Biological function of Viral Interleukin-6 Encoded by a Rhesus Monkey Herpesvirus Genetically Related to Human Herpesvirus 8

by

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TABLE OF CONTENTS

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"...*the* thing that gives people courage is an idea".

- Georges Clemenceau, 1841-1929

"...research, though toilsome, is easy; imagination, though delightful, is difficult".

[Oxford Lectures on Poetry]
- A.C. Bradley, 1851-1935
Practically Speaking

[and....]

"...we can never achieve absolute truth, but we can live hopefully by a system of calculated probabilities that give science the unity of life we seek".

[Education for a New Morality]
- Agnes Meyer, 1887-1970
Statistically Speaking

I dedicate this dissertation to my mother, **N. Kasiri Specioza Kaleeba**. You are so strong and generous in spirit and you will always be my finest hero in a world that is interminably needy of compassion. You have continued to inspire me without limit ***to keep my head up, to look at the world in the face and to try my immeasurable best, each time trusting in God.*** Thank you for your love and kindness.

List of Tables

List of Figures

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1. **Kaleeba, J. A. R.** A rhesus herpesvirus related to Kaposi's sarcoma-associated herpesvirus (KSHV) encodes a cellular homologue of interleukin-6. **ORAL PRESENTATION**, 15th Annual Student Research Forum, Oregon Health Sciences University, May 7-8, 1998.

2. **Kaleeba, J. A. R.,** E. P. Bergquam, R. P. Searles, and S. W. Wong. (1998) Characterization of gene expression by a herpesvirus (Rhγ2HV) related to Kaposi's sarcoma-associated herpesvirus (KSHV). **ORAL PRESENTATION**, 1st Annual meeting on KSHV and related Rhadinoviruses. July 25-28, 1998. Santa Cruz, CA., USA.

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Preamble

Many seasonal as well as epidemic virus-associated illnesses (e.g., "flu", rashes, measles, chicken pox, mumps, Ebola, AIDS) remind us of how vulnerable we are to virus infection. Unfortunately, this reality is likely to endure for several years to come, because previously unknown viruses as well as new strains of known viruses continue to be described. Currently, basic studies of how viruses survive, propagate or induce disease in the infected host are generally carried out using well-controlled virus infections in vitro. However, in vitro studies reveal a very limited view of the infective power of viruses, because "life" in rich culture medium in the presence of millions of identical permissive cells is a luxury never encountered in the hostile environment of hosts with complex homeostatic systems and multi-factorial immune defenses.

Therefore, to fully understand the complexity of virus/host interactions in vivo, in vitro studies of virus infection need to be complemented by live-animal studies of how virus offense defeats host defense to induce disease. Animal models of virus infection not only can provide insight into critical features of viral pathogenesis but they can also unveil frameworks for development of novel therapeutic strategies for infectious disease. Work described in this thesis is part of an effort to develop a non-human primate model for the infectious process of human herpesvirus 8 (HHV-8) in humans, using rhesus macaque rhadinovirus (RRV), a recently described HHV-8-related herpesvirus that infects rhesus macaques. We believe that development of a non-human primate infection system that mimics the defining features of HHV-8 disease in humans represents a major milestone in virological research.

ABSTRACT

The recently described rhesus macaque rhadinovirus (RRV) is genetically related to human herpesvirus 8 (HHV-8). In HIV-infected and otherwise immunocompromized individuals, HHV-8 is etiologically linked to a number of cell growth abnormalities that are thought to result from HHV-8-induced deregulation of angiogenic and pro-inflammatory cytokines like interleukin-6 (IL-6). Similarly, in rhesus macaques with SIV-AIDS that is clinically analogous to HIV-AIDS, RRV co-infection is associated with lymphoproliferative disease (LPD) marked by B cell hyperplasia, splenomegaly, and follicular lymphadenopathy that resembles the plasma-cell variant of HHV-8 associated multicentric Castleman's disease (MCD). Because RRV-associated syndromes are patho-physiologically analogous to the IL-6-related disorders in HIV/HHV-8 co-infected individuals, we hypothesized that HHV-8 and RRV might share several pathogenetic features, including deregulation of IL-6 bioactivity.

Both HHV-8 and RRV encode functional homologues of cellular IL-6 (vIL-6 and RvIL-6, respectively) with ability to stimulate growth of the IL-6-dependent B9 cell line. Here, we show that in addition to B9 cell stimulation, RvIL-6 also prevented apoptosis and induced neurite outgrowth in NGF-starved PC12 neuronal cells. In both the B9 and PC12 assays, RvIL-6 was generally two-to-three-fold less potent than rhesus macaque IL-6 (MacIL-6) and produced an additive signal when used in conjunction with either human (huIL-6) or MacIL-6. RvIL-6 bioactivity was readily blocked by anti-RvIL-6 and by anti-gp130; however, anti-IL-6R was relatively less effective against RvIL-6 compared to huIL-6 or MacIL-6. Interestingly, RvIL-6 activated gp130 on BAF-130 cells in the absence of IL-6R, and appeared to attenuate MacIL-6 signaling when added to IL-

$6R^+/gp130^+$ B9 cells prior to adding MacIL-6. MacIL-6 did not have the same effect on RvIL-6 signaling, suggesting that direct binding of RvIL-6 to gp130 may affect gp130-mediated transduction of a signal initiated by the MacIL-6/IL-6R complex. Therefore, RvIL-6 and MacIL-6 display significant functional homology but may exhibit differences in the requirement for, and/or mechanism of interaction with the IL-6R/gp130 receptor system. Additionally, we show that blood plasma of SIV/RRV co-infected animals that developed LPD, but not plasma from non-LPD animals infected with SIV or RRV alone contained bioactive RvIL-6 but did not contain detectable rhesus IL-6 protein. Plasma IL-6-like bioactivity was both inhibited and affinity-depleted by polyclonal anti-RvIL-6, but not by polyclonal anti-human IL-6 that cross-reacts with rhesus macaque IL-6. We also show that NIH 3T3 cells that were transduced with RvIL-6 displayed features of altered growth — serum independence and focus formation in soft-agar cultures — and also became more responsive to exogenous RvIL-6 but not to MacIL-6.

In summary, RvIL-6 stimulates B cell growth, prevents apoptosis, circulates in plasma of SIV/RRV co-infected animals with lymphoproliferative disease, and may be available to stimulate hyperplastic expansion not only of cells within the B cell compartment but also other non-immune $gp130^+$ cells that do not traditionally express IL-6R. Based on these observations, the contribution of RvIL-6 towards proliferative syndromes in vivo can now be addressed directly using an already constructed RvIL-6-deletion mutant of RRV that also carries enhanced GFP inserted in the RvIL-6 locus. The mutant virus will be used in animal infection studies to separate the lymphoproliferative disease phenotype of RRV from RvIL-6-minus RRV in the context of SIV-induced immunodeficiency.

