Multiple cell populations, including NK cells, macrophages, a variety of T-lymphocyte populations, and lymphokines generated by these cells, play a part in the host's defenses against HSV infections (27,29-32,41-43,57,76-81,94-96). In animals, passive transfer of primed lymphocytes confers protection from subsequent challenge. Maximal protection usually requires the activation of multiple T-cell subpopulations. *In vivo* studies in mice suggest that class I restricted T cells are important in limiting the spread of virus to the brain, whereas class II restricted responses are needed to eliminate virus from the lungs and liver, as well as to provide help for antibody production. *In vitro*, HSV stimulates proliferation and cytotoxicity by human class II restricted T cells from immune adults, and class I restricted responses have been detected in the small number of patients studied. Immunodepletion studies have shown the importance of CTLs in resolving cutaneous disease. Adoptive transfer of HSV-immune $CD8^+$ or HSV-immune $CD4^+$ T cells reduce viral replication or provide protection upon challenges (29,61,76-78).

1.2.12 Th Responses in HSV Infection

Th cells belong to the $CD4^+$ subset and their activation may be followed by proliferation and selected lymphokine release. $CD4^+$ T-helper cells through their cytokines play a major role in the development and effectiveness of both humoral and cell mediated immunities (97-100). Both major subsets of Th1 and Th2 cells participate in recovery from HSV (42). There is predominance of $CD4^+$ T cells at cutaneous sites, in humans and in Balb/c mouse model. In a mouse model for HSV, Th responses provide protection against low doses of HSV. Such anti-HSV protection conferred by MHC class II-

restricted cells is long term. Successful immunity against higher doses of HSV requires anti-HSV specific $CD8^+$ cytotoxic T lymphocytes (CTL) activity, which are also regulated by $CD4^+$ T lymphocytes. Thus, the relative capacity of $CD4^+$ T lymphocytes to recognize and respond to Ag is a central factor in host immunity to HSV (27,42,43,80,81,95,101). In mice, HSV glycoproteins gB and gD can elicit protective immunity to lethal challenge of HSV by induction of Th lymphocyte responses (30,43).

1.2.13 IL-2 in HSV Infection

IL-2 is a major cytokine responsible for intercellular regulation of immunity. Murine studies have indicated that IL-2 is primarily produced by a subset of $CD4^+$ T lymphocytes, termed Th1 cells, during activation by specific Ag and mitogens. The functions of IL-2 are quite broad, and include upregulation of helper and cytotoxic T lymphocytes, NK cells, and macrophages (97-100,102).

Studies have shown that IL-2 treatment alone and together with HSV immune lymphocytes or other cytokines can protect animals from lethal HSV infection, although it does not prevent subsequent recurrences of HSV infection. This protection is associated with different effector mechanisms, including augmentation of NK cell lysis, as previously described. Lymphocytes derived from lymph nodes of HSV-infected mice produce IL-2 after *in vitro* expansion and stimulation with HSV Ag (27,101). It has been shown that removal of $Ly2^+$ cells prior to stimulation *in vitro* augments IL-2 production. This suggests that $CD8^+$suppressor T cells are operative in regulation of IL-2 production during HSV infection (27).

1.2.14 IFN- γ in HSV Infection

Actual mechanisms by which viral clearance is achieved by innate or immune cells are unresolved but considerable evidence suggest that interferons, especially IFN-γ, is intricately involved. In support of a protective effect, is the detection of a higher level of IFN-γ in stimulated peripheral blood mononuclear cells (PBMC) and in recurrent vesicles of patients having greater interval between attacks (103,104). Initial studies demonstrated that *in vitro* induction of IFN-γ by HSV is immune specific. It was recognized, however, that IFN-α was also produced by the HSV-stimulated PBMC from HSV seronegative and seropositive individuals. It has been shown that the interferon produced in the stimulated cultures is primarily alpha at 2 days and a mixture of alpha and gamma by 5 days. Further studies showed that IFN-γ production does not vary with the inducing strain of HSV. It does depend on the concentration of monocytes in the cultures, which could be related to the Ag processing functions of these cells (103).

It has been demonstrated that low levels of both spontaneous (unstimulated) and HSV Ag-induced IFN-γ in PBMC cultures correlate with time of recurrence of herpes virus infection. Further evidence linking IFN-γ with HSV infection is that high titers of this cytokine are present in herpetic lesion fluids, and are predictive of longer disease-free intervals between recurrences (80,81).

1.2.15 IL-4 in HSV Infection

IL-4 has been described as an immunosuppressive cytokine produced by Th2 cells and $CD8^+$ type 2 T cells. Down-regulatory activities of IL-4 on the cell-mediated immune

responses are well known. Numerous cell types proliferate and/or differentiate in response to IL-4. IL-4 has also been shown to be the major regulator of lymphokine-producing phenotype of $CD4^+$ T lymphocytes and to facilitate differentiation to Th2 cells (105).

In a mouse model for HSV-1 encephalitis, it has been demonstrated that a small amount of exogenous IL-4 increases the severity of encephalitis through the increased production of IL-4 from local $CD4^+$ T cells (106). It has also been reported that the highly enriched gD-specific $CD4^+$ T cells are specific to the NH_2-terminal 15-23 amino acids of gD protein, bear TCR Vβ8.2 determinants, and produce IL-4. In a mouse model for HSV stromal keratitis, IL-4 is shown to exacerbate the symptoms (44).

1.2.16 IL-10 in HSV Infection

IL-10 is a pleotropic cytokine produced by Th2 cells, B cells, mast cells, and monocytes. IL-10 possesses a broad spectrum of biological activities. IL-10 may contribute to the polyclonal B cell proliferation and is a promoter of proliferation of IL-2 activated cytotoxic T cells. IL-10 has potent inhibitory functions, which act partially via its inhibitory effect on APC. Human IL-10 inhibits Ag-specific proliferation of Th0, Th1, and Th2 cells, as well as Ag-stimulated cytokine synthesis by PBMC and NK cells in a macrophage/monocyte dependent manner (107-109). These effects of IL-10 might at least in part be as a result of its downregulation of expression of class II MHC and expression of co-stimulatory molecules, which are necessary for T cell and NK cell activation. IL-10 is a strong modulator of monocytes and downregulates the production of prostaglandin E2 as well as production of pro-inflammatory cytokines such as TNF-α,

IL-1, and IL-6. IL-10 inhibits the expression of B7 and ICAM-1 molecules, while it enhances the expression of soluble TNF receptors. IL-10 has been associated with the immunopathogenesis of a number of diseases including HSV, septic shock, lymphoproliferative disorders, and autoimmune diseases (44,107-110).

1.2.17 Strategies for Immune Escape

HSV appears to have mechanisms to overcome the immune responses of the host thereby making it difficult to understand the immunopathogenesis of HSV disease. These mechanisms include genes whose products prevent apoptosis in differentiated cells by interacting with antibody and complement, and by preventing induction of $CD8^+$ CTLs (10).

HSV expresses an immediate early (ie) protein, ICP47, which blocks presentation of viral peptides to MHC class I-restricted cells. ICP47 binds to transporter associated with antigen processing (TAP) and prevents peptide translocation into the endoplasmic reticulum (11). In humans, HSV is shown to interfere with the provision by monocytes of co-stimulatory factors that are essential for T cell stimulation. This effect of HSV may be due to secondary shut-off mechanisms that decrease host protein synthesis or secretion after HSV infection (12).

Expression of HSV ICP0 protein is shown to inhibit the induction of interferon-stimulated genes that are required for antiviral response, indicating that ICP0 is capable of overcoming an already established antiviral state (13).

1.3 Cytomegalovirus (CMV)

1.3.1 General Description

The cytomegaloviruses are the principal members of *betaherpesviruses* subgroup of *Herpesviridae* family. They are characterized by strict species specificity, salivary gland tropism, slow growth in cultured cells, and have a cytopathology-involving characteristic nuclear as well as cytoplasmic inclusions. CMVs share many characteristics, such as virion and genome structure, and the ability to establish persistent and latent infection (2,5,111). The importance of human CMV also designated as human herpesvirus 5, has increased over the past two decades as immunosuppressive post transplant therapies, as well as AIDS and other immunodeficiency states have become major medical concerns. These conditions predispose individuals to primary CMV infection or to reactivation of latent infection resulting in fulminant, life-threatening disease (4-8).

1.3.2 Genomic Organization

CMVs have the largest genome ranging from 180 Kbp in equine CMV to 230-240 Kbp for human, simian, guinea pig, and murine CMVs. There have been a number of reports of cellular DNA or protooncogene sequences carried in the human or simian CMV genome that is due to cross-hybridization of G + C-rich DNA or DNA containing relatively simple dinucleotide repeats. The HCMV genome (AD 169 strain) and MCMV (Smith strain) have been completely sequenced (111-114). HCMV is the only betaherpesvirus known to have a class E genome structure consisting of complex arrangement of unique and inverted repeats. HCMV strain AD 169 genome contains 208

predicted open reading frames (ORFs) of greater than 100 amino acids (AAs) in length. ORFs are designated by their location within the unique and repeated regions of the viral genome (TRL, UL, IRL, IRS, and US) and are numbered sequentially (111-114). Few of these predicted ORF products have been directly identified as proteins. The ORFs on the HCMV genome are largely collinear with those on the MCMV genome, and these viruses share a core set of herpesvirus-common proteins with other members of the herpes virus family. Approximately 33 of the 208 ORFs of HCMV show a substantial level of amino acid similarity to ORFs in HSV-1, VZV, and EBV. Additional ORFs are located within a cluster that exhibits ORF length and orientation similarity in all herpesvirus genomes, and other ORFs have low amino acid similarity but appear to perform similar function in all herpesviruses, such that a total of 46 ORFs may be arranged as conserved blocks. These conserved sequence blocks have a different order in the genomes of betaherpesviruses, gammaherpesviruses, and alphaherpesviruses. The functions encoded by these conserved blocks appear to have roles in DNA replication, DNA repair, nucleotide metabolism, or virion structure (111-114).

Although analysis of individual gene functions in human or other animal CMVs is not complete, the large coding capacity of HCMV includes functions that are essential as well as functions that are dispensable for viral growth in cultured cells. Through comparison of different strains, adventitious deletions, and deletion mutagenesis, approximately 41 ORFs have been identified as being dispensable for growth in human fibroblast (HF) cells (111,115-117).

1.3.3 Life Cycle

To initiate infection, the virus attaches to the cell surface receptors, fuse its envelope to the plasma membrane, and the de-enveloped capsid is transported to the nuclear pores. DNA is released into the nucleus of the cell where transcription, replication of the viral DNA, and assembly takes place. CMV transcription and protein synthesis is highly ordered. The transcription is characterized by the expression of three gene classes: alpha (immediate-early), beta (early), and gamma (late) genes, although there can be some overlap between these classes. In an active infection, the immediate early (α) phase of gene transcription commences upon entry into the cell during which time the positive and negative regulators of early gene transcription as well as proteins necessary for gene replication are expressed (111,118). The alpha genes are responsible for the initiation of replication. Next delayed early (β) gene expression occurs. The onset of expression of beta genes coincides with both the decline in the rate of expression of alpha genes and an irreversible shut-off of host cellular macromolecular protein synthesis. The gamma genes are heterogeneous and are differentiated from beta genes solely by their requirement for viral DNA synthesis for maximal expression (111,118,119).

Assembly of the virus begins in the nucleus, with acquisition of the envelope as capsid buds through the inner lamella of the nuclear membrane. Virus is transported through the cytoplasm to the plasma membrane, where release of progeny virions occurs. In addition to having an active replication cycle, CMV can also latently infect cells. Neither the molecular mechanism involved in CMV's transmission from active to the latent state (and vice versa) nor the location in which latency takes place is well-understood (111,118).

1.3.4 Animal Models

HCMV belongs to β family of herpes viruses whose members share the common feature of being highly species specific. Strict species specificity makes it difficult to study HCMV in any animal model. Several animals such as mouse, rat, guinea pig, rabbit, etc., have been used to investigate the pathogenesis and biology of CMV infection (120-123). Currently, the most accepted animal model for CMV infection involves using mice and MCMV. HCMV and MCMV share many homologous genes and have similar life cycles. As with other herpes viruses, after primary infection, both HCMV and MCMV persists in the host for life in a latent state from which reactivation can occur. The genomes of both HCMV and MCMV have been sequenced. Both HCMV and MCMV have a highly restricted host range and cause severe infection in the immunocompromised and immunologically immature host, resulting in similar clinical syndromes. In addition, both HCMV and MCMV are susceptible to the antiviral agent 9-(1,3-dihydroxy-2-propoxymethyl) guanine (113,123-125). However, MCMV and HCMV have some biological differences, as transplacental transmission of MCMV has not been demonstrated and mouse models of fetal infection involve direct inoculation of MCMV into the central nervous system or uterus (126,127). The similarities between the two viruses as well as the availability of a vast pool of genetically defined strains of mice have made MCMV an attractive model for studying HCMV virulence and pathogenesis. Therefore, a complete understanding of the biology of MCMV and the function of its genes can provide insight into the pathogenesis of HCMV.

1.3.5 Tn-Mediated Mutagenesis of MCMV Genes

One of the most powerful approaches to identify the function of viral-encoded genes is to introduce mutations into the viral genome and to screen viral mutants in both tissue culture and animals to determine the *in vitro* and *in vivo* function of viral genes. The methods to generate CMV mutants by site directed homologous recombination and transposon-mediated insertion have been well-established (128-133). These approaches certainly provide a very useful and convenient strategy to study the functions of viral genes in tissue culture and in animals.

1.3.6 Epidemiology

Humans are the sole reservoir for HCMV. Transmission of the virus occurs by direct or indirect person to person contact. Spread within susceptible populations is enhanced by the prolonged excretion of infectious virus following primary infection. In fact, prolonged replication of HCMV can follow primary infection in older children and adults, and recurrences are associated with intermittent shedding of HCMV from many sites in a seropositive young adults. HCMV infection is endemic and is present throughout the year. The prevalence of HCMV is quite astounding. In developed countries, as many as 80 % of children are infected before puberty. In developing nations, this value increases to nearly 100 % (134,135).

Despite this extraordinarily high prevalence, one does not see an epidemic of CMV-related illness because it is a disease of neonates and immunocompromised individuals. CMV causes opportunistic infections in AIDS patients and patients

undergoing transplantation. It has been estimated that 90-100 % of immunocompromised population will develop active HCMV infection in their lifetime. Ill-defined socioeconomic factors predispose to higher infection rate, both by intrauterine and extrauterine transmission. Between 0.2 % and 2.2 % of infants born in the United States are infected in utero. Another 8 % to 60 % become infected during the birth or following breast feeding (7,134). Sexual transmission of HCMV represents another major route of virus transmission in the adult population. HCMV is shed from the genital tract from both males and females (136). HCMV also causes ~40,000 cases of illness in neonates making it the most prevalent congenital viral infection (134,137). Human CMV infection rates in normal individuals have been best defined in pregnant women because of interest in understanding intrauterine transmission of this virus. In different populations, primary infection has varied from 0.7 % to 4 % per gestation among susceptible seronegative women. Poor hygiene and closeness of contacts within population groups appear to be important factors (134,135).

1.3.7 Pathology

HCMV characteristically infects ductal epithelial cells and rarely involves fibroblasts. Experiments done in murine and guinea pig model has shown that salivary gland involvement is probably chronic and most likely results from subclinical congenital and perinatal infection. In humans, the virus prefers various type of cells including macrophages, monocytes, endothelial cells, and arterial smooth muscle cells. With severe disseminated disease, evidence of HCMV involvement can be found in virtually all organ systems. Typically, HCMV-infected cells, as seen under light microscopy following

routine histologic staining, are large with limited cytoplasm and prominent central nuclei. The nuclei often contain marginated chromatin and prominent intranuclear inclusions surrounded by a clear halo. The presence of multiple inclusions has given the term owl's eyes inclusions (121,122,135-140). Viruria is a consistent feature of HCMV infection in all age groups and results from viral replication in the genitourinary tract (8). The other organ systems involved are gastrointestinal, respiratory tract, and Central nervous system (5,137,141-143).

1.3.8 Pathogenesis

Transmission of CMV occurs mainly through person-to-person contact and occasionally through person-to-object-to-person contact. After primary infection the virus persists in the body. Although, the exact localization of the latent virus is unknown it is clear that during latency viral DNA is present in many organs. By immune suppression the virus can reactivate from its latent state. During viraemia the virus is present in mononuclear cells by which it is transported through the body. In neonates and in immuno-suppressed animals the infection results in multiorgan failure, leading to death (120,121,127,137). The infection of the endothelial cells in the microvasculature and mononuclear cells seems to be important in the pathogenesis of the CMV-induced disease. In addition to the direct complications attributable to the virus itself, CMV has modulating effects on the immune response. Although in some instances the infection leads to immune suppression, the virus infection can also accelerate inflammatory and immune responses (2,5,14,122).

Developmental disorders induced by congenital CMV infection mainly involve the central nervous system. The specific components of the immune system that are

responsible for preventing reactivation disease have not been identified. Reactivation disease in humans involves multiple tissues, including the retina, liver, lungs, and gastrointestinal tract (2,5,137,139,141,144). In apparently immunocompetent hosts, CMV has been implicated in the genesis of atherosclerosis, rapidly progressive coronary artery disease and endothelialitis in cardiac transplant patients, coronary stenosis after angeoplasty, and inflammatory aortic diseases (122,140). Furthermore, CMV has been proposed as an etiological agent in the pathogenesis of certain malignancy, and insulin-dependent diabetes mellitus (145,146). Several *in vitro* properties of CMV, including slow replication, restricted cell tropism, and limited cell to cell spread, may contribute to its limited pathogenicity in normal hosts. The limited growth of HCMV *in vivo* also reflects the apparent protective responses of a normal immunity (2,137).

1.3.9 CMV Immunology

The role of the innate and adaptive immune systems in controlling acute MCMV infection has been characterized (2,34,40,137,147-150).

1.3.10 Innate Immunity

NK cells are important in controlling virus until specific immunity develops. Although less well documented, NK cell activity has been demonstrated against HCMV-infected cells. NK cells play a significant role in the control of acute infection with MCMV, and at least one mechanism for NK cell protection is secretion of IFN-γ (33,149). Macrophages also play an important role in CMV pathogenesis. Macrophages under certain

activation/differentiation conditions are permissive for HCMV or MCMV infection. Macrophage activation occurs *in vivo* during MCMV infection (31).

1.3.11 Adaptive Immunity

1.3.11.1 Humoral Immunity

Early reports suggested that the neutralizing antibodies do not play a primary role in recovery because infection is generally cleared before any obvious increase in antibody levels. Further, HCMV can be transmitted in the presence of passively acquired antibodies. However, recent evidence suggests that neutralizing antibody may play a protective role in tempering the severity of infection in some patient populations (147,148).

1.3.11.2 Cell Mediated Immunity

Various aspects of cellular immunity are antigen recognition, lymphokine production, and cytotoxic kill of the infected cells. These reactions are mediated by $CD4^+$ Th, $CD8^+$ CTL, and NK cells (31-35,40,147-150).

1.3.12 $CD8^+$ T Cell Immunity in CMV Infection

$CD8^+$ T lymphocytes represent the major protective principle. In MCMV, cell-mediated immune clearance of virus is largely carried out by cytotoxic T cells. The presence of CMV-reactive cytotoxic T cells (CTLs) has been correlated with protection from CMV disease. Adoptive transfer of CMV specific CTLs holds promise for therapy of post-

transplant disease caused by CMV. In a murine model for CMV disseminated cytopathic infection of the lungs, fatal outcome was observed only when reconstituting $CD8^+$ T cells were depleted and persistence of protective pulmonary $CD8^+$ T cells infiltrate occurred after clearance of acute infection. Humans mount a cytotoxic T cell response against CMV that may be MHC class I or class II restricted. Human carry class I-restricted cytotoxic T cells reactive to HCMV immediate early 1 gene product as well as to late gene products (35,151,152).

1.3.13 Th Responses in CMV Infection

Although, $CD8^+$ T lymphocytes have been suggested to be the major protective principle, these cells can not prevent horizontal infection in the absence of $CD4^+$ T lymphocytes. It has been reported that in the absence of $CD8^+$ T cells, $CD4^+$ cells can fully replace their functions and provide virus clearance. Adaptive transfer of $CD4^+$ T cells from the spleens of immune mice can have a modulating effect on viral infection, either by a direct cytotoxicity or through helper role. It has been shown that the $CD4^+$ T lymphocytes and not $CD8^+$ T cells play a pivotal role in controlling viral replication in the salivary gland. Levels of IFN-γ mainly influence this control (34,37-40,150-154). Ag-specific Th cells recognize processed Ag in association with class II MHC genes expressed on the surface of macrophages as well as on other cell types; such as activated B cells, dendritic cells, epidermal Langerhans cells, and human dermal fibroblasts. EBV lymphocyte cell lines are also capable of presenting CMV Ag to CMV-specific Th clones in MHC-restricted fashion. However, the efficiency of presentation is better by autologous mononuclear cells than the lymphoblastoid cell lines. A defect in proliferation

of Th cells not related to defective Ag presentation has been shown in infants who are congenitally infected with CMV (151,155). $CD4^+$ T cells against HCMV proteins have also been described, and significant response towards IE1, the major immediate early DNA-binding protein has been reported. The precursor frequencies of IE1-specific $CD4^+$ T cells are high in latently infected individuals, and cytokines such as IFN-γ and TNF-α found in the supernatant of $CD4^+$ T cells clones specific for IE1 significantly reduce HCMV replication (39,152).

1.3.14 Strategies for Immune Escape

The lifelong persistence of CMV in the face of a vigorous and repeatedly boosted immune response must require a strategic interaction between the virus and the host immune system. First, CMVs can abortively infect cells and establish latency. During the latent state of infection, the viral gene expression occurs minimally which is believed to prevents immune recognition. Secondly, CMV replicates at privileged sites in the body, for example salivary glands where productive infection is maintained for longer periods of time in a manner poorly controlled by $CD8^+$ T cells. Thirdly, highly adapted viral gene functions (eg. vIL-10) allow the virus to subvert detection by the immune system (14-20).

The $CD4^+$ Th cell immunity is suspected to be down regulated as HCMV is shown to inhibit IFN-γ induced upregulation of HLA class II expression (18). A mechanism of JAK1 proteolysis by HCMV has been described to explain the inhibition of IFN-γ-mediated augmentation of HLA class II (19). In mice, MCM inhibits the transcription of expression of MHC class II-related genes. Escape of HCMV from HLA-DR-restricted $CD4^+$ T cell response is mediated by repression of IFN-γ-induced class II

transactivator expression (14,19,155-158). It has also been reported that HCMV gpUL40 enhances the surface expression of HLA-E (MHC class I molecule) which is an inhibitor of NK cells and thereby represent an escape route for HCMV (14,159).

1.3.15 Counter-Balance by Immune Mechanisms

Complete evasion from immune control would result in the uncontrolled replication of the virus leading to the death of the host and limiting the dissemination of the virus. Experimental evidence suggests that the viral immune evasion mechanisms operate *in vivo* with only a limited efficacy. First, there is evidence that the degree of inhibition by both MCMV and HCMV is subject to regulation by distinct cytokines *in vitro*. Of these, IFN-γ is certainly the most potent and can efficiently restore Ag presentation of infected fibroblasts, although this has also been reported for TNF-α and type I IFNs (14,36). Second, adoptive transfer experiments have shown that the antiviral function of $CD8^+$ T cells has a strict requirement for IFN-γ, suggesting that this cytokine also regulate Ag presentation of infected cells *in vivo*. In addition, the quantitative assessment of antigenic viral peptides from MCMV-infected organs has demonstrated the pivotal role of IFN-γ for Ag processing. Final evidence for counteraction by host immune system is the presence of certain cell types that are resistant to the subversive effects of CMV on MHC class I. These cells are the professional antigen presenting cells such as macrophages (32,36-39,158).

1.4 T Cell Anergy and Costimulation

T Lymphocytes require two signals for optimal proliferation and induction of effector functions. Signal one is delivered by the engagement of the T cell antigen receptor (TCR) with the peptide-MHC complex. The second signal is antigen independent and provided by the interaction between co-stimulatory B7 molecules expressed on antigen presenting cells and their ligand present on T cells. MHC/Ag-TCR binding in the absence of costimulation leads to Ag-specific anergy (160-162).

1.4.1 B7-1 and B7-2 Molecules

B7 surface receptors exist in at least three distinct forms B7-1 (CD80), B7-2 (CD86), and B7-3. B7-1 and B7-2 isoforms are expressed on a variety of APC including B cells, dendritic cells, Langerhans cells, and monocytes. Expression of B7-1 and B7-2 on monocytes and dendritic cells can be rapidly induced following activation (163,164). The most extensively characterized pathway of costimulation has been the interaction of CD28 and CTLA4 on the T cell with B7-1 on APC. The B7-1 molecule is also known as the CD80 differentiation antigen. Engagement of T cell CD28 by monoclonal antibody (MoAb) or by interactions with CD80 results in enhanced production of cytokines by activated T cells and enhance stimulation of anti-tumor immunity. Blockage of this costimulatory pathway during T cell activation results in development of antigen-specific tolerance referred to as anergy. Interactions between CD28 on T cells and CD80 on activated B cells also regulate B-cell differentiation. CD80 is expressed by activated B lymphocytes, interferon-treated monocytes, activated T cells, and HTLV transformed T

cells. T cells also express a cell-surface molecule called CTLA-4 (cytotoxic-T-lymphocyte antigen-4 or CD152) that is structurally related to CD28, which also binds CD80 (163-165).

Occupancy of CTLA4 negatively regulates the activation of mouse T lymphocytes. In humans the interaction of CTLA4 with its functional ligands, B7-1 or B7-2, can down-regulate human T cell responses, probably by intracellular signaling events and independent of CD28 occupancy (166,167). The co-stimulatory effects of both B7-1 and B7-2 are dependent on CD28 cross-linking, based on complete inhibition of proliferation by CD28 antibody Fab fragments. Both B7-1 and B7-2 can maintain long term expansion of $CD4^+$ T cells. Co-stimulation with B7-1 and B7-2 induces high levels of cytokine secretion by T cells, and the effects of B7-1 and B7-2 can not be distinguished. Both B7-1 and B7-2 can elicit IL-4 secretion from $CD4^+$ T cells while, anti-CD28 Ab induced substantially less IL-4 secretion. Furthermore, both B7-1 and B7-2 can stimulate high levels of IFN-γ and IL-4 from $CD4^+CD45RO^+$ T cells, while neither B7 receptor can co-stimulate IFN-γ and IL-4 secretion from $CD4^+CD45RA^+$ T cells. B7-1 and B7-2 can, however, co-stimulate $CD4^+CD45RA^+$ T cells to secrete IL-2. It has been suggested that there are no intrinsic differences between B7-1 and B7-2 in their ability to co-stimulate $CD4^+$ T cells (168).

B7-2 belongs to the Ig superfamily and consists of an extracellular region containing a V- and a C- type Ig-like domain, a hydrophobic transmembrane domain, and a cytoplasmic tail (163). Comparison of the expression and function of B7-1 and B7-2 indicate that: (i) B7-1 and B7-2 can be expressed by multiple cell types, including B cells, T cells, macrophages, and dendritic cells, all of which are therefore candidate

populations for delivering co-stimulatory signals mediated by these molecules; (ii) Stimulating B cells with either LPS or anti-IgD-dextran induce expression of both B7-1 and B7-2, and peak expression of both co-stimulatory molecules occurs after 18-42 hours of culture (expression of B7-2 on these B cell populations is significantly higher than expression of B7-1 at all times assayed after stimulation); (iii) Blocking B7-2 co-stimulation inhibits antigen driven T cell proliferation and cytokine production, without affecting early consequences of TCR signaling such as induction of CD69 or IL-2 receptor alpha (IL-2R alpha) and; (iv) Expression of both B7-1 and of B7-2 can be regulated by a variety of stimuli. Moreover, expression of B7-1 and B7-2 can be independently regulated by the same stimulus, adding to the complexity of mechanism for regulating costimulation and immune response (163,167,169). In a mouse model for HSV-2, the blockage of B7 costimulation leads to enhanced disease signifying the role of B7 costimulation in HSV pathogenesis (170).

1.4.2 B7 and Th Differentiation

The relative statement of B7 isoforms B7-1 and B7-2 on antigen presenting cells have been suggested to be important in determining the nature and extent of the immune response (171-173). Immunoregulatory cytokines may affect the development of Th1/Th2 response by altering the levels of B7 isoforms on APC. IL-10 and IL-12 have been reported to elicit their biological effects via binding of B7 isoforms with their ligand CD28 on T cells (174,175). It has also been shown that Th2 cytokines IL-4 and IL-10 inhibit the expression of B7-2 and moderately enhance the expression of B7-1 (176).

Further, it has been suggested that Th1 inducing cytokine IFN-γ enhances the expression of both B7-1 and B7-2 isoforms on APC (176).

B7-1 (CD80) and B7-2 (CD86) have been implicated as differential determinants of Th1- vs. Th2-type cytokine profiles (167). Using TCR transgenic T cells stimulated by peptide in association with splenic APCs (obtained from knockout mice that selectively lack expression of either the CD80 or CD86 molecules), it has been suggested that CD86 and to a lesser extent CD80, can make significant contributions to the production of both IL-4 and IFN-γ (168). However, neither molecule plays an obligatory role in priming for the production of either effector cytokine. Furthermore, B7-1 and B7-2 contribute to the magnitude of T cell activation, but do not appear to selectively regulate Th1 vs. Th2 differentiation (168). It has been shown that B7 is a critical and potent stimulator of Th2 differentiation, and that anti-CD28 prevents this effect (172). It has been suggested that the interaction between T helper precursor (Th P) cells and B7-2 favor the development of Th2 cells (172). It has also been reported that the relative expression of B7 isoforms, B7-1 and B7-2 may be critical in determining the nature and extent of the immune response and both B7-1 and B7-2 are potent and similar co-stimulators of T lymphocytes (173). Therefore, B7-1 and B7-2 may determine the nature of immune response by differential expression on APC (164,171,176).

1.5 Hypothesis and Objectives

Primary infection with HSV-2 or HCMV leads to specific antiviral immune response that effectively suppresses the viral replication but does not eliminate the virus from the host. The virus persists in the host in a latent form or in selective tissues where its replication remains undetectable by the immune system. The latent virus is however periodically reactivated causing recurrent disease. The immunological basis for such reactivation of viral replication is unknown. $CD4^+$ T helper response to HSV-2 and CMV plays a pivotal role in the development of immunity and control of replication of these viruses *in vivo*. The broad objective of this thesis is therefore to determine the nature of T helper immune responses to HSV-2 and CMV and the viral factors associated with the modulation of such responses in order to understand the immunological basis for the imperfection in the immunity to these viruses. The specific objectives of this thesis are discussed below.

1.5.1 Determination of T Cell and Cytokine Responses to HSV-2 during HSV-2 Infection

As discussed before, the appearance and the nature of T helper response are central to the development of effective cellular and humoral immunity against pathogenic viruses such as HSV (41,42,80,81,91,92). The observations of generalized immunosuppression during HSV infection (10-12) and an association between frequent HSV recurrences and certain HLA types (177) suggest an immunological basis for herpesvirus reactivation. Both the T helper response to viral antigens and nature of such response determine the development and the duration of protective immunity to viruses. For example, a transient or permanent loss of T helper response to a viral region that is essential for replication or pathogenesis

is expected to abrogate protective immunity transiently or permanently, respectively. Likewise, transition from Th1 to Th2 type immune response may also alter the course of protective immunity to HSV. However, changes in T helper response during the course of HSV infection have not been fully investigated. In case of HSV-2 infection, it is not known whether episodes of viral recurrence or remission of genital lesions are associated with changes in the immunity to a specific viral antigen or Th1/Th2 transition. A possible association between Th1 or Th2 immune response during HSV-2 latency, reactivation or disease progression in humans has not been examined. A critical balance in the regulation of Th1/Th2 cytokines may be necessary for the maintenance of a symptom-free remission period. **The first objective** of this study was therefore to examine the proliferative response to HSV-2 antigen and to analyze the production of Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10) cytokines in HSV-2-stimulated PBMC cultures from HSV-2 seropositive individuals categorized into various disease stages according to disease symptoms.

1.5.2 Analysis of B7 Expression on Monocytes during HSV-2 Infection

Co-stimulatory actions of B7 molecules play an important role in T cell activation and generation of immune response to antigens (160-164,172-176). The antigenic stimulation of T cells in the absence of B7 co-stimulation leads to T cell anergy. The expression of B7 isoforms, B7-1 (CD80) and B7-2 (CD86), on APC such as monocytes may therefore regulate immune response *in vivo*. Further, immunoregulatory cytokines such as IFN-γ and IL-10 are also known to affect the development of specific Th1/Th2 response by altering relative levels of the two B7 isoforms on APC (172-176). These cytokines may

regulate the expression of B7 isoforms on monocytes during HSV-2 infection and thus modify the nature of immune response to HSV-2. Further, several viral genes are known to directly influence the development or nature of immune response *in vivo*. Therefore, **the second objective** of this research was to determine the effects of IFN-γ, IL-10, and HSV antigens on B7-1 and B7-2 expression on monocytes of HSV-2 infected individuals.

1.5.3 Examination of Cytokine Response during CMV Infection and Effect of Specific Viral Genes

Members of the Herpesviridae family share several common structural and functional features that include a common genetic organization, high degree of nucleotide sequence homology in several genes, an orderly expression of immediate early, early, and late viral genes in the host, and cycles of latency and reactivation in hosts (50,51,111). The nature of immune response of these viruses has not been closely compared. To understand the underlying principles that govern the immunity to herpesviruses, the knowledge of the common features of immune response to these viruses is essential. Such knowledge must be corroborated by identification of viral genes that contribute to the common features of the immune response. These goals can be achieved by comparing the nature of T helper response to viruses belonging to different subfamilies of the Herpesviridae family, and by adopting animal models where mutant viruses with specific gene disruption can be used. Thus, **the third objective** of this study was to determine the cytokine response to MCMV infection in mice and identify specific viral genes associated with the generation of Th1 or Th2 type immune response *in vivo* by using well-characterized MCMV mutants.

Chapter 2

MATERIALS AND METHODS

2.1 Patients and Collection of Samples

This study was approved by the Research Ethics Board of the Ottawa Hospital, University of Ottawa, Ottawa, ON, Canada. Thirty-six individuals with recurrent genital herpes, seven asymptomatic HSV-2 seropositive individuals, and six HSV-seronegative healthy controls participated in this research. All the HSV-2 infected individuals recruited for this study were previously tested for AIDS and other sexually transmitted diseases and were otherwise healthy. The selection of individuals for this study was based on their symptoms, clinical history, and absence of HIV and any other sexually transmitted diseases. For asymptomatic seropositive cases spouse or sexual partners of patients with genital HSV disease were examined and blood drawn for analysis.

At the first patient visit, informed consent was obtained, clinical information was recorded, a blood and a genital swab sample were taken, and patients agreed to report all genital HSV-2 outbreaks to the clinic staff. Additional blood and genital swab samples were taken from those patients who were seen several times at different disease stages. Blood samples were obtained in polyethylene tubes containing heparin. All swabs were placed in 1 ml of Kult-Sure^{TM} viral collection and transport medium (NCS Diagnostics Inc., Etobioke, ON, Canada). According to the routine clinical grouping and based on the previous study (65) the participants were divided into following four groups:

(i) Active disease patients (AD) had clinically detectable genital lesions within 2 weeks preceding examination.

(ii) Patients with non-active disease (NAD) were in a recurrence-free period and had a history of genital lesions but absence of clinically detectable lesions in the 2 weeks preceding the examination.

(iii) Asymptomatic seropositive patients (AS) had no clinical history of genital lesions but had serologic evidence of anti-HSV-2 antibodies by Western blot analysis.

(iv) Asymptomatic seronegative subjects (NEG) were healthy adult controls.

The demographic information for each group is provided in Table 1 on the following page.

Disease Stage	Total Number (Samples/ Patients)	Age Group (Years)	Last Episode	Number of Recurrences/Year
Active Disease (AD)	16/14	26 to 50	1 day to 2 weeks	4 to 12
Non-Active Disease (NAD)	38/22	24 to 79	3 weeks to 7 months	3 to 12
Asymptomatic Seropositive (AS)	7/7	NA	Nil	Nil
Seronegative (NEG)	7/6	22 to 48	Nil	Nil

NA = data not available

Table 1. Demographics of participants of the HSV-2 study.

2.2 Preparation of HSV-1, HSV-2, and Control Ag

HSV-1 and HSV-2 antigen were prepared as described earlier (178). Human fetal lung cells HFL # 62 were obtained from American Tissue Culture Collection (Rockville, MD). These cells were cultured in T150 tissue culture flasks (Falcon labware, Oxnard, CA) containing heat inactivated fetal calf serum (Gibco BRL, Grand Island, NY). Confluent monolayers of HFL# 62 were infected with HSV-1 or HSV-2 (kindly provided by Dr. K. Rosenthal, McMaster University, Hamilton, Canada).

When the majority of cells exhibited cytopathic effect, the cells were harvested and the culture supernatants were transferred into 50 ml centrifuge tubes (Falcon labware, Oxnard, CA) and centrifuged at 600 x g for 15 minutes. The cells were sonicated and the cell lysate was combined with the supernatant. The tubes were centrifuged to pellet the cell debris.

The supernatants were incubated at 56 °C for 2 hours to inactivate the virus. Viral inactivation was confirmed by inoculation of the antigen preparation in tissue culture. The antigen preparation was then aliquoted and stored at -80 °C. The control antigen was prepared using uninfected HFL # 62 cells and same procedure as described above for the HSV antigen.

2.3 Isolation and Culture of PBMC

Blood samples were collected in polyethylene tubes containing heparin as anticoagulant. The PBMC were isolated by density gradient centrifugation in Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) as described earlier (179). In 50 ml polystyrene tubes (Corning, Oxnard, CA), blood was layered on half the volume of Ficoll-Hypaque ie 15 ml for 30 ml of blood. The tubes were centrifuged for 30 minutes at 400 x g. The cell layer consisting mainly of mononuclear cells was collected, washed three times in sterile phosphate buffered saline (PBS; GIBCO Laboratories, Grand Island, NY) and resuspended in Iscove's Modified Dulbecco's Medium (IMDM; Sigma Chemical Company, Saint Louis, MO), containing 10 % fetal calf serum (FCS; GIBCO Laboratories, Grand Island, NY), 100 U/ml penicillin, and 100 µg/ml gentamicin. All cultures were incubated at 37 °C in 5 % CO_2 in a humidified incubator.

2.4 Cell Proliferation Assay

PBMC (1×10^6 cells/ml) were stimulated with anti-CD3 antibodies (OKT-3), American Tissue Culture Collection (ATCC, Rockville, MD; 1:200 final dilution) for 48 hours and with HSV-1 or HSV-2 antigens (1:10 final dilution) and the control antigen i.e. supernatant from the cells not infected with the HSV-1 or HSV-2 virus (1:10 final dilution) for 5 days. Stimulation experiments were done in triplicate in a final volume of 200 µl in 96 well plates (Falcon Labware, Oxnard, CA). The cells were then pulsed with 0.5 µCi [^{3}H] thymidine (Amersham, Arlington Heights, IL) and cultured for a further 16

hours followed by cell harvesting and measurement of [^{3}H] thymidine incorporation (liquid scintillation counter, 1450 Microbeta Wallac, Turku, Finland).

The stimulation index (SI) was calculated as a ratio of [^{3}H] thymidine incorporation (cpm) by PBMC stimulated in the presence of antigen/mitogen to that of PBMC cultured in the absence of antigen/mitogen or PBMC cultured in the presence of HSV antigen to that cultured in the presence of control antigens. SI of more than 3 was considered a positive response.

2.5 Collection of Supernatant for Measurement of Cytokines

To determine the ability of PBMC from HSV-2-seropositive individuals and heathy controls to produce Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-10) cytokines, the PBMC (1 x 10^6/ml) were stimulated with either HSV-1 or HSV-2 antigen or control antigens (1:10 final dilution) in 24 well tissue culture plates (Falcon Labware, Oxnard, CA). The supernatants were harvested after 5 days and stored at –70 °C. The choice of antigen concentration and number of days of HSV stimulation was based on dose-response and time-response experiments showing that PBMC exhibit maximal [^{3}H] thymidine incorporation 5 days after stimulation with HSV-1 or HSV-2 antigens.

2.6 Measurement of Th1 and Th2 Cytokines during HSV-2 Infection

The standard enzyme linked immuno sorbant assay (ELISA) described previously (179) was employed to measure the Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-10) cytokines in the supernatants obtained after stimulation of PBMC from HSV-2 seropositive and seronegative individuals.

2.6.1 IL-2

IL-2 was quantitated by sandwich ELISA using the human IL-2 Quantikine™ reagents (R&D Systems Inc., Minneapolis, MN), as described by the manufacturer. Briefly, the primary anti-human IL-2 antibody (#MAB602, R&D Systems Inc., Minneapolis, MN) was used at a concentration of 4 μg/ml for coating the 96-well plates at 4 °C overnight. The plates were washed with 0.05 % Tween 20 in PBS and blocked with 10 % FCS in PBS for 2 hours at room temperature. Recombinant human IL-2 was used as a standard. Anti-IL-2 biotinylated antibody (#BAF202, R&D Systems Inc., Minneapolis, MN) was used at a concentration of 25 ng/ml as detection antibodies. Streptavidin-peroxidase conjugated with horseradish peroxidase was used at a final dilution of 1:1000. The color reaction was developed by adding the stop solution containing o-phenylenediamine dihydrochloride and hydrogen peroxide. The plates were read at 450 nm using the ELISA reader. The sensitivity of IL-2 assay was 8 pg/ml.

2.6.2 IFN-γ

Measurement of IFN-γ produced by PBMC was performed by a sandwich ELISA using monoclonal antibody (MoAb) that recognizes distinct epitopes. Briefly, the primary anti-human IFN-γ antibody (clone 25718.11, R&D Systems Inc., Minneapolis, MN) was used at a concentration of 4 μg/ml for coating the 96-well plates (Nunc Immune-modules, Denmark) at 4 °C overnight. The plates were washed with PBS-Tween 20 and blocked with 5 % skim milk in PBS-Tween 20 (0.05 %). Recombinant IFN-γ (Endogen, Woburn, MA) was used as a standard. Anti-IFN-γ biotinylated antibody (#BAF285, R&D Systems Inc., Minneapolis, MN) was used at a concentration of 200 ng/ml as detection antibody. Streptavidin-peroxidase conjugated with horseradish peroxidase (Jackson Immuno-Research, West Grove, PA) was used at a final dilution of 1:1000. The color reaction was developed by o-phenylenediamine dihydrochloride (OPD, Sigma) and hydrogen peroxide. The plates were read at 450 nm on ELISA reader. The sensitivity of IFN-γ assay was 8 pg/ml.

2.6.3 IL-4

IL-4 was quantitated by sandwich ELISA using the human IL-4 Quantikine reagents (R&D Systems Inc., Minneapolis, MN), as described by the manufacturer. Briefly, the primary anti-human IL-4 antibody (#MAB604, R&D Systems Inc., Minneapolis, MN) was used at a concentration of 4 μg/ml for coating the 96-well plates at 4 °C overnight. The plates were washed with 0.05 % Tween 20 in PBS and blocked with 10 % FCS in PBS for 2 hours at room temperature. Recombinant human IL-4 was used as a standard.

Anti-IL-4 biotinylated antibody (#BAF204, R&D Systems Inc., Minneapolis, MN) was used at a concentration of 12.5 ng/ml as detection antibody. Streptavidin-peroxidase conjugated with horseradish peroxidase was used at a final dilution of 1:1000. The color reaction was developed by adding the stop solution containing o-phenylenediamine dihydrochloride and hydrogen peroxide, and the plates were read at 450 nm. The sensitivity of IL-4 assay was 8 pg/ml.

2.6.4 IL-10

Measurement of IL-10 produced by PBMC was performed by a sandwich ELISA using monoclonal antibody (MoAb) that recognize distinct epitopes. Briefly, 96-well plates (Nunc Immunomodules, Denmark) were coated with purified anti-IL-10 mAb (clone JES3-9D7, PharMingen, Mississauga, ON, Canada) used at a concentration of 5 μg/ml and incubated overnight at 4 °C. The plates were washed with PBS-Tween 20 and blocked with PBS-10 % FCS. Recombinant IL-10 (PharMingen, Mississauga, ON, Canada) was used as a standard. Anti-IL-10 biotinylated antibody (clone JES3-12G8, PharMingen) was used at concentration of 4 μg/ml as detection antibody. Streptavidin-peroxidase (Jackson Immuno-Research, West Grove, PA) was used at a final dilution of 1:1000. The color reaction was developed by *o*-phenylenediamine dihydrochloride (OPD, Sigma) and hydrogen peroxide. The plates were read at 450 nm. The sensitivity of IL-10 assay was 8 pg/ml.

2.7 Detection of Viral DNA by PCR Assay

To determine the correlation between viral shedding and Th cell response, detection of HSV-2 DNA in genital and oral swabs was done by PCR and ELISA quantitation (experiments performed by Dr. Peihua Cheng). Viral DNA was isolated by using a monophase solution containing guanidine thiocyanate and phenol (TRI Reagent solution, Molecular Research Center Inc., Cincinnati, OH), as described previously (180). HSV was detected in the DNA isolated from clinical samples by PCR analysis using both a gel system (178) and the Digene Sharp Signal System (Digene Diagnostics Inc., Beltsville, MD, USA) and HSV-1 and HSV-2 primers, as described previously (178).

The colorimetric Digene Sharp Signal System was used for the detection and quantitation of the amplified PCR products as per the manufacturer protocol (Advanced Biotechnologies Inc., Columbia, MD). Briefly, 10 µl of DNA isolated from the clinical samples was added into a 90 µl of PCR reaction mixture solution. Meanwhile, 10 µl of serial 10 fold dilutions of the standard HSV-1 or HSV-2 DNA copies ($1x10^7$ copies/µl) provided by the manufacturer were added to 90 µl of PCR reaction mixture solution to make standard quantitative curves.

HSV-1 and HSV-2 types were distinguished via distinct 3′ primers, p3-1 and p3-2, which are specific for HSV-1 and HSV-2 respectively (181-183). The 5' HSV primer recognized the common sequences for both HSV-1 and HSV-2. For quantitation of HSV copies, the 5′ end of HSV primers were biotinylated (Digene Diagnostics Inc.). PCR was carried out for 30 cycles in a programmable DNA thermal cycler (480; Perkin-Elmer), as follows: DNA denaturation at 94 °C for 60 second, primer annealing at 65 °C for 1 minute 30 second, and primer extension at 72 °C for 2 minutes. For the last cycle, the

DNA molecules were allowed to extend for 10 minutes at 72 °C. The amplified PCR products were detected by electrophoresis on a 1.5 % agarose gel and were stained with ethidium bromide. The sequence of primers p3-1 and p3-2 were as follows: Primer p3-1, sense 5′-CCT CGC GTT CGT CCT CGT CCT CC-3′ and primer p3-2, sense 5′-CCT CCT TGT CGA GGC CCC GAA AC-3′. By using these primers, the presence of HSV-1 and HSV-2 was distinguished on the basis of the PCR product size, 469 and 391 bp, respectively (181-183).

To determine that the DNA isolated from the clinical samples was not degraded, PCR for β-actin gene was performed. The primers for β-actin were as follows: sense (n-1038) 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3' (n-1067), and antisense (n-1876) 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3' (n-1905). Clinical samples from patients with genital lesions but negative by PCR were spiked with HSV-2 DNA to rule out the presence of PCR inhibitors.

The amplified PCR products were transferred onto nylon filters (Amersham) and were hybridized with the oligonucleotide probe HSV-P3 (181-183). The sequence for HSV-P3 is as follows: sense (nt 1561) 5'-GGC GTA GTA GGC GGG GAT GTC GCG-3' (nt 1583). This oligonucleotide probe was labeled with alkaline phosphatase using the E-Link™ and E-Link Plus™ oligonucleotide labeling kit according to the manufacturer recommendations (Genosys, Woodlands, TX). The filters were prehybridized with 10 ml of prehybridization buffer for 4 hours at 42 °C. Hybridization was performed at 42 °C overnight with 10 ml of prehybridization buffer containing 20 µl of the alkaline phosphatase labeled oligonucleotide probe. After successive washes with 2 x SSC plus 0.1 % SDS, 0.5 x SSC plus 0.1 % SDS, and buffers A (100 mM Tris, pH 7.4, 150 mM

NaCl), buffer B (2 % blocking buffer in A), and buffer C (100 mM Tris pH 9.5, 100 m NaCl and 50 mM $MgCl_2$), the damp filters were sprayed two or three times with LumiPhos 530 (Boehringer Mannheim) and were exposed to X-ray film (X-Omat, Kodak) at 37 °C for between 30 minutes and 2 hours.

2.8 ELISA Quantitation of PCR Products

Quantitation of the amplified PCR products obtained as above was performed as per the manufacturer's recomendation (Digene Sharp Signal System, Advanced Biotechnologies Inc.). Briefly, 25 µl of PCR reaction mixture containing 5'-biotinylated products was hybridized with 25 µl (500 pmol/liter) of specific single-stranded RNA probe complementary to the biotinylated strand of the PCR product at 65 °C for 30 minutes. The resultant RNA:DNA hybrids were captured through biotin onto the surface of streptavidin-coated microwells. The hybridized contents were transferred onto the streptavidin-coated microwells and incubated at 25 °C for 30 minutes on a rotary shaker (1200 rpm/min). The immobilized hybrids were then reacted with detection reagent containing anti-RNA-DNA antibody conjugated to alkaline phosphatase at 25 °C for 30 minutes on a rotary shaker followed by washing with a wash buffer. The PCR products were detected with a colorimetric substrate (para-nitrophenylphenol, PNPP) at 37 °C for 2 hours. The microwell plates were read at 405 nm using the microplate ELISA reader. The intensity of the color generated was proportional to the amount of biotinylated PCR product in each reaction. Ten-fold dilutions of the PCR controls were utilized to calculate the amount of HSV-1 and HSV-2 DNA copies contained in the clinical specimens.

2.9 Western Blot Analysis

HSV-1 and HSV-2 specific antibodies in the serum of subjects who participated in this study were detected by Western blot analysis as described previously (184). The various steps involved are described in the following.

2.9.1 Preparation of HSV-1 and HSV-2 Antigen

The HSV-1 and HSV-2 antigen for sepharose was prepared by infecting monolayers of human diploid fibroblast (HDF) cells obtained from American Tissue Culture Collection (Rockville, MD) with HSV-1 and HSV-2. The cells were harvested. After pouring off the growth media the monolayer of cells was rinsed with 25 ml cold 10 x PBS (Gibco Laboratories, Grand Island, NY). After adding 50 glass beads and 15-20 ml cold PBS (Gibco Laboratories, Grand Island, NY) the cells were dislodged by swirling motion. The cells were transferred into a 50 ml conical screw cap centrifuge tubes and 15 ml cold PBS was added to wash cells off the beads. Cells were again washed with wash solution containing 0.5 % Tween in PBS in 50 ml conical tubes and centrifuged at 1200 rpm. The pellet was resuspended in 15 ml coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH to 8.6 with solid Na_2CO_3). The cells were sonicated and lysate aliquoted and stored at –80 °C.

2.9.2 Preparation of Sepharose

Sepharose was prepared using 1 gm CNBr-activated sepharose (Sigma Chemical company, Saint Louis, MO) per 5 ml protein. First sepharose was allowed to swell in 1 mM HCl/gm sepharose and then it was added to each 50 ml conical tube containing HSV-1 and HSV-2 lysate by mixing it on an orbital rotor for 2 hours at room temperature at 1500 rpm for 5 minutes. The remaining active groups on sepharose were blocked with blocking solution containing 0.2 % glycine pH adjusted to 8 with NaOH. The pellet was washed with coupling buffer in a ratio of 1:1 and then with TST buffer containing 50 mM Tris, 0.9 % NaCl, 0.01 % Thimersal, pH 7.5 in a ratio of 1:1. After Centrifugation the sepharose pellet was resuspended in TST buffer in a ratio of 2:1.

2.9.3 Preparation of Western Blot Test Strips

Western blot test strips were prepared using lysates from cells infected with HSV-1 and HSV-2. Aliquots of HSV-1 and –2 lysates stored in microcentrifuge tubes (1 ml per tube) were boiled for 2 minutes. HSV-1 and HSV-2 crude protein extracts (50 μg) were subjected to SDS-PAGE electrophoresis (8 %) followed by transfer of proteins on to the Hybond^{TM}-C extra nitrocellulose membranes (Amersham). The nitrocellulose was then cut into 3 mm strips which were stored at 4 °C and used in the Western blot assay to detect antibodies to HSV-1 and HSV-2 antigens present in the test sera.

2.9.4 Immunostaining of HSV-1 and HSV-2 Blots

HSV Western blot screening was done by immunostaining of HSV-1 and HSV-2 blots. For each set of strips 20 µl of control serum in 1 ml of blotto per strip was run. The control serum used were HSV-1, HSV-2, HSV-1, and HSV-2 positive pooled sera; HSV seronegative control serum and; HSV-1 cross reactive control serum. The strips were washed with wash solution (0.05 % Tween in PBS) and incubated overnight with blotto and serum. The strips were washed three times with wash solution followed by one wash with PBS. The strips were then incubated for 75 minutes with 1 ml of horse reddish peroxidase conjugated with anti-human IgG. The strips were washed and allowed to develop in 4CN solutions A and B (1:1) for 7 minutes.

2.9.5 Analysis of Blots

The serum containing HSV-1 antibodies alone after adsorption with HSV-1 coated beads did not exhibit any HSV-1-specific banding pattern either on the HSV-1 or the HSV-2 carrying membrane strips, respectively. However, HSV-1 specific bands were detected on HSV-1 carrying strips and not on HSV-2 carrying strips after adsorption with HSV-2 coated beads. Inversely, HSV-2 specific bands for HSV-2 patients were detected on HSV-2 carrying strips after adsorption of serum with HSV-1 beads. Patient's serum containing both HSV-1 and HSV-2 antibodies exhibited bands on the membranes carrying HSV-1 proteins after adsorption with HSV-2 beads, and HSV-2 specific bands were detected on HSV-2 carrying strips after adsorption with HSV-1 beads. This protocol was useful to detect patients exposed specifically to HSV-1, HSV-2 or with both the virus

strains reproducibly. Only those patients who had antibodies for HSV-2 or both HSV-1 and HSV-2 were included in this study.

2.10 Flow Cytometric Analysis

2.10.1 Regulation of B7 Statement on Monocytes in HSV-2 Infection

To analyze the regulation by IL-10, IFN-γ, and HSV antigens of statement of B7 isoforms on the monocytes from HSV-2 symptomatic patients and HSV-seronegative subjects, unstimulated PBMC (3 x 10^6 /ml) were cultured in the presence of either IL-10 (1 ng/ml), IFN-γ (1 ng/ml), HSV-1 or HSV-2 antigens (1:10 dilution). The monocytes were analyzed for B7-1 and B7-2 expression after 24 hours of stimulation as described earlier (154). Briefly, cells were washed once with PBS/0.05 % sodium azide and distributed into flow cytometry tubes (Sarstedt, Numbrecht, Germany). Cells were stained with 5 μl of either PE labeled anti-B7-1 (Becton Dickinson, Lincoln Park, NJ) or PE labeled anti-B7-2 (PharMingen) monoclonal antibodies and 2.5 μl of FITC labeled anti-CD14 monoclonal antibody (Becton Dickinson, Lincoln Park, NJ). Autofluorescence and isotype controls, IgG1 for B7-1 and IgG2b for B7-2 (Becton Dickinson, Lincoln Park, NJ) were included.

For PBMC, two distinct populations (lymphocytes and monocytes) were visualized by light scatter properties. For analysis of B7 statement on monocytes in PBMC, cells were gated in the monocytic gate. The gates were set by forward angle and 90° light scatter data from cells stained with isotype matched control antibodies. B7-1

and B7-2 expressing $CD14^+$ monocytes were observed in clusters distinct from lymphocytes and median fluorescence intensity (MFI) was obtained. Data were acquired on a Becton Dickinson FACScan flow cytometer by counting 5,000 events. Validity of comparison in the expression level of B7-1 and B7-2 isoforms between different patient populations was ensured through the use of Calibrite™ Beads (Becton Dickinson, Lincoln Park, NJ). Data was analyzed using the WinMDI software package (J. Trotter, Scripps Institute, San Diego, CA).

2.10.2 Effect of Endogenously Produced IFN-γ on B7-1 and B7-2 Statement during HSV-2 Infection

To determine if HSV-2 induced statement of B7-1 and B7-2 is mediated by endogenously produced IFN-γ, effect of endogenously produced IFN-γ on the statement of B7-1 and B7-2 on $CD14^+$ monocytes following stimulation with HSV-1 and HSV-2 antigens was analyzed. PBMC from six HSV-2 infected individuals were stimulated with HSV-2 antigens in the presence or in the absence of neutralizing anti-IFN-γ antibodies for 24 hours. $CD14^+$ monocytes were analyzed for the statement of B7-1 and B7-2 as described above.

2.11 Preparation of Wild Type and Mutant MCMV Stocks

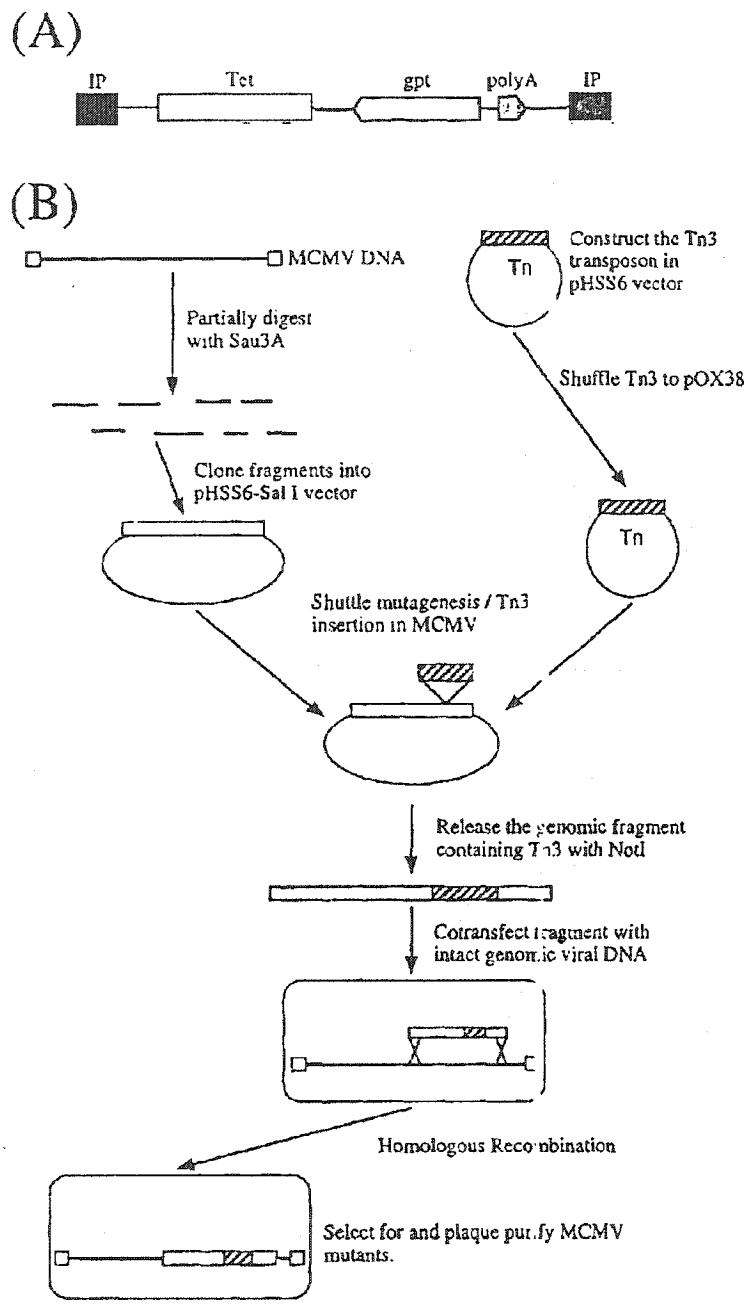
To determine *in vivo* role of viral genes in modulation of immune response of the host during infection with herpesviruses having similarities in genetic composition, disease stages and predominant IFN-γ response, the previously established mutagenesis approach was employed. The MCMV mutants RvM25, RvM27, RvM43, and Rvm09 having Tn3 transposon inserted into the open reading frame of M25, M27, M43, and m09 genes, respectively, were provided by Dr. Xiaoyan Zhan and Manfred Lee.

Figure 1 presents the schematic representation of method for construction of these mutants, as described previously by Zhan et al. (130). Briefly, the plasmid pOX38 containing transposon was inserted into the library of MCMV genomic DNA in *Escherichia coli.* Sequence analysis was done to determine whether the pool contained MCMV fragments that had random insertion of transposon into the coding region of open reading frames of viral genes. The regions bearing an insertion mutation were then transferred to the MCMV genome by homologous recombination. The Tn3-based mutants obtained by this approach have been previously used to study the *in vitro* and *in vivo* function of several MCMV genes in viral replication in tissue culture and various organs of immunocompetent and immunodeficient animals (130-133).

Stocks of wild type MCMV (smith strain) obtained from American Tissue Culture Collection (Rockville, MD) and the recombinant MCMV RvM25, RvM27, RvM43, and Rvm09 having Tn3 transposon inserted into the ORF of MCMV M25, M27, M43, and m09 genes, respectively, were obtained by propagating these viruses in NIH3T3 cells (American Tissue Culture Collection ATCC CRL1658) in complete Dulbecco's modified

Eagle medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 10 % Nuserum (Collaborative Biomedical Products, Bedford, MA), essential and nonessential amino acids, penicillin and streptomycin (Life Technologies, Grand Islands, NY). When most of the cells were infected, the cells were harvested, and virus stocks prepared by adding an equal volume of 10 % skim milk (50:50, by vol.), followed by sonication using a 550 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) and stored at –80 °C.

Figure 1. Schematic representation of the construction of MCMV mutants by Tn3 transposon mutagenesis approach, as described in Zhan et al. 2000 (130). **(A)** Represents the plasmid construct used for mutagenesis. **(B)** represents the procedure used for construction of MCMV mutants that contained random transposon insertions. IP, inverted repeat; Tet, tetracycline resistance gene; gpt, gene that encodes guanine phosphoribosyltransferase (*gpt*); poly(A), transcription termination signal; pOX38, plasmid containing two 38 nucleotides terminal inverted repeats, a sequence of the loxP and loxR.



Zhan et al., Figure 1

Figure 1

2.12 Measurement of Viral Titers

Virus titers were measured by plaque assay as described earlier (130). Briefly, NIH3T3 cells were propagated in complete DMEM in each well of six well tissue culture plates (Falcon labware, Oxnard, CA) in triplicate. These cells were then infected with a serial 10 fold dilution of the virus and incubated at 37 °C and 5 % CO_2 for 1.5 hour. The cells were washed with DMEM media and overlaid with 0.5 % agarose in DMEM and incubated at 37 °C and 5 % CO_2. The titers of the viral stocks were determined by counting the number of plaques formed.

2.13 Preparation of Viral and Control Antigen

Viral antigen for each virus type was prepared by using the previously described method (178). Briefly, NIH3T3 cells were infected with wild type MCMV and recombinants RvM25, RvM27, RvM43, and Rvm09 in a T150 tissue culture flask (Falcon labware, Oxnard, CA). When most of the cells were infected, cells were harvested and supernatants were collected and centrifuged at 600 x g for 10 minutes. Cells were sonicated using a 550 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) and mixed in the supernatant. The samples were then incubated in a water bath at 56 °C for two hours. Viral inactivation was confirmed by innoculation of the antigen in NIH3T3 cell cultures. Control antigen was prepared similarly using uninfected NIH3T3 cells.

2.14 Isolation of Splenocytes

Three-week-old Balb/c mice (Jackson Laboratory, Bar Harbor, ME) were injected intraperitonially with $1x10^4$ PFU of wild type (Smith strain) and recombinant MCMVs (RvM25, RvM27, RvM43, and Rvm09). The control mice were injected with sonicated uninfected NIH3T3 cells in PBS. On 1, 3, 7, 10, and 14 days post infection (dpi) spleen samples were obtained from a set of 3 mice from each group and the splenocytes were isolated using Medimachine mechanical desegregation system (Becton Dickinson, San Jose, CA) according to manufacturer's instructions. Briefly, spleen samples were washed in 1x Hank's balanced buffer (Gibco BRL, Grand Island, NY) and inserted into a 50 μm medicon together with 1 ml Hank's buffer. The medicon was then inserted into the medimachine and the machine was started for about 1 minute. As the spleen was desegregated, the medicon was removed from the medimachine. The cell suspension was recovered using a syringe in the syringe port. The medicon was rinsed with Hank's buffer to ensure maximum cell recovery. The cell suspension was then passed through 70 μm filcon to obtain spleen cells.

The spleen cells were washed three times with Hank's buffer and resuspended in RPMI media (Gibco BRL, Grand Island, NY) containing 10 % fetal calf serum and Penicillin and streptomycin in a 50 ml tube (Falcon labware, Oxnard, CA). The cells were centrifuged at 1000 rpm for 10 minutes and supernatant removed. 5 ml of Tris ammonium chloride (0.16M NH_4Cl and 0.17M Tris, pH 7.2) was added to lyse the red blood cells. After incubating for 5 minutes Hank's buffer was added and cells centrifuged at 1000 rpm for 10 min. Cells were washed twice with Hank's buffer and resuspended in RPMI media containing 10 % FCS.

2.15 Standardization of Antigen Concentration

In order to get comparable results for Th1 and Th2 cytokine production during infection with wild type and recombinant MCMVs, it is essential to determine the optimum concentration of respective antigen required for stimulation of spleen cells from mice infected with wild type and recombinant MCMVs. The Cell Proliferation assay was done using Cell Titer 96 ® Non-Radioactive Cell Proliferation Assay (Promega Corporation, Madison, WI) using manufacturer's protocol. Briefly, the number of viable cells from each sample was counted using trypan blue. $1x10^5$ cells/well in a 96 well plate were stimulated with various concentration of the respective viral antigen. The plates were incubated at 37 °C and 5 % CO_2 incubator. After 5 days, 15 µl of the dye solution was added in each well and the plates were incubated at 37 °C for 4 hours in a humidified, 5 % CO_2 incubator. After 4 hours, 100 µl of solubilization/stop solution provided with the kit was added in each well. The plates were kept overnight in a sealed container with a humidified atmosphere. The absorbance was recorded at 540 and 690 nm wavelength using a 96 well plate reader. Stimulation index (SI) was calculated as a ratio of formazan produced by cells stimulated with the viral antigen to that of cells cultured in the presence of control antigen.

2.16 Collection of Cell Supernatant for Cytokine Analysis

To determine the ability of the cells from mice infected with the wild type and recombinant MCMV and uninfected controls to produce Th cytokines, the splenocytes

($2x10^6$ cells/ml) were stimulated with the respective antigen (1:5 and 1:10 final dilution) in a 24 well tissue culture plates (Falcon labware, Oxnard). On fifth day after stimulation, supernatants were collected and stored at –80 °C for cytokine analysis by ELISA at a later date.

2.17 Measurement of Cytokines during CMV Infection

2.17.1 IL-2

IL-2 was quantitated by sandwich ELISA using the murine IL-2 Quantikine™ M Kit (R&D systems Inc., Minneapolis, MN), as described by the manufacturer. Briefly, 50 µl assay diluent provided with the kit was added in each well of the 96 well microplates coated with polyclonal antibody specific for mouse IL-2. 50 µl of various dilutions of standard (recombinant mouse IL-2), control (mouse IL-2) and cell supernatants were added and incubated for 2 hours at room temperature. The plates were washed with wash buffer and 100 µl of mouse IL-2 conjugate containing antibody against mouse IL-2 conjugated to horseradish peroxidase was added to each well. After 2 hours of incubation at room temperature, the plates were washed and 100 µl of substrate solution containing equal volume of stabilized hydrogen peroxide and stabilized chromogen was added. Plates were incubated in dark for 30 min. 100 µl of stop solution containing hydrochloric acid was added per well and optical density (OD) was read at 450 nm. The sensitivity of the assay was 3 pg/ml.

2.17.2 IFN-γ

IFN-γ was quantitated by sandwich ELISA using the murine IFN-γ Quantikine™ M Kit (R&D systems Inc., Minneapolis, MN), as described by the manufacturer. Briefly, 50 µl assay diluent provided with the kit was added in each well of the 96 well microplates coated with monoclonal antibody specific for mouse IFN-γ. 50 µl of various dilutions of standard (recombinant mouse IFN-γ), control, and cell supernatants were then added and incubated for 2 hours at room temperature. The plates were washed with a wash buffer and 100 µl of mouse IFN-γ conjugate containing antibody against IFN-γ conjugated to horseradish peroxidase was added to each well. After 2 hours of incubation at room temperature, the plates were washed and 100 µl of substrate solution containing equal volume of stabilized hydrogen peroxide and stabilized chromogen was added. Plates were incubated in dark for 30 min. 100 µl of stop solution containing hydrochloric acid was added per well and OD was read at 450 nm. The sensitivity of the assay was 2 pg/ml.

2.17.3 IL-4

IL-4 was quantitated by sandwich ELISA using the murine IL-4 Quantikine™ M Kit (R&D systems Inc., Minneapolis, MN), as described by the manufacturer. Briefly, 50 µl assay diluent provided with the kit was added in each well of the 96 well microplates coated with a monoclonal antibody specific for mouse IL-4. 50 µl of various dilutions of standard (recombinant mouse IL-4), control (mouse IL-4) and cell supernatants were then added and incubated for 2 hours at room temperature. The plates were washed with wash buffer and 100 µl of mouse IL-4 conjugate containing antibody against IL-4 conjugated

to horseradish peroxidase was added to each well. After 2 hours of incubation at room temperature, the plates were washed and 100 µl of substrate solution containing equal volume of stabilized hydrogen peroxide and stabilized chromogen was added. Plates were incubated in dark for 30 min. 100 µl of stop solution containing hydrochloric acid was added per well and OD was read at 450 nm. The sensitivity of the assay was 2 pg/ml.

2.17.4 IL-10

IL-10 was quantitated by sandwich ELISA using the murine IL-10 Quantikine™ M Kit (R&D systems Inc., Minneapolis, MN), as described by the manufacturer. Briefly, 50 µl assay diluent provided with the kit was added in each well of the 96 well microplates coated with polyclonal antibody specific for mouse IL-10. 50 µl of various dilutions of standard (recombinant mouse IL-10), control (mouse IL-10) and cell supernatants were then added and incubated for 2 hours at room temperature. The plates were washed with wash buffer and 100 µl of mouse IL-10 conjugate containing antibody against mouse IL-10 conjugated to horseradish peroxidase was added to each well. After 2 hours of incubation at room temperature, the plates were washed and 100 µl of substrate solution containing equal volume of stabilized hydrogen peroxide and stabilized chromogen was added. Plates were incubated in dark for 30 min. 100 µl of stop solution containing hydrochloric acid was added per well and OD was read at 450 nm. The sensitivity of the assay was 4 pg/ml.

2.18 Statistical Analysis

Mean of SI and cytokine values for responders from AD, NAD, AS were compared with nonresponders from AD, NAD, AS, and with NEG by the Student's *t*-test. The amount of Th1 and Th2 cytokines produced by wild type (WT) and mutant MCMV were also compared by the Student's paired *t*-test function using SigmaPlot® 2000. The results are presented as mean ± standard error. Linear regression analysis was performed using the Statistical Analysis function of Microsoft Excel.

Chapter 3

RESULTS

3.1 Proliferative Response of PBMC to HSV-2 Antigen

To determine the proliferative response of PBMC at various stages of HSV-2 disease, the PBMC from HSV-2 seropositive individuals belonging to AD, NAD, AS groups and HSV-seronegative healthy controls (NEG) were analyzed for proliferation *in vitro* after stimulation with HSV-1 antigen, HSV-2 antigen, and control antigen, or anti-CD3 antibody. SI was calculated as described in Materials and Methods.

The proliferative response of the PBMC to HSV-1 antigen, HSV-2 antigen, and anti-CD3 antibody are presented in Table 2. Out of 43 seropositive individuals included in this study, 9 were HSV-2 seropositive and remaining 34 were HSV-1 and HSV-2-seropositive. 61 blood samples were obtained from 43 HSV-2-seropositive patients at various stages (AD, NAD, AS) of HSV-2 disease. As described earlier in Materials and Methods section, in some cases with the consent of the individuals the blood was drawn several times (at the time of active disease and during follow up visits). There were 5 individuals who had blood drawn when they had AD at one time point but NAD at another time point and therefore were included in more than one group. 47 samples (77 %) from 32 individuals had HSV-2 antigen SI of more than 3, and were categorized as responders. Out of these 47 samples with positive response, 14 samples were obtained

from AD patients (n = 12), 30 samples from NAD patients (n = 17), and 3 samples from AS patients (n = 3), as presented in Table 2.

Two blood samples from 2 AD patients, 8 samples from 5 NAD patients, and 4 samples from 4 AS patients had a SI of less than 3, and were categorized as non-responders. None of the 7 PBMC samples obtained from six healthy HSV-seronegative controls responded to either HSV-1 antigen or HSV-2 antigen (Table 2).

All the samples had a positive response to anti-CD3 antibodies. However, as seen in Table 2, the samples from patients with recurrent genital herpes demonstrated a trend towards higher SI in response to anti-CD3 antibodies compared to the asymptomatic seronegative controls, although the difference was not statistically significant ($p = 0.08$). In 3 cases (6 samples) with recurrent HSV-2 disease the PBMC proliferated in response to HSV-2 antigen. However, when examined later during the course of disease one sample from each patient (examined on different days) showed lack of responsiveness to HSV-2 antigen.