CHAPTER 1

INTRODUCTION

I. HERPESVIRUSES

Herpesviruses are a family of DNA viruses that are known to infect humans, non-human primates, and other animals. About 100 herpesviruses have been isolated to date, and many more are likely to be discovered. The known herpesviruses share a number of biological features (409). (i) Herpesviruses encode enzymes that are involved in nucleic acid metabolism, viral DNA synthesis, and processing of viral proteins. (ii) Synthesis of viral DNA and assembly of the herpesvirus capsid occur in the nucleus of infected cells. (iii) Release of virus progeny is usually accompanied by lysis of the infected cell, as infectious virus particles must "bud" out of either the nuclear or plasma membranes. (iv) Herpesviruses can persist throughout the life span of their hosts and are characterized by ability to remain latent in specific cell types. Latent viral genomes usually exist as circular episomes maintained either by cellular factors and/or a small subset of virus-encoded latency-associated proteins. Little or no lytic cycle gene expression occurs during herpesvirus latency.

1. Virion

Herpesvirus particles (or virions) are complex, and range from 180-200-nm in size. The herpesvirus genome is linear and comprises of 105-235 kilo-bases of double-stranded DNA that is wound around a proteinaceous core. The core is surrounded by an icosahedral capsid, about 95-105nm in diameter with 162 hexagonal capsomers. Outside the capsid is the tegument, a protein-filled region that appears amorphous in electron micrographs; all herpesviruses are almost indistinguishable in electron micrographs. On

the outside of the particle is the envelope, which contains numerous glycoproteins that may mediate various aspects of the virus life cycle, including attachment, entry, and spread.

2. Genome

Herpesvirus genomes are large (up to 235-kbp DNA). Although the genomes of herpesviruses may be organized in similar gene blocks, the genomic sequence and encoded proteins of individual herpesviruses may differ substantially. Thus, all herpesvirus genomes are characteristically flanked by inverted repeats and contain multiple repeated sequences positioned at unique regions within the genome; depending on the number of these repeated sequences, the genome size of various isolates of a particular herpesvirus can vary by up to 10-kilo-base pairs. In cases where minor differences are detected in the size of individual isolates of the same virus, such differences are often due to variations in the number of internal and terminal repeat regions. Therefore, herpesvirus DNA can be distinguished not only on the basis of size, but also on genomic composition and structural arrangement of unique as well as re-iterated blocks of sequences in the genome (Fig. 1.1A).

3. Classification

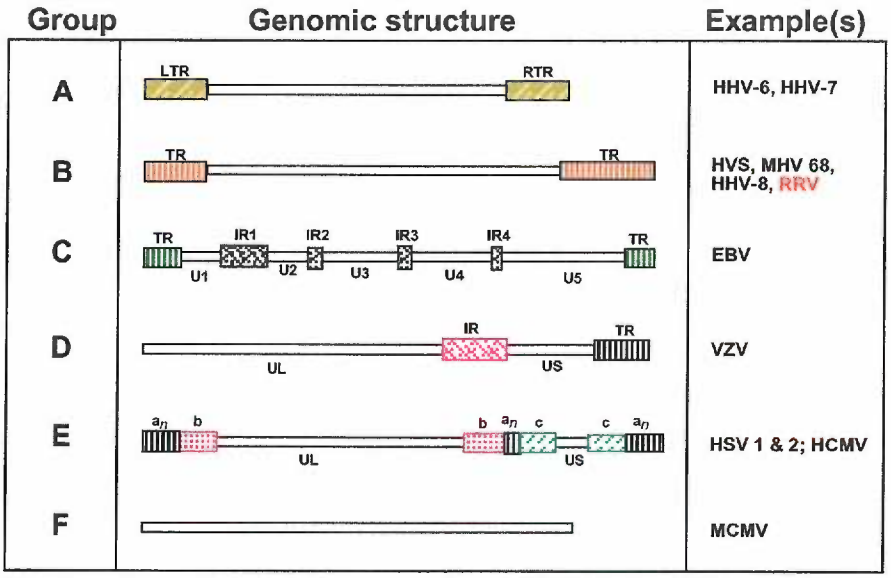
Herpesviruses are divided into six structurally distinct groups A to F, based on presence and location of repeated DNA sequences at least 100 base pairs in length (Fig. 1.1A). Group A herpesviruses (including HHV-6 and HHV-7) contain a large left

terminal repeat (LTR) sequence that is directly duplicated at the right terminus to form the right terminal repeat (RTR). In the genomes of group B viruses (e.g., HHV-8), the terminal sequence is directly repeated several times at both ends of the genome. In contrast, group C genomes (e.g. EBV) possess both terminal and internal repeat sequences that subdivide the genome into five unique regions. In group D (e.g., VZV), the terminal region is internally repeated in an inverted orientation, so that the short unique region between the terminal repeat and this inverted form can assume two possible orientations relative to the long unique sequence. Accordingly, virions isolated from cells infected with group C viruses consist of two genome populations that exist in equimolar ratios. The genomes of group E viruses (e.g., HSV-1 and HSV-2) are perhaps the most complex. In group E viruses, sequences from both the left and right termini (i.e., $\mathbf{a}_n\mathbf{b}$ and $\mathbf{a}_n\mathbf{c}$, where "**a**" is repeated "*n*" times in each of the left and right blocks) are reiterated in an inverse orientation and then juxtaposed internally. This organization creates a genomic structure where $\mathbf{b'a'}_n$ (derived from the left end) is juxtaposed to $\mathbf{a'}_n\mathbf{c'}$ (derived from the right end), dividing the genome into two components — a unique-long (U_l) component flanked by $\mathbf{a}_n\mathbf{b}$ and $\mathbf{a'}_n\mathbf{b'}$ sequences, and a unique-short (U_s) component flanked by $\mathbf{a'}_n\mathbf{c'}$ and $\mathbf{a}_n\mathbf{c}$ sequences. Both U_l and U_s segments are therefore separated by "*n*" inverted repeats derived from either terminus. The "a'_n" repeats allow rearrangements of the U_l and U_s, sequences that can then be organized in two distinct orientations relative to each other, resulting in four isomeric genome populations of virions. Finally, group F viruses, exemplified by murine herpesvirus 1, may have the simplest genome structure with no repeat regions anywhere in their genomes.