Disease Stage	Total Number (Samples/ Patients)	*SI to α CD3 Ab	*SI to HSV-1	*SI to HSV-2	Responders (Samples/ Patients)	Non-responders (Samples/ Patients)
Active Disease	16/14	R: 108.2 ± 25.1 NR: 105.2 ± 44.0	R: 6.1 ± 0.9 NR: 1.5 ± 0.5	R: 6.5 ± 1.0 NR: 1.0 ± 0.2	14/12	2/2
Non-Active Disease	38/22	R: 96.2 ± 15.9 NR: 138.4 ± 61.7	R: 17.3 ± 4.5 NR: 2.7 ± 0.5	R: 9.9 ± 1.5 NR: 1.1 ± 0.2	30/17	8/5
Asymptomatic Seropositive	7/7	ND	ND	R: 75.6 ± 32.2 NR: 1.4 ± 0.1	3/3	4/4
Seronegative	7/6	NR: 45.6 ± 5.7	NR: 1.2 ± 0.2	NR: 1.0 ± 0.1	0	7/6

PBMC (1 x 10^6 /ml) were stimulated with anti-CD3 antibody (1:200 final dilution of culture supernatant of anti-CD3 monoclonal antibody hybridoma), HSV-1 antigen or HSV-2 antigen. Patients were classified as responders (R) (SI > 3) and non-responders (NR) (SI < 3) on the basis of proliferative response to HSV-2 antigen. SI = stimulation index, *Mean ± standard error, ND = not done.

Table 2. Proliferative response of PBMC from HSV-2-seropositive and HSV-seronegative individuals to anti-CD3 antibody, HSV-1 and HSV-2 antigen.

3.2 Production of Th1 and Th2 Cytokines by HSV-2 Stimulated PBMC

Responder and non-responder PBMC samples from HSV-2 infected individuals may secrete distinct patterns of Th cytokines. In order to study Th responses in HSV-2 disease, the production of Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-10) cytokines was analyzed in the supernatants of PBMC harvested five days after stimulation with HSV-2 antigen. Th1 and Th2 cytokine secretion by responders and non-responders were compared in HSV-2 seropositive (AD, NAD, AS) individuals and with HSV seronegative controls (NEG).

Figure 2 shows that PBMC from HSV-2 seropositive responders from AD, NAD, and AS groups secreted significantly higher levels of IFN-γ when compared to non-responders from these groups ($p = 0.002$) and HSV seronegative controls ($p = 0.02$). Also, PBMC from AS individuals in the responders category secreted higher levels of IFN-γ compared to responders from AD and NAD patients ($p = 0.049$) and AS nonresponders ($p = 0.019$). As discussed in next chapter, this increased level of IFN-γ may be responsible in maintaining the symptom free phase in these individuals.

Figure 3 suggests that the responders from AD and NAD group produced higher levels of IL-2 (Th1) as compared to those from nonresponder group ($p = 0.02$). Figure 3 also shows that there was negligible amount of IL-2 produced by PBMC from AS and NEG individuals.

Figure 4 illustrates that PBMC from HSV-2 seropositive responders from AD and NAD groups secreted significantly higher levels of IL-10 (Th2) cytokine as compared to non-responders ($p = 0.007$) and HSV-2 seronegative controls ($p = 0.03$). However, the

level of IL-10 produced by PBMC from the responders in the AS group did not differ significantly when compared to levels produced by PBMC from AS non-responders ($p = 0.188$). Furthermore, PBMC from non-responders in the AD and NAD groups did not secrete significantly higher levels of IL-10 compared to non-responders from the AS group ($p = 0.14$), see Figure 4.

Figure 5 shows that HSV-2-stimulated PBMC from HSV-2 patients produced very low levels of another Th2 cytokine, IL-4. Trend similar with other published data obtained by using HSV type specific glycoproteins, as discussed later. Also, the levels of IL-4 produced by PBMC were not significantly different in responders and nonresponders categories of AD, NAD and AS groups of patients.

Finally, HSV-2-stimulated PBMC from asymptomatic seronegative healthy controls had very low levels of IFN-γ (Figure 2), IL-2 (Figure 3), IL-10 (Figure 4), and IL-4 (Figure 5).

Figure 2. Production of IFN-γ by PBMC from HSV-2-seropositive responders and non-responders at different stages of HSV-2 disease and HSV-2-seronegative controls. PBMC (1×10^6 /ml) obtained from patients with AD (n = 16), NAD (n = 38), AS (n = 7), and NEG (n = 7) were stimulated with HSV-2 antigen for 5 days, and the supernatants analyzed for IFN-γ production by ELISA, as described in Materials and Methods. The results presented are mean ± standard error.

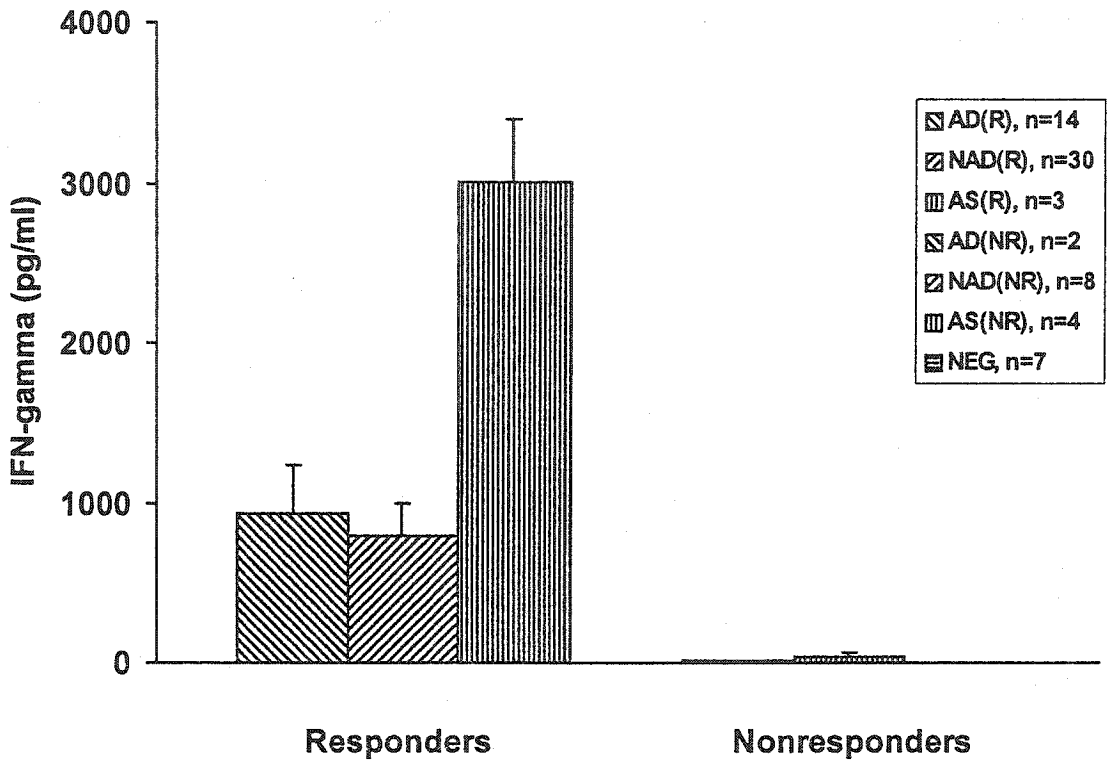

4000
3000
2000
1000
0
IFN-gamma (pg/ml)
AD(R), n=14
NAD(R), n=30
AS(R), n=3
AD(NR), n=2
NAD(NR), n=8
AS(NR), n=4
NEG, n=7
Responders
Nonresponders

Figure 2

Figure 3. Production of IL-2 by PBMC from HSV-2-seropositive responders and non-responders at different stages of HSV-2 disease and HSV-2-seronegative controls. PBMC (1×10^6 /ml) obtained from patients with AD (n = 16), NAD (n = 38), AS (n = 7), and NEG (n = 7) were stimulated with HSV-2 antigen for 5 days, and the supernatants analyzed for IL2 production by ELISA, as described in Materials and Methods. The results presented are mean ± standard error.

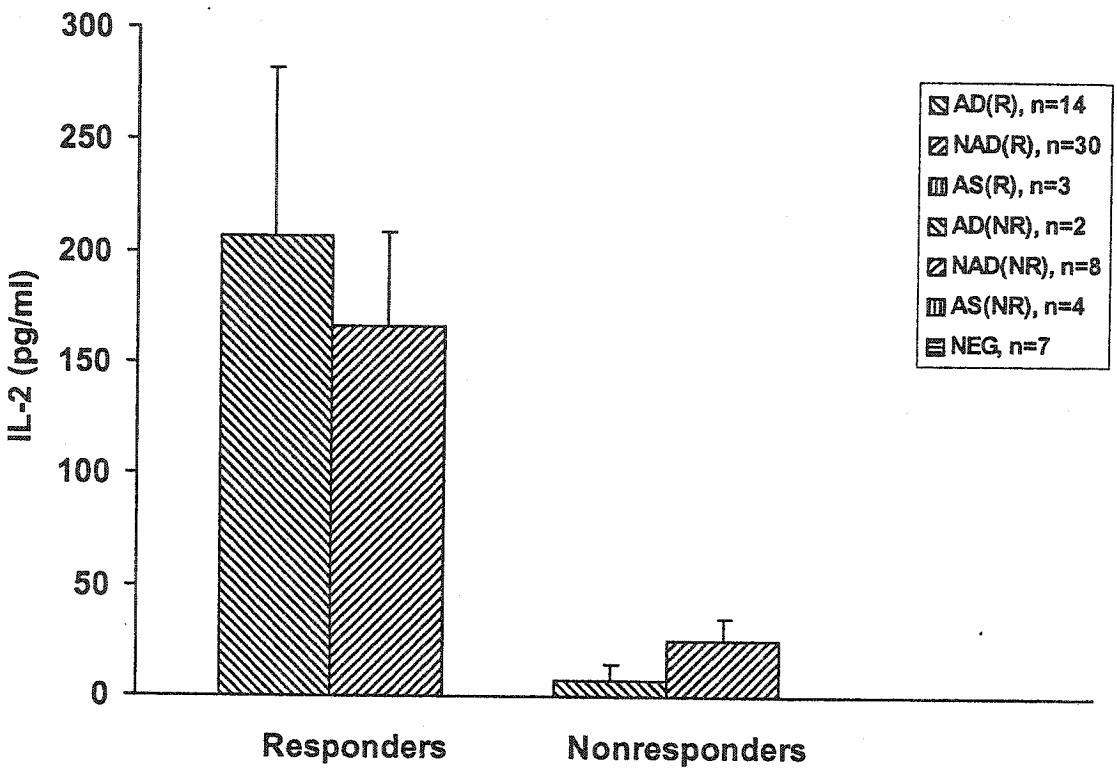

300
250
200
150
100
50
0
IL-2 (pg/ml)
AD(R), n=14
NAD(R), n=30
AS(R), n=3
AD(NR), n=2
NAD(NR), n=8
AS(NR), n=4
NEG, n=7
Responders
Nonresponders

Figure 3

Figure 4. Production of IL-10 by PBMC from HSV-2-seropositive responders and non-responders at different stages of HSV-2 disease and HSV-2-seronegative controls. PBMC (1×10^6 /ml) obtained from patients with AD (n = 16), NAD (n = 38), AS (n = 7), and NEG (n = 7) were stimulated with HSV-2 antigen for 5 days, and the supernatants analyzed for IL-10 production by ELISA, as described in Materials and Methods. The results presented are mean ± standard error.

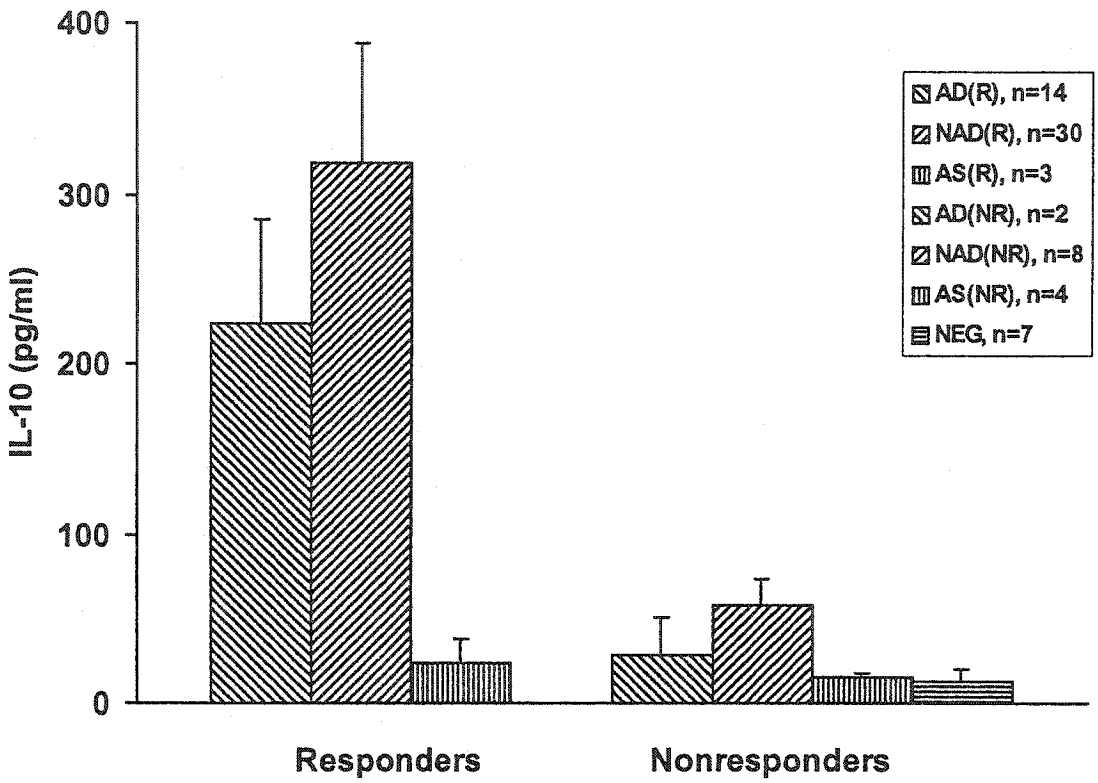

400
300
200
100
0
IL-10 (pg/ml)
AD(R), n=14
NAD(R), n=30
AS(R), n=3
AD(NR), n=2
NAD(NR), n=8
AS(NR), n=4
NEG, n=7
Responders
Nonresponders

Figure 4

Figure 5. Production of IL-4 by PBMC from HSV-2-seropositive responders and non-responders at different stages of HSV-2 disease and HSV-2-seronegative controls. PBMC (1×10^6 /ml) obtained from patients with AD (n = 16), NAD (n = 38), AS (n = 7), and NEG (n = 7) were stimulated with HSV-2 antigen for 5 days, and the supernatants analyzed for IL-4 production by ELISA, as described in Materials and Methods. The results presented are mean ± standard error.

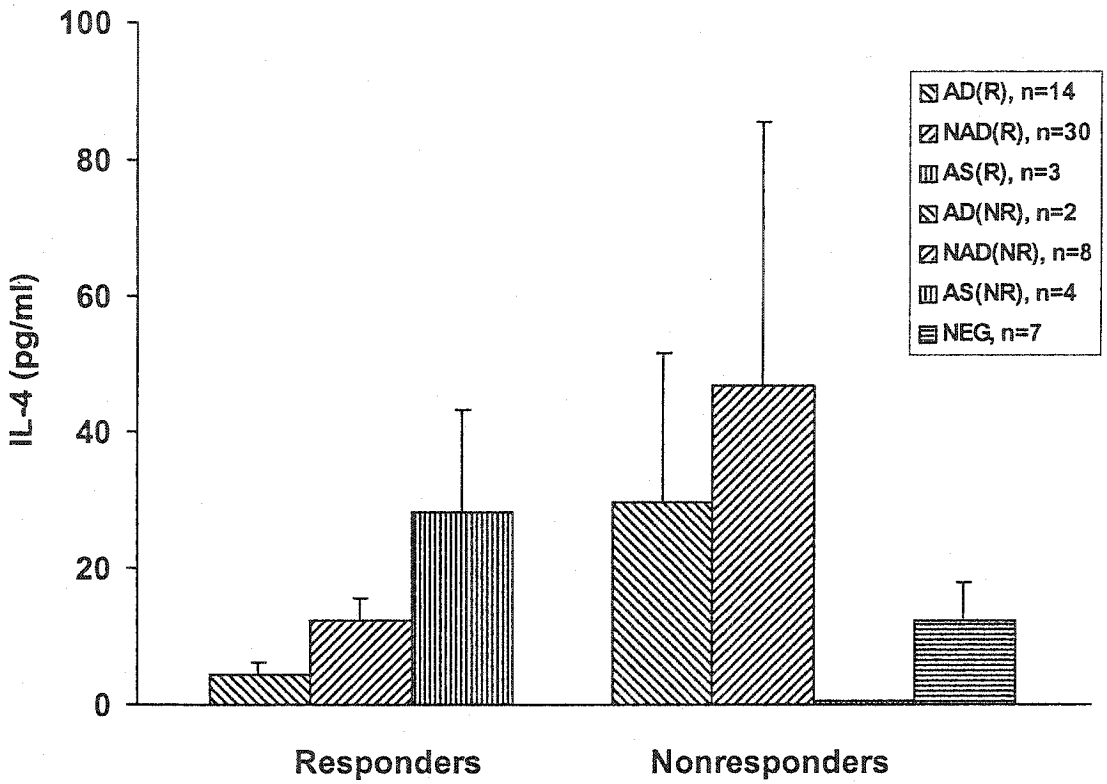

100
80
60
40
20
0
IL-4 (pg/ml)
AD(R), n=14
NAD(R), n=30
AS(R), n=3
AD(NR), n=2
NAD(NR), n=8
AS(NR), n=4
NEG, n=7
Responders
Nonresponders

Figure 5

3.3 Th1/Th2 Response at Different Stages of HSV-2 Disease

The above results suggest that the level of Th1 and Th2 cytokines is different in various categories of HSV-2 patients. To determine if the levels of Th cytokines produced at different times following infection may also have an impact on the disease process, the production of IFN-γ, IL-2, and IL-10 by HSV-2 stimulated PBMC from patients was analyzed in 44 samples obtained from 23 patients with recurrent disease. In 12 cases samples were obtained at different time points from their last HSV-2 genital outbreak (as per the patient's clinical chart).

Figure 6 (A-L) illustrates IFN-γ response from 12 HSV-2 symptomatic individuals from whom more than one blood samples (n = 33) were drawn for analysis. In 8 out of 12 (67%) individuals, the levels of IFN-γ decreased over time following a recurrence. As seen in Figures 7 to 9, peak levels of IFN-γ, IL-10, and IL-2 production were noted around the time of a recurrence of active disease. Also, as presented in Figure 7, IFN-γ levels showed a decreasing trend during the recurrence-free phase ($p = 0.046$). However, as seen in Figure 8, the high levels of IL-10 produced during the period of active disease persisted (after a modest decline) throughout the study period following a recurrence, and unlike IFN-γ, a progressive decrease was not observed ($p = 0.71$). Figure 9 illustrates that the levels of IL-2 production decreased over time but the association was not very significant ($p = 0.24$).

Figure 6. Kinetics of IFN-γ production by PBMC from 12 patients with recurrent HSV-2 disease from whom more than 1 blood samples were obtained at various times before and after the onset of a genital HSV-2 recurrence. PBMC (1×10^6/ml) obtained from HSV-2 patients were stimulated with HSV-2 antigen for 5 days and the supernatants analyzed for IFN-γ production by ELISA as described in Materials and Methods.

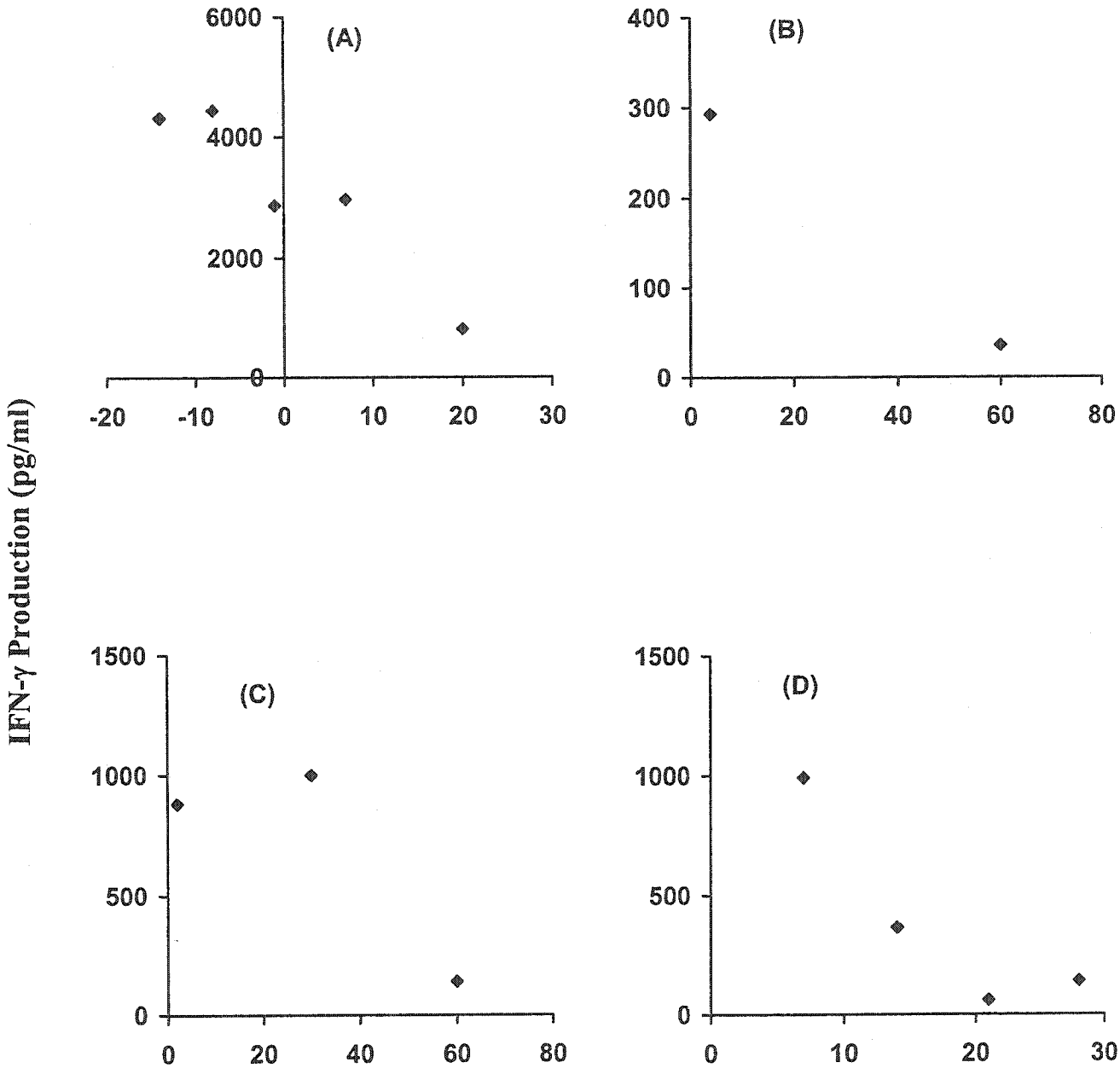

IFN-γ Production (pg/ml)
(A)
6000
4000
2000
0
-20
-10
0
10
20
30
(B)
400
300
200
100
0
0
20
40
60
80
(C)
1500
1000
500
0
0
20
40
60
80
(D)
1500
1000
500
0
0
10
20
30
Days Since Last Recurrence

Figure 6

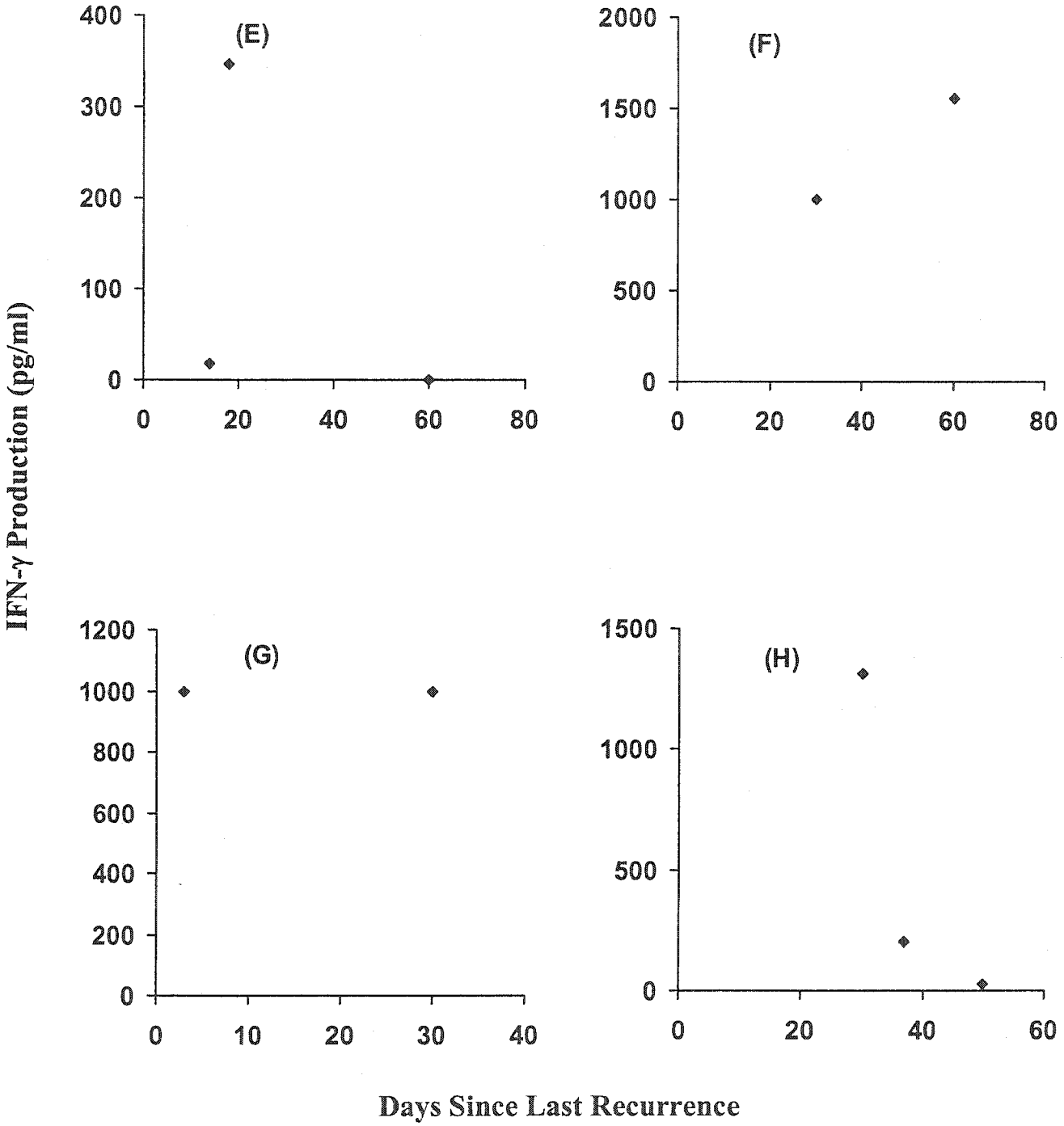

IFN-γ Production (pg/ml)
(E)
400
300
200
100
0
0
20
40
60
80
(F)
2000
1500
1000
500
0
0
20
40
60
80
(G)
1200
1000
800
600
400
200
0
0
10
20
30
40
(H)
1500
1000
500
0
0
20
40
60
Days Since Last Recurrence

Figure 6

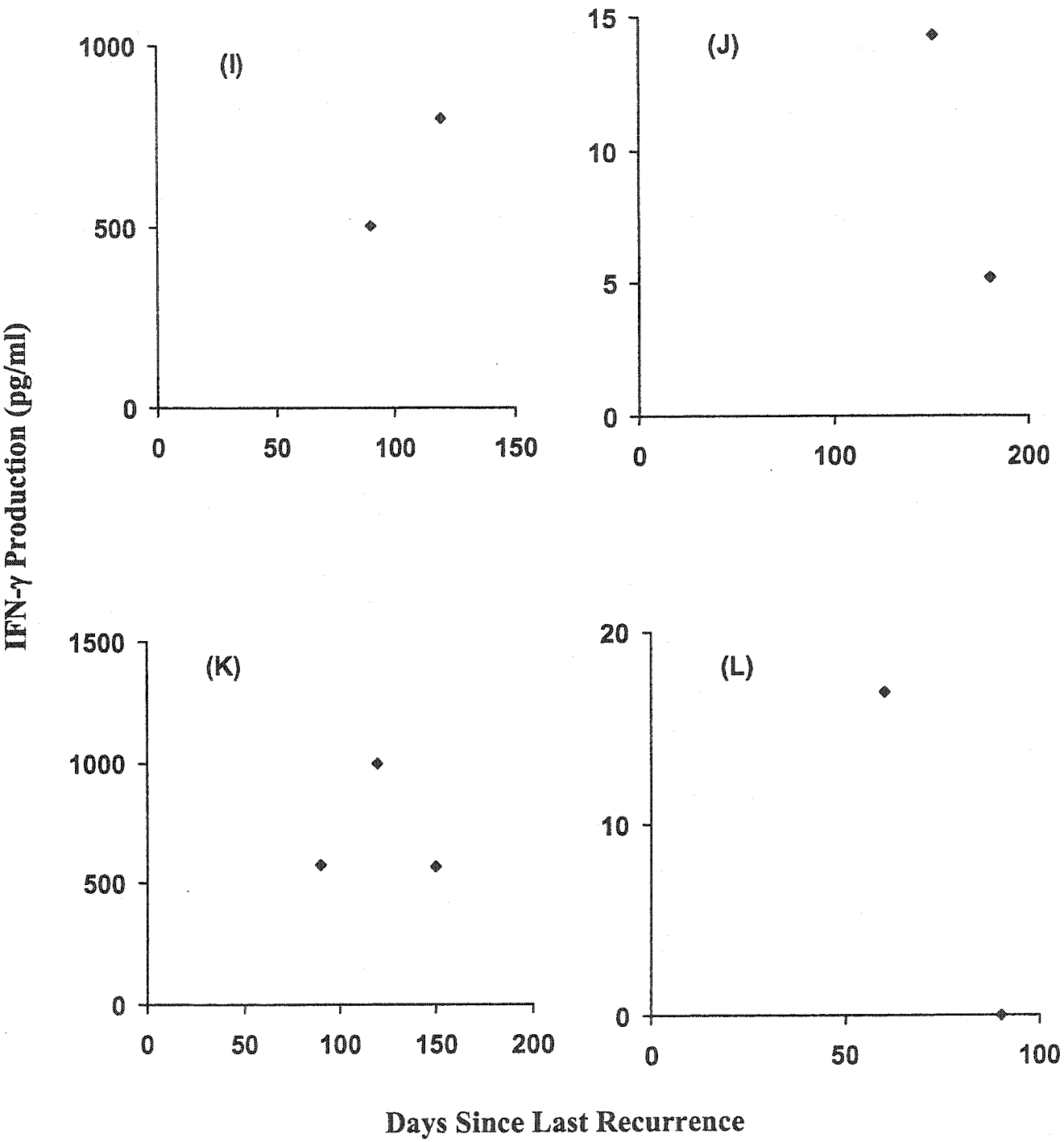

IFN-γ Production (pg/ml)
(I)
1000
500
0
0
50
100
150
(J)
15
10
5
0
0
100
200
(K)
1500
1000
500
0
0
50
100
150
200
(L)
20
10
0
0
50
100
Days Since Last Recurrence

Figure 6

Figure 7. Kinetics of IFN-γ production by PBMC from 23 patients with recurrent HSV-2 disease at various times before and after the onset of a genital HSV-2 recurrence. PBMC (1×10^6 /ml) obtained from HSV-2 patients were stimulated with HSV-2 antigen for 5 days and the supernatants analyzed for IFN-γ production by ELISA as described in Materials and Methods.

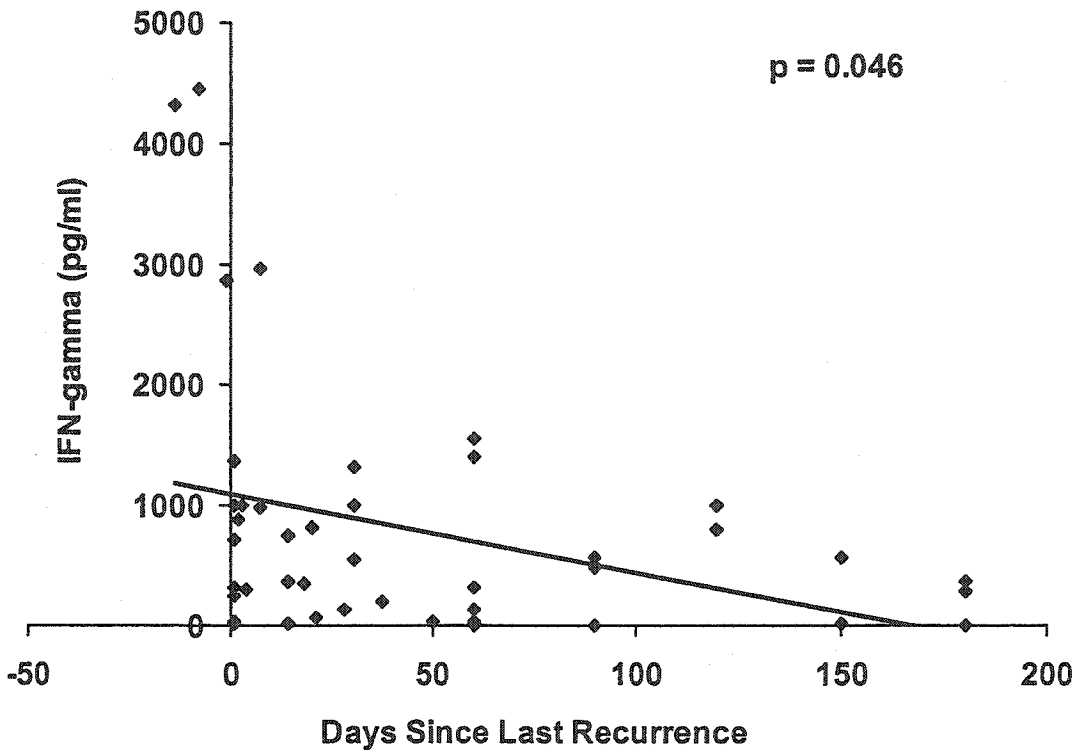

p = 0.046
IFN-gamma (pg/ml)
5000
4000
3000
2000
1000
0
-50
0
50
100
150
200
Days Since Last Recurrence

Figure 7

Figure 8. Kinetics of IL-10 production by PBMC from 23 patients with recurrent HSV-2 disease at various times before and after the onset of a genital HSV-2 recurrence. PBMC (1×10^6 /ml) obtained from HSV-2 patients were stimulated with HSV-2 antigen for 5 days and the supernatants analyzed for IL-10 production by ELISA, as described in Materials and Methods.

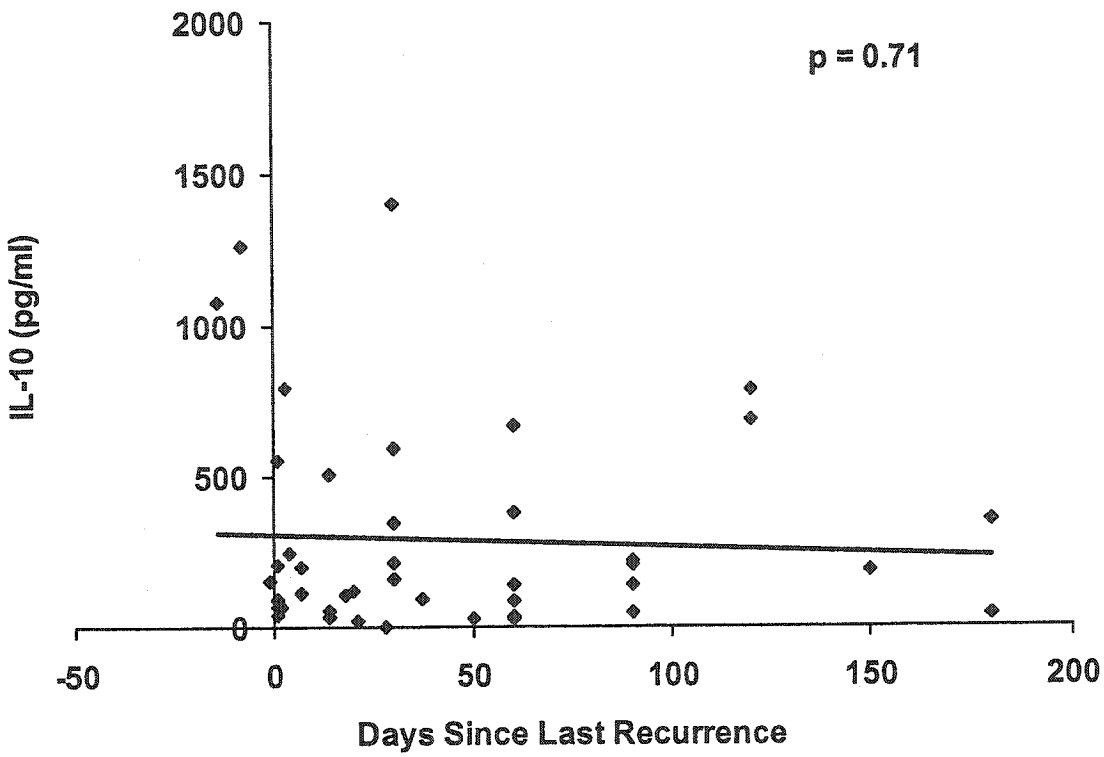

2000
1500
1000
500
0
IL-10 (pg/ml)
p = 0.71
-50
0
50
100
150
200
Days Since Last Recurrence

Figure 8

Figure 9. Kinetics of IL-2 production by PBMC 44 samples from 23 patients with recurrent HSV-2 disease at various times before and after the onset of a genital HSV-2 recurrence. PBMC (1 x 10^6 /ml) obtained from HSV-2 patients were stimulated with HSV-2 antigen for 5 days and the supernatants analyzed for IL-2 production by ELISA, as described in Materials and Methods.

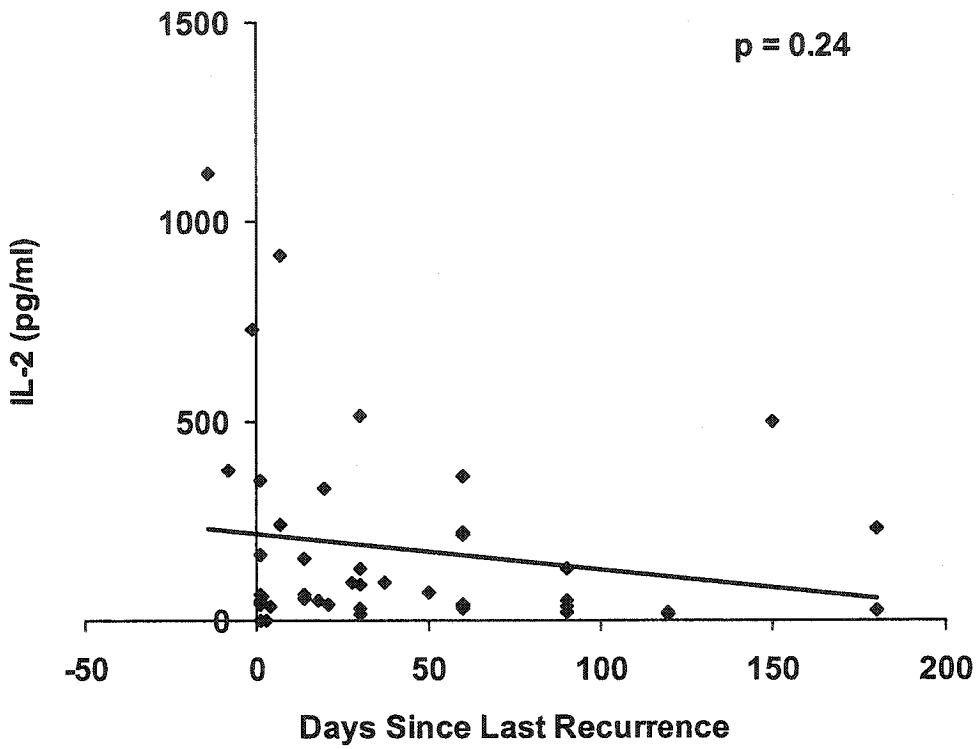

1500
1000
500
0
p = 0.24
IL-2 (pg/ml)
-50
0
50
100
150
200
Days Since Last Recurrence

Figure 9

3.4 Viral Copy Numbers in Genital Swabs

Several *in vitro* studies and experimental animal models have suggested that IFN-γ inhibits HSV replication (86,92). To investigate whether an association exists between Th cytokine production by HSV-2-stimulated PBMC and the presence of virus in genital lesions, quantitative HSV-2 specific PCR was performed on extracts from 34 genital swabs from 20 patients with recurrent genital HSV-2 infection (AD and NAD). HSV-2 DNA was detected in 15 swabs (44 %) from 13 patients, in the remaining 7 patients, HSV-2 DNA was not detected at the time of recurrence.

As seen in figure 10, these numbers declined sharply in 4-6 months thereafter, as did IFN-γ levels as presented in Figure 7. Comparison of IFN-γ production by HSV-2 stimulated PBMC with HSV-2 copy number present in the genital swabs revealed a significant correlation ($r = 0.44$; $p = 0.048$).

Figure 10. Analysis of HSV-2 copy number in the genital swabs obtained from HSV-infected individuals. HSV-2 was quantified by HSV-type specific PCR in genital swabs (n = 34) from AD and NAD individuals. The HSV-2 was detected in the DNA isolated from clinical samples by PCR analysis using the Digene Sharp Signal System with HSV-1 and HSV-2 primers, as described in Materials and Methods.

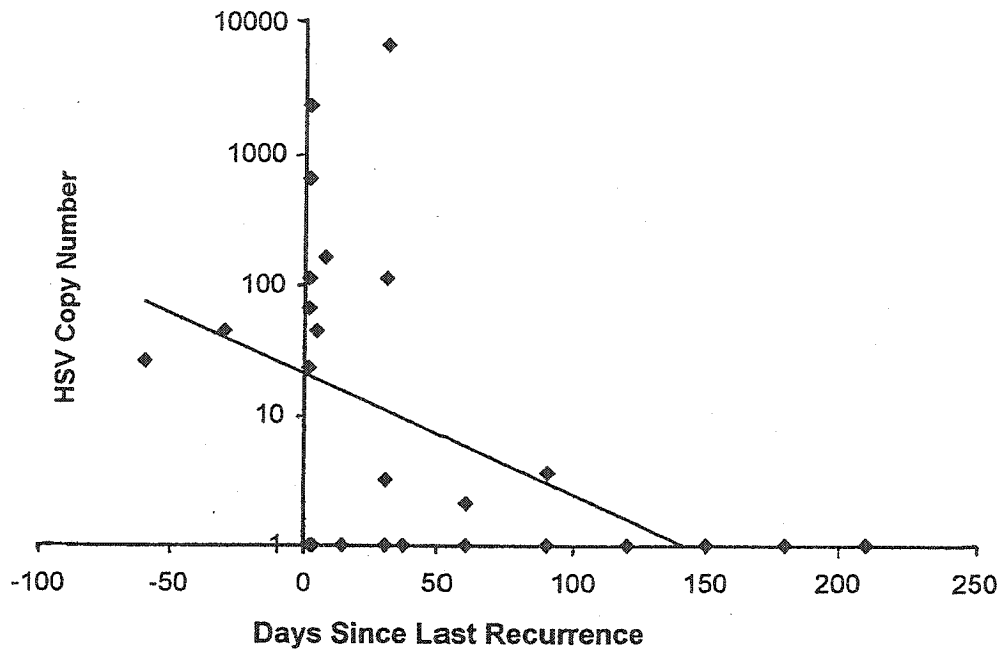

10000
1000
100
10
1
HSV Copy Number
-100
-50
0
50
100
150
200
250
Days Since Last Recurrence

Figure 10

3.5 Regulation of B7 in HSV-2 Infection by IL-10, IFN-γ, and HSV Antigen

As indicated earlier in chapter 1, MHC/Ag-TCR binding in the absence of proper signal from costimulatory molecules such as B7-1 and B7-2 leads to antigen specific T cell anergy (160-162). It has been previously shown that IFN-γ upregulates expression of both B7-1 (CD80) and B7-2 (CD86) on monocytes (176). The above results showing lack of responsiveness by PBMC from a subpopulation of HSV-2 patients and difference in the levels of IFN-γ and IL-10 production by HSV-2 stimulated PBMC suggest that regulation of B7 expression on monocytes of these patients may be altered.

Therefore, unstimulated PBMC from 9 HSV-2 patients; 6 responders (4 AD, 2 NAD) and 3 non-responders (1 AD, 2 NAD), and 4 HSV-2 negative healthy individuals were stimulated with IFN-γ, IL-10, HSV-1 or HSV-2 antigen. The monocytes were analyzed for B7-1 (CD80) and B7-2 (CD86) expression after 24 hours of stimulation. Prior to IL-10 and IFN-γ treatment there were no significant differences in the levels of B7-1 and B7-2 expression on monocytes of HSV-2 infected patients compared to HSV-seronegative controls.

Figure 11 shows that IL-10 slightly enhanced expression of B7-1 and downregulated the expression of B7-2 on monocytes of both HSV-2 patients and HSV-seronegative controls. In agreement with previous observation (176), Figures 12 and 13 illustrate that IFN-γ consistently upregulated the expression of both B7-1 and B7-2 on monocytes from HSV-seronegative individuals. In contrast, IFN-γ failed to show similar augmentation in the expression of B7-1 in 8 out of 9 and B7-2 in 6 out of 9 HSV-2 infected individuals examined, as seen in Figures 13 (a) and (b).

Figures 14 to 16 show that stimulation of PBMC with HSV-1 or HSV-2 antigen enhanced the expression of B7-1 and B7-2 on monocytes from HSV-2 infected individuals and to a lesser extent in HSV-negative controls. After treatment with HSV-2 antigen the level of B7-1 (CD80) and B7-2 (CD86) increased in 9 out of 9 HSV-2 patients, see Figures 16 (a) and (b).

The difference in median fluorescence intensity (MFI) for B7-1 and B7-2 in unstimulated cells and cells stimulated with IFN-γ, HSV-1 or HSV-2 antigen are shown in Figures 13 (a,b), 15 (a,b) and 16 (a,b), respectively. The first four samples in the bar charts were from AD responders, samples 5 and 6 were from NAD responders. The last three samples were from nonresponders sample 7 from AD nonresponder and samples 8 and 9 were from NAD nonresponders. As shown in figures Figures 13 (a,b), 15 (a,b) and 16 (a,b), there was no significant difference observed in the statement of B7-1 (CD 80) and B7-2 (CD86) after stimulation with IFN-γ, HSV-1 or HSV-2 antigen in HSV-2-seropositive responders (first 6 samples in the bar charts) and nonresponders (samples 7-9 in the bar charts).

Figure 11. Histogram representation for the effect of IL-10 on B7-1 (CD80) and B7-2 (CD86) statement on $CD14^+$ monocytes from HSV-2 patients and HSV-2-seronegative controls. PBMC from 9 HSV-2 patients from AD and NAD groups (6 responders and 3 nonresponders) and 4 HSV-seronegative controls were cultured in the presence or absence of IL-10 antigen for 24 hours. PBMC were stained with either PE-labeled anti-B7-1 or PE-labeled anti-B7-2 antibodies and counter stained with FITC-labeled anti-CD14 antibodies for monocytes, as described in Materials and Methods. The cells in the monocytic gate were analyzed for the statement of B7-1 (CD80) and B7-2 (CD86). The comparability in the statement levels of B7-1 and B7-2 isoforms between different patient populations was ensured through the use of standard brite flow cytometric fluorescence intensity standardization beads.

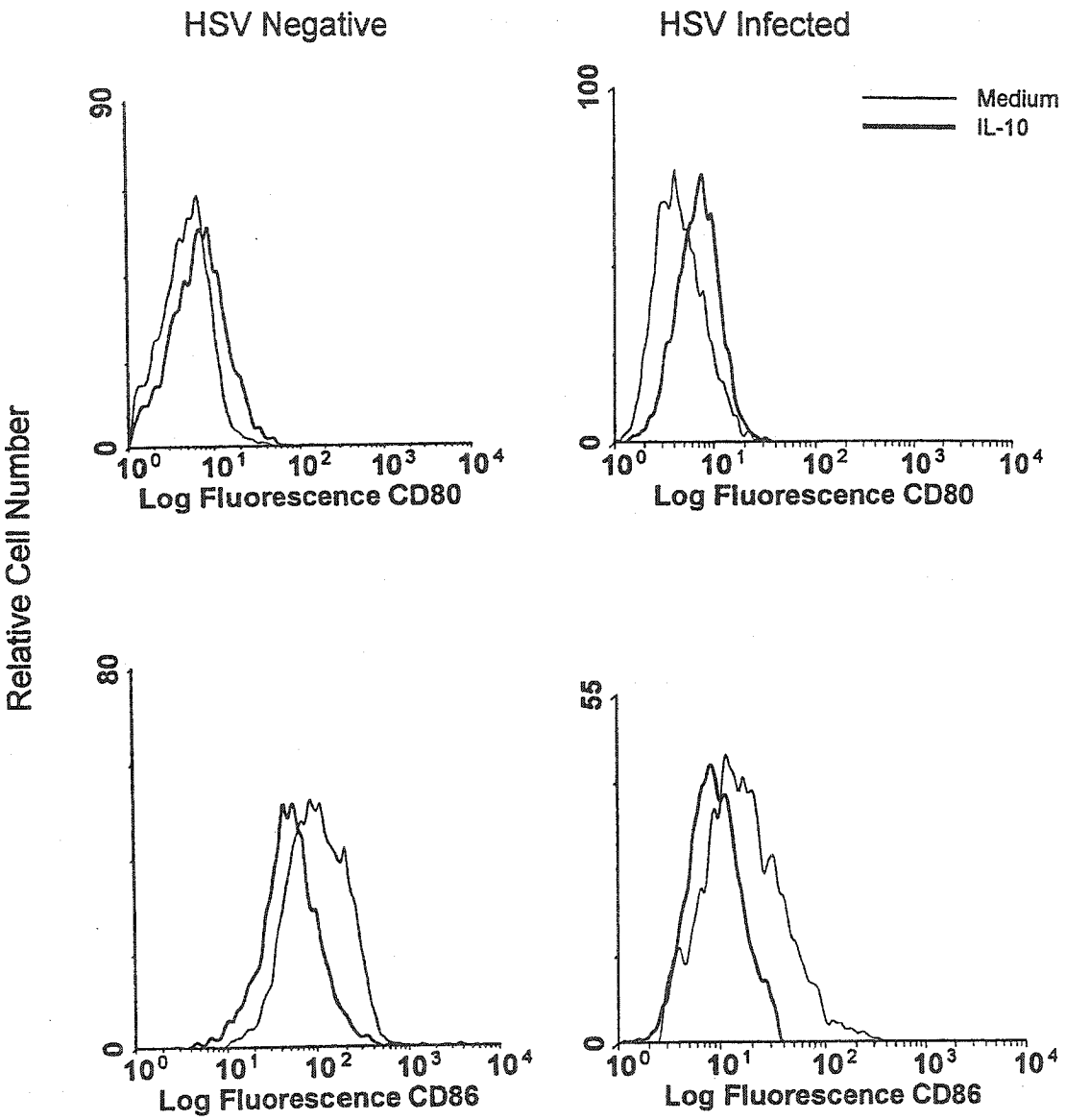

HSV Negative
HSV Infected
Medium
IL-10
Relative Cell Number
90
0
100
0
80
0
55
0
10^0 10^1 10^2 10^3 10^4
Log Fluorescence CD80
Log Fluorescence CD86

Figure 11

Figure 12. Histogram representation for the effect of IFN-γ on B7-1 and B7-2 statement on $CD14^+$ monocytes from HSV-2 patients and HSV-2-seronegative controls. PBMC from 9 HSV-2 patients from AD and NAD groups (6 responders and 3 nonresponders) and 4 HSV-seronegative controls were cultured in the presence or absence of IFN-γ for 24 hours. PBMC were stained with either PE-labeled anti-B7-1 or PE-labeled anti-B7-2 antibodies and counter stained with FITC-labeled anti-CD14 antibodies for monocytes, as described in Materials and Methods. The cells in the monocytic gate were analyzed for the statement of B7-1 (CD80) and B7-2 (CD86). The comparability in the statement levels of B7-1 and B7-2 isoforms between different patient populations was ensured through the use of standard brite flow cytometric fluorescence intensity standardization beads.

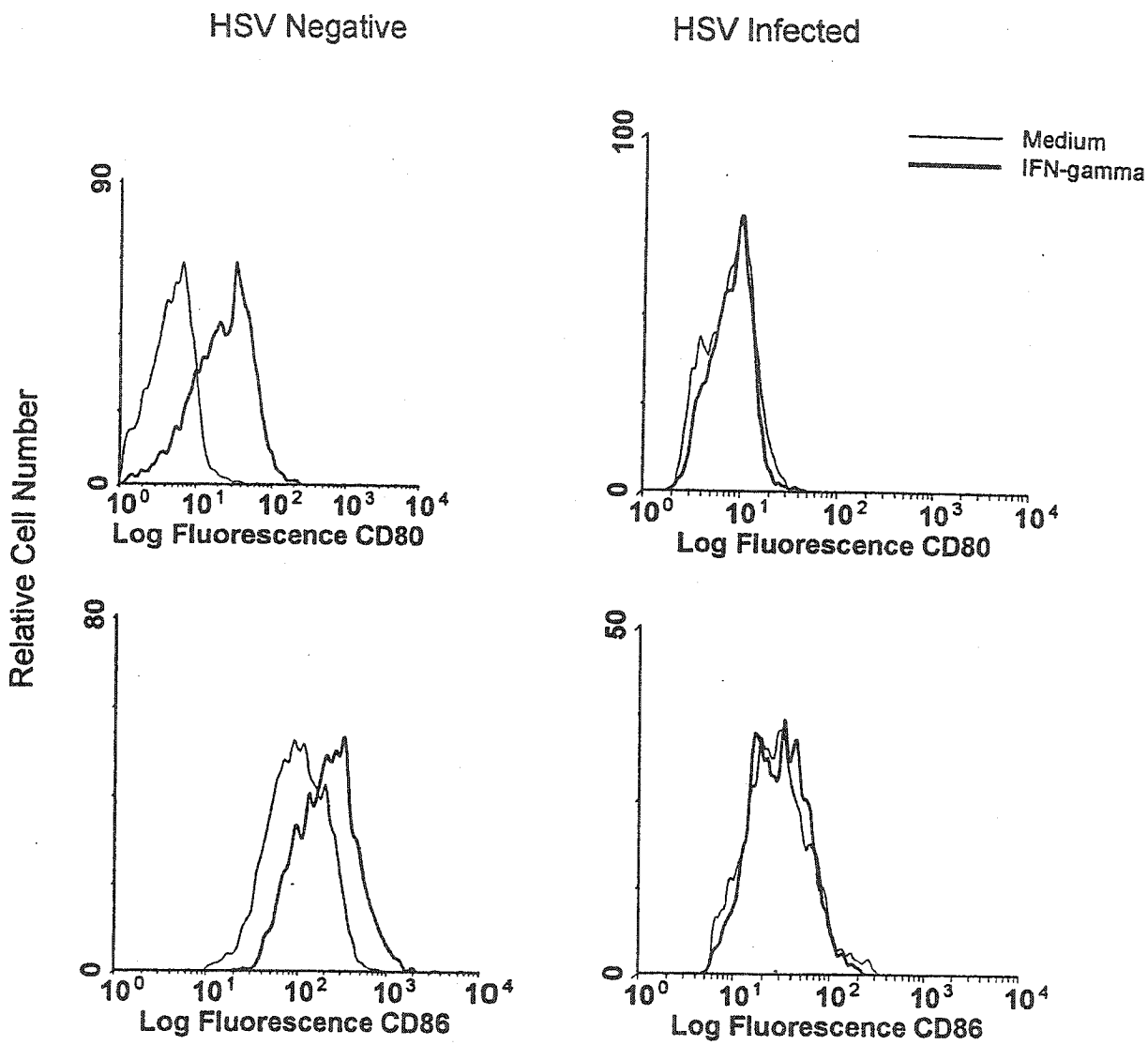

HSV Negative
HSV Infected
Medium
IFN-gamma
Relative Cell Number
90
0
100
0
80
0
50
0
10^0 10^1 10^2 10^3 10^4
Log Fluorescence CD80
Log Fluorescence CD86

Figure 12

Figure 13. Bar chart representation for the difference in median fluorescence intensity (MFI) value for **(a)** CD80 and **(b)** CD86 expression on $CD14^+$ monocytes before and after IFN-γ treatment. PBMC from 9 HSV-2 patients from AD and NAD groups (6 responders and 3 nonresponders) and 4 HSV-seronegative controls were cultured in the presence or absence of IFN-γ for 24 hours. PBMC were stained with either PE-labeled anti-B7-1 or PE-labeled anti-B7-2 antibodies and counter stained with FITC-labeled anti-CD14 antibodies for monocytes, as described in Materials and Methods. The cells in the monocytic gate were analyzed for the statement of B7-1 (CD80) and B7-2 (CD86). The comparability in the statement levels of B7-1 and B7-2 isoforms between different patient populations was ensured through the use of standard brite flow cytometric fluorescence intensity standardization beads.

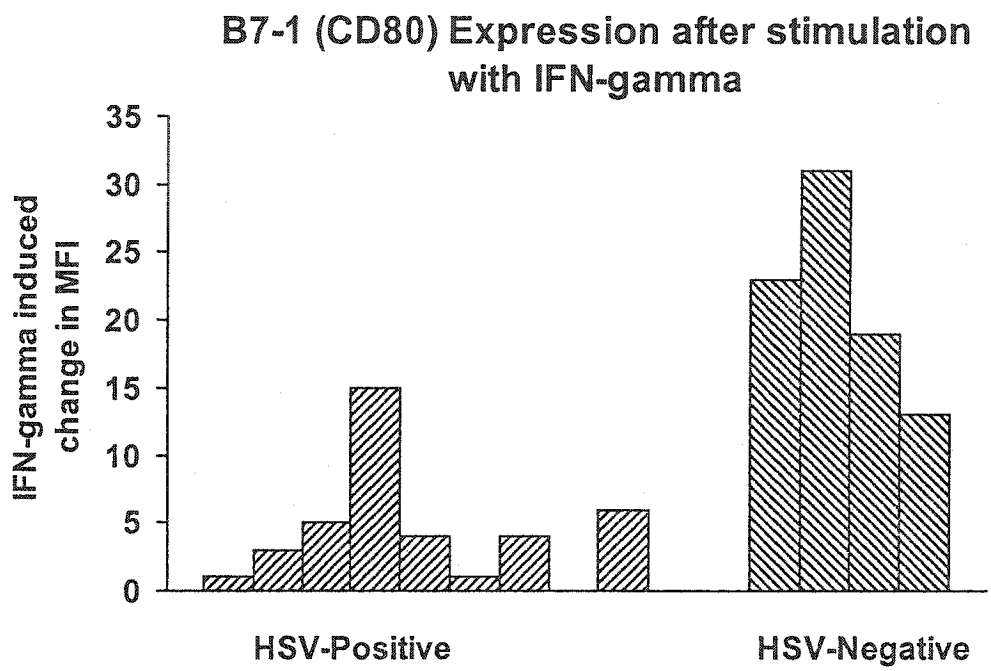

B7-1 (CD80) Expression after stimulation with IFN-gamma
IFN-gamma induced change in MFI
35
30
25
20
15
10
5
0
HSV-Positive
HSV-Negative

Figure 13 (a)

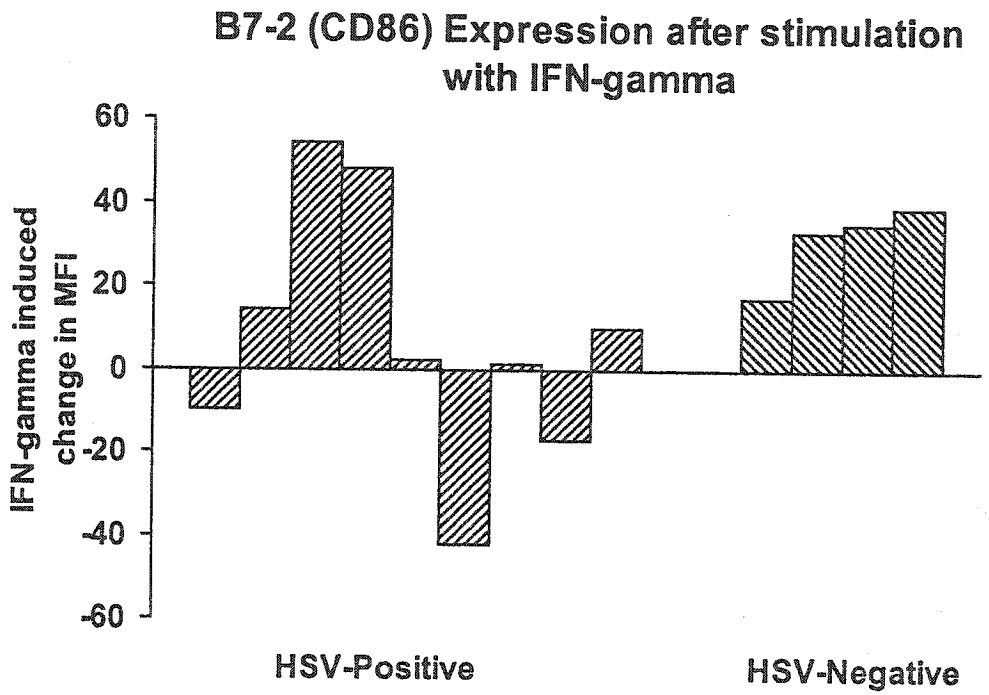

B7-2 (CD86) Expression after stimulation with IFN-gamma
IFN-gamma induced change in MFI
60
40
20
0
-20
-40
-60
HSV-Positive
HSV-Negative

Figure 13 (b)

Figure 14. Histogram representation of the effect of HSV-1 and HSV-2 antigen on B7-1 (CD80) and B7-2 (CD86) statement on $CD14^{+}$ monocytes from HSV-2 patients and HSV-2-seronegative controls. PBMC from 9 HSV-2 patients from AD and NAD groups (6 responders and 3 nonresponders) and 4 HSV-seronegative controls were cultured in the presence or absence of HSV-1 antigen for 24 hours. PBMC were stained with either PE-labeled anti-B7-1 or PE-labeled anti-B7-2 antibodies and counter stained with FITC-labeled anti-CD14 antibodies for monocytes, as described in Materials and Methods. The cells in the monocytic gate were analyzed for the statement of B7-1 (CD80) and B7-2 (CD86). The comparability in the statement levels of B7-1 and B7-2 isoforms between different patient populations was ensured through the use of standard brite flow cytometric fluorescence intensity standardization beads.

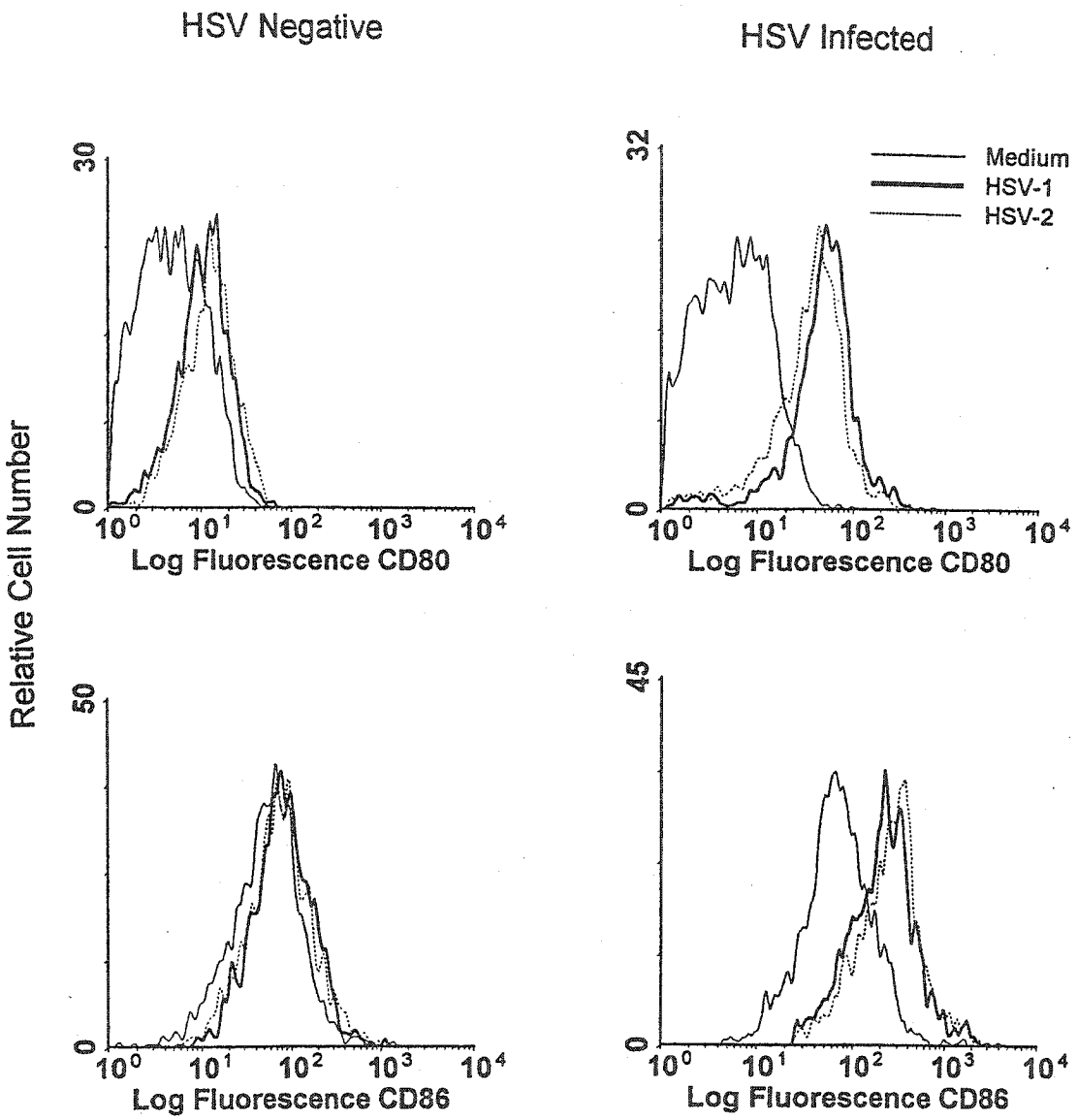

HSV Negative
HSV Infected
Relative Cell Number
30
0
32
0
Medium
HSV-1
HSV-2
10^0 10^1 10^2 10^3 10^4
Log Fluorescence CD80
50
0
45
0
Log Fluorescence CD86

Figure 14

Figure 15. Bar chart representation for the difference in median fluorescence intensity (MFI) value for **(a)** CD80 and **(b)** CD86 expression on $CD14^+$ monocytes before and after stimulation with HSV-1 antigen. PBMC from 9 HSV-2 patients from AD and NAD groups (6 responders and 3 nonresponders) and 4 HSV-seronegative controls were cultured in the presence or absence of HSV-1 antigen for 24 hours. PBMC were stained with either PE-labeled anti-B7-1 or PE-labeled anti-B7-2 antibodies and counter stained with FITC-labeled anti-CD14 antibodies for monocytes, as described in Materials and Methods. The cells in the monocytic gate were analyzed for the statement of B7-1 (CD80) and B7-2 (CD86). The comparability in the statement levels of B7-1 and B7-2 isoforms between different patient populations was ensured through the use of standard brite flow cytometric fluorescence intensity standardization beads.

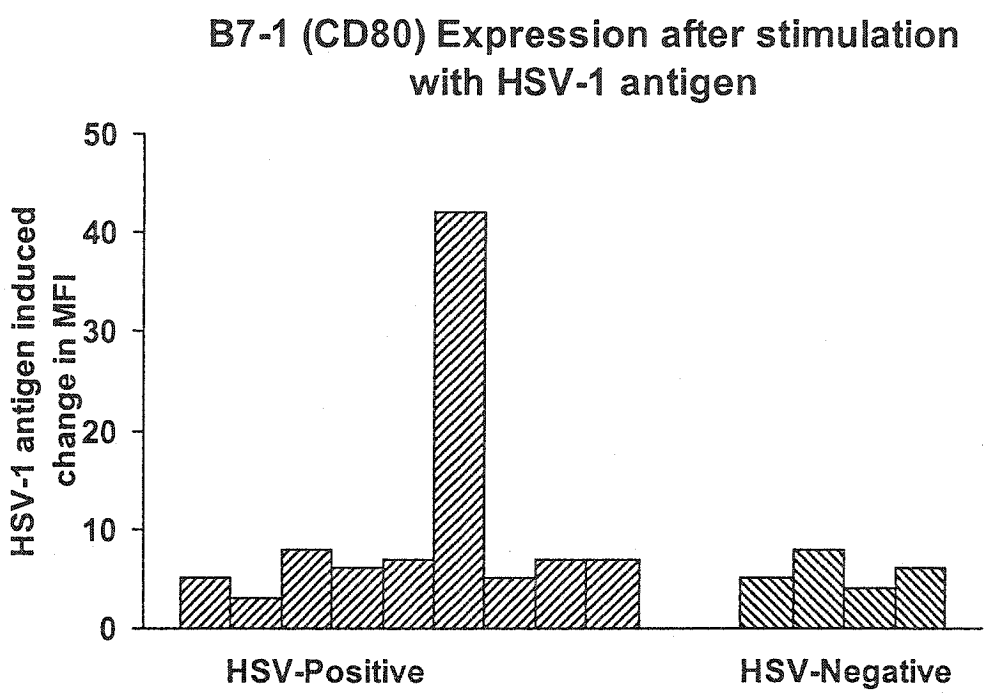

B7-1 (CD80) Expression after stimulation with HSV-1 antigen
HSV-1 antigen induced change in MFI
50
40
30
20
10
0
HSV-Positive
HSV-Negative

Figure 15 (a)

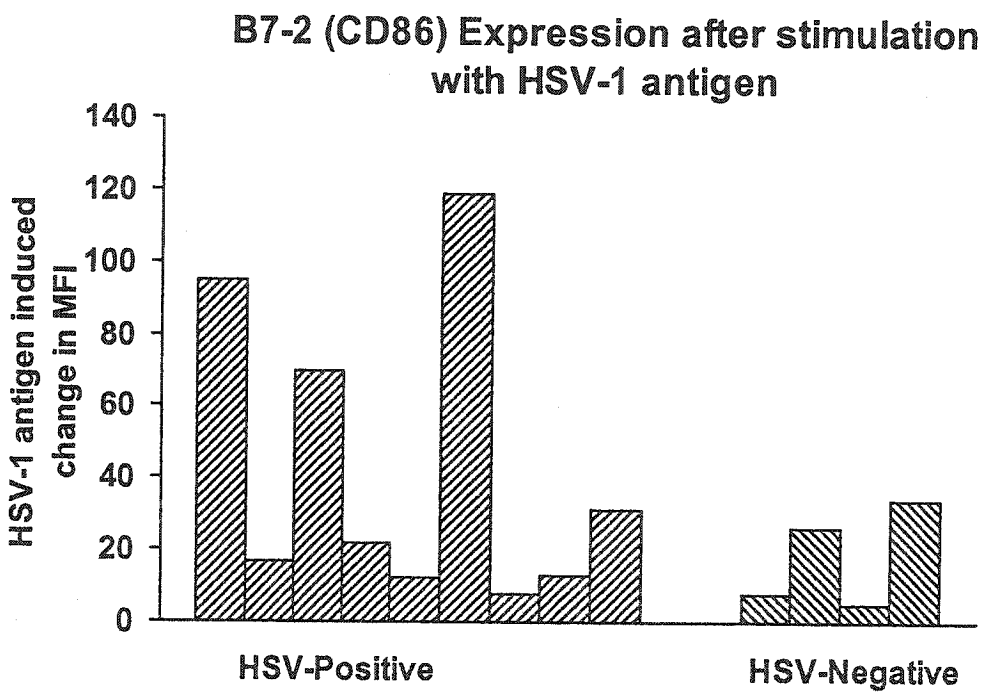

B7-2 (CD86) Expression after stimulation with HSV-1 antigen
HSV-1 antigen induced change in MFI
140
120
100
80
60
40
20
0
HSV-Positive
HSV-Negative

Figure 15 (b)

Figure 16. Bar chart representation for the difference in median fluorescence intensity (MFI) value for **(a)** CD80 and **(b)** CD86 expression on $CD14^+$ monocytes before and after stimulation with HSV-2 antigen. PBMC from 9 HSV-2 patients from AD and NAD groups (6 responders and 3 nonresponders) and 4 HSV-seronegative controls were cultured in the presence or absence of HSV-2 antigen for 24 hours. PBMC were stained with either PE-labeled anti-B7-1 or PE-labeled anti-B7-2 antibodies and counter stained with FITC-labeled anti-CD14 antibodies for monocytes, as described in Materials and Methods. The cells in the monocytic gate were analyzed for the statement of B7-1 (CD80) and B7-2 (CD86). The comparability in the statement levels of B7-1 and B7-2 isoforms between different patient populations was ensured through the use of standard brite flow cytometric fluorescence intensity standardization beads.