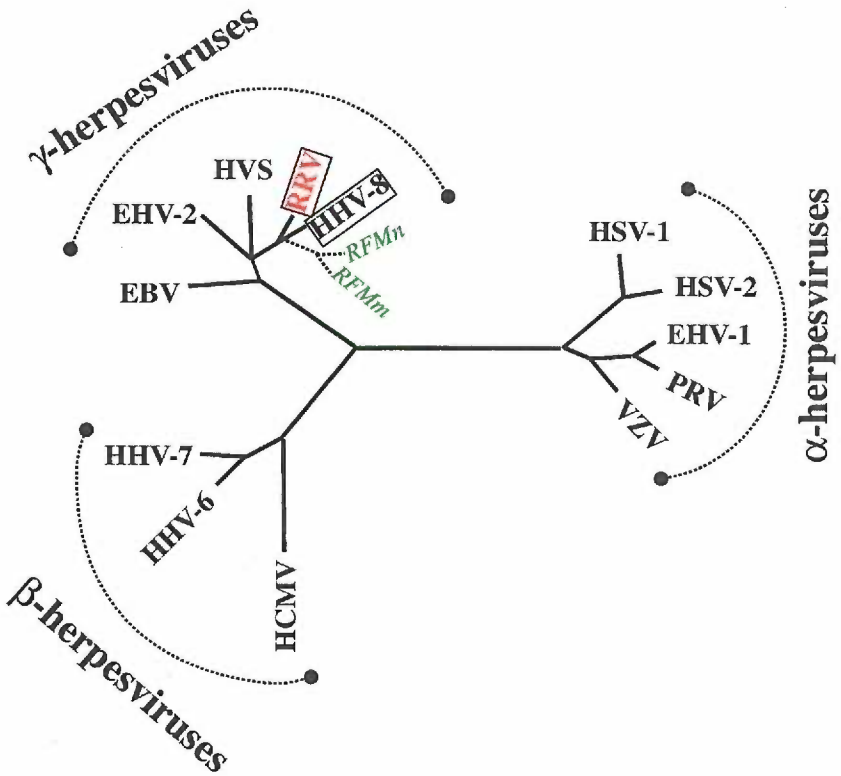
Fig 1.1: Herpesvirus genome classifications — Panel **A** shows taxonomic differences between herpesvirus genomes. LTR, left terminal-repeat; RTR, right terminal-repeat; TR, terminal-repeat; IR, internal repeat; U, unique sequence; UL, long unique sequence; US, short unique sequence; HV, herpesvirus. Designations $\mathbf{a}_n\mathbf{b}$ and $\mathbf{a'}_n\mathbf{b'}$ represent sequences "a" and "b", where "a" is repeated "n" times and then juxtaposed to sequence blocks "b" and b' that flank the UL region. Sequences $\mathbf{b'a'}_n$ and $\mathbf{a'}_n\mathbf{c'}$ represent internal, inverse orientations of sequence blocks $\mathbf{a}_n\mathbf{b}$ and $\mathbf{a}_n\mathbf{c}$ that divide the genome into a U_l and a U_s component. *Based on the original figure in ref. (266).*

B, phylogenetic classification of herpesviruses based on comparison of aligned amino acid sequences either from single genes or combined gene sets. The tree, derived from bootstrap analysis, is shown in un-rooted form with branch lengths representing divergence between the nodes that divide each branch. The phylogenetic distances between RRV, *RFMn*, and *RFMm* are arbitrarily drawn in, and are merely based on limited sequence data for the DNA polymerase gene that is conserved among the known gamma-herpesviruses (47, 414). Please see the "Selected Abbreviations" section of this thesis for definitions of HSV, EHV, PRV, VZV, HCMV, RRV, *RFMn*, and *RFMm*. *The tree was adapted from the original figure in ref. (181).*

a


Group
Genomic structure
Example(s)
A
LTR
RTR
HHV-6, HHV-7
B
TR
TR
HVS, MHV 68, HHV-8, RRV
C
TR
IR1
IR2
IR3
IR4
TR
U1
U2
U3
U4
U5
EBV
D
IR
TR
UL
US
VZV
E
an
b
b
an
c
c
an
UL
US
HSV 1 & 2; HCMV
F
MCMV

b


γ-herpesviruses
HVS
RRV
HHV-8
EHV-2
RFMn
RFMm
EBV
HSV-1
HSV-2
EHV-1
PRV
VZV
α-herpesviruses
HHV-7
HHV-6
HCMV
β-herpesviruses

Based on genomic organization, biological characteristics, and tissue tropism, herpesviruses are classified into three taxonomic subfamilies — alpha-herpesviruses (α), beta-herpesviruses (β), and gamma-herpesviruses (γ) (Fig. 1.1B). The three (α, β, and γ) subfamilies are further subdivided into genera based on DNA sequence homology, similarities in genomic sequence arrangement and relatedness of virus-encoded proteins (409).

The **alpha-herpesvirinae** includes the genera *Simplexvirus* (e.g., human simplex virus, HSV-1 and HSV-2) and *Varicellovirus* (e, g., varicella zoster virus, VZV). Members of the α-herpesvirinae subfamily have a broad host range and may infect other, related species in addition to the natural host species. The α-herpesviruses exhibit a relatively short productive cycle in epithelial cells and spread very efficiently in tissue culture. A major hallmark of the α-herpesvirus subfamily is the capacity to establish latent infection primarily, but not exclusively, in neurons (412).

The **beta-herpesvirinae** subfamily is exemplified by the genera *Cytomegalovirus* that includes human cytomegalovirus (HCMV) and murine cytomegalovirus (MCMV). The β-herpesviruses have a restricted host range but can replicate in a variety of cell types in vivo including epithelial cells, although the range of permissive host cells is more restricted in vitro. Beta-herpesvirus infections progress slowly and are usually accompanied by cell enlargement (cytomegaly) as well as appearance of characteristic nuclear inclusion bodies. These inclusion bodies are formed by the accumulation of defective particles containing enveloped viral proteins without DNA or assembled capsids. The β-herpesviruses can be maintained in a latent form in various tissues,

including secretory glands and kidneys.

The **gamma-herpesviruses** are known to replicate and persist in lymphoid cells and tissue, although some of these viruses are capable of lytic replication in epithelial cells and fibroblasts. The γ-herpesvirus subfamily consists of two genera: *Lymphocryptoviruses* (or gamma-1 herpesviruses) include Epstein-Barr virus (EBV, or HHV-4) and related viruses of Old World primates such as *Herpesvirus pan* of chimpanzees, *Herpesvirus orangutan* of orangutans, and *Herpesvirus papio* of baboons. γ-1 herpesviruses share tropism for B cells and several of the structural and non-structural proteins encoded by γ-1 herpesviruses are antigenically related, especially among the primate viruses (90, 175, 258). The second genera of γ-herpesviruses, the *Rhadinoviridae* (or γ-2 herpesviruses), is typified by *Herpesvirus saimiri* (HVS) of squirrel monkeys, *Equine herpesvirus* (EHV) of horses, ateles virus of the spider monkey, and *Murine herpesvirus 68* (MHV-68). Human herpesvirus 8 (HHV-8), also known as *Kaposi's sarcoma-associated herpesvirus* (KSHV), and the most recently described *Rhesus monkey rhadinovirus* (RRV) are classified as γ-2 herpesviruses because the genomes of these viruses are similar to that of HVS, the prototypic member of the *Rhadinoviridae* genera (Fig. 1.1B).