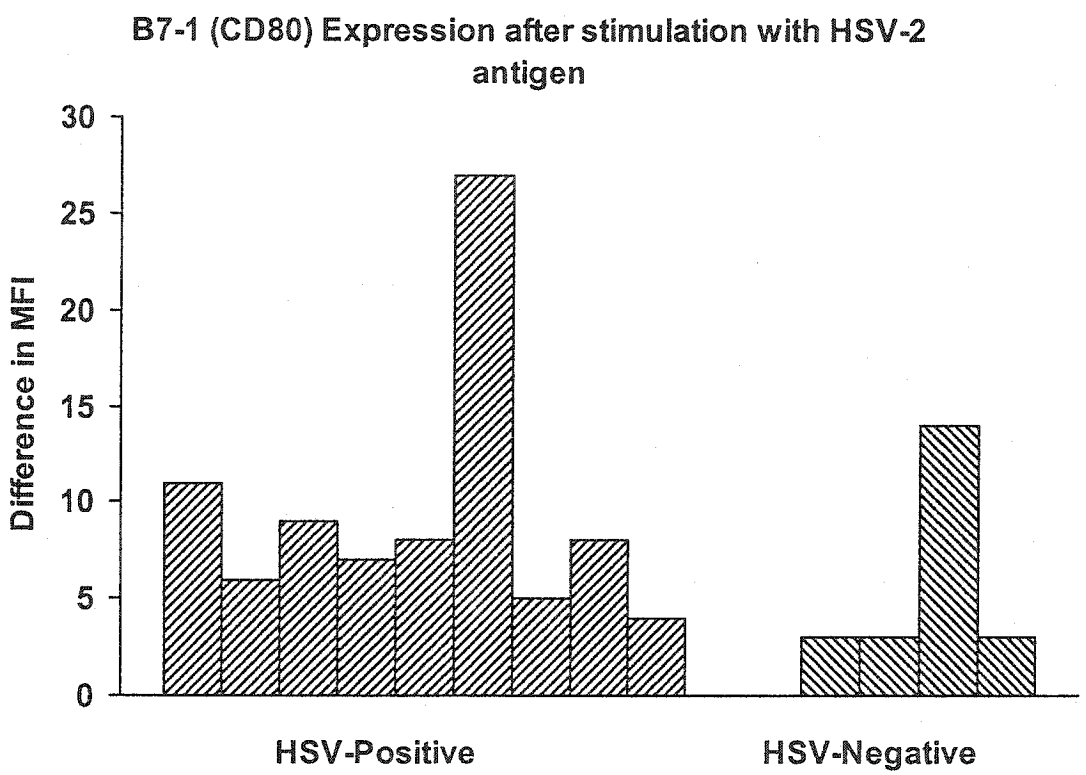

B7-1 (CD80) Expression after stimulation with HSV-2 antigen
Difference in MFI
30
25
20
15
10
5
0
HSV-Positive
HSV-Negative

Figure 16 (a)

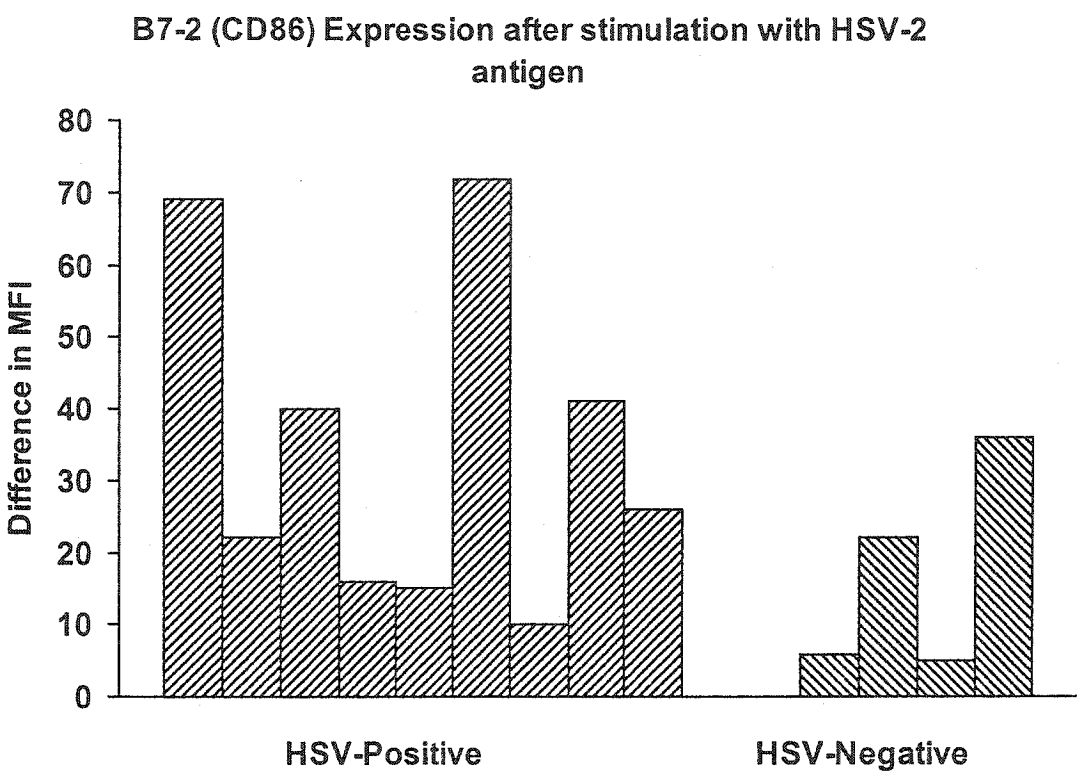

B7-2 (CD86) Expression after stimulation with HSV-2 antigen
Difference in MFI
80
70
60
50
40
30
20
10
0
HSV-Positive
HSV-Negative

Figure 16 (b)

3.6 Effect of Endogenously Produced IFN-γ on Expression of B7 Isoforms on Monocytes

The above results suggest that IFN-γ and HSV-2 antigen have opposing effects on the expression of B7-1 and B7-2 on monocytes from HSV-2 infected individuals. Since HSV-2 stimulation of PBMC from HSV-2 patients induces IFN-γ production, HSV-induced statement of B7-1 and B7-2 may be mediated by endogenously produced IFN-γ. Hence, PBMC stimulated with HSV-1 and HSV-2 antigen were cultured in the presence or absence of neutralizing anti-IFN-γ antibody, as described in materials and methods. Figure 17 shows that anti-IFN-γ antibody did not influence the statement of either B7-1 or B7-2 on unstimulated or HSV-1 and HSV-2-stimulated $CD14^+$ monocyte.

Figure 17. Effect of endogenously produced IFN-γ on the statement of B7-1 and B7-2 on $CD14^+$ monocytes following stimulation with HSV-1 and HSV-2 antigens. PBMC from six HSV-2 infected individuals were stimulated with HSV-2 antigens in the presence or absence of neutralizing anti-IFN-γ antibodies for 24 hours. PBMC were stained with either PE-labeled anti-B7-1 or PE-labeled anti-B7-2 antibodies and counter stained with FITC-labeled anti-CD14 antibodies for monocytes, as described in Materials and Methods. The cells in the monocytic gate were analyzed for the statement of B7-1 (CD80) and B7-2 (CD86). The comparability in the statement levels of B7-1 and B7-2 isoforms between different patient populations was ensured through the use of standard brite flow cytometric fluorescence intensity standardization beads.

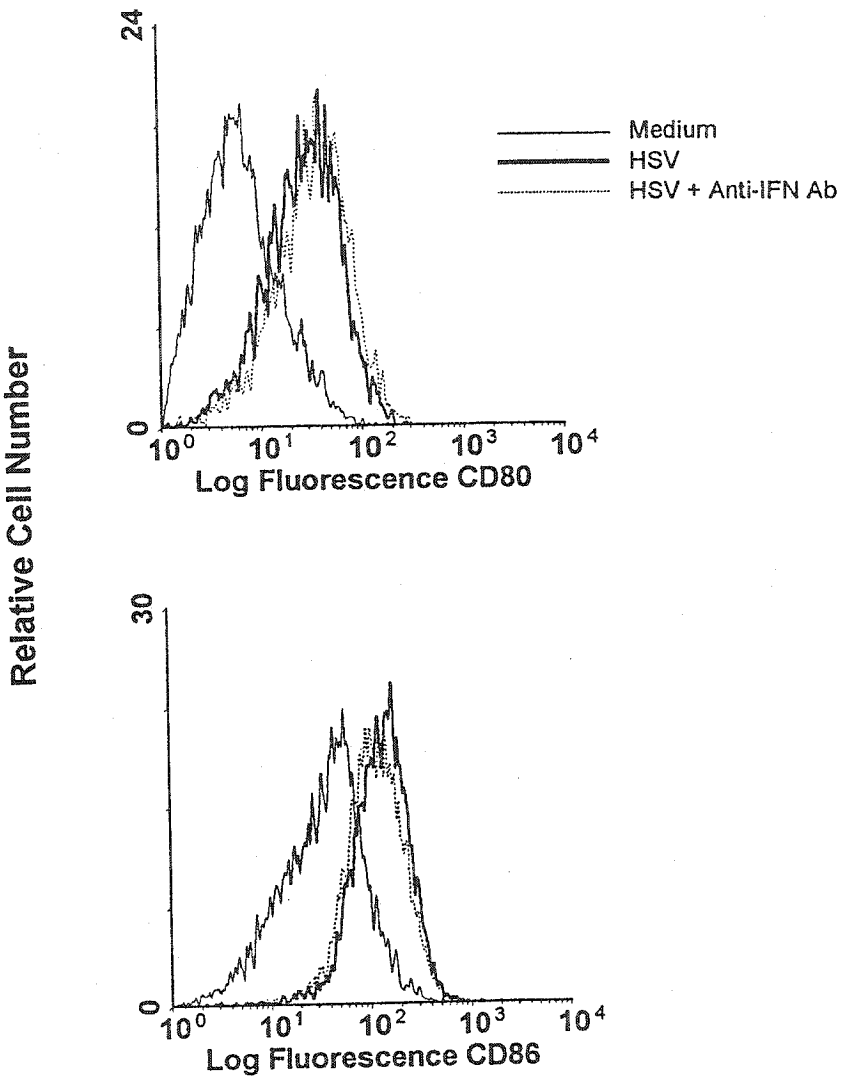

Relative Cell Number
24
0
10^0 10^1 10^2 10^3 10^4
Log Fluorescence CD80
Medium
HSV
HSV + Anti-IFN Ab
30
0
10^0 10^1 10^2 10^3 10^4
Log Fluorescence CD86

Figure 17

3.7 Th Responses during Infection with Wild Type and MCMV mutants

The above results show that during HSV-2 infections there is secretion of both Th1 and Th2 cytokine with occurrence of predominant Th1 response. Previous study using type specific glycoproteins of HSV-1 and HSV-2 has also suggested that the predominant Th response during HSV-2 infection is Th1 (45). Similar results have been observed during infections caused by CMV, another prominent member of herpesvirus family (47-49). Both HSV and CMV persist in the host for life after primary infection and causes acute, and persistent infection that includes latency, and reactivation disease (1,5,74,111). Further, these viruses have similarity in their genetic organization with immediate early, early and late genes, as described earlier (50,51,74,111). Both HSV-2 and CMV are reported to modulate immune responses leading to either evasion of the host immune responses (10-25) or protection against infection (26-37). $CD4^+$ Th cells through production of various Th1 and Th2 cytokines play an important role in providing immunity against these infections (37-44,79,155,152,185).

To identify if there was a genetic element responsible for the predominent Th1 response during infection caused by these herpesviruses, the previously established mutagenesis approach was employed to study the role of MCMV M25, M27, M43, and m09. The choice of the MCMV recombinants was based on the results of preliminary experiments conducted using a mouse model and MCMV with disruption at the ORF of m09, m155, M25, M27, M37, M43, M48, M78, and M83. These early experiments and other studies performed in Dr. Liu's laboratory at UC Berkeley had indicated that MCMV mutants RvM25, RvM27, RvM43, and Rvm09 having transposon inserted into

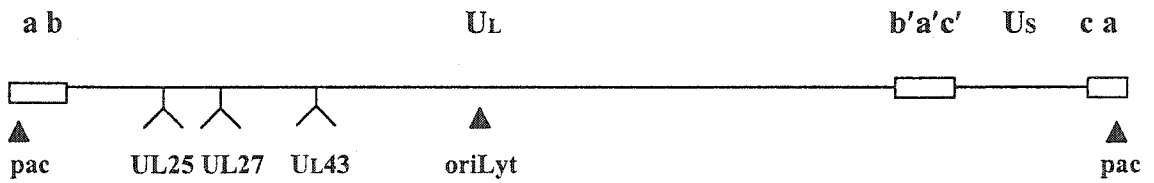
the ORF of M25, M27, M43, and m09 respectively, might have some role in modulating host Th1 and Th2 responses. Therefore, the *in vivo* role of M25, M27, M43, and m09 genes in modulation of Th1 and Th2 responses were analyzed using MCMV mutants RvM25, RvM27, RvM43 and Rvm09 having transposon inserted into the ORF of M25, M27, M43, and m09 genes. As shown in figure 17, the MCMV genes M25, M27, and M43 have counter parts in human CMV and HSV (UL25, UL27, and UL43, respectively) (111,113).

To identify the *in vivo* role of these genes in modulation of Th responses, the Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-10) cytokines were measured in the supernatants of splenocytes obtained from wild type (WT) and recombinant MCMVs at various days post infection (dpi) and cultured in the presence of 1:10 and 1:5 dilution of antigen and harvested five days after stimulation. The choice of antigen concentration and number of days was based on dose-response and time-response experiments described earlier in the Materials and Methods.

Figure 18. Linear map of MCMV, HCMV, and HSV genome representing the location of MCMV m09, M25, M27, and M43 genes along with the HCMV and HSV counterparts for M25, M27, and M43 (UL25, UL27, and UL43, respectively) and the packaging signal (pac); origins of DNA replication (oriLyt, oriL and oriS).

MCMV

HCMV



HSV

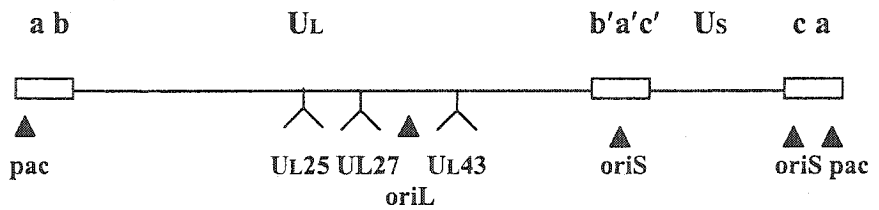


Figure 18

3.7.1 Th1 Response during Wild Type and Mutant MCMV Infection

Results of Th1 cytokine production at different dpi are graphically presented in Figures 19 and 20. Figures 19 (a) to (c) show that for all recombinants and WT, IFN-γ appeared on day 3 post infection and showed an increasing trend up to day 14. There was increased production of IFN-γ with no significant difference in the levels during infection with wild type (WT) and recombinant MCMVs Rvm09, RvM25, RvM27, and RvM43. Similar to IFN-γ, Figures 20 (a) and (b) illustrate that there was insignificant difference in the levels of another Th1 cytokine IL-2 produced during infection with wild type and recombinant MCMVs. Further, the level of IL-2 produced was very low in case of infection with all the virus types.

To determine if the mean values of two data sets were significantly different, p-values were calculated by Student's paired *t*-test for WT vs. RvM43 data. For IFN-γ, at 14 dpi the differences was statistically insignificant ($p = 0.355$). The similar trend of IFN-γ production by spleen cells after stimulation with 1:10 and 1:5 dilution of antigen as seen in Figure 19 (a) and (b) suggest the consistency and reproducibility of the results. This is also depicted in Figure 19 (c) where mean ± standard error is plotted for results from both the dilutions of antigen ($n = 6$).

Figure 19. Production of IFN-γ by splenocytes from Balb/c mice infected with the wild type (WT) MCMV, MCMV mutants (RvM25, RvM27, RvM43, and Rvm09) and uninfected controls stimulated. On day 1, 3, 7, 10, and 14 days post infection (dpi), $2x10^6$ /ml spleen cells from a batch of three mice were stimulated with **(a)** 1:10 and **(b)** 1:5 dilutions of respective antigen for 5 days and the supernatants analyzed for IFN-γ production by ELISA. The results shown are mean ± standard error of results from three mice. **(c)** The results shown are mean ± standard error for both dilutions of antigen (n = 6).

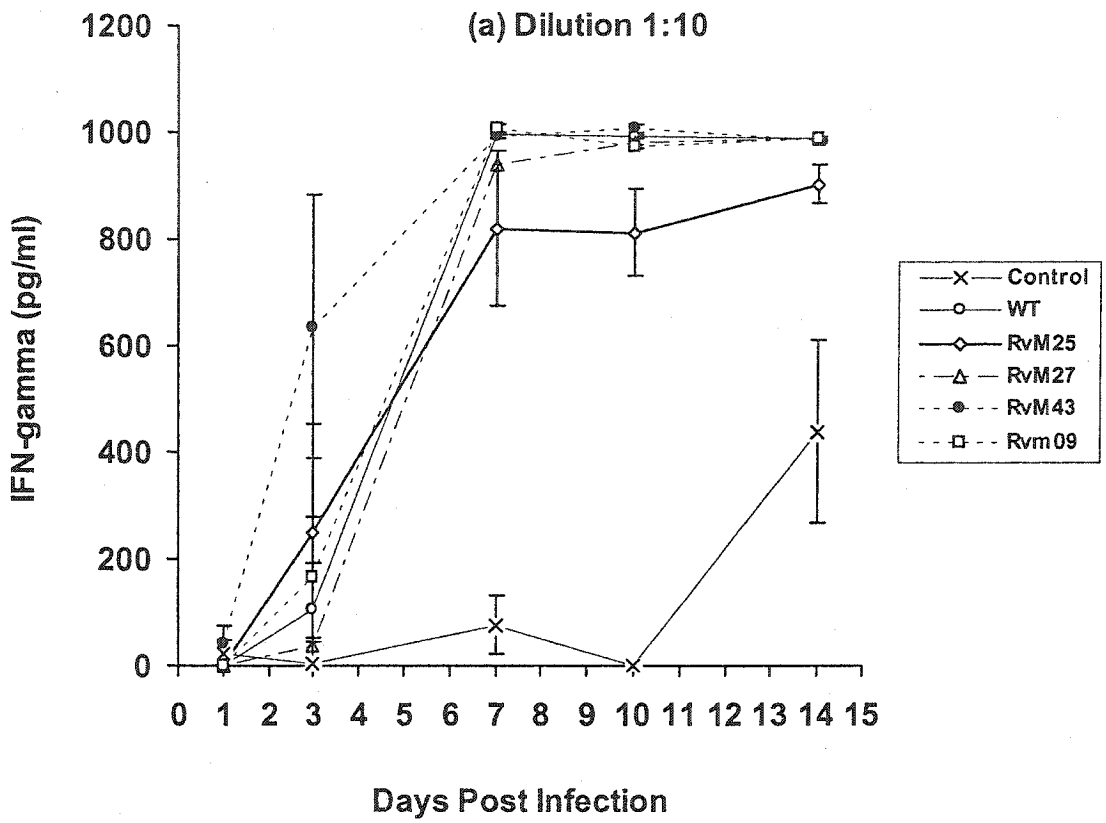

(a) Dilution 1:10
IFN-gamma (pg/ml)
1200
1000
800
600
400
200
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days Post Infection
Control
WT
RvM25
RvM27
RvM43
Rvm09

Figure 19 (a)

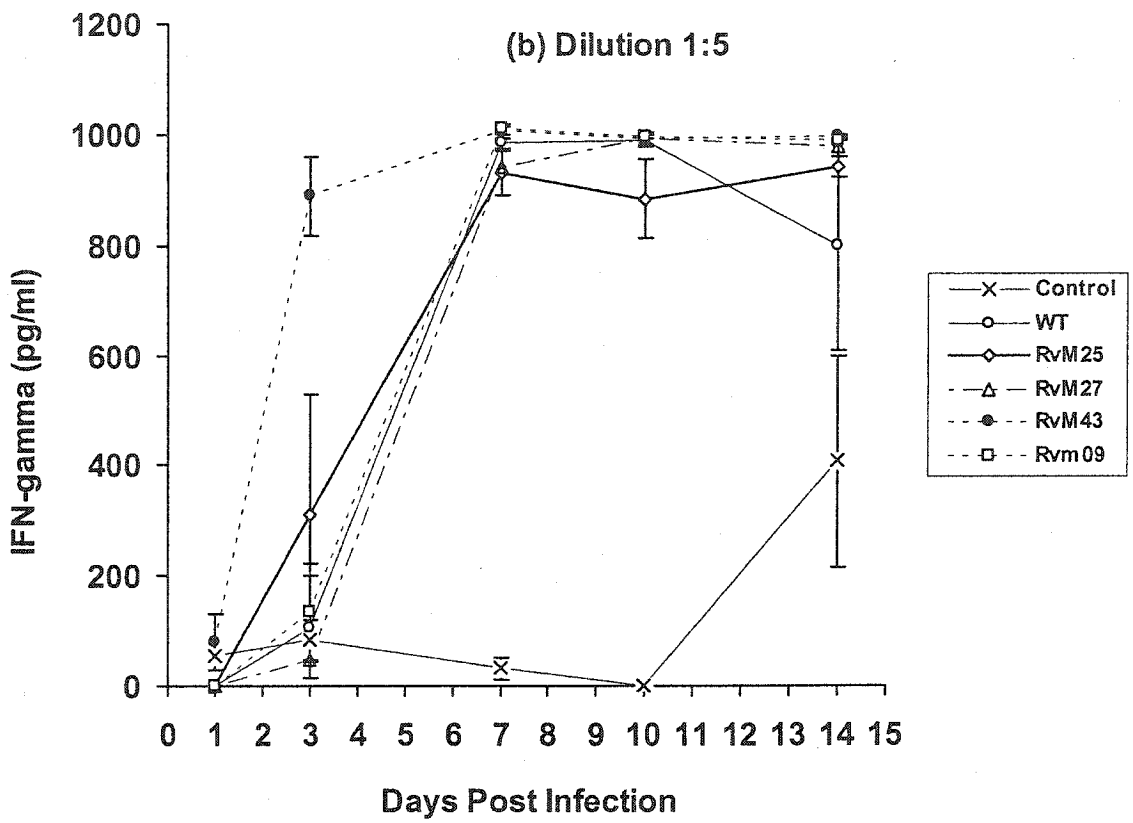

(b) Dilution 1:5
IFN-gamma (pg/ml)
1200
1000
800
600
400
200
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days Post Infection
Control
WT
RvM25
RvM27
RvM43
Rvm09

Figure 19 (b)

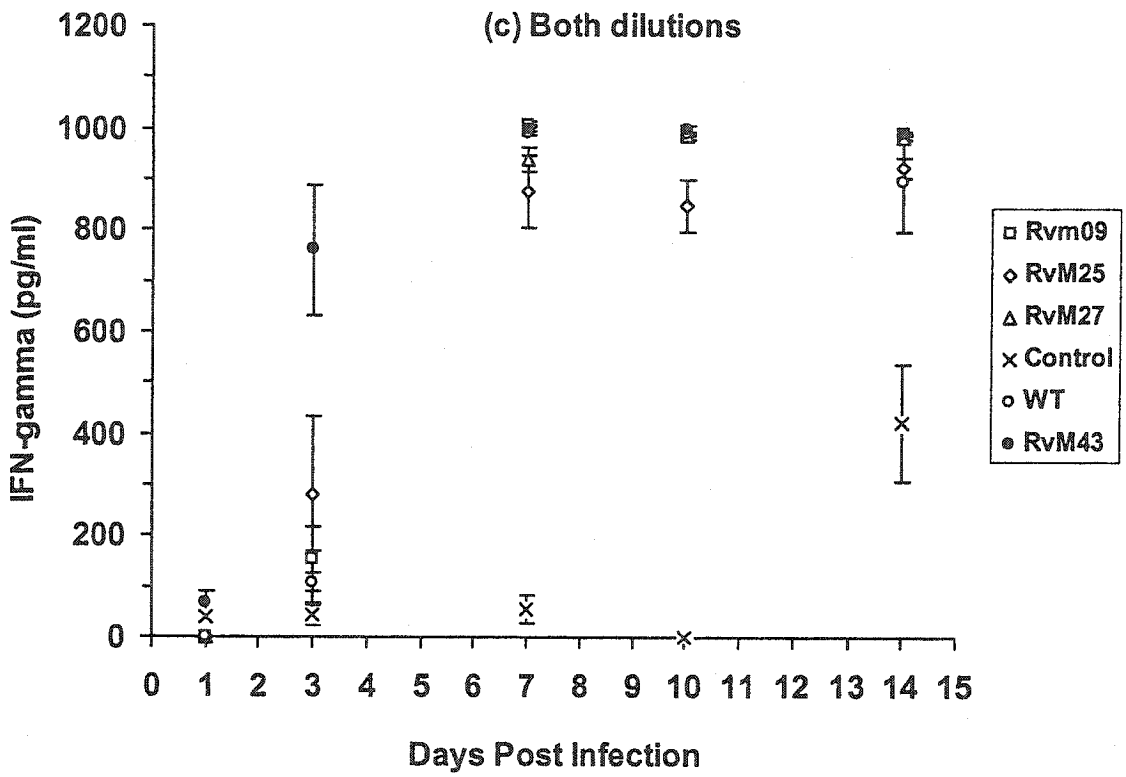

(c) Both dilutions
IFN-gamma (pg/ml)
1200
1000
800
600
400
200
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days Post Infection
Rvm09
RvM25
RvM27
Control
WT
RvM43

Figure 19 (c)

Figure 20. Production of IL-2 by spleen cells from Balb/c mice infected with the wild type (WT) MCMV, MCMV mutants (RvM25, RvM27, RvM43, and Rvm09) and uninfected controls. On day 1, 3, 7, 10, and 14 days post infection (dpi), $2x10^6$ /ml spleen cells from a batch of three mice were stimulated with **(a)** 1:10 and **(b)** 1:5 dilutions of respective antigen for 5 days and the supernatants analyzed for IL-2 production by ELISA. The results shown are mean ± standard error of results from three mice.

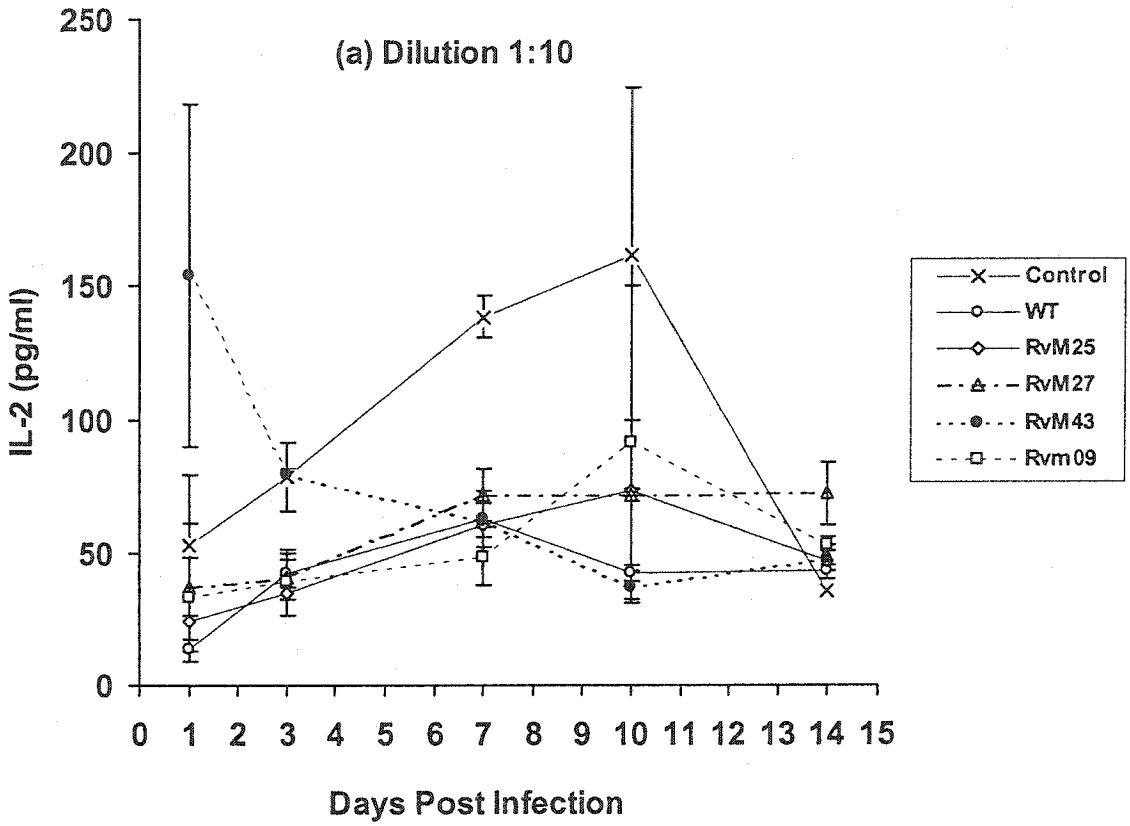

(a) Dilution 1:10
IL-2 (pg/ml)
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days Post Infection
Control
WT
RvM25
RvM27
RvM43
Rvm09

Figure 20 (a)

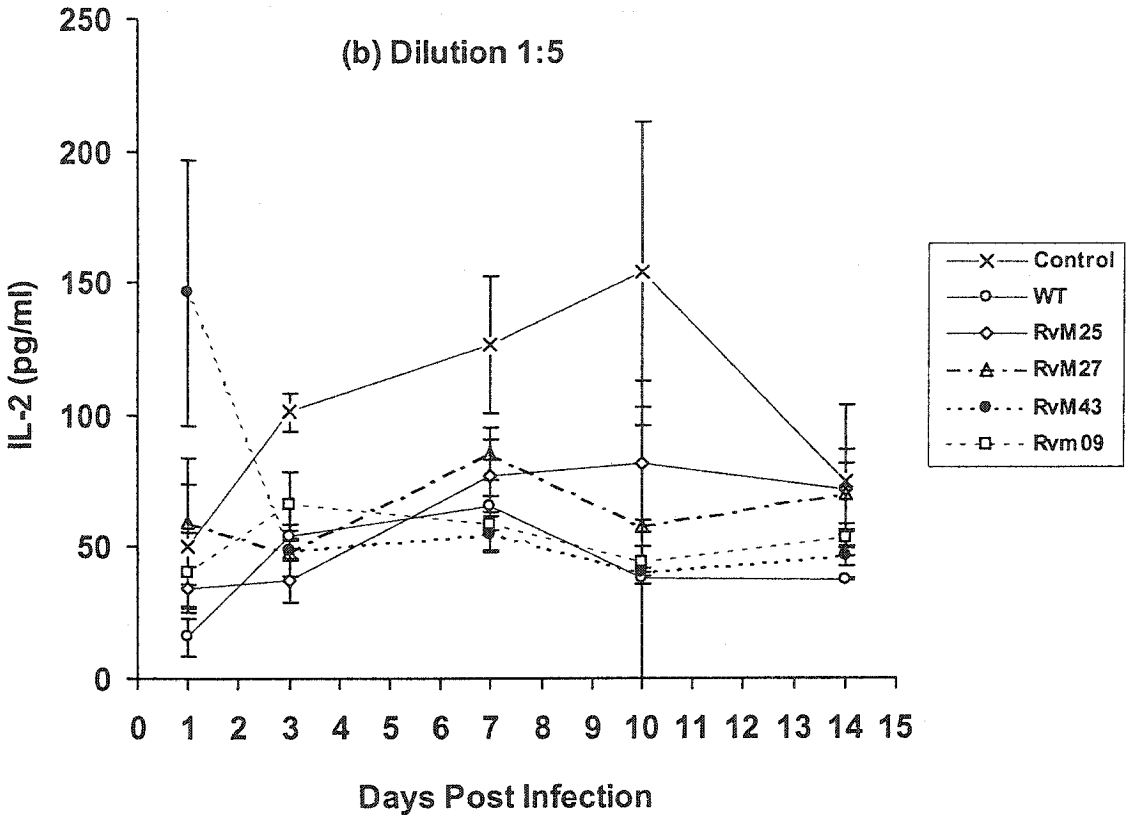

(b) Dilution 1:5
IL-2 (pg/ml)
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days Post Infection
Control
WT
RvM25
RvM27
RvM43
Rvm09

Figure 20 (b)

3.7.2 Th2 Response during Wild Type and Mutant MCMV Infection

Figures 21 (a) to (c) illustrate that the amount of Th2 cytokine IL-4 produced by splenocytes was very low in case of infection with WT and all the recombinants except RvM43. It can also be seen that in case of infection with RvM43, the level of IL-4 on 14 dpi was significantly higher as compared to that during infection with WT and other recombinant MCMVs.

Figures 22 (a) to (c) demonstrate that the level of IL-10 production was much higher in case of infection with RvM43, as compared to that during infection with WT and other recombinants. However, the increasing trend of IL-10 production was similar in case of infection with RvM43 and WT MCMV. In contrast to early appearance of IFN-γ, IL-10 appeared on 7 dpi and had an increasing trend up to day 14. There was increased production of IL-4 ($p = 0.0002$) and IL-10 ($p = 0.015$) during infection with RvM43 as compared with that during infection with WT.

The similar trend of IL-4 and IL-10 production by spleen cells after stimulation with 1:10 and 1:5 dilution of antigen as seen in Figure 21 and 22 (a and b) suggest the consistency and reproducibility of the results. This is also depicted in Figure 21 and 22 (c) where mean ± standard error is plotted for results from both the dilutions of antigen ($n = 6$). These results illustrate that infection with RvM43 uniquely turns on the production of IL-4 cytokine suggesting that the absence of M43 gene of MCMV may lead to skewing of the Th responses towards Th2 type or in other words MCMV M43 gene may play a role in suppressing the production of Th2 cytokine IL-4.

Figure 21. Production of IL-4 by spleen cells from Balb/c mice infected with the wild type (WT) MCMV, MCMV mutants (RvM25, RvM27, RvM43, and Rvm09) and uninfected controls. On day 1, 3, 7, 10, and 14 days post infection (dpi), $2x10^6$ /ml spleen cells from a batch of three mice were stimulated with **(a)** 1:10 and **(b)** 1:5 dilutions of respective antigen for 5 days and the supernatants analyzed for IL-4 production by ELISA. The results shown are mean ± standard error of results from three mice. **(c)** The results shown are mean ± standard error for both dilutions of antigen (n = 6).