4. Herpesvirus replication

Herpesvirus replication occurs in the infected cell nucleus, so that herpesviruses must infect a target cell to multiply. Herpesviruses are therefore referred to as obligate intracellular parasites. A number of steps are involved in the replication cycle of all

herpesviruses (412). **Attachment**/adsorption of the viral envelope to either cell-specific or widely expressed receptors on the plasma membrane of the target cell precedes fusion events between the viral envelope and the cellular membrane. Examples of cellular receptors used by herpesviruses include CD46 for HHV-6 (427), the C3d receptor (CR2)/CD21 for EBV (146, 154), a 60/56K cellular mediator for BHV-1 (483, 504), CD4 for HHV-7 (283), and herpesvirus entry mediator (HVEM) for HSV-1 (289). Viral attachment is followed by **penetration**, un-coating and release of the capsid (a DNA-protein complex) into the infected cell cytoplasm. The DNA-protein complex is then translocated into the cell nucleus where viral DNA is **transcribed.** The synthesized messenger RNA (mRNA) transcripts are subsequently transported to the rough endoplasmic reticulum (ER) where **translation** of viral regulatory and structural proteins occurs. **Replication** of viral nucleic acid, facilitated largely by virus-encoded enzymes, occurs in the nucleus and replicated DNA is then extruded into preformed immature nucleocapsids that subsequently undergo maturation and **assembly** into mature capsids. Capsids acquire an envelope by "budding" either through the nuclear membrane and/or through other cell membranes containing pre-synthesized viral as well as cellular glycoproteins. Some viral envelope glycoproteins mediate virus attachment to cellular receptors on target cells; therefore, the capacity of virus progeny to infect new cells is acquired during **budding**, a process that is also referred to as **egress**. Because of the unspecific nature of the budding event, some cellular glycoproteins may become incorporated into the viral envelope when progeny virus is finally **released** into the extracellular environment. Conceivably, cellular proteins that are incorporated into the

virion in this manner could play a beneficial role for the virus either as cognate partners for receptors on target cells, or as fortuitous "decoys" for immunological escape since, as "self" components, these cellular proteins would be "ignored" by the immune system.

Herpesviruses encode several proteins (kinases and other enzymes) that are involved in the complete replication of the genome, although other components of the host machinery may also facilitate the replication process at various stages of the virus life cycle. During the initial stages of infection, herpesvirus genes are transcribed in a coordinated or "temporal" fashion that follows a cascade divided into immediate early (IE), early (E) and late (L) programs (409-411). The IE or *alpha* genes are those that are transcriptionally activated only a few hours following infection, and therefore do not require *de novo* protein synthesis. The protein products of IE genes often function as enzymatic regulators of subsequent viral gene expression. On the other hand, E or *beta* genes are transcriptionally activated only following prior (i.e., IE) viral gene expression. The E genes are expressed prior to or concomitant with viral DNA replication, and protein products of E genes are directly involved in replication of the viral genome. Finally, the late (L) or *gamma* genes are those that are transcriptionally activated only after the on-set of viral DNA replication. L gene products generally make up the structural components of mature virus progeny but may occasionally play a role in the maturation process itself.

Because of the temporal nature of gene expression, herpesvirus genes involved in lytic replication can be empirically defined for each virus by dissecting those genes that are transcribed within the IE, E and L programs. This determination is achieved by treatment of

infected cells (at defined time points post-infection) with drugs that specifically block certain biochemical features of viral DNA replication and/or viral protein synthesis, followed by Northern blot analysis of total or poly-adenosine-selected RNA transcripts isolated from infected cells. Drugs that are typically used in such studies include cycloheximide (CHX) and phosphonoacetic acid (PAA). CHX inhibits protein translation (86, 187, 535) and therefore inhibits downstream viral replication and maturation events that rely on *de novo* synthesis of viral or cellular proteins involved in these processes. On the other hand, PAA inhibits virus-induced DNA polymerase and therefore blocks viral DNA replication (11, 208, 375). The use of these drugs not only makes permits classification of viral genes into the IE, E, and L categories but also facilitates determination of genes that are dispensable for lytic replication in vitro. Northern analysis and drug-inhibition data from these studies can then be used to construct replication-competent deletion mutants of viruses that can be grown to high titer in vitro and then used in animal studies to define the pathogenetic relevance of the deleted gene(s) in vivo. This approach has been used as a standard for delineating viral genes that may not required for growth in culture but are necessary for viral pathogenesis in vivo (88, 114, 125, 145, 309, 310).

5. Herpesvirus interactions with the host

The symptoms of viral diseases are the culmination of a series of interactions between the virus and the host, determined both by viral and host factors. Virus infection of a host is normally followed by two possible outcomes — lytic and/or persistent infections, both of which may or may not occur during infection with a single virus.

During lytic infection, virus replication results in the death of the host cell, while persistent infection usually preserves the host cell. Persistent infection is further divided into (a) "chronic" or productive infection, in which infectious virus is continuously produced and can be recovered by conventional methods, and (b) latent infection, in which replicative viral genomes can be detected but infectious virus is not produced except in some rare cases of re-activation.

Because preservation of the infected cell is very critical for virus survival, inherently persistent herpesviruses should also be capable of minimizing overt cytolysis of the infected cell, mainly by regulating viral lytic (productive) potential. In addition, herpesviruses as a family have also evolved various strategies for interaction with the host, mainly by targeting components of the immune system in order to survive for long periods of time in the harsh environment of host immunity (389, 458, 459, 462, 519, 521).

6. Regulation of cytokine function by viruses

Compelling evidence from a number of experimental systems indicates that cytokines play an important role in host anti-viral responses (142, 184, 255, 399). In turn, many viruses have evolved several elaborate mechanisms to circumvent, bias, or interfere with cytokine-mediated responses in order to enhance virus survival in the host. These include: (i) expression of cytokine-like molecules ("virokines") that may play an important role in the virus life-cycle (253), (ii) expression of viral proteins that interact with intracellular cytokine signaling pathways (106), and (iii) induction of host proteins

that bias or antagonize cytokine responses (228, 445). Some of the viral proteins that have been reported to interfere with host cytokine (or chemokine) function include the Epstein-Barr virus (EBV) protein BCRF1 (viral IL-10) (323), viral IL-17 (530), viral IL-8 receptors (3), as well as viral chemokine receptor homologues (3, 66, 165, 308, 452). Some viruses encode cytokine-binding proteins ("viroceptors") that resemble the corresponding cellular cytokine receptors and are therefore able to bind and sequester the active cytokines in vivo (307, 308). Viroceptors may also function to inhibit the local inflammatory response elicited to kill virus-infected cells, consequently prolonging the life of persistently infected cells. The importance of "viroceptors" to virus pathogenesis is further underscored by the finding that recombinant viruses lacking these "cytokine receptor-like" proteins display decreased virulence in vivo (452, 458).

Although many of the above strategies may be thought of as generally beneficial to the virus, virus-induced perturbations in host homeostasis can also result in a number of pathological expressions that may lead to morbidity and eventual mortality of the host (237, 324, 371, 468). For instance, during immunosuppression when the host response to viral infection is most impaired, some herpesvirus infections are associated with syndromes that may not necessarily be the "intended objective" of the virus but instead result from a physiological imbalance caused by the infection process itself. Therefore, identification of virus-encoded proteins with functional implications for virus survival and pathogenesis may lead not only to elucidation of the basic biology of these viruses but also to development of therapeutic strategies against infectious disease.

7. Virus-induced oncogenesis: the gamma-herpesviruses

More than any other herpesvirus subfamily, gamma-herpesvirus infections have been associated with overt malignancy, lymphoproliferative disease, as well as oncogenesis in experimental animals (12). Mechanisms by which individual gamma-herpesviruses alter the growth properties of the infected cell may vary from virus to virus (99), although recent studies reveal three general themes in this regard. Firstly, gamma-herpesviruses encode homologues of host genes that are normally involved in promoting cell growth (107, 346, 356, 420). Secondly, gamma-herpesviruses express viral proteins that are not themselves homologues of host genes but function to target specific host signaling pathways involved in regulation of cell growth and/or survival. Examples of the latter theme include STP (or saimiri transformation-associated protein) of herpesvirus saimiri (HVS) (125), LMP1 of Epstein-Barr virus (EBV) (117, 217, 332, 495), K1 of HHV-8 (261, 262), and R1 of rhesus rhadinovirus (RRV) (98, 101), all of which have been shown to exhibit signaling functions. Interestingly, all of these genes are located at the left or right ends of the respective virus genomes but share little structural relatedness, perhaps because they might have been acquired independently during evolution. One exception is that K1 of HHV-8 and R1 of RRV that are positional analogues also share substantial structural homology (10, 420, 442), reflecting the possibility that RRV is the rhesus monkey homologue of HHV-8. A third mechanism for gamma-herpesvirus-induced alteration of cell growth is direct induction of host regulatory proteins. For example, EBV infection is associated with increased expression of host IL-6, cyclin D, IL-12, complement-control protein (CCP) and IL-8 receptor, all of which are involved in

promotion of cell growth and/or prevention of complement-mediated lysis of virus-infected cells (42, 66, 450, 451, 489). This strategy contrasts that employed by HVS, HHV-8 and RRV that encode functional homologues of the same genes that EBV induces in the infected cell (9, 10, 80, 358, 420, 442). Among these, a notable observation is that EBV, HVS, HHV-8 and RRV have evolved various strategies to ensure IL-6 bioactivity during the infectious process, underscoring the importance of IL-6 in viral pathogenesis. Together, the above observations reveal how gamma-herpesviruses have evolved shared, as well as unique strategies to achieve the same objective — survival and persistence in the infected host. In spite of these shared biological properties, however, gamma-herpesviruses may also display pathogenetic differences in the respective natural infection systems (see below).

Herpesvirus saimiri (HVS)

HVS is the prototypical non-human primate gamma-herpesvirus. Although HVS remains latent in lymphocytes of the natural host (the squirrel monkey) without causing disease (115), HVS can cause lymphoproliferation and other rapidly progressing lymphomas in other New World primates, such as the common marmosets, tamarins, and owl monkeys (114, 115, 124, 394). Because of the availability of a permissive cell culture system for HVS infection in vitro (394) and for transformation assays in vivo (490), HVS has been used as a paradigm for studying mechanisms of virus-induced oncogenesis (224). For instance, the transforming potential of HVS is contained in the actions of two HVS-encoded genes, STP and Tip, that are not required for virus replication or

persistence but are essential not only for transformation of T lymphocytes to IL-2-independent growth in vitro but are also necessary for lymphoma induction in common marmosets (125).

Epstein-Barr virus (EBV)-induced oncogenesis

Primary EBV infection in humans is usually asymptotic but a proportion of EBV-infected individuals develop infectious mononucleosis (IM), Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin's disease (HD) and T cell lymphomas (238). EBV-dependent malignancies are consistently EBV-positive and, as in HVS-induced transformation, may result from EBV-induced changes in cell growth (392, 433). EBV LMP1 has been widely studied as a transforming protein that is essential for immortalization of primary B cells to lymphoblastoid cell lines (511, 512). The carboxyl-terminal domain of LMP1 is capable of interacting with TNF receptor-associated factors (TRAFs) and with TNF receptor-associated death domain (TRADD) (117, 118). This interaction is thought to activate the NF-κB pathway that regulates cell activation (217, 218, 235). As such, LMP1 appears to mimic some actions of the B cell co-stimulatory antigen CD40 that, upon interaction with CD40 ligand also associates with cytosolic cell growth-control proteins, stimulating down-stream events that ultimately result in B cell activation (178, 191). However, unlike CD40, transduction of signals initiated by interactions between LMP1 and TRAFs or TRADD occurs in the absence of extracellular ligand or cross-linking. Instead, the LMP1-induced signal is caused by multimerization of the LMP1 protein through the transmembrane domains in a manner that mimics ligand-

induced CD40 aggregation. Hence, LMP1 contributes to EBV-induced alteration of B cells using the CD40 signaling pathway but without involvement of a ligand.

Murine herpesvirus 68 (MHV-68)

MHV-68 is also a member of the γ2-herpesvirus subfamily but is more closely related to HVS and HHV-8 than to EBV (130, 508). Intranasal administration of MHV-68 results in acute productive infection of lung alveolar epithelial cells and eventually leads to a latent infection in B cells (467, 468). A major hallmark of MHV-68 infection is induction of an inflammatory infiltrate in the lungs and enlargement of the lymph nodes and spleen, presenting as alveolar effusions, lymphadenopathy and splenomegaly (469). Infectious virus is then cleared from the lungs by a T cell-mediated process about one-to-two weeks after infection (134). In keeping with one of the intriguing properties of gamma-herpesviruses, MHV-68 infection is also associated with high level expression of IL-6 (429). Interestingly, as demonstrated with IL-6 deficient mice, IL-6 is not essential for clearance of infectious MHV-68 or for establishment of viral latency (428). Therefore, the pathogenic benefit of MHV-68-induced expression of IL-6 remains unclear, although IL-6 protein induced as such may stimulate growth of B cells that are thought to serve as reservoirs for latent virus (469).