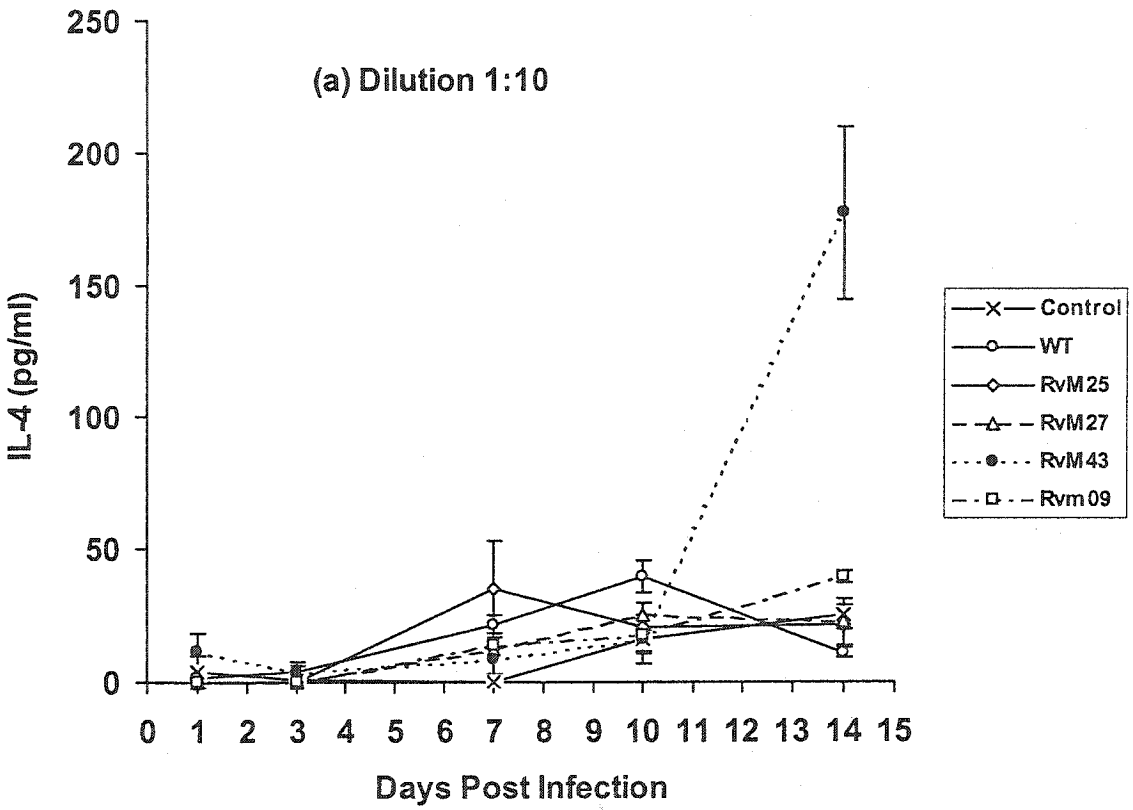

(a) Dilution 1:10
IL-4 (pg/ml)
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days Post Infection
Control
WT
RvM25
RvM27
RvM43
Rvm09

Figure 21 (a)

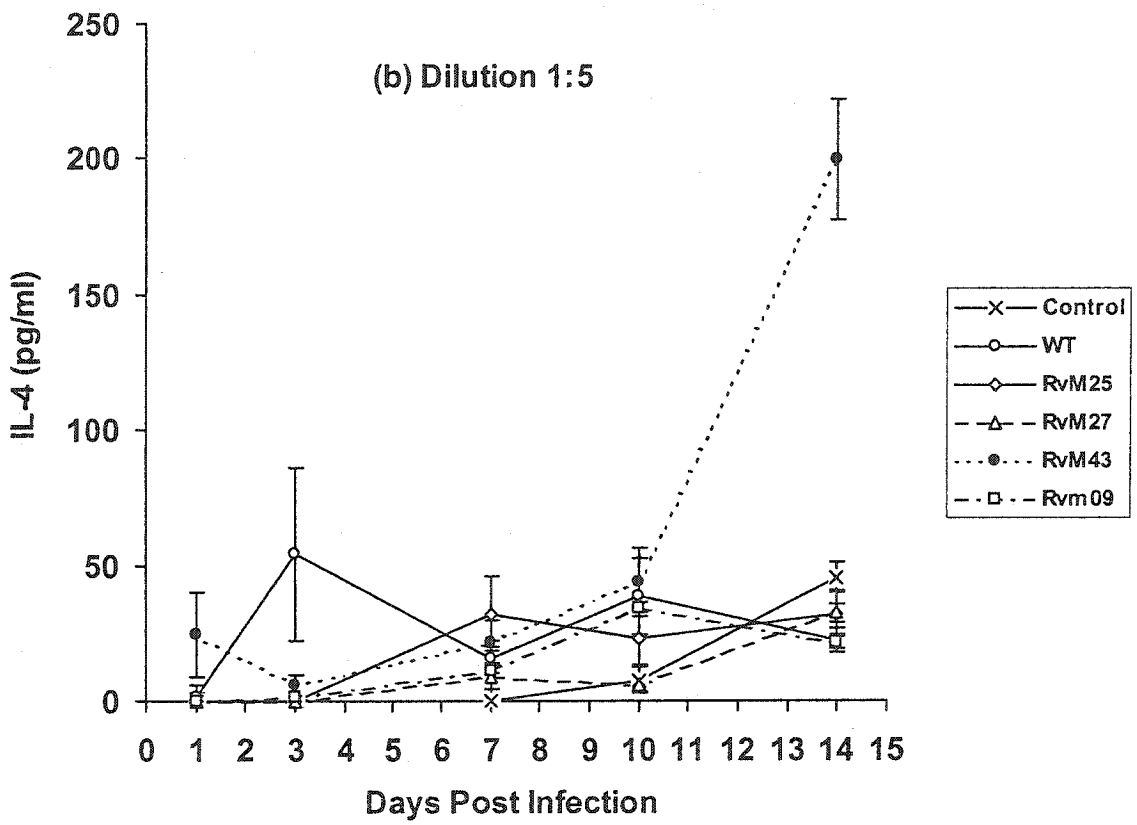

(b) Dilution 1:5
IL-4 (pg/ml)
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days Post Infection
Control
WT
RvM25
RvM27
RvM43
Rvm09

Figure 21 (b)

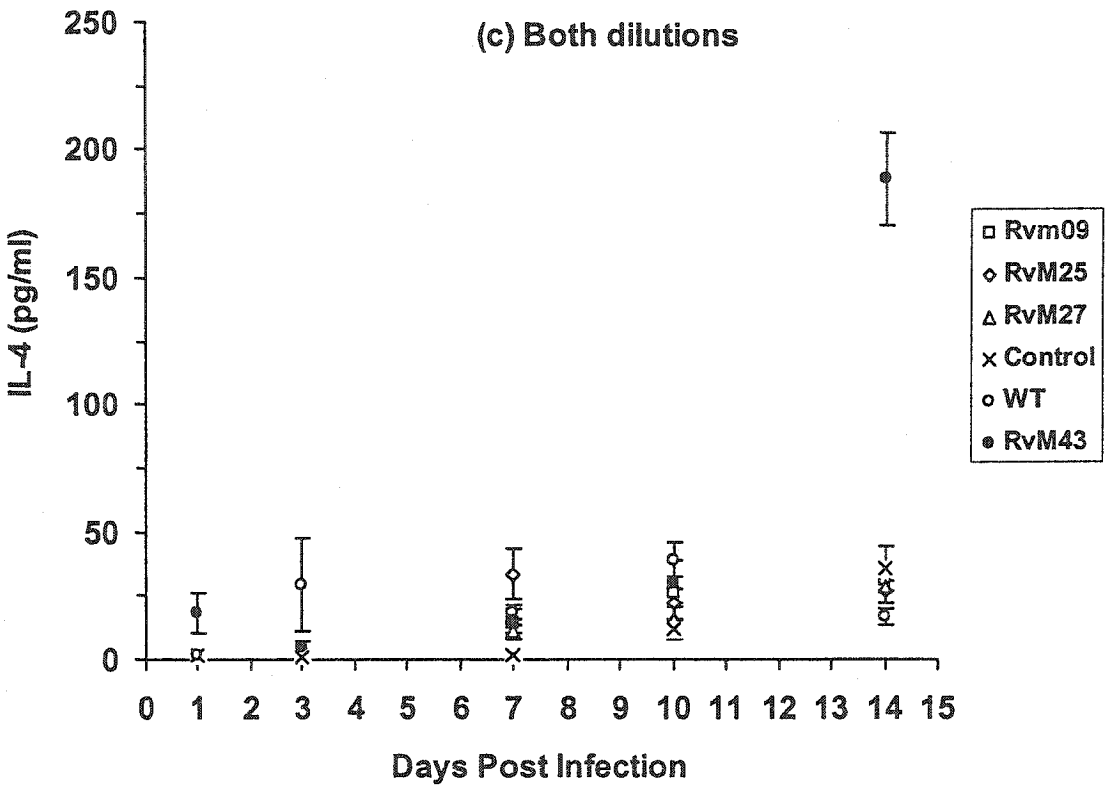

(c) Both dilutions
IL-4 (pg/ml)
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days Post Infection
Rvm09
RvM25
RvM27
Control
WT
RvM43

Figure 21 (c)

Figure 22. Production of IL-10 by spleen cells from Balb/c mice infected with the wild type (WT) MCMV, MCMV mutants (RvM25, RvM27, RvM43, and Rvm09) and uninfected controls. On day 1, 3, 7, 10, and 14 days post infection (dpi), $2x10^6$/ml spleen cells from a batch of three mice were stimulated with **(a)** 1:10 and **(b)** 1:5 dilutions of respective antigen for 5 days and the supernatants analyzed for IL-10 production by ELISA. The results shown are mean ± standard error of results from three mice. **(c)** The results shown are mean ± standard error for both dilutions of antigen (n = 6).

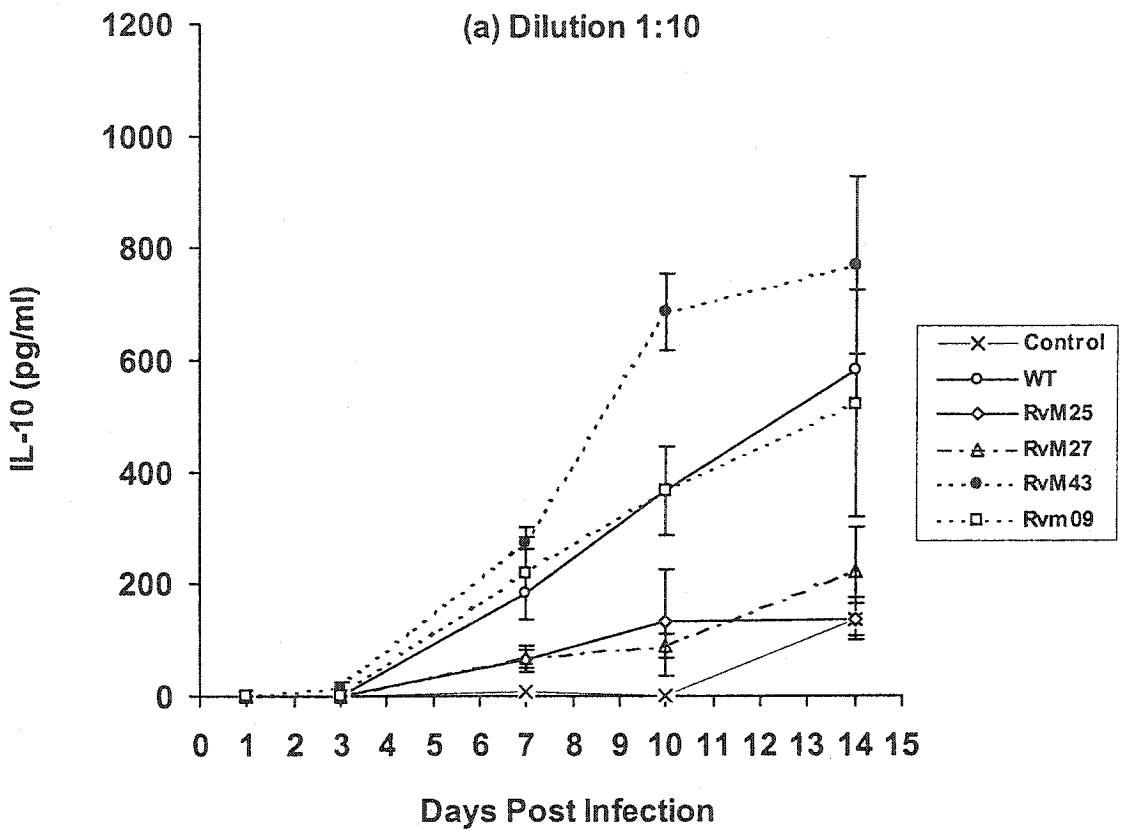

(a) Dilution 1:10
IL-10 (pg/ml)
1200
1000
800
600
400
200
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days Post Infection
Control
WT
RvM25
RvM27
RvM43
Rvm09

Figure 22 (a)

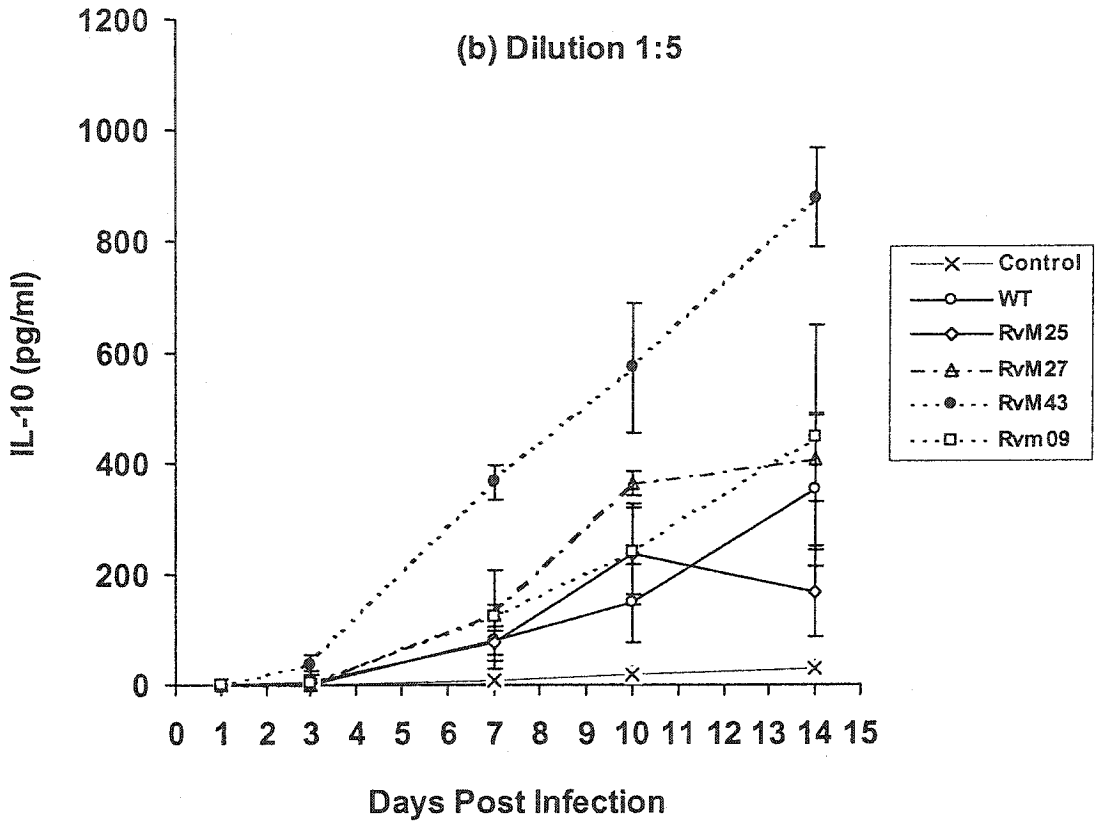

(b) Dilution 1:5
IL-10 (pg/ml)
1200
1000
800
600
400
200
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days Post Infection
Control
WT
RvM25
RvM27
RvM43
Rvm09

Figure 22 (b)

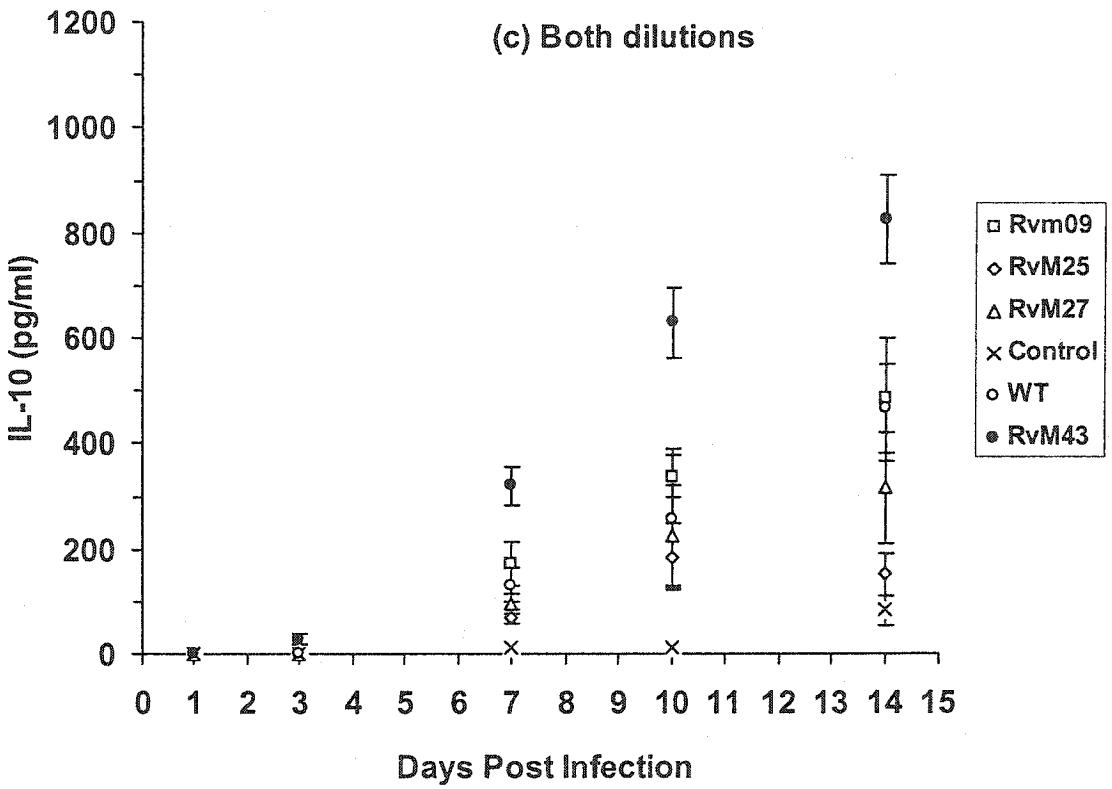

(c) Both dilutions
IL-10 (pg/ml)
1200
1000
800
600
400
200
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days Post Infection
Rvm09
RvM25
RvM27
Control
WT
RvM43

Figure 22 (c)

Chapter 4

DISCUSSION

To have a better understanding of the immunity and immunopathogenesis of herpesviruses that persist in the host for life, this research involves study of two herpesviruses, HSV-2 and MCMV, which belong to the alphaherpesvirinae and betaherpesvirinae subfamilies of the Herpesviridae family, respectively. Two types of systems were employed to characterize the cellular and viral factors involved in modulation of immune response during infection with these viruses. The first involved determination of T helper response in HSV-2 seropositive individuals that were categorized on the basis of clinical symptoms. The second utilized a mouse model in which the T helper response to MCMV was monitored after infection with WT MCMV and mutant MCMVs with specific gene disruption.

The hallmark of herpesvirus infection is viral persistence after the establishment of latency from which the virus reactivates to cause recurrent infection (1,2,5,50,51,74,111). A large number of clinical and experimental studies have shown that the status of the host immune system largely determines the outcome of herpesvirus infection. The immune response to herpesviruses has been suggested to influence the acquisition of infection, severity of disease, resistance to development and maintenance of latency, and the frequency of recurrences (1-9,27-35,57,61,65,74,185,186). The cell-mediated immunity is known to play a major role in control of herpesvirus infection (27-

42). However, the virologic factors that influence the cell-mediated immune response to these viruses have not been satisfactorily explored.

$CD4^+$ T cells are known to play a major role in the development of immunity to herpesviruses (37-43). The principal function of $CD4^+$ T cells is to provide critical helper function in the form of cytokines that play important roles in both, the development and regulation of immune response by controlling the proliferation, differentiation and the effector function of immune cells. According to the profile of cytokine production, $CD4^+$ T cell clones can be separated into two major subsets, Th1 and Th2. These two subsets are capable of cross-regulating each other and participating in the development of humoral and cell mediated immunities (37-46,97,98,109,187,188). A balance between Th1 and Th2 cells and their secreted cytokines may therefore be important regulatory mechanisms that control immune response during HSV or CMV infection.

Several molecular mechanisms involving specific cellular and viral gene products can modulate the antiviral host immune responses or subvert detection of HSV and CMV by the immune system (10-26). A complete evasion of the immunological control would result in the uncontrolled replication of HSV and CMV leading to death of the host and a limited dissemination of the virus. Experimental evidence suggests that viral immune evasion mechanisms operate *in vivo* with a limited efficacy and are counter-balanced by various cellular responses (26-37). Thus, understanding the cellular and viral factors that lead to suppression or evasion of the host immunity or to viral clearance are intellectually the most challenging aspects of herpesvirus infection.

This study has addressed some of the above questions by analyzing the nature and the role of T helper responses during HSV-2 genital infection through the examination of

the proliferative response of PBMC to HSV-2 antigen and production of Th cytokines at various stages of infection. Since the statement of B7 isoforms, B7-1 (CD80) and B7-2 (CD86), on APC plays an important role in T cell activation and development of specific Th1/Th2 responses (160-162,171-172,176), the regulation of B7 isoforms on monocytes from HSV-2 infected individuals was also determined. Further, to investigate the genetic basis for the nature of immune response during herpesvirus infection, we took advantage of a mouse model for CMV and previously established MCMV mutants.

4.1 Absence of T Cell Responsiveness to HSV-2 Antigen during HSV-2 Infection

As discussed earlier, $CD4^+$ T helper cells belong to two major T helper subsets. Their activation causes release of IL-2 and other selected cytokines, which is followed by T cell proliferation (97,98). PBMC cultures from a majority of HSV-2 seropositive individuals proliferate and produce cytokines in response to HSV-2 antigen (responders). However, the cultures from a sizable subpopulation (23 %) of HSV-2 seropositive samples fail to proliferate in respose to HSV-2 antigen but proliferate normally in response to anti-CD3 antibody (non-responders) (Table 2). Such failure of HSV-2-specific T cell response is accompanied by lower level of IL-2 production in response to HSV-2 antigen (Figure 3). This finding shows a selective loss of HSV-2-specific T cell function during HSV-2 infection. The mechanism of this loss of response to HSV-2 Ag is presently unknown. Its potential causes may include the loss (deletion) of HSV-2-specific T cells due to lytic infection by HSV-2 or HSV-2-mediated apoptosis, and anergy of HSV-2-specific T cells.

In three cases, with positive response to HSV-2 Ag, subsequent samples (one per patient) showed lack of response to HSV-2 Ag. This suggests that at some stage of the disease the patients immune cells may become unresponsive which may be advantageous for the viral replication and existence in the host. Throughout the course of this study, none of the nonresponders changed to responder. For the period of this study (3-4 months) the nonresponders could not be distinguished from responders with respect to clinical status. However, the disease progression in these individuals may vary later in the disease.

The non-responders among asymptomatic individuals (Table 2) may represent a transitional stage during which a transient loss of T cell responsiveness to HSV-2 antigen is associated with the appearance of clinical symptoms. This view is supported by the fact that 2 out of 4 asymptomatic non-responders turned symptomatic within several months of examination.

4.2 HSV-2 Infection leads to a Th1 Immune Response

Upon stimulation with HSV-2 antigen, the cultures of PBMC from HSV-2 seropositive responders produce significantly higher levels of Th1 cytokine IFN-γ than Th2-inducing cytokine IL-4, regardless of whether PBMC are derived from individuals with active or non-active disease (Figures 2, 5). This is indicative of a predominant Th1 type immune response during HSV-2 infection. Neither the onset of symptoms nor the recurrence of disease was associated with transition from Th1 to Th2 immune response. The PBMC cultures from symptomatic responders also produce high levels of IL-10 (Figure 4), a pleotropic Th2 cytokine produced by Th2 cells, B cells, mast cells, and monocytes (107-

109). The striking discordance between the levels of IL-4 and IL-10 production in PBMC cultures from symptomatic and asymptomatic responders (Figures 4, 5) indicates that IL-10 in these cultures might not have originated from typical Th2 type $CD4^+$ T cells. The development of a predominant Th1 immune response against HSV-2 may be associated with the emergence of protective immunity presumably responsible for suppression of virus replication and the duration of either symptom-free or non-active disease. However, this immunity is not perfect as individuals with anti-HSV-2 immune response are afflicted by recurrent AD.

IL-2 is a major cytokine responsible for intercellular regulation of immunity and the prime growth factor for T cell proliferation (100,102,189). IL-2 production is potentially a useful measure of HSV-specific T cell response in humans (189,190). HSV-2-stimulated PBMC cultures from symptomatic, but not asymptomatic, responders showed high levels of IL-2 (Figure 3). The reason for this discrepancy is unclear. The early period of helper cell proliferation, IL-2 production and accumulation in cultures is followed by IL-2 consumption by rapidly proliferating cells (189). Therefore, one can speculate that the low level of IL-2 production in AS responders could be due to rapid consumption and exhaustion of IL-2 in cultures from AS responders that exhibited 8 to 10-fold higher rate of T cell proliferation in response to HSV-2 antigen (Table 2).

4.3 Symptomatic HSV-2 Infection is associated with Suboptimal IFN-γ Production

When stimulated with HSV-2, the PBMC cultures from asymptomatic responders exhibit approximately 3-fold higher levels of IFN-γ than such cultures from symptomatic

responders (Figure 2). Individuals producing lower IFN-γ were symptomatic where as those producing greater than IFN-γ were usually asymptomatic. The asymptomatic non-responders produce very low IFN-γ and, as stated above, may be in transition from asymtomatic to symptomatic disease. The protective effects of IFN-γ during HSV infection are well documented. Both HSV-infected neonates and postpartum women exhibit decreased IFN-γ response and increased susceptibility to severe HSV disease (84,191). In cardiac transplant recipients, low IFN-γ responses to HSV antigen are associated with both severe and prolonged HSV infection (27,30). IFN-γ has been shown to mediate protective effects in HSV infection in both *in vitro* and *in vivo* experimental animal models (41,80,81,192). Mice depleted of IFN-γ have been highly susceptible to HSV infection (83, 184,192). IFN-γ inhibits HSV replication in mouse macrophages, induces lysis of HSV-infected cells, augments MHC class II gene expression, and activates antigen-specific and non-specific effector cells (NK cells, macrophages, CTL) (103,104). It also affects macrophage functions, for example, by upregulating potential antiviral activities such as TNF-α and NO production. It is suggested that NO may inhibit HSV replication (31,83,91,92). The analyses of IFN-γ production and action are therefore important in assessing the host immunity to HSV.

Other investigations have shown that defects in HSV-stimulated IFN-γ production may predispose certain individuals to increased frequency of recrudescent oral herpes infections (80,81,104). The sensitivity of HSV replication to inhibitory effects of IFN-γ may be of equal importance to host resistance. The antiviral action of IFN-γ differs as to the type of infected cells. HSV replication in human epithelioid cells is relatively more sensitive to the inhibitory effects of IFN-γ than in macrophages and lymphocytes (103).

As seen in Figure 2, the asymptomatic HSV-2 seropositive responders had highest level of IFN-γ. Further, the kinetic experiments shown in Figure 7 suggest that high levels of IFN-γ production occur around the onset of HSV-2 disease but show a decreasing trend during the recurrence free phase. Among HSV-2-infected individuals, the decline in IFN-γ production was noted to follow a similar pattern to the decline in HSV copy number in the genital lesions. These results also suggest an important role of IFN-γ in HSV-2 pathogenesis.

4.4 Opposing Effects of HSV-2 Infection on B7-1 and B7-2 Expression on Monocytes

B7 costimulation is shown to affect the production of Th1 and Th2 cytokines depending on the system studied (167,171-173). Conversely, immunoregulatory cytokines such as IL-10 and IFN-γ influence the development of Th1 versus Th2 response by altering the levels of B7 isoform expression on APC (94,176). IFN-γ has been shown to induce Th1 type response, perhaps through modulation of B7 expression on APCs (176). These facts warrant determination of changes in B7-1 and B7-2 expression on monocytes from HSV-2 patients after treatment with IFN-γ and IL-10. Further, since some viral proteins resemble the structure or mimic the function of immunoregulatory cytokines, the effects of HSV-1 and HSV-2 antigens on B7-1 and B7-2 expression on monocytes was also examined.

These investigations revealed two opposing effects of HSV-2 infection on B7 expression on monocytes. First, HSV-2 infection renders monocytes refractory to IFN-γ-induced upregulation of B7-1 and B7-2 expression (Figures 12,13). Second, treatment of

PBMC with HSV-2 upregulates the expression of B7-1 and B7-2 on monocytes (Figures 14,16) in a manner independent of IFN-γ (Figure 17). Treatment of PBMC with IL-10 had no effect on B7-1 or B7-2 expression on monocytes (Figure 11). The effects of IFN-γ or HSV-2 on B7 isoform expression were not significantly different on monocytes derived from HSV-2-infected responders and HSV-2-infected non-responders, which suggests that the observed loss of T cell response to HSV-2 in symptomatic non-responders is unrelated to IFN-γ- or HSV-2-induced modulation of B7-1 or B7-2 expression on monocytes.

The abrogation of IFN-γ-induced upregulation of B7-1 and B7-2 expression on monocytes was observed in both symptomatic responders and non-responders. This may be due to a defect in the normal IFN-γ signaling pathway in monocytes during HSV-2 infection. It is possible that HSV-2 proteins directly disrupt this pathway as a part of the immune escape mechanism. The immunopathological consequence of this immunological defect is presently unclear but it may have an impact on IFN-γ-mediated inhibition of HSV replication (92). The dysregulation of B7 isoforms on monocytes may also influence the production of other Th cytokines in HSV-2 infected individuals and play an important role in HSV-2 disease.

Expression of B7 isoforms, B7-1 and B7-2, on monocytes, dendritic cells and T cells can be rapidly upregulated following activation (163,164). The two B7 isoforms can be regulated independently on monocytes. Stimulation with bacterial lipopolysaccharides (LPS) upregulates the expression of B7-1 and downregulates B7-2 expression, whereas stimulation with Neisserial porins selectively upregulates the expression of B7-2 (193). The downregulation of B7-2 expression by LPS is mediated by endogenously produced

IL-10 (194). Infection of monocytes with *Mycobacterium tuberculosis* and *Leishmania donovani* inhibits B7-1 expression (195,196). The B7-1 expression is upregulated on T cells and APC in diseases such as experimental allergic encephalitis that show a predominant Th1 response (171). This study shows a significant upregulation of B7-1 and B7-2 expression on monocytes after exposure to HSV-1 or HSV-2 regardless of whether monocytes are derived from HSV-2 seropositive or normal healthy individuals. It occurs independent of any endogenous IFN-γ (Figure 17). Such augmentation of B7-1 and B7-2 expression may be responsible for sustained and predominant Th1 response during HSV-2 infection, despite the failure of IFN-γ-induced upregulation of the B7 isoforms on monocytes. Engagement of T cells by B7-1 or B7-2 together with TCR/CD3 ligation results in enhanced cytokine production that can maintain long-term expansion of $CD4^+$ T cells. The observations of elevated B7-2 expression with high IL-10 production in PBMC cultures from symptomatic responders suggest that the inhibitory effects of IL-10 be countered during HSV-2 infection. The two opposing effects of HSV-2 infection on B7 expression may be a part of the viral strategy to persist in immunocompetent hosts.

4.5 Th1/Th2 Paradigm in HSV-2 Disease Progression

As seen in Figure 2, HSV-2 stimulation of PBMC cultures from HSV-2 seropositive asymptomatic individuals exhibit approximtely 3-fold higher levels of IFN-γ production than cultures from symptomatic (AD plus NAD) individuals. In contrast, the former cultures produced only $1/10^{th}$ to $1/15^{th}$ levels of IL-10 than the latter cultures (Figure 4). This indicates an inverse relationship between the two Th1 (IFN-γ) and Th2 (IL-10)

cytokine responses during HSV-2 infection. Individuals producing higher levels of IFN-γ and lower levels of IL-10 seem to be protected from developing symptoms, whereas those producing lower IFN-γ and higher IL-10 are not. This may lead to the speculation that the conversion from asymptomatic to symptomatic disease may be associated with the downregulation of IFN-γ and a concomitant upregulation of IL-10 response. No such relationship could be seen when the levels of IL-4, a Th2-inducing cytokine, were compared with IFN-γ levels (Figure 5). This may partly be due to variations in IL-4 levels and overall low levels of IL-4 production.

Once the symptomatic disease occurs, the new combination of lower IFN-γ and higher IL-10 response fails to prevent the onset of recurrences. As seen in Figures 2 and 4, these two cytokine (IFN-γ and IL-10) responses differ only marginally during, and in between, the symptomatic disease (AD and NAD). The onset of AD is associated with neither a change in IFN-γ, IL-10, IL-4 or IL-2 response, nor a change in the response of any combination of these cytokines. The trigger for the onset of AD therefore remains unknown. The loss of protective effect of IFN-γ and its failure to prevent the onset of AD in symptomatic patients may be due to the presence of suboptimal IFN-γ levels or a deterioration of IFN-γ-signaling mechanism or both. The latter possibilities are supported by frequent observations of the failure of IFN-γ-induced upregulation of B7-1 and B7-2 expression on monocytes (Figures 11,12).

In symptomatic HSV-2 patients, the time course of IFN-γ response shows a steady decline in IFN-γ production after the recurrence of AD (Figure 7), roughly in parallel with a decline in the copies of HSV-2 in genital swabs of the patient (Figure 10). This suggests that, during the symptomatic disease when the existing IFN-γ response fails to

prevent onset of AD, the IFN-γ production is driven by HSV-2 replication. The IFN-γ response during symptomatic disease may therefore be a passive effect of stimulation by HSV-2 rather than protective response. The conversion of HSV-2 seropositive symptomatic responders into non-responders represents a relatively late event in HSV-2 disease progression that may also be unrelated to changes in Th1/Th2 cytokine profile. It is presumably caused by HSV-2-induced death or anergy of HSV-2-specific T cells. Based on the results showing very low levels of IFN-γ in asymptomatic nonresponders as compared to asymptomatic responders (Figure 2) and the conversion of two of the asymptomatic nonresponders into HSV-2 symptomatic patients several months later we believe that the asymptomatic non-responder patients are likely to be a transient subset.

HSV-2-stimulated PBMC cultures from HSV-2 seropositive individuals produce relatively low levels of the Th2-inducing cytokine, IL-4. In this regard, infection with HSV-2 is fundamentally different from infection with measles or respiratory syncytial virus (RSV) and from allergic conditions such as atopic dermatitis, which are associated with elevated IL-4 production followed by skewing of the immune response towards Th2 type (197-199). These observations raise two important questions. First, what will be the course of HSV-2 disease with respect to change from asymptomatic to symptomatic disease or to AD-NAD-AD cycle if the IL-4 response is experimentally elevated either by eliminating HSV-2 genes responsible for the repressed IL-4 response or by adoptive transfer of IL-4-producing cells? The adaptive transfer of IL-4-producing Th2 cells into herpetic stromal keratitis (HSK) susceptible mice accelerates the onset and accentuates the severity of the HSK (44,106). Second, what will be the consequences of the HSV-2 infection on the course of measles or RSV disease, and on conditions leading to allergies?

The answers to these questions may be of great importance not only in understanding the basis of immunity to HSV-2 however imperfect that may be, but also to the development strategies to treat and manage Th2 cytokine-mediated viral and environmental diseases.

IL-10, a pleotropic cytokine associated with the development of Th2 response, is produced by HSV-2-stimulated PBMC cultures from symptomatic but not asymptomatic responders (Figure 4). IL-10 may induce immune suppression through its inhibitory effects on antigen-specific proliferation of Th0, Th1, and Th2 cells, as well as antigen-stimulated monocytes/macrophage-dependent cytokine synthesis by PBMC and NK cells. The analysis of IL-10 secretion has been important in understanding the immunopathogenesis of diseases in which T cell immunity plays a critical role, such as septic shock, lymphoproliferative disorders, and autoimmune diseases (107,200-205). IL-10 has also been suggested to contribute to defective antigen responses during HIV infection (206). These effects of IL-10 might, at least in part, be mediated by the downregulation of the expressions of class II MHC and co-stimulatory molecules necessary for T cell and NK cell activation. These activities of IL-10 may therefore dictate the clinical course of disease. Locally produced IL-10 has been suggested to act in an autocrine/paracrine fashion to downregulate the production of proinflammatory mediators, and thus limit the corneal inflammation in mouse model for herpes stromal keratitis (107-109).

4.6 MCMV Infection also triggers a Predominant Th1 Immune Response *in vivo*

The observations of a predominant Th1 immune response during HSV-2 infection may not be unique to infection by this member of the Herpesviridae family since similar Th1

immune responses have been reported during HCMV infection (49). Although belonging to different Herpesviridae subfamilies, both HSV-2 and HCMV persist in host for life, undergo latency after primary infection, cause recurrent disease by reactivation of the latent virus, employ similar mechanisms to evade host immunity, and share a common genetic organization that includes extensive nucleotide sequence homologies (1-5,10,14, 50). It is thus conceivable that these, and presumably other, herpesviruses carry common genes that are responsible for a predominant Th1 type immune response in the host that allows only limited windows of opportunity for viral replication. In order to study such genetic basis for a predominant Th1 type immune response, which could distinguish herpesviruses from measles or RSV that elicit a predominant Th2 type immune response, a mouse model for MCMV infection was adopted to enable study of specific viral genes.

The mouse model for CMV infection was chosen due to the obvious difficulties in performing *in vivo* studies in human subjects. Animal models have proven to be very useful in unraveling the mechanisms involved in persistence and immunopahtogenesis by human viruses. Strict species specificity requirement makes it difficult to study HCMV in any animal model. However, the similarities between HCMV and MCMV (discussed in Introduction) and the availability of a pool of genetically defined MCMV mutants, make MCMV infection of mice an attractive model for studying CMV immunopathogenesis and viral virulence. This is further aided by the knoweledge of the complete nucleotide sequences of both HCMV and MCMV genome.

Like HSV-2, the infection with WT MCMV also leads to a predominant Th1 type immune response characterized by high levels of IFN-γ (Figures 19 a,b,c) and very low levels of IL-4 (Figures 21 a,b,c) production by splenocyte cultures from MCMV-infected

mice in response to MCMV stimulation. The IL-10 response is notably different from IL-4 response in that the former gradually but steadily rises to significantly high levels 14 days after MCMV infection (Figures 22 a,b,c), despite an ongoing robust IFN-γ response. Therefore, as suggested in the case of HSV-2 infection, the IL-10 response during MCMV infection may be afforded by a non-T cell source. The gradual appearance of IL-10 response during MCMV infection is consistent with the presence of IL-10 response during symptomatic but not asymptomatic HSV-2 disease (Figure 4). The nature of the T helper cytokine response during MCMV and HSV-2 infection is therefore fairly similar, and fundamentally different from the T helper cytokine response during measles or RSV infection. This justifies the utilization of the mouse model for MCMV infection to search for viral genetic elements that may be responsible for a predominant Th1 type immune response during herpesvirus infection.