II. HUMAN HERPESVIRUS 8 (HHV-8)

Using a PCR-based technique called "representational difference analysis" (271) that is designed to identify rare differences between two complex genomes, Chang et al. (81) were the first to detect DNA sequences of a novel human herpesvirus in Kaposi's sarcoma (KS) specimens from AIDS patients. In keeping with the nomenclature adopted for the two other recently discovered human herpesviruses (i.e., HHV-6 and HHV-7), the formal designation "human herpesvirus 8" (HHV-8) was proposed by the herpesvirus subcommittee of the International Committee on the Taxonomy of Viruses (344). The first hint that KS might be caused by an infectious agent came from epidemiological studies that showed KS to be 20-fold more common in homosexual or bisexual HIV-infected patients than in HIV-infected hemophiliacs (39). This observation suggested that a factor besides HIV was critical in the development of KS. During the past decade, compelling evidence based on epidemiological, serological, molecular biological, and immunohistochemical analysis has accumulated supporting the notion that infection with HHV-8 is a risk factor for development of all forms of KS (81, 328, 465). As such, HHV-8 has also been given the informal name "Kaposi's sarcoma-associated herpesvirus (KSHV)" (267) to reflect the etiological link between HHV-8 and KS. HHV-8 has also been linked to other AIDS- and non-AIDS-related lymphoproliferative diseases (78) such as body cavity-based lymphomas (BCBL), also known as primary effusion lymphomas (PEL), lymphoblastic variants of multicentric Castleman's disease (MCD), and possibly to multiple myeloma (MM) (74, 162). Interestingly, BCBL-PEL, MCD and MM are principally or exclusively of B cell origin, suggesting that these syndromes may result

from HHV-8-induced alteration in the growth/survival patterns of a cell population in which the virus likely established a persistent infection (280, 311, 322).

(a) THE HHV-8 GENOME

HHV-8 represents the first "human" member of the γ_2-herpesvirus subfamily and has a genomic structure similar to that of other rhadinoviruses (see subgroup B, Fig. 1.1A). The HHV-8 genome consists of a contiguous 140.5-kb long-unique coding region (LUR) of low-GC content (53.3% G+C, L-DNA) with at least 89 predicted open-reading frames (420). The LUR is flanked on either side by a terminal sequence of variable length composed of repeat units approximately 800 base pairs long with an 84.5% G:C content. Because most of the ORFs in the HHV-8 LUR have homologues in other oncogenic herpesviruses, ORFs in HHV-8 were assigned names according to the homologous genes in HVS (9). Other ORFs that are unique to HHV-8 were assigned a "K" prefix.

The HHV-8 LUR has blocks of core genes that are conserved among all herpesviruses, such as structural and viral DNA replication-associated proteins. Between the conserved gene blocks lie sub-blocks of genes that are unique to HHV-8 or are found only in the Rhadinoviridae. The majority of genes in this category share sequence homology with cellular genes and display functional activity similar to that of their cellular counterparts, leading to the idea that these genes might have been "acquired" from the host at some point during virus evolution. These genes include viral homologues of cytokines and cytokine receptors, as well as genes associated with cell proliferation and prevention of apoptosis. Thus, HHV-8 encodes a complement-binding protein (ORF

4, also present in HVS), interleukin-6 (ORF K2), and three copies of macrophage inflammatory proteins (MIP, encoded by ORFs K4, K4.1 and K6). HHV-8 also encodes a homologue of bcl-2 (ORF 16), interferon regulatory factor (IRF, encoded by K9), a D-type cyclin (ORF 72), FLICE-inhibitory protein (FLIP), the adhesion molecule OX-2 (ORF K14), and a chemokine receptor (ORF 74). Other HHV-8-encoded proteins with in vitro transforming potential include a type-I transmembrane protein encoded by K1, and a small hydrophobic protein called kaposin/T0.7 (K12) (262, 343). Also found in HHV-8 is the latency-associated nuclear antigen (LANA, encoded by HHV-8 ORF 73) that is also found in HVS, BHV-4, and MHV-68, but is absent in EHV-2. HHV-8 LANA has been reported to interact with a nuclear regulator of gene expression (388), and to tether HHV-8 DNA to chromosomes during mitosis (24). As such, LANA is postulated to promote persistence and efficient segregation of episomal HHV-8 DNA in dividing cells. At least three HHV-8-encoded genes: vIL-6, a G protein-coupled receptor (GpCR), and MIP-I/II) have been shown to induce angiogenesis (15, 21, 48), an observation that has pathogenic significance since angiogenic proliferation is one of the defining features of a number of HHV-8-associated abnormalities, including KS.

(b) SERO-EPIDEMIOLOGY OF HHV-8

HHV-8 establishes a latent or persistent infection and can be reactivated under immunosuppressive conditions. Therefore, detection of antibodies against HHV-8 proteins in various populations provides important information with respect to the mode of transmission and the general epidemiology of infection. Using recombinant viral

proteins associated with lytic or latent infection, as well as HHV-8-infected lymphoblastoid cell lines, a number of sero-epidemiological studies have been performed on various patient groups and cohorts around the world (83, 264, 400). Based on several of these antibody studies, HHV-8 seroprevalence ranges from 30-100% in sub-Saharan Africa (168), approximately 10% in Mediterranean countries, 2-4% in Northern Europe, Southeast Asia, and the Caribbean, and 5-20% in the United States (83, 264, 400, 448). Remarkably, regardless of geographic location, HHV-8 seroprevalence rates are greater than 90% in people with KS and/or other HHV-8 associated diseases such as MCD and BCBL (448), consistent with a causative role for HHV-8 in all of these disorders. In HIV-1-infected persons with no signs of KS (the so-called risk group), HHV-8 seroprevalence rates are also elevated (20-50%) above those in the general population except in Southeast Asia and the Caribbean where no AIDS-associated KS has been reported (168, 448). Collectively, these serological data demonstrate that in contrast to most other human herpesviruses, HHV-8 is not ubiquitous in the general healthy population, and that HHV-8 sero-conversion may be a predictive factor for development of KS (168).

(c) ABNORMALITIES ASSOCIATED WITH HHV-8 INFECTION

HHV-8-associated abnormalities (i.e., BCBL-PEL, MCD, MM and KS) display a strikingly preponderant involvement of cells of the hematopoietic lineage (15, 193, 464, 498). Although these syndromes may have distinct patho-physiological parameters, a unifying feature among them is the propensity to occur in immunocompromized individuals especially those infected with HIV. This link suggests that HHV-8 replication

and/or gene expression may be necessary for triggering the various forms of HHV-8 disease particularly in the context of immunodeficiency (464).

(i) Body cavity-based lymphomas (BCBL)

Body cavity based lymphomas (BCBL), also known as primary effusion lymphomas (PEL) are among the less common malignant lymphomas. Most cases of BCBL-PEL have been described primarily in severely immunocompromized individuals, consistent with the notion that emergence of this class of lymphoma is favored by lack of immunosurveillance. BCBL-PELs develop in body cavities as pleural, peritoneal, and pericardial lymphomatous effusions (76, 347) without formation of a tumor mass, a phenotype that is possibly due to absence of "homing" markers on the surface of lymphoma cells (232). A B cell origin for BCBL-PEL is indicated by presence of clonal Ig gene re-arrangement (249) and expression of monotypic kappa (κ) and lambda (λ) mRNA in the cell cytoplasm (347). However, BCBL-PEL do not exhibit the molecular defects commonly associated with other neoplasms of mature B cells, such as activation of the proto-oncogenes *c-myc*, *bcl-2*, *bcl-6*, N-*ras*, and K-*ras* (70, 71, 79, 347), and cell lines derived from BCBL-PEL express the same immunological markers of the original lymphomas (19, 69, 163, 233, 405, 425). IL-6R is expressed in BCBL-PEL and the level of IL-6, a cytokine involved in B cell proliferation and differentiation, is 340-16000-fold higher in BCBL-PEL than in normal human plasma (250), suggesting that IL-6 may be part of a mechanism for persistent B cell proliferation that results in overt malignancy.

An etiological role for HHV-8 in BCBL-PEL has been suggested based on the

finding that most BCBL-PEL cells contain multiple episomal copies of HHV-8 and also express vIL-6 mRNA (76, 79, 249, 347), suggesting that vIL-6 may also contribute to the overall IL-6 bioactivity responsible for propagation of BCBL-PEL.

(ii) Multicentric Castleman's disease (MCD)

MCD, also referred to as angiofollicular hyperplasia of the lymph nodes, is a rare form of lymphoproliferative disease. The histopathological hallmarks of MCD include vascular alterations in the lymph nodes, hyperplastic expansion of the lymphoid follicles, plasmacytic lymphadenopathy as well as polyclonal hyperimmunoglobulinemia (73, 85, 158, 534). Two distinct variants of MCD with different clinical characteristics have been described.. The more common hyaline vascular type presents primarily as a solitary mass most frequently in the retroperitoneum (373), while the rare plasma cell variant is characterized by generalized lymphadenopathy (333).

Several lines of evidence support a role for IL-6 in MCD. IL-6 is present at high levels in biopsy samples from patients with MCD, and peripheral blood mononuclear cells from MCD patients secrete high levels of IL-6 (534), providing an explanation for the IL-6-related plasmacytic lymphadenopathy characteristic of MCD. Retroviral-mediated transfer of interleukin 6 into haematopoietic cells of mice also results in a Castleman's disease-like syndrome accompanied by anemia, polyclonal hypergammaglobulinemia, splenomegaly and peripheral lymphadenopathy (58, 59). Moreover, treatment of MCD with a monoclonal antibody against IL-6 has been reported to have therapeutic benefits (365), further supporting IL-6 deregulation as necessary for

induction/propagation of MCD.

Both HIV-infected and uninfected individuals can develop MCD (77), although lymph nodes of HIV patients with MCD show a high rate of HHV-8 infection (61). In fact, the strong association between MCD and KS in AIDS patients is consistent with a shared etiological entity — HHV-8 — in both these syndromes (369, 378, 457). HHV-8 may act as a cofactor for MCD perhaps by deregulating IL-6 bioactivity (158) since, as in BCBL-PEL, HHV-8 vIL-6 has been suggested to act as an autocrine or paracrine factor in the lymphoproliferative processes of MCD (378, 464). Moreover, the recent finding that vIL-6 induces production of host IL-6 in established cell lines and in MCD-derived cells provides further support for the pathogenic role of this viral cytokine in MCD (330).

(iii) Multiple myeloma (MM)

MM is a neoplasm that affects the late B cell differentiation stage, the plasma cell (536). MM is characterized by the accumulation of immunoglobulin (Ig)-secreting plasma cells in the bone-marrow and by the presence of a monoclonal Ig produced by the malignant plasma cells in biological fluid (e.g., urine and serum) of affected individuals (188). Fluorescent in situ hybridization, spectral karyotyping and comparative genomic hybridization have revealed that chromosomal abnormalities exist in the malignant cells of most, if not all, myeloma patients, and this discovery has led to the identification of a number of oncogenes that may play a major role in the transition to MM (62, 128, 220, 268, 536).

Factors that control the growth and survival of myeloma cells are still under

investigation. However, recent research in the biology of multiple myeloma (MM) has yielded evidence for the pathogenic role of cytokines such as interleukin-6 (IL-6) in the conversion of monoclonal gammopathy of undetermined significance (MGUS) to MM and in the proliferation and survival of myeloma cells themselves (13, 447). Secretory products of the bone-marrow microenvironment, such as cytokines released from the stromal cells that are themselves not part of the malignant population, may also serve as paracrine support for stimulation of myeloma cell growth (13).

What is the role of HHV-8 in MM? In all the disorders to which HHV-8 has been linked, viral DNA sequences have been localized to the pathogenic cells. In MM, however, a viral etiology has been much less demonstrable and therefore more controversial than for other syndromes (108, 162, 284, 313, 438, 531). Using nested PCR, HHV-8 genes ORF K7 (T 1.1) and ORF 72 (v-Cyclin D) were detected in long-term bone marrow stromal cells from 92% of MM patients (84). In addition, Rettig et al. (406) detected HHV-8 DNA in the adherent non-malignant dendritic cell population of long-term bone-marrow cultures from 15 patients with MM, and in one out of four patients with monoclonal gammopathy of undetermined significance (MGUS), but not in myeloma cells themselves. Subsequently, HHV-8 was demonstrated in bone-marrow biopsy specimens and in peripheral blood from most patients with myeloma using primer pairs from many different HHV-8 ORFs, including vIL-6 (84, 166, 397, 423). Because growth and survival of MM cells as well as MM-derived cell lines rely on the function of IL-6 (196, 447), vIL-6 expression in bone-marrow of myeloma patients suggests that this viral protein may contribute to the mechanism whereby the interaction between the bone

marrow microenvironment, B cells, and HHV-8-infected vIL-6-expressing dendritic cells may provide the relevant viral stimuli for myeloma cell growth. Based on this model, clinical features of MM and secondary diseases such as hemolytic autoimmune anemia and monoclonal/polyclonal gammopathies could result from B cell expansion due to vIL-6 bioactivity. In support of this model, vIL-6 was found to exert proliferative effects on a myeloma-derived cell line (65); demonstration of a similar in vivo function will await further investigation. Although the evidence regarding the etiological link between HHV-8 and MM is still controversial (32, 33, 108, 190, 319, 438, 480, 531), localization of HHV-8 in bone-marrow dendritic cells of MM patients may provide a local source of vIL-6 that could, along with other factors in the bone-marrow microenvironment, support MM growth and survival.

(iv) Kaposi's Sarcoma (KS)

KS was first described by the Austro-Hungarian dermatologist Moritz Kaposi in 1872 as a multi-pigmented skin disease (231). KS is now referred to as an angio-proliferative disorder usually appearing as brownish-purple lesions on extremities but may, in more aggressive forms of the disease progress to involve organs such as lungs, lymph nodes and the gastro-intestinal tract. A variety of clinical forms of KS have been described (63) (also see Fig. 1.2). Thus, "iatrogenic" KS is associated with prolonged immunosuppression and usually breaks out in solid organ-transplant patients (380, 381), while "endemic" KS is common in parts of Central Africa. On the other hand, "classical" KS is found in older men of Eastern Europe or of Mediterranean origin and usually shows an indolent clinical course, while "AIDS-associated KS" (AIDS-KS) is common in

individuals infected with HIV (416).