The development of a predominant IFN-γ immune response against CMV may be associated with the emergence of protective immunity presumably responsible for suppression of virus replication. IFN-γ has a number of functions that probably play a role in controlling acute MCMV infection. These include activation of macrophages during MCMV infection, enhancement of MHC class I-dependent antigen presentation by infected cells to $CD8^+$ T cells, and inhibition of lytic MCMV replication and gene expression. Administration of IFN-γ can protect against lethal infection, and administration of anti-IFN-γ can both decrease the effectiveness of anti-MCMV $CD8^+$ and $CD4^+$ T cells (37-39,103,104). It has been shown that IFN-γ is necessary to regulate MCMV-associated elastic arteritis and latency *in vivo* and reactivation of a herpesvirus from latency *in vitro*. Further, the levels of IFN-γ play an important role in controlling

viral replication in salivary glands (37-39,153). Therefore, analysis of IFN-γ production and action is important in assessing CMV infection and effects on host immunity. The actual mechanism is still unclear, but it has been suggested that IFN-γ mediates antiviral activity through dynamic modulation of TNF-related apoptosis inducing ligand and its recptor expression (207).

4.7 Th2 Responses during MCMV Infection

Splenocytes from mice infected with wild type MCMV produced very low levels of Th2-inducing cytokine IL-4. However, IL-10, another cytokine associated with the development of Th2 response (107,108), was produced by antigen stimulated spleen cells from mice infected with wild type MCMV. As discussed earlier, IL-10 is a pleotropic cytokine produced by Th2 cells, B cells, mast cells, and monocytes. IL-10 possesses a broad spectrum of biological activities and has been associated with the immunopathogenesis of a number of diseases and induction of IL-10 in certain infections is suggested to be a strategy employed by pathogens to evade the host immune system (200-205).

It has been reported that HCMV harbors its own unique IL-10 homologue that provides a range of counter measures developed by CMV to circumvent detection and destruction by the host immune system (208). Further, it has also been suggested that MCMV infection of macrophages results in a decreased expression of MHC class II expression on the cells, very early in the inflammatory response. This downregulation of MHC class II statement is brought about by IL-10 by interfering with exocytosis and recycling of proteins (209). This delineates a strategy by which MCMV infection

immediately begin to interfere with the immune response of the host and impair the ability of macrophages to present antigen to CD4+ T cells. In addition, IL-10 has several other immunosuppressive effects including inhibition of Ag specific proliferation of Th1 and production of inflammatory cytokines. This effect of IL-10 again demonstrates the remarkable variety of CMV strategies for weakening the host immune responses. CMV is known to have strategies to interfere with the host cell expression of MHC class I. In this regard, it is important to note that IL-10 has been reported to cause down-regulation of MHC class I in studies with tumor cells and B cell lines (210,211).

Thus, induction of IL-10 very early in MCMV infection would certainly help the virus to escape detection by the host adaptive immune responses by reducing the capacity of the infected macrophages to present antigenic peptides to $CD4^+$ T cells. IL-10 has also been reported to be important in infections with other intracellular pathogens such as *Listeria monocytogenes* and *Mycobacterium bovis* (212). Thus IL-10 mediated pathways may be of great significance in intracellular infections such as that caused by CMV and HSV viruses. It has been suggested that locally produced IL-10 can act in an autocrine/paracrine fashion to down-regulate the production of proinflammatory mediators (107,108). Thus, IL-10 may on one hand assist CMV in evading the host immune response but on the other hand it may limit inflammation caused due to CMV infection. CMV infections such as interstitial pneumonitis, retinitis, colitis, hepatitis and encephalitis are common during immune suppression (6,7). Thus anti-inflammatory properties of IL-10 may be useful in reducing inflammation during these diseases caused by CMV. This shows that the role of cytokines in viral infection is complex and yet to be fully characterized.

4.8 Identification of MCMV Gene that Modulates Th Responses *in vivo*

One of the most powerful approaches to identify the function of a viral gene or its product is to introduce specific mutations into the viral gene followed by screening of the replication-competent viral mutants in tissue culture or animals to determine the function of the viral gene. Transposon-mediated mutagenesis has been widely used to study gene functions in viruses, bacteria, yeast, and mammalian cells (130-133,213,214). The use of Tn3-based system for mutagenesis to study the function of MCMV genes has been previously established (see Figure 1). A number of such MCMV mutants have been generated and used in the past (130-133). These include RvM25, RvM27, RvM43 and Rvm09, which represent Tn3-based mutations in M25, M27, M43, and m09 genes of the MCMV, respectively. In this study, the role of MCMV M25, M27, M43 and m09 genes in modulating the Th cytokine response during MCMV infection in mice was analyzed at various times for up to 14 days post infection (dpi).

As seen in Figure 21, infection with RvM43 gene uniquely turned on the production of Th2 inducing cytokine, IL-4. IL-4 has been described as an immunosuppressive cytokine and the down-regulatory activities of IL-4 on CMI responses are well known. Numerous cell types proliferate and/or differentiate in response to IL-4. Proliferation of quiescent T cells as well as B cells in the presence of anti-IgM antibodies is induced by IL-4. IL-4 also mediates transcription of unrearranged IgE and IgG_1 constant regions, leading to isotype class switching and subsequent biosynthesis (105). The infection with mutant RvM43 also lead to increased production of IL-10 as compared to that during wild type infection (Figure 22 a,b,c). However, the

trend of increase was similar during infection with both WT and RvM43. During infection with RvM43 the increased levels of Th2 cytokines, IL-4 and IL-10 were present along with high levels of IFN-γ suggesting that the cross-regulatory mechanism of these cytokines was not operative.

Open reading frame M43 and its human counterpart, UL43, belong to the MCMV M23 and HCMV US22 gene family, respectively (112-114). The role of M43 is not known. It has been published that a transcript of about 5,000 nucleotides is expressed from the M43 ORF and M43 has been suggested to be a viral determinant for replication in the salivary glands (133). The viral tropism for salivary gland and shedding of virus in saliva are believed to be the major routes for CMV transmission among healthy human population.

The increased production of IL-4 in the absence of M43 gene suggest that this gene may play an important role in altering Th responses by suppressing *in vivo* production of IL-4. This is a valuable novel finding that provides a genetic basis for the regulation of cellular immune response in virus infected hosts. This finding is corroborated by several observations of suppression of Th2 immune response in the presence of CMV genome (47-49) and provides a long-awaited explanation for such observations. Further, this finding can also explain the occurrence of an inverse relationship between the incidence of certain infectious disease and allergy, during which there is increased production of IL-4 cytokine (197-199).

4.9 Role of MCMV M25 Gene in Modulation of Th Response

M25 belongs to the CMV UL25 gene family, which also includes M35 open reading frame (114) and has been shown to encode a tegument protein (215). Its human counterpart, the UL25 protein of HCMV, also encodes a tegument protein (216). It has been reported that MCMV with a mutation at the tegument protein gene M25 is dispensable for viral growth and the presence of the transposon sequence in the viral genome does not significantly affect viral replication *in vivo* (130). It has also been shown that MCMV with a mutation at tegument protein gene M25 is attenuated in virulence and growth in immunodeficient SCID mice. This suggests that disruption of the M25 open reading frame diminishes viral virulence and that M25 plays an important role in MCMV virulence in SCID mice (130). The results of present study show that there was no significant difference in the cytokine profile during infection with wild type and RvM25. Since these experiments were performed using immunocompetent Balb/c mice the results of this study suggest that MCMV M25 gene may not play a role in immunomodulation during MCMV pathogenesis in immunocompetent Balb/c mice.

4.10 Role of MCMV M27 Gene in Modulation of Th Response

A viral transcript of ~ 3000 nucleotides is expressed from the M27 regions in cells infected with the wild type virus (130). It has been suggested that the M27 carboxyl-terminal sequence is dispensable for viral replication *in vitro*. Further, it has been suggested that a disruption of M27 expression results in reduced viral growth and

attenuated viral virulence *in vivo* in infected animals (132). However, there was no significant difference in the cytokine production during infection with wild type and recombinant RvM27 suggesting that MCMV M27 gene may not play a role in altering the Th1/Th2 responses in the host.

4.11 Role of MCMV m09 Gene in Modulation of Th Response

Open reading frame m09 belongs to MCMV m09 gene family and is believed to encode a membrane protein (113). However, neither the transcript nor the protein product coded by this open reading frame has been reported. The growth rate of Rvm09 in NIH3T3 cells is not significantly different from that of the wild type MCMV (131).

It has been shown that Rvm09 replicates as equally well as the wild type in the salivary glands, lungs, spleen, liver and kidneys of both Balb/c and CB17 SCID mice (131). There was not much difference in the cytokine profile during infection with wild type and Rvm09, which is in agreement with the above findings.

4.12 Summary and Conclusions

Herpesviruses persist in hosts and cause a variety of serious illnesses in humans. This study was undertaken to characterize the nature of T helper cytokine response during herpesvirus infection and to identify the viral genetic elements associated with such response, using two herpesviruses, HSV-2 and MCMV, which belong to different

subfamilies of the Herpesviridae family yet share extensive genetic, biological, and pathological properties. The main findings of this research are summarized below:

1. Unlike infection with RSV or measles virus, the infection with HSV-2 or MCMV leads to a predominant Th1 (IFN-γ) cytokine response.
2. A sizeable number of samples (~ 23 %) from a subpopulation of HSV-2-infected individuals fail to exhibit T cell proliferation upon stimulation by HSV-2 antigen and the lack of responsiveness is associated with diminished IL-2 production.
3. The samples from asymptomatic responders (n = 3) showed elevated IFN-γ but low IL-10 response compared to samples from symptomatic responders (n = 44) suggesting a protective effect of this combination in responders from HSV-2 seropositive individuals.
4. Neither the onset nor the disappearance of AD is associated with changing IFN-γ or IL-10 response. The trigger for the onset of AD remains unknown.
5. Symptomatic HSV-2-infected individuals fail to exhibit similar levels of IFN-γ-induced upregulation of B7-1 and B7-2 expression on monocytes as observed in HSV-negative controls.
6. Exposure of monocytes to HSV-1 or HSV-2 antigen however causes a significant increase in both B7-1 and B7-2 expression in a manner independent of IFN-γ.
7. The M43 gene of MCMV is a potent suppressor of IL-4 (Th2) response *in vivo*. Disruption of this gene causes the appearance of IL-4 response without inhibiting and in the presence of a strong IFN-γ (Th1) response, overriding the cross-regulatory effects of these cytokines within the Th1/Th2 paradigm. The sequence of M43 gene is

conserved among herpesviruses and therefore it may be responsible for a predominant Th1 immune response during the course of infection with these viruses.

In summary, this study has identified two important immunological deficiencies during the course of HSV-2 infection potentially related to HSV-2 disease progression. The first renders monocytes refractory to IFN-γ-upregulation of B7-1 and B7-2 expression, and the second causes a selective loss of T cell responsiveness to HSV-2 antigens. The virus also independently augments B7-1 and B7-2 expression on monocytes and may thus confer a partially protective immunity in symptomatic patients. Infection with HSV-2, a member of alphaherpesvirinae, or MCMV, a member of betaherpesvirinae, leads to a predominant and permanent Th1 (IFN-γ) cytokine response. This study has identified MCMV M43 gene to be suppressive to IL-4 response, suggesting an important role of this gene and its counterparts in other herpesviruses in the maintenance of Th1 (IFN-γ) cytokine response.

4.13 Significance and Implications

Together, HSV and HCMV contribute to the majority of herpesvirus-related diseases in humans and are therefore subjects of public health concern. Genital infection by HSV is one of the most common sexually transmitted diseases affecting individuals regardless of their immune status whereas HCMV primarily afflicts immunocompromised or immunodeficient individuals such as transplant and HIV-infected patients and infants with developing immune system. HCMV is also implicated in the pathogenesis of birth defects, cardio vascular diseases, certain malignancy, and insulin-dependent diabetes mellitus (134,140,145,146). The ability to prevent these viral infections and related

diseases are limited largely by the understanding of mechanism of immunopathogenesis by these viruses. The above mentioned medical problems suggest that both HSV and CMV infections have substantial public health implications, and require increased awareness of the disease and its prevalence. Together with better use of diagnostic tests, a better knowledge of its immunopathogenesis, may be one step towards more effective management and infection control.

Previous studies have shown that HSV and CMV are not simply conventional Ags but represent a paradigm for persistent viruses that actively manipulate and exploit several aspects of the immune response (10-25). The identification and analysis of individual viral gene functions would certainly help to identify the targets for modulation and would help in elucidation of specific immune control mechanisms involved. This study provides useful insights into the role of cellular and viral factors in the pathogenesis of herpesvirus infections. The important findings of this study are presented in an empirical model in Figure 23. As depicted in the model, after infection with the virus, (with the exception of some individuals; nonresponders), host's immune cells get activated and produce various cytokines that help in the development of cell mediated (Th1) and humoral immunity (Th2). A proper balance between IFN-γ (Th1) and IL-10 (Th2) production may help in the maintenance of symptom-free stage. The results of this study also suggest that the viral antigens (HSV) via upregulation of B7 expression and viral genes such as MCMV M43 (by modulation of Th cytokine production) may play a role in the maintenance of predominant Th1 response during these herpesvirus infections. This study therefore helps in the understanding of herpesvirus pathogenesis and role played by various factors in viral persistence and recurrences after primary infection.

Figure 23. An empirical model for the role of cellular and viral factors in herpesvirus pathogenesis.

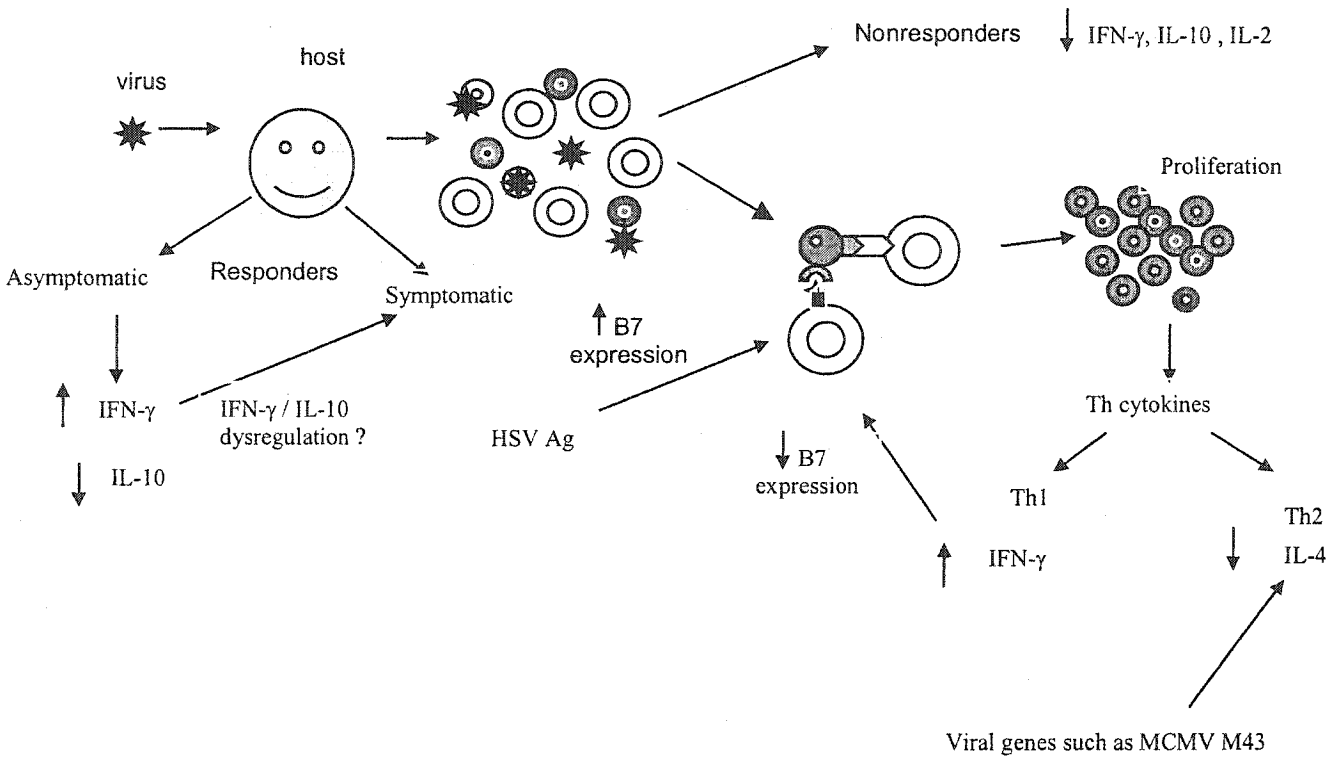

virus
host
Nonresponders
IFN-γ, IL-10 , IL-2
Proliferation
Asymptomatic
Responders
Symptomatic
B7
expression
IFN-γ
IFN-γ / IL-10
dysregulation ?
HSV Ag
Th cytokines
IL-10
B7
expression
Th1
Th2
IL-4
IFN-γ
Viral genes such as MCMV M43

Figure 23

4.14 Future Studies

The findings of this study have provided new grounds for future research both to enhance our understanding of the herpesvirus immunopathogenesis and to improvise applications for the treatment and control of herpesvirus infection and Th2 cytokine-mediated diseases such as allergies. The following future studies may help achieve these objectives.

1. **Mechanism of loss of T cell responsiveness to HSV-2:** It will be interesting to determine whether the specific loss of T cell responsiveness to HSV-2 during HSV-2 infection is due to depletion or to anergy of HSV-2-specific T cells. This can be achieved by the detection and quantitation of HSV-2-specific T cells using tetramer staining, in symptomatic patients transitioning from a responder to a non-responder state. This may be followed by the determination of mechanism of HSV-2-induced depletion or anergy of HSV-2-specific T cells, as the case may be.

2. **Identity of IL-10-producing cells and strategy for inhibition of IL-10 production:** The transition from asymptomatic to symptomatic HSV-2 infection is associated with a marked reduction in IFN-γ production with a concomitant rise in IL-10 production presumably from a non-T cell source. Can inhibition of IL-10 production prevent this transition? The identity of IL-10-producing cells may assist in developing a strategy to inhibit IL-10 production. The cell identity can be determined by flow cytometry after intracellular staining of IL-10 together with staining for cell surface markers, before and after stimulation of PBMC with HSV-2. Once the identity is established, the IL-10-producing cell may be targeted *in vitro* to develop a drug for use *in vivo*.

3. **Examination of IFN-γ-signalling pathway during symptomatic HSV-2 infection:** The examination of the functioning of IFN-γ-signaling pathway in monocytes of HSV-2 infected symptomatic patients may help establish the cause of this immunodeficiency and its potential remedy. This may prove to be an important target for intervention.

4. **Mechanism of HSV-2-upregulation of B7 on monocytes:** Using an *in vitro* system, it should be determined whether other human herpesviruses augment B7 expression on monocytes. The identity of HSV-2 gene(s) essential for the upregulation of B7 should be determined by using specific viral mutants.

5. **Can MCMV M43 gene alone suppress Th2 response *in vivo*:** In order to determine or improvise therapeutic applications of M43 gene, the effect of M43 (or another related) gene on IL-4 response must be examined *in vivo* and the mechanism of the effect determined. It would be very interesting to study the effect of introducing M43 gene on pathogenesis of viral infections that lead to a predominant Th2 response such as infection with RSV or measles. Also, the determination of the effect of M43 gene on the development of Th2 cytokine-mediated allergic conditions such as atopic dermatitis will be of immense value.

REFERENCES

1. Taylor, T.J., Brockman, M.A., McNamee, E.E., and D.M., Knipe. 2002. Herpes Simplex Virus. Front. Biosci. 7: 752-764.

2. Sinzger, C., and G. Jahn. 1996. Human cytomegalovirus cell tropism and pathogenesis. Intervirol. 39: 302-309.

3. U. Desselberger. 1998. Herpes simplex virus infection in pregnancy: diagnosis and significance. Intervirol. 41(4-5): 185-190.

4. Mbizro, E.M., Msuya Sia, E., Stray-Pedersen, B., Chirenje, M.Z., Munjoma, M., and A. Hussain. 2002. Association of herpes simplex virus type 2 with the human immunodeficiency virus among urban women in Zimbabwe. Int. J. STD AIDS. 13(5): 343-348.

5. Koshnevis, M. and S.K., Tyring. 2002. Cytomegalovirus infections. Dermatol. Clin. 20(2): 291-299.

6. Klatt, E.C., and D. Shibata. 1988. Cytomegalovirus infection in the acquired immunodeficiency syndrome. Arch. Pathol. Lab. Med. 112: 540-544.

7. Stagno, S., Pass, R.F., Dworsky, M.E., and C.A. Alford. 1982. Maternal cytomegalovirus infection and perinatal transmission. Clinical obstetrics and gynecology. Ed. Knox GE, Philadelphia: JB Lippencott. pp. 563-576.

8. Gnann, J.W., Ahlmen, J., Svalander, C., Olding, L., Oldstone, M.B., and J.A. Nelson. 1988. Inflammatory cells in transplanted kidneys are infected by human cytomegalovirus. Am. J. Pathol. 132: 239-248.

9. P.D. Griffiths. 1996. Herpesviruses and AIDS. Scand. J. Infect. Dis. Suppl. 100: 3-7.

10. Mossman, K.L., Macgregor, P.F, Rozmus, J.J, Goryachev, A.B., Edwards, A.M., and J.R. Smiley. 2001. Herpes simplex virus triggers and then disarms a host antiviral response. J. Virol. 75(2): 750-758.

11. Hill, A., Jugovic, P., York, I., Russ, G., Bennink, J., Yewdell, J., Ploegh, H., and D. Johnson. 1995. Herpes simplex virus turns off the TAP to evade host immunity. Nature 375: 411-415.

12. Hayward, A.R., Read, G.S., and M. Cosyns. 1993. Herpes simplex virus interferes with monocyte accessory cell function. J. Immunol. 150: 190-196.

13. Edison, K.M., Hobbs, W.E., Manning, B.J., Carlson, P., and N.A. DeLuca. 2002. Expression of herpes simplex virus ICP0 inhibits the induction of interferon-stimulated genes by viral infection. J. Virol. 76(5): 2180-2191.

14. Hengel, H., Brune, W., and U.H. Koszinowski. 1998. Immune evasion by cytomegalovirus-survival strategies of a highly adapted opportunist. Trends in Microbiol. 6(5): 190-197.

15. Miller, D.M., and D.D. Sedmak. 1998. Cytomegalovirus persistence: escape from cell mediated immunosurveillance. In: Sholtz M, Rabenau HF, Doerr HW, Cinatl J Jr, eds., CMV related immunopathology. Monographs in Virology. Basel. Karger 21: 1-11.

16. Wiertz, E., Hill, A., Tortorella, D., and H.L. Ploegh. 1997. Cytomegaloviruses use multiple mechanisms to elude the host immune response. Immunol. Lett. 57: 213-216.

17. Hengel, H., and U.H. Koszinowski. 1998. Inhibition of MHC Class I function by cytomegalovirus. Herpesviruses and immunity, eds. Medveczky et al., Plenum Press, New York. pp. 247-264.

18. Sedmak, D.D., Chaiwiriyakul, S., Knight, D.A., Birmingham, D.J., Huang, E.H., and W.J. Waldman. 1994. Cytomegalovirus inhibits major histocompatibility class II expression on infected endothelial cells. Am. J. Pathol. 144: 683-692.

19. Miller, D.M., Rahill, B.M., Boss, J.M., Lairmore, M.D., Durbin, J.E., Waldman, J.W., and D.D. Sedmak. 1998. Human cytomegalovirus inhibits Major Histocompatibility Complex class II expression by disruption of the JAK/Stat pathway. J. Exp. Med. 187: 675-683.

20. S. Michelson. 1999. Human cytomegalovirus escape from immune detection. Intervirolog. 42: 301-307.

21. Del Val, M., Hengel, H., Hacker, H., Hartlaub, U., Ruppert, T., Lucin, P., and U.H. Koszinowski. 1992. Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-golgi compartment. J. Exp. Med. 176: 729-738.

22. DeVal, M., Münch, K., Reddehase, M.J., and U.H. Koszinowski. 1989. Presentation of cytomegalovirus immediate-early antigens to cytotoxic T lymphocytes is selectively blocked by viral genes expressed in the early phase. Cell 58: 305-315.

23. Jones, T.R., Hanson, L.K., Sun, L., Slater, J.S., Stenberg, R.M., and A.E. Campbell. 1995. Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chain. J. Virol. 69: 4830-4841.

24. Scott, E.S., Malcomber, S., and P. O'Hare. 2000. Nuclear translocation and activation of the transcription factor NFAT is blocked by herpes simplex virus infection. 75(20): 9955-9965.

25. Redpath, S., Ghazal, P., and N.R. Gascoigne. 2001. Hijacking and exploitation of IL-10 by intracellular pathogens. Trends Microbiol. 9(2): 86-92.

26. Long, D., Madara, T.J., Ponce de Leon, M., Cohen, G.H., Montgomery, P.C., and R.J. Eisenberg. 1984. Glycoprotein D protects mice against lethal challenge with herpes simplex virus types 1 and 2. Infect. Immun. 43: 761-764.

27. Rinaldo, C.R. Jr., and D.J. Tropey III. 1993. Cell-mediated immunity and immunosuppression in herpes simplex virus infection. Immunodeficiency 5: 33-90.

28. Harandi, A.M., Svennerholm, B., Holmgren, J., and K. Eriksson. 2001. Differential roles of B cells and IFN-gamma-secreting CD4(+) T cells in innate and adaptive immune control of genital herpes simplex virus type 2 infection in mice. J. Gen. Virol. 82(4): 845-853.

29. Schmid, D.S., and B.T. Rouse. 1992. The role of T cell immunity in control of herpes simplex virus. Curr. Top. Microbiol. Immunol. 179: 57-74.

30. Rasmussen, L., and C.M. Thomas. 1978. Role of T lymphocytes in cellular immune responses during herpes simplex virus infection in humans. Proc. Natl. Acad. Sci. USA 75(8): 3957-3961.

31. Paludan, S.R., and S.C. Mogensen. 2001. Virus-cell interactions regulating induction of tumor necrosis factor alpha production in macrophages infected with herpes simplex virus. J. Virol. 75(21): 10170-10178.

32. Heise, M.T., and H.W. Virgin. 1995. The T cell independent roll of IFN-gamma and TNF-alpha in macrophage activation during murine cytomegalovirus and herpes simplex virus infection. J. Virol. 69: 904-909.

33. Tay, C.H., and R.M. Welsh. 1997. Distinct organ-dependent mechanisms for the control of murine cytomegalovirus infection by natural killer cells. J. Virol. 71: 267-275.

34. Koszinowski, U.H., Del Val, M., and M.J. Reddehase. 1990. Cellular and Molecular Basis of the protective immune response to cytomegalovirus infection. Curr. Top. Microbiol. Immunol. 154: 189-220.

35. L. Rasmussen. 1990. Immune response to human cytomegalovirus infection. Curr. Top. Microbiol. Immunol. 154: 221-254.

36. Boes, B., Hengel, H., Ruppert, T., Multhaup, G., Koszinowski, U.H., and P.M. Kloetzel. 1994. Interferon gamma stimulation modulates the proteolytic activity and cleavage site preference of 20S mouse proteasomes. J. Exp. Med. 179(3): 901-909.

37. Hengel, H., Lucin, P., Jonjic, S., Ruppert, T., and U.H. Koszinowski. 1994. Restoration of cytomegalovirus antigen presentation by gamma interferon combats viral escape. J. Virol. 68: 289-297.

38. Geginat, G., Ruppert, T., Hengel, H., Holtappels, R., and U.H. Koszinowski. 1997. IFN-gamma is a prerequisite for optimal antigen processing of viral peptides in vivo. J. Immunol. 158: 3303-3310.

39. Lucin, P., Pavic, I., Polic, B., Jonjic, S., and U.H. Koszinowski. 1992. Gamma-interferon dependent clearance of cytomegalovirus infection in salivary glands. J. Virol. 66: 1977-1984.

40. Jonjic, S., Pavic, I., Lucin, P., Rukavina, D., and U.H. Koszinowski. 1990. Efficacious control of cytomegalovirus infection after long term depletion of CD8+ lymphocytes. J. Virol. 64: 5457-5464.

41. Smith, P.M., Wolcott, R.M., Chervenak, R., and S.R. Jennings. 1994. Control of acute cutaneous herpes simplex virus infection: T cell-mediated viral clearance is dependent upon interferon-gamma. Virol. 202: 76-88.

42. Spruance, S.L., Evans, T.G., McKeough, M.B., Thai, L., Araneo, B.A., Daynes, R.A., Mishkin, E.M., and A.S. Abramovitz. 1995. Th1/Th2-like immunity and resistance to herpes simplex labialis. Antiviral Res. 28(1): 39-55.

43. Chan, W-L., Lukig, M.L., and F.Y. Liew. 1985. Helper T cells induced by an immunopurified herpes simplex virus type 1 (HSV-1) 115-kilodalton glycoprotein (gB) protect mice against HSV-1 infection. J. Exp. Med. 162: 1304-1318.

44. Jayaraman, S., Heiligenhaus, A., Rodriguez, A., Soukiasian, S., Dorf, M.E., and C.S. Foster. 1993. Exacerbation of murine herpes simplex virus-mediated stromal keratitis by Th2 type T cells. J. Immunol. 151(10): 5777-5789.

45. Carmack, M.A., Yasukawa, L.L., Chang, S.Y., Tran, C., Saldana, F., Arvin, A.M., and C.G. Prober. 1996. T cell recognition and cytokine production elicited by common and type- specific glycoproteins of herpes simplex virus type 1 and type 2. J. Infect. Dis. 174(5): 899-906.

46. Stumpf, T.H., Shimeld, C., Easty, D.L., and T.J. Hill. 2001. Cytokine production in a murine model of recurrent herpetic stromal keratitis. Invest. Opthalmol. Vis. Sci. 42(2): 372-378.

47. Raz, E., Tighe, H., Sato, Y., Corr, M., Dudler, J.A., Roman, M., Swain, S.L., Spiegelberg, H.L., and D.A. Carson. 1996. Preferential induction of a Th_1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. Proc. Natl. Acad. Sci. USA, 93: 5141-5145.

48. Wu, C.A., Puddington, L., Whiteley, H.E., Yiamouyiannis, C.A., Schramn, C.M., Mohammadu, F., and R.S. Thrall. 2001. Murine cytomegalovirus infection alters Th1/Th2 cytokine expression, and enhances mucus production in allergic airway disease. J. Immunol. 167(5): 2798-2807.

49. Zhu, J., Shearer, G.M., Marincola, F.M., Norman, J.E., Rott, D., Zou, J.P., and S.E. Epstein. 2001. Discordant cellular and humoral immune responses to cytomegalovirus infection in healthy blood donors: existence of a Th1-type dominant response. Int. Immunol. 13(6): 785-790.

50. B. Roizman. Herpesviridae. 1996. Fields Virology, Third edition Raven Press, New York. pp. 2221-2230.

51. Roizman, B., Desrosiers, R.C., Fleckenstein, B., Lopez, C., Minson, A.C., and M.J. Studdert. 1992. The family Herpesviridae: an update. Arch. Virol. 123: 424-449.

52. K. Nazerian. 1974. DNA configuration in the core of Marek's disease virus. Virol. 13: 1148-1150.

53. Wildy, P., and D.H. Watson. 1963. Electron microscopic studies on the architecture of animal viruses. Quant. Biol. Cold spring harbor Symp. 27: 25-47.

54. Spear, P.G., and B.P. Roizman. 1972. Proteins specified by herpes simplex virus V. Purification and structural proteins of the herpesvirion. J. Virol. 9: 431-439.

55. Karlin, S., Mocarski, E.S., and G.A. Sachachtel. 1994. Molecular evolution of herpesviruses: genomic and protein comparisons. J. Virol. 68: 1886-1902.

56. Roizman, B., Carmichael, L.E., Deinhardt, F., Nahmias, A.J., Plowright, W., Rapp, F., Sheldrick, P., Takahashi, M., and K. Wolf. 1981. Herpesviridae: definition, provisional nomenclature and taxonomy. Intervirol. 16: 201-217.

57. L.R. Stanberry. 1992. Pathogenesis of herpes simplex virus infection and animal models for its study. Curr. Top. Microbiol. Immunol. 179: 15-30.

58. Corey, L., and P.G. Spear. 1986. Infections with herpes simplex viruses. N. Engl. J. Med. 314: 686-691.

59. Roizman, B., and P.R Jr. Roane. 1961. A physical difference between two strains of herpes simplex virus apparent in cesium chloride. Virol.15: 75-79.

60. Nahmias, A.J., and W.R. Dowdle. 1968. Antigenic and biologic differences in herpesvirus hominis. Prog. Med. Virol. 10: 110-159.

61. R.J. Whitley. 1996. Herpes Simplex Viruses. Fields Virology, Third edition Raven Press, New York. pp. 2297-2342.

62. Buchman, T.G., Roizman, B., Adams, G., and B.H. Stover. 1978 Restriction endonuclease fingerprinting of herpes simplex DNA: A novel epidemiological tool applied to a nosocomial outbreak. J. Infect. Dis. 138: 488-498.

63. Arvin, A., and R.J. Whitley. 1995 Neonatal herpes simplex virus infection. In: Remington J., Klein J., eds., Infectious diseases of the fetus and newborn infant. Philadelphia. Saunders W. B. pp. 354-376.

64. Corey, L., and H.H. Handsfield. 2000. Genital herpes and public health: addressing a global problem. JAMA. 283(6): 791-794.

65. Benedetti, J., Corey, L., and R. Ashley. 1994. Recurrence rates in genital herpes after symptomatic first-episode infection. Ann. Intern. Med. 121(11): 847-854.

66. Brugha, R., Keersmaekers, K., Renton, A., and A. Meheus. 1997. Genital herpes Infection: a review. Int. J. Epidemiol. 26(4): 698-709.

67. Miller, R.L., Tomai, M., Harrison, C.J., and D.I. Berstein. 2002. Immunomodulation as a treatment strategy for genital herpes: review of evidence. Int. Immunopharmacol. 2(4): 443-451.

68. Nesburn, A.B., Cook, M.L., and J.G. Stevens. 1972. Latent herpes simplex virus: isolation from rabbit trigeminalganglia between episodes of recurrent occular infection. Arch. Opthalmol. 88: 412-417.

69. Fleming, D.T., McQuillan, G.M., Johnson, R.E., Nahmias, A.J., Aral, S.O., Lee, F.K., and M.E. ST Louis. 1997. Herpes simplex virus type 2 in the United States, 1976-1994. N. Engl. J. Med. 337: 1105-1111.

70. Van Gelder, R.N., Willig, J.L., Holland, G.N., and H.J. Kaplan. 2001. Herpes simplex virus type 2 as a cause of acute retinal necrosis syndrome in young patients. Opthalmol. 108(5): 869-876.

71. Cone, R.W., Swenson, P.D., Hobson, A.C., Remington, M., and L. Corey. 1993. Herpes simplex virus detection from genital lesions: a comparative study using antigen detection (Herpchek) and culture. J. Clin. Microbiol. 31(7): 1774-1776.

72. Armstrong, G. L., Schillinger, J., Markowitz, L., Nahmias, A.J., Johnson, R.E., McQuillan, G.M., and M.E. St Louis. 2001. Incidence of herpes simplex virus type 2 infection in the United States. Am. J. Epidemiol. 153(9): 912-920.

73. T. Schacker. 2001. The role of HSV in the transmission and progression of HIV. Herpes. 8(2): 46-49.

74. Roizmam, B., and A.E. Sears. 1996. Herpes Simplex Viruses and their replication, Fields Virology, Third edition Raven Press, New York. pp. 2231-2295.

75. Bukowski, J.F., and R.M. Welsh. 1986. The role of natural killer cells and interferon in resistance to acute infection of mice with herpes simplex virus type 1. J. Immunol. 136(9): 3481-3485.

76. Koelle, D.M., Chen, H.B., Gavin, M.A., Wald, A., Kwok, W.W., and L. Corey. 2001 CD8 CTL from genital herpes simplex lesions: recognition of viral tegument and immediate early proteins and lysis of infected cutaneous cells. J. Immunol. 166(6): 4049-4058.

77. Milligan, G.N., Bourne, N., and K.L. Dudley. 2001. Role of polymorphonuclear leukocytes in resolution of HSV-2 infection of the mouse vagina. J. Reprod Immunol. 49(1): 49-65.

78. Milligan, G.N., Bernstein, D.I., and N. Bourne. 1998. T lymphocytes are required for protection of the vaginal mucosae and sensory ganglia of immune mice against reinfection with herpes simplex virus type 2. J. Immunol. 160(12): 6093-6100.

79. Sheridan, J.F., and L. Aurelian. 1983. Immunity to herpes simplex virus type 2. Risk of recurrent disease following primary infection: modulation of T cell subsets and lymphokine production. Diagn. Immunol. 1: 245-256.

80. Cantin, E.M., Hinton, D.R., Chen, J., and H. Openshaw. 1995. Gamma interferon expression during acute and latent nervous system infection by herpes simplex virus type 1. J. Virol. 69: 4898-4905.

81. Cantin, E., Tanamachi, B., and H. Openshaw. 1999. Role for gamma interferon in control of herpes simplex virus type 1 reactivation. J. Virol. 73(4): 3418-3423.

82. Sullender, W.M., Miller, J.L., Yasukawa, L.L., Bradley, J.S., Black, S.B., Yeager, A.S., and A.M. Arvin. 1987. Humoral and cell mediated immunity in neonates with herpes simplex virus infection. J. Infect. Dis. 155: 28-37.

83. Hendrzak, J.A., and P.S. Morahan. 1994. The role of macrophages and macrophage cytokine in host resistance to herpes simplex virus. Immunol. Ser. 60: 601-617.

84. S. Kohl. 1989. The neonatal human's immune response to herpes simplex virus infection: a critical review. Pediatr. Infect. Dis. J. 8(2): 67-74.

85. Keadle, T.L., Morris, J.L., Pepose, J.S., and P.M. Stuart. 2002. CD4(+) and CD8 (+) cells are key participants in the development of recurrent herpetic stromal keratitis in mice. Microb. Pathog. 32 (6): 255-262.

86. Feduchi, E., Alonso, M.A., and L. Carrasco. 1989. Human gamma interferon and tumor necrosis factor exert a synergistic blockade on the replication of herpes simplex virus. J. Virol. 63: 1354-1359.

87. Kohl, S., Strynadka, N.C.J., Hodges, R.S., and L. Pereira. 1990. Analysis of the role of antibody-dependent cellular cytotoxic antibody activity in murine neonatal herpes simplex virus infection with antibodies to synthetic peptides of glycoprotein D and monoclonal antibodies to glycoprotein B. J. Clin. Invest. 86: 273-278.

88. Prober, C.G., Sullender, W.M., Yasukawa, L.L., Au, D.S., Yeager, A.S., and A.M. Arvin. 1987. Low risk of herpes simplex virus infection in neonates exposed to the virus at the time of vaginal delivery to mothers with recurrent genital herpes simplex virus infections. N. Engl. J. Med. 316: 240-244.

89. Hashido, M., and T. Kawana. 1997. Herpes simplex virus-specific IgM, IgA and IgG subclass antibody response in primary and non primary genital herpes patients. Microbiol. Immunol. 41(5): 415-420.

90. Handa, K., Suzuki, R., Matsui, H., Shimizu, Y., and K. Kumagai. 1983. Natural killer (NK) cells as a responder to interleukin 2 (IL-2): IL-2 induced interferon gamma production. J. Immunol. 130: 988-992.

91. Le Page, C., Genin, P., Baines, M.G., and J. Hiscott. 2000. Interferon activation and innate immunity. Rev. Immunogenet. 2(3): 374-386.

92. Komatsu, T., Bi, Z., and C.S. Reiss. 1996. Interferon-gamma induced type I nitric oxide synthase activity inhibits viral replication in neurons. J. Neuroimmunol. 68: 101-108.

93. Kohl, S., Adam, E., Matson, D.O., Kaufman, R.H., and G.R. Dreesman. 1982. Kinetics of human antibody responses to primary genital herpes simplex infection. Intervirol. 18: 164-168.

94. Thomas, J., and B.T. Rouse. 1997. Immunopathogenesis of herpetic ocular disease. Immunol. Res. 16(4): 375-386.

95. Seid, M., Leung, K-N., Pye, C., Phelan, J., Nash, A.A., and H.P. Godfrey. 1987. Clonal analysis of the T-cell response to mice to herpes simplex virus: correlation between lymphokine production in vitro and the induction of delayed-type hypersensitivity and antiviral activity in vivo. Viral Immunol. 1: 35-44.

96. Young, C., Lehner, T., and C.G. Barnes. 1988. CD4 and CD8 cell responses to herpes simplex virus in Behcet's disease. Clin. Exp. Immunol. 73(1): 6-10.

97. Mossman, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., and R.L. Coffman. 1986. Two types of murine helper T cell clone definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136: 2348-2357.

98. Abbas, A.K., Murphy, K., and A. Sher. 1996. Functional diversity of helper T lymphocytes. Nature 383, 787-790.

99. Mossmann, T.R., and S. Sad. 1996. The expanding universe of T cell subsets: Th1, Th2, and more. Immunology today. 17(3): 138-146.

100. Paul, W.E., and R.A. Seder. 1994. Lymphocyte responses and cytokines. Cell 76: 241-251.

101. Prymowicz, D., Moore, R.N., and B.T. Rouse. 1985. Frequency of herpes simplex virus-specific helper T lymphocyte precursors in the lymph node cells of infected mice. J. Immunol. 134: 2683-2688.

102. S.L. Gaffen. 2001. Signaling domains of the interleukin 2 receptor. Cytokine 14(2): 63-77.

103. A. Billiau. 1996. Interferon-gamma: biology and role in pathogenesis. Adv. Immunol. 62: 61-130.

104. Farrar, M.A., and R.D. Schreiber. 1993. The cell biology of interferon-gamma and its receptor. Annu. Rev. Immunol. 11: 571-611.

105. Gessner, A., and M. Rollinghoff. 2000. Biologic functions and signaling of the interleukin-4 receptor complex. Annu. Rev. Immunol. 201: 285-307.

106. Ikemoto, K., Pollard, R.B., Fukomoto, T., Morimatsu, M., and F. Suzuki. 1995. Small amounts of exogenous IL-4 increase the severity of encephalitis induced in mice by the intranasal infection of herpes simplex virus type 1. J. Immunol. 155(3): 1326-1333.

107. T.R. Mossmann. 1994. Properties and functions of interleukin-10. Adv. Immunol. 56: 1-26.

108. Moore, K.W., de Waal, M., Coffman, R.L., and A. O'Garra. 2001. IL-10 and IL-10 receptor. Annu. Rev. Immunol. 19: 683-765.

109. Yan, X.T, Zhuang, M., Oakes J.E., and R.N. Lausch. 2001. Locally produced IL-10 can act in an autocrine/paracrine fusion to downregulate the production of

proinflammatory mediators and thus limit corneal inflammation. J. Leuko. Biol. 69(1): 149-157.

110. Zak Prelich, M., Hallidy, K.E., Walker, C., Yates, C.M., Norval, M., and R.C. Mckenze. 2001. Infection of murine keretinocytes with herpes simplex type 1 induces the expression of interleukin-10, but not interleukin-1 alpha or tumor necrosis factor alpha. Immunol. 104(4): 468-475.

111. E.S. Jr. Mocarski. 1996. Cytomegalovirus and their replication. Fields Virology, Third edition Raven Press, New York. pp. 2447-2492.

112. Chee, M.S., Bankier, A.T., Beck, S., Bohni, R., Brown, C.M., Cerny, R., Horsnell, T., Hutchison, C.A., Kouzarides, T., and J.A. Martigenetti. 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD 169. Curr. Top. Microbiol. Immunol. 154: 125-169.

113. Rawlinson, W.D., Farrell, H.E., and B.G. Barrell. 1996. Analysis of the complete DNA sequence of murine cytomegalovirus. J. Virol. 70: 8833-8849.

114. Rawlinson, W.D., Farrell, H.E., and B.G. Barrell. 1993. Global comparison of the DNA sequences of HCMV (AD 169) and MCMV (Smith)-preliminary analysis. In Multidisciplinary approaches to understanding cytomegalovirus disease. Amsterdam: Elsevier. pp. 55-62.

115. Takekoshi, M., Ihara, S., Tanaka, S., Maeda-Takekoshi, F., and Y.A. Watanabe. 1987. New human cytomegalovirus isolate has an invertible subsegment within its L component producing eight genome isomers. J. Gen. Virol. 68: 765-776.

116. Kollert-Jons, A., Bogner, E., and K. Radsak. 1991. A 15-kilobase-pair region of the human cytomegalovirus genome which includes US1 through US13 is dispensable for growth in cell culture. J. Virol. 65: 5184-5199.

117. Browne, H., Churcher, M., and T. Minson. 1992. Construction and characterization of a human cytomegalovirus mutant with the UL18 (class I homologue) gene deleted. J. Virol. 66: 6784-6787.

118. J.M. DeMarchi. 1983. Post-transcriptional control of human cytomegalovirus gene expression. Virol. 124: 390-402.

119. J.M. DeMarchi. 1983. Correlation between stimulation of host cell DNA synthesis by human cytomegalovirus and lack of expression of a subset of early virus genes. Virol. 129: 274-286.

120. Bruggeman, C.A., Li, F., and F.S. Stals. 1995. Pathogenecity: animal models. Scand. J. Infect. Dis. Suppl. 99: 43-50.

121. Sha, M., Griffith, B.P., Isom, H.C., Ward, D.C., and G.D. Hsiung. 1987. Detection of guinea pig cytomegalovirus nucleic acids in cultured cells with biotin-labelled hybridization probes. Virus Res. 6: 317-329.

122. Span, A.H.M., Grauls, G., Bossman, F., Van Boven, C.P.A., and C.A. Bruggeman. 1992. Cytomegalovirus induces vascular injury in the rat. Atherosclerosis 93: 41-52.

123. J.B. Hudson. 1979. The murine cytomegalovirus as a model for the study of viral pathogenesis and persistent infections. Arch. Virol. 62: 1-29.

124. Pomeroy, C., Hilleren, P.J., and M.C. Jordan. 1991. Latent murine cytomegalovirus DNA in spleenic stromal cells of mice. J. Virol. 65: 3330-3334.

125. Shanley, J.D., Morningstar, J., and M.C. Jordan. 1985. Inhibition of murine cytomegalovirus lung infection and interstitial pneumonitis by acycovir and 9-(1,3-dihydroxy-2-propoxymethyl) guanine. Antimicob. Agents Chemother. 28: 172-175.

126. Kashiwai, A., Kawamura, N., Kadota, C., and Y. Tsutsui. 1992. Susceptibility of mouse embryo to murine cytomegalovirus infection in early and midgestation stages. Arch. Virol. 127: 37-48.

127. Tsutsui, Y., Kashiwai, N., Kawamura, N., and C. Kadota. 1993. Microphathalmia and cerebral atrophy induced in mouse embryos by infection with murine cytomegalovirus in midgestation. Am. J. Pathol. 143: 804-813.

128. Kemble, G., Duke G., Winter R., and R. Spate. 1996. Defined large scale alterations of the human cytomegalovirus genome constructed by cotransfection of overlapping cosmids. J. Virol. 70: 2044-8.

129. Spaete, R.R., and E.S Mokarski. 1987. Insertion and deletion mutagenesis of the human cytomegalovirus genome. Proc. Natl. Acad. Sci. USA. 84:7213-7.

130. Zhan, X., Lee, M., Abenes, G., Von Reis, I., Kittinunvorakoon, C., Ross-Macdonald, P., Snyder, M., and F. Liu. 2000. Mutagenesis of murine cytomegalovirus using a Tn3-based transposon. Virol. 266: 264-274.

131. Zhan, X., Lee, M., Xiao, J., and F. Liu. 2000. Construction and characterization of murine cytomegaloviruses that contain transposon insertions at open reading frames m09 and M83. J. Virol. 74(16): 7411-7421.

132. Abenes, G., Lee, M., Haghjoo, E., Tong, T., Zhan, X., and F. Liu. 2001. Murine cytomegalovirus open reading frame M27 plays an important role in growth and virulence in mice. J. Virol. 75(4): 1697-1707.

133. Xiao, J., Tong, T., Zhan, X., Haghjoo, E., and F. Liu. 2000. In vitro and in vivo characterization of a murine cytomegalovirus with a transposon insertional mutation at open reading frame M43. J. Virol. 74(20): 9488-97.

134. Fowler, K.B., Stagno, S., Pass, R.F., Britt, W.J., Boll, T.J., and C.A. Alford. 1992. The outcome of congenital cytomegalovirus infection in relation to maternal antibody status. N. Eng. J. Med. 326: 663-667.

135. Gold, E., and G.A. Nankervis. 1982. Cytomegalovirus. Viral Infections of humans: epidemiology and control. 2nd ed. New York Plenum Press. pp. 167-186.

136. Chandler, S.H., Holmes, K.K., Wentworth, B.B., Gutman, L.T., Wiesner, P.J., Alexander, E.R., and H.H. Hansfield. 1985. The epidemiology of cytomegaloviral infection in women attending a sexually transmitted disease clinic. J. Infect. Dis. 152: 597-605.

137. Britt, W.J., and C.A. Alford. 1996. Cytomegalovirus and their replication, Fields Virology, Third edition Raven Press, New York. pp. 2493-2523.

138. Henson, D., and A.J. Strano. 1972. Mouse cytomegalovirus: necrosis of infected and morphologically normal submaxillary gland acinar cells during termination of chronic infection. Am. J. Pathol. 68: 183-202.

139. Danker, W.M., McCutchan, J.A., Richman, D.D., Hirata, K., and S.A. Spector. 1990. Localization of human cytomegalovirus in peripheral blood leukocytes by in situ hybridization. J. Infect. Dis. 161: 31-36.

140. Persoons, M.C.J., Daemen, M.J.A.P., Bruning, J.H., and C.A. Bruggeman. 1994. Active cytomegalovirus infection of arterial smooth muscle cells in immunocompromised rats: a clue to herpesvirus-associated atherogenesis. Circ. Res. 75: 214-220.

141. Rohrer, T., Rinaldi, D., Bulb, R., Engelcke, G., Di Gallo, A., and C. Rudin. 1999. Combined treatment with zidovudine, lamivudine, nelfinavir and ganciclovir in an infant with human immunodeficiency virus type 1 infection and cytomegalovirus encephalitis: case report and review of the literature. Pediatr. Infect. Dis. J. Pediatr. 18(4): 382-386.

142. Cottone, M., Pietrosi, G., Martorana, G., Casa, A., Pecoraro, G., Oliva, L., Orlando, A., Rosselli, M., Rizzo, A., and L. Pagliaro. 2001. Prevalence of cytomegalovirus infection in severe refractory ulcerative and crohn's colitis. Am. J. Gastroenterol. 96(3): 773-775.

143. Bonkowsky, H.L., Lee, R.L., and G. Klatskin. 1975. Acute granulomatous hepatitis: occurrence in cytomegalovirus mononucleosis. JAMA 233: 1284-1288.

144. Collins, T., Pomeroy, C., and M.C. Jordan. 1993. Detection of latent cytomegalovirus DNA in diverse organs of mice. J. Infect. Dis. 168: 725-729.

145. Spector, D.H., and S.A. Spector. 1984. The oncogenic potential of human cytomegalovirus. Prog. Med. Virol. 29: 45-89.

146. Pac, C.Y., Eun, H.M., McArthur, R.G., and J.W. Yoon. 1988. Association of cytomegalovirus infection with autoimmune type I diabetes. Lancet 1: 1-4.

147. Farrell, H.E., and G.R. Shellam. 1991. Protection against murine cytomegalovirus infection by passive transfer of neutralizing and non-neutralizing monoclonal antibodies. J. Gen. Virol. 72: 149-156.

148. Klein, M., Schoppel, K., Amvrossiadis, N., and M. Mach. 1999. Strain-specific neutralization of human cytomegalovirus isolates by human sera. J. Virol. 73(2): 878-886.

149. Orange, J.S., Wang, B., Terhorst, C., and C.A. Biron. 1995. Requirement for natural killer cell produced interferon-gamma in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. J. Exp. Med. 182: 1045-1056.

150. Jonjic, S., Mutter, W., Weiland, F., Reddehase, M.J., and U.H. Koszinowski. 1989. Site-restricted persistent cytomegalovirus infection after selective long-term depletion of CD4+ T lymphocytes. J. Exp. Med. 169: 1199-1212.

151. Liu, Y., Faud, S., and R. Gehrz. 1987. Epstein-Barr virus transformed lymphoblastoid cell lines as antigen presenting cells and augmenting cells for human CMV-specific Th clones. Cell Immunol. 108: 64-75.

152. Davignon, J-L., Michelson, S., and C. Davrinche. 1995. Presentation of human cytomegalovirus immediate early (IE1) protein by astrocytoma cells to a CD4+ T cell clone. J. Immunol. 41: 247-255.

153. Presti, R.M., Pollock, J.L., Dal Canto, A.J., O'Guin, A.K., and H.W. Virgin IV. 1998. Interferon γ regulates acute and latent murine cytomegalovirus infection and chronic disease of the great vessels. J. Exp. Med. 188: 577-588.

154. Gehrz, R., and T. Leonard. 1985. Cytomegalovirus (CMV)-specific lymphokine production in congenital CMV infection. Clin. Exp. Immunol. 62: 507-514.

155. Gehrz, R., Liu, Y., Peterson, E., and S. Faud. 1987. Role of antigen-presenting cells in congenital cytomegalovirus-specific immuneodeficiency. J. Infect. Dis. 156: 198-202.

156. J. Pieters. 1997. MHC class II restricted antigen presentation. Curr. Opin. Immunol. 9: 89-96.

157. Geginat, G., Ruppert, T., Hengel, H., Holtappels, R., and U.H. Koszinowski. 1997. IFN-gamma is a prerequisite for optimal antigen processing of viral peptides in vivo. J. Immunol. 158: 3303-3310.

158. Hengel, H., Lucin, P., Jonjic, S., Ruppert, T., and U.H. Koszinowski. 1994. Restoration of cytomegalovirus antigen presentation by gamma interferon combats viral escape. J. Virol. 68: 289-297.

159. Tomasec, P., Veronique, B.M., Rickards, C., Powell, M.B., McSharry, B.P., Gadola, S., Cerundolo, V., Borysiewicz, L.K., McMichael, A.J., and G.W.G. Wilkinson. 2000. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. Science. 287: 1031-1033.

160. P. Bretscher. 1992. The two signal model of lymphocyte activation twenty one years later. Immunol. Today. 13: 74-76.

161. R.H. Schwartz. 1990. A cell culture model for T lymphocyte clonal anergy. Science 248: 1349-1356.

162. R.H. Schwartz. 1996. Models for T cell anergy: Is there a common molecular mechanism? J. Exp. Med. 184: 1-8.

163. Engel, P., Gribben, J.G., Freeman, G.J., Zgou, L-J., Nozawa, Y., Abe, M., Nadler, L.M., Wakasa, H., and T.F. Tedder. 1994. The B7-2 (B70) costimulatory molecule expressed by monocytes and activated lymphocytes is the CD86 differentiation antigen. Blood 84(5): 1402-1407.

164. Azuma, M., Yssel, H., Phillips, J.H., Spits, H., and L.L. Lanier. 1993. Functional expression of B7/BB1 on activated T lymphocytes. J. Exp. Med. 177: 845-850.

165. June, C.H., Bluestone, J.A., Nadler, L.M., and C.B. Thompson. 1994. The B7 and CD28 receptor families. Immunol. Today 15: 321-331.

166. Boussiotis, V.A., Freeman, G.J., Gribben, J.G., Daley, J., Gray, G., and L.M. Nadler. 1993. Activated human B lymphocytes express three CTLA4 counter-receptors which costimulate T cell activation. Proc. Natl. Acad. Sci. USA 90: 11059-11063.

167. C.B. Thompson. 1995. Distinct roles for the costimulatory ligands B7-1 and B7-2 in T helper cell differentiation? Cell 81(7): 979-982.

168. Schweitzer, A.N., Borriello, F., Wong, R.C., Abbas, A.K., and A.H. Sharpe. 1997. Role of costimulators in T cell differentiation studies using antigen-presenting cells lacking expression of CD80 or CD86. J. Immunol. 158(6): 2713-2722.

169. Hathcock, K.S., Laszlo, G., Pucillo, C., Linsley, P., and R.J. Hodes. 1994. Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. J. Exp. Med. 180: 631-640.

170. Thebeau, L.G., and L.A. Morrison. 2002. B7 costimulation plays an important role in protection from herpes simplex virus type 2-mediated pathology. J. Virol. 76: 2563-2566.

171. Kuchroo, V.K., Das, M.P., Brown, J.A., Ranger, A.M., Zamvil, S.S., Sobel, R.A., Weiner, H.L., Nabavi, N., and L.H. Glimcher. 1995. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. Cell 80(5): 707-718.

172. Ranger, A.M., Das, M.P., Kucroo, V.K, and L.H. Glimcher. 1996. B7-2 (CD86) is essential for the development of IL-4 producing T cells. Int. Immunol. 8(10): 1549-1560.

173. Broeren, C.P., Gray, G.S., Carreno, B.M., and C.H. June. 2000. Costimulation light: activation of CD4+ T cells with CD80 or CD86 rather than anti-CD28 leads to a Th2 cytokine profile. J. Immunol. 165(12): 6908-6914.

174. Ding, L., Linsley, P.S., Huang, L.Y., Germain, R.N., and E.M. Shevach. 1993. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 statement. J. Immunol. 151(3): 1224-1234.

175. Kubin, M., Kamoun, M., and G. Trinchieri. 1994. Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. J. Exp. Med. 180(1): 211-222.

176. Creery, W.D., Diaz-Mitoma, F., Filion, L., and A. Kumar. 1996. Differential modulation of B7-1 and B7-2 isoform statement on human monocytes by cytokines which influence the development of T helper cell phenotype. Eur. J. Immunol. 26(6): 1273-1277.

177. Lekstrom-Himes, J.A., Hohman, P., Warren, T., Wald, A., Nam, J.M., Simonis, T., Corey, L., and S.E. Straus.1999. Association of major histocompatibility complex determinants with the development of symptomatic and asymptomatic genital herpes simplex virus type 2 infections. J. Infect.Dis. 179(5): 1077-1085.

178. Diaz-Mitoma, F., Ruben, M., Sacks, S., MacPherson, P., and G. Caissie. 1996. Detection of viral DNA to review outcome of antiviral treatment of patients with recurrent genital herpes. J. Clin. Microbiol. 34(3): 657-663.

179. Kumar, A., Angel, J.B., Aucoin, S., Creery, W.D., Daftarian, M.P., Cameron, D.W., Filion, L., and F. Diaz-Mitoma. 1999. Dysregulation of B7.2 (CD86) statement on monocytes of HIV-infected individuals is associated with altered production of IL-2. Clin. Exp. Immunol. 117: 84-91.

180. Kryworuchko, M., Gee, K., Diaz-Mitoma, F., and A. Kumar. 1999. Regulation of CD44-hyaluronan interactions in Burkitt's lymphoma and epstein-barr virus-transformed lymphoblastoid B cells by PMA and interleukin-4. Cell Immunol. 194: 54-66.

181. Kimura, H., Shibata, M., Kuzushima, K., Nishikawa, K., Nishiyama, Y., T. Morishima. 1990. Detection and direct typing of herpes simplex virus by polymerase chain reaction. Med. Microbiol. Immunol. 179: 177-184.

182. Tsurumi, T., Maeno, K., and Y. Nishiyama. 1987. Nucleotide sequence of the DNA polymerase gene of herpes simplex virus type 2 and comparison with the type 1 counterpart. Gene. 52:129-37.

183. Gibbs, J.S., Chiou, H.C., Hall, J.D., Mount, D.W., Retondo, M.J., Weller, S.K., and D.M. Coen. 1984. Sequence and mapping analyses of the herpes simplex virus DNA polymerase gene predict a C-terminal substrate binding domain. Proc. Natl. Acad. Sci. USA. 82: 7969-7973.

184. Ashley, R.L., Militoni, J., Lee, F., Nahmias, A., and L. Corey. 1988. Comparison of Western blot (immunoblot) and glycoprotein G-specific immunodot enzyme assay for detecting antibodies to herpes simplex virus types 1 and 2 in human sera. J. Clin. Microbiol. 26(4): 662-667.

185. Williams, A.J., Duong, T., McNally, L.M., Tookey, P.A., Masters, J., Miller, R., Lyall, E.G, and D.M. Gibb. 2001. Pneumocystis carinii pneumonia and cytomegalovirus infection in children with vertically acquired HIV infection. AIDS 15(3): 335-339.

186. Sisson, J.G., and A.J. Carmichael. 2002. Clinical aspects and management of cytomegalovirus infection. J. Infect. 44(2): 78-83.

187. Keadle, T.L., Usi, N., Laycock, K.A., Kumano, Y., Pepose, J.S., and P.M. Stuart. 2001. Cytokine expression in murine corneas during recurrent herpetic stromal keratitis. Ocul. Immunol. Inflamm. 9(3): 193-205.

188. Sester, M., Sester, U., Gartner, B., Kubuschok, B., Girndt, M., Meyerhans, A., and H. Kohler. 2002. Sustained high frequencies of specific CD4 T cells restricted to a single persistent virus. J. Virol. 76(8): 3748-3755.

189. S.L. Swain. 1991. Lymphokines and the immune response: the central role of interleukin-2. Curr. Opin. Immunol. 3(3): 304-310.

190. Ghiasi, H., Wechsler, S.L., Kaiwar, R., Nesburn, A.B., and F.M. Hofman. 1995. Local expression of tumor necrosis factor alpha and interleukin-2 correlates with protection against corneal scarring after ocular challenge of vaccinated mice with herpes simplex type 1. J. Virol. 69(1): 334-340.

191. Burchett, S.K., Corey, L., Mohan, K.M., Westall, J., Ashley, R., and C.B. Wilson. 1992. Diminished interferon-gamma in neonatal and postpartum primary herpes simplex virus infection. J. Infect. Dis. 165(5): 813-818.

192. Bouley, D.M., Kanangat, S., Wire, W., and B.T. Rouse. 1995. Characterization of herpes simplex virus type-1 infection and herpetic stromal keratitis development in IFN-gamma knockout mice. J. Immunol. 155: 3964-3971.

193. Wetzler, L.W., Ho, Y., and H. Reiser. 1996. Neisserial porins induce B lymphocytes to express costimulatory B7-2 molecules and to proliferate. J. Exp. Med. 183(3): 1151-1159.

194. Lim, W., Ma, W., Gee, K., Aucoin, S., Nandan, D., Diaz-Mitoma, F., Kozlowski, M., and A. Kumar. 2002. Distinct roles of p38 and c-Jun N-terminal kinase in IL-10-dependent and IL-10 independent regulation of the costimulatory molecule B7.2 in lipopolysaccharide-stimulated human monocytic cells. J. Immunol. 168(4): 1759-1769.

195. Kaye, P.M., Rogers, N.J., Curry, A.J., and J.C. Scott. 1994. Deficient statement of co-stimulatory molecules on Leishmania- infected macrophages. Eur. J. Immunol. 24(11): 2850-2854.

196. Saha, B., Das, G., Vohra, H., Ganguly, N.K., and G.C. Mishra. 1994. Macrophage-T cell interaction in experimental mycobacterial infection. Selective regulation of co-stimulatory molecules on Mycobacterium-infected macrophages and its implication in the suppression of cell-mediated immune response. Eur. J. Immunol. 24: 2618-2624.

197. Baumgarth, N., Brown, L., Jackson, D., and A. Kelso. 1994. Novel features of the respiratory tract T-cell response to influenza virus infection: lung T cells increase expression of gamma interferon mRNA in vivo and maintain high levels of mRNA expression for IL-5 and IL-10. J. Virol. 68: 7575-7581.

198. P.C. Doherty. 1994. Vaccines and cytokine-mediated pathology in RSV infection. Trends Microbiol. 2(5): 148-149.

199. Chan, S.C., Brown, M.A., Willcox, T.M., Li, S.H., Stevens, S.R., Tara, D., and J.M. Hanifin. 1996. Abnormal IL-4 gene expression by atopic dermatitis T lymphocytes is reflected in altered nuclear protein interactions with IL-4 transcriptional regulatory element. Journal of Investigation Dermatology 106, 1131-1136.

200. Eo, S.K., Chun, S., Lee, S., and B.T. Rouse. 2000. On the mechanism of T cell silencing by IL-10 DNA: direct and indirect inhibition of T cell functions. Cell Imunol. 206(1): 59-69.

201. Del Prete, G., De Carli, M., Almerigogna, F., Giudizi, M.G., Biagiotti, R., and S. Romagnani. 1993. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. J. Immunol. 150(2): 353-360.

202. de Waal Malefyt, R., Yssel, H., and J. De Vries. 1993. Direct effects of IL-10 on subsets of human CD4+ T cell clones and resting T cells. Specific inhibition of IL-2 production and proliferation. J. Immunol. 150: 4754-4765.

203. de Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M.G., te Velde, A.X.F.C., Johnson, K., Kastelein, R., Yssel, H., and J.E. de Vries. 1991. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen- specific human T cell proliferation by diminishing the antigen- presenting capacity of monocytes via downregulation of class II major histocompatibility complex statement. J. Exp. Med. 174(4): 915-924.

204. Gerard, C., Bruyns, C., Marchant, A., Abramowicz, D., Vandenabeele, P., Delvaux, A., Fiers, W., Goldman, M., and T. Velu. 1993. Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. J. Exp. Med. 177(2): 547-550.

205. Silva, J.S., Morrissey, P.J., Grabstein, K.H., Mohler, K.M., Anderson, D., and S.G. Reed. 1992. Interleukin 10 and interferon gamma regulation of experimental Trypanosoma cruzi infection. J. Exp. Med. 175(1): 169-174.

206. Kumar, A., Angel, J.B., Daftarian, M.P., Parato, K., Cameron, W.D., Filion, L., and F. Diaz-Mitoma. 1998. Differential production of IL-10 by T cells and monocytes of HIV- infected individuals: association of IL-10 production with CD28- mediated immune responsiveness. Clin. Exp. Immunol. 114(1): 78-86.

207. Sedger, L.M., Shows, D.M., Blanton, R.A., Peschon, J.J., Goodwin, R.G., Cosman, D., and S.R. Wiley. 1999. IFN-gamma mediates a novel antiviral activity through dynamic modulation of TRAIL and TRAIL receptor expression. Journal of Immunology 163, 920-926.

208. Kotenko, S.V., Saccani, S., Izotova, L.S., Mirochnitchenko, O.V., and S. Pestka. 2000. Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). Proc. Nat. Acad. Sci. 97(4): 1695-1700.

209. Redpath, S., Angulo, A., Gascoigne, N.R.J., and P. Ghazal. 1999. Murine cytomegalovirus infection down-regulates MHC class II expression on macrophages by induction of IL-10. J. Immunol. 162: 6701-6707.

210. Matsuda, M., Salazar, F., Petersson, M., Masucci, G., Hansson, J., Pisa, P., Zhang, Q.-J., Masucci, M.G., and R. Kiessling. 1994. Interleukin 10 pretreatment protects target cells from tumor and allo-specific cytotoxic T cells and downregulates HLA class I expression. J. Exp. Med. 180: 2371-2376.

211. Petersson, M., Charo, J., Salazar-Onfray, F., Noffz, G., Mohaupt, M., Quin, Z., Klien, G., Blankenstein, T., and R. Kiessling. 1998. Constitutive IL-10 production accounts for the high NK sensitivity, low MHC class I expression, and poor transporter associated with antigen processing (TAP)-1/2 function in the proto-type NK target YAC-1. J. Immunol. 161: 2099-2105.

212. Flesch, I.E., and S.H. Kaufmann. 1994. Role of macrophages and αβ T lymphocytes in early interleukin 10 production during Listeria monocytogenes infection. Int. Immunol. 6: 463-468.

213. Seifert, H.S., Ajioka, R.S., Paruchuri, D., Heffron, F., and M. So. 1990. Shuttle Mutagenesis of Neisseria gonorrhoeae pitin null mutations lower DNA transformation competence. J. Bacteriol. 172: 40-46.

214. Burns, N., Grimwade, B., Ross-Macdonald, P.B., Choi, E.Y., Finberg, K., Roeder, G.S., and M. Snyder. 1994. Large scale analysis of gene expression, protein localization, and gene disruption in Saccromyces Cerevisiae. Genes Dev. 8: 1087-1105.

215. Dallas, P.B., Lyons, P.A., Hudson, J.B., Scalzo, A.A., and G.R. Shellam. 1994. Identification and characterization of a murine cytomegalovirus gene with homology to the UL25 open reading frame of human cytomegalovirus. Virol. 200: 643-650.

216. Zini, N., Battista, M.C., Santi, S., Riccio, M., Bergamini, G., Landini, M.P., and N.M. Maraldi. 1999. The novel structural protein of human cytomegalovirus, pUL25, is localized in the viral tegument. J. Virol. 73: 6073-6075.

CURRICULUM VITAE

Rekha Singh

5610 Salvia Common
Fremont, CA 94538
E-mail: singh_rek@yahoo.com

POSTGRADUATE EDUCATION AND RESEARCH

UNIVERSITY OF CALIFORNIA, BERKELEY, USA
Visiting Ph.D. Student and Post Graduate Researcher
Dept. of Infectious Diseases and Immunity, School of Public Health 1999-2003

UNIVERSITY OF OTTAWA, OTTAWA, CANADA
Ph.D. Student and Research Assistant
Dept. of Biochemistry, Microbiology and Immunology 1996-2003
Courses Taken: Virology, Immunology, Advanced Topics in Virology, Immuno-Chemistry, Ph.D. Comprehensive Exam

UNIVERSITY OF MANITOBA, WINNIPEG, CANADA
M.Sc. Student and Research Assistant
Dept. of Pathology, Faculty of Medicine 1994-1996
Courses Taken: Inter Biochemistry, Molecular Biology, Microbial Physiology, Biochemistry of Nucleic Acids, Recombinant DNA Technology, Advances in Microbial Genetics, Recombinant DNA Methodology, General Pathology

PROFESSIONAL QUALIFICATION

UNIVERSITY OF RAJASTHAN, AJMER, INDIA
JLN MEDICAL COLLEGE AND HOSPITALS
Bachelor of Medicine and Bachelor of Surgery (M.B.B.S.) 1981 - 1986
Courses Taken: Anatomy, Embryology, Physiology and Biochemistry, Pharmacology, Pathology and Microbiology, Forensic Medicine, Medicine including Pediatrics, Surgery including Orthopaedics, Obstetrics and Gynaecology, Family Planning and Nutrition, Ophthalmology, E.N.T., Community Medicine

PROFESSIONAL EXPERIENCE

KAMLA NEHRU MEMORIAL HOSPITAL, ALLAHABAD, INDIA
Medical Officer and House Surgeon (Obstetrics & Gynaecology) 1988 – 1990

UNIVERSITY OF RAJASTHAN, AJMER, INDIA
JLN MEDICAL COLLEGE AND HOSPITALS
Rotary Intern 1986 - 1987

AWARDS AND MEMBERSHIPS

- Registered medical practitioner, Uttar Pradesh Medical Council, Lucknow, India
- Registered medical practitioner, Rajasthan Medical Council, Jaipur, India
- Honors in Chemistry, Biology, and Physics in secondary and higher secondary
- Merit certificate and scholarships for being class prefect and for general English in secondary and higher secondary
- Merit certificates in the Basketball league tournaments at St. Mary's Convent
- Ex associate member, American Association for Cancer Research, USA

PUBLICATIONS AND RESEARCH CONTRIBUTIONS

Singh, R., Kumar, A., Ruben, M., Creery, W.D., and F. Diaz-Mitoma. 2003. Dysregulated expression of IFN-γ and IL-10 and impaired IFN-γ-mediated responses at different disease stages in patients with genital Herpes Simplex Virus-2 infection. *Clinical and Experimental Immunology*. 133(1): 97-107.

Singh, R., Haghjoo, E., and F. Liu. 2003. Cytomegalovirus M43 gene modulates T helper cell response. *Immunology Letters*. 88(1): 31-35.

Singh, R., Kumar, A., and F. Diaz-Mitoma. 2003. Augmentation of B7 Expression by Herpes Simplex Virus Antigen. *Human Immunology*. 64(8): 780-786.

Singh, R. 2003. Cellular Immune Responses in HSV and CMV Infections. Ph.D. Thesis. Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada.

Diaz-Mitoma, F., **Singh, R.**, Kumar, A., and P. Cheng. 1998. Cytokine Responses in Herpes Simplex Virus Type 2 Infections. In *Proceedings of the 10th International Congress of Immunology*, New Delhi, India.

Diaz-Mitoma, F., **Singh, R.**, Kumar, A., and P. Cheng. 1998. T Helper Responses in Herpes Simplex Virus Type 2 Infections. In *Proceedings of the 38th Interscience Conference on Antimicrobial Agents and Chemotherapeutics*, San Diego, California, USA

Watson, P.H., **Singh, R.**, and A.K. Hole. 1996. Influence of c-*myc* on the Progression of Human Breast Cancer. In *Current Topics in Medical Microbiology, "Attempts to understand metastasis formation."* U Gunthert and W Birchmeirer Eds., Springer Verlag, pp. 267-283.

Singh, R., and P.H. Watson. 1996. Quantitative reverse transcription polymerase chain reaction analysis of c-*myc* expression in breast cancer. In *Proceedings of the 87th Annual Meeting of the American Association for Cancer Research*, Washington, DC, USA.

Singh, R. 1996. Quantitation of c-*myc* and estrogen receptor gene expression in human breast cancer by non-isotopic competitive RT-PCR. M.Sc. Thesis. Department of Pathology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada.

CONTRIBUTION OF COLLABORATORS

The contributions of Dr. Pehua Cheng, Claudia Galvis, and Erik Haghjoo for their assistance in experimentation are gratefully acknowledged.