All the necessary co-factorial components underlying the different forms of KS are still unclear, although these components may be reflected in the diverse epidemiological expressions of the lesion. For instance, unlike endemic or classical KS whose co-factorial determinants are less clear, both iatrogenic and AIDS-KS show a more aggressive clinical course. In fact, AIDS-KS is the most common proliferative complication in HIV-AIDS patients, perhaps because immunological debilitation subsequent to HIV infection provides the proper setting for development of KS. The preponderance of AIDS-KS in young homosexual men was indeed one of the first signs of the AIDS epidemic (35, 39, 94, 174, 183, 225, 422). The more aggressive course of AIDS-KS has been attributed to the effects of HIV-1 Tat protein that is known to enhance growth of cultured KS cells (139, 140). The role of HIV-1 Tat in this regard is supported by epidemiological findings that although HHV-8 seroprevalence is high in the Gambia (where HIV-2, but not HIV-1, is the common strain), KS is rarely seen in Gambian AIDS patients compared to Central Africa where HIV-1 is the common strain (18). Accordingly, the few cases of KS in Gambia are found only in individuals infected with HIV-1, rather than HIV-2, implying that infection with HIV-1 may provide a more suitable co-factorial background for development of KS. There is no explanation as to why Tat of HIV-2 would not provide the same regulatory effect as Tat of HIV-1 in this context.

The extent to which HHV-8 may be involved in the development of KS is still a matter of debate. In one model, HHV-8 may act only as a "by-stander" virus around an

already-formed tumor, perhaps indirectly recruited to the site as an episome within the infected mononuclear cells that characteristically infiltrate the tumor (322). In another model, HHV-8 could act as a transforming virus (440), although this model would be inconsistent with what is known of most tumor viruses (e.g., EBV, HPV) (393) that are relatively more widespread than the tumors they cause; KS is rare except in situations of immunosuppression. In addition, the polyclonal proliferative process in KS differs from the virus-transformed cell paradigms of other transforming viruses, where cell growth is usually monoclonal; therefore, HHV-8 does not exhibit the traditional properties of a transforming virus (329). As such, a causative role for HHV-8 in KS and other diseases to which it is etiologically linked (126) will only be established by experimental scrutiny of the expression patterns of HHV-8-encoded genes in these syndromes, most suitably in an animal model of HHV-8 infection.

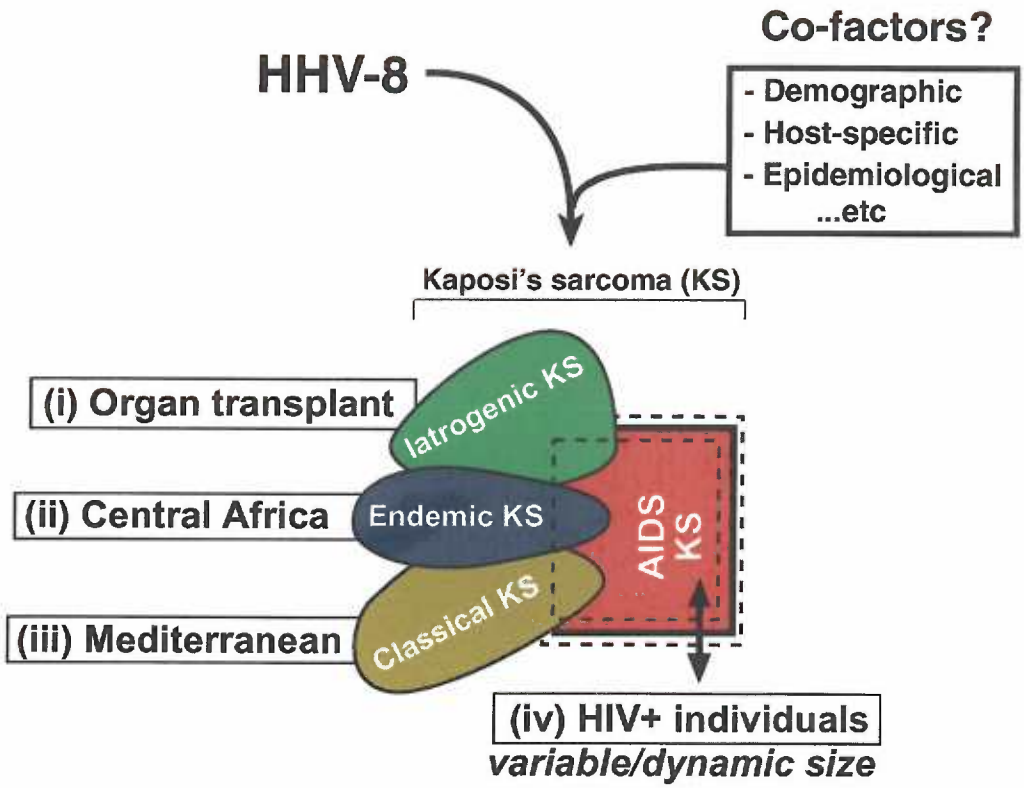
Cell biology of KS

The KS lesion is predominantly comprised of elongated spindle cells surrounded by irregular slit-like vascular spaces. Consistent positive staining of KS tissue for endothelial cell antigens supports an endothelial origin for KS (417), and strengthens the belief that spindle cells in the lesion represent differentiated endothelial cells following activation by angiogenic and pro-inflammatory cytokines (537) (also see Fig. 1.3). However, presence of other cells in KS suggests a multi-lineage origin for the lesion (416, 494, 516). For instance, the KS lesion stains positive for markers of lymphatic tissue at the early patchy stage (518), for vascular endothelium at a later stage (30, 421),

and for smooth muscle at the final plaque or angiofollicular stage (486). While such phenotypic transitions may provide critical information about the genesis of KS, a potential multi-lineage origin of the tumor raises the question as to whether cells of different lineages can adopt spindle-like morphology in the presence of the appropriate stimuli.

Fig. 1.2 — Clinico-epidemiological forms of KS. Although infection with HHV-8/KSHV may be critical for KS histogenesis, emergence of the various KS histotypes appears to be influenced by demographic and epidemiological co-factors. Iatrogenic KS breaks out in a finite number of people under generalized immunosuppression during and/or following organ transplant surgery. The co-factors underlying endemic and classical KS are still not known, but HIV may not be required. On the other hand, HIV is a critical co-factor in the more aggressive AIDS-KS. Because of the HIV component in AIDS-KS, the number of people presenting with this form of KS may vary over time (i.e., "dynamic") due to (i) undocumented cases of HIV infection, (ii) indeterminate changes in sexual behavior, or (iii) improved availability of highly active anti-retroviral therapy (HAART), all of which are likely to impact the rate of HIV dissemination.

Clinico-epidemiological forms of Kaposi's sarcoma



Patho-physiology of KS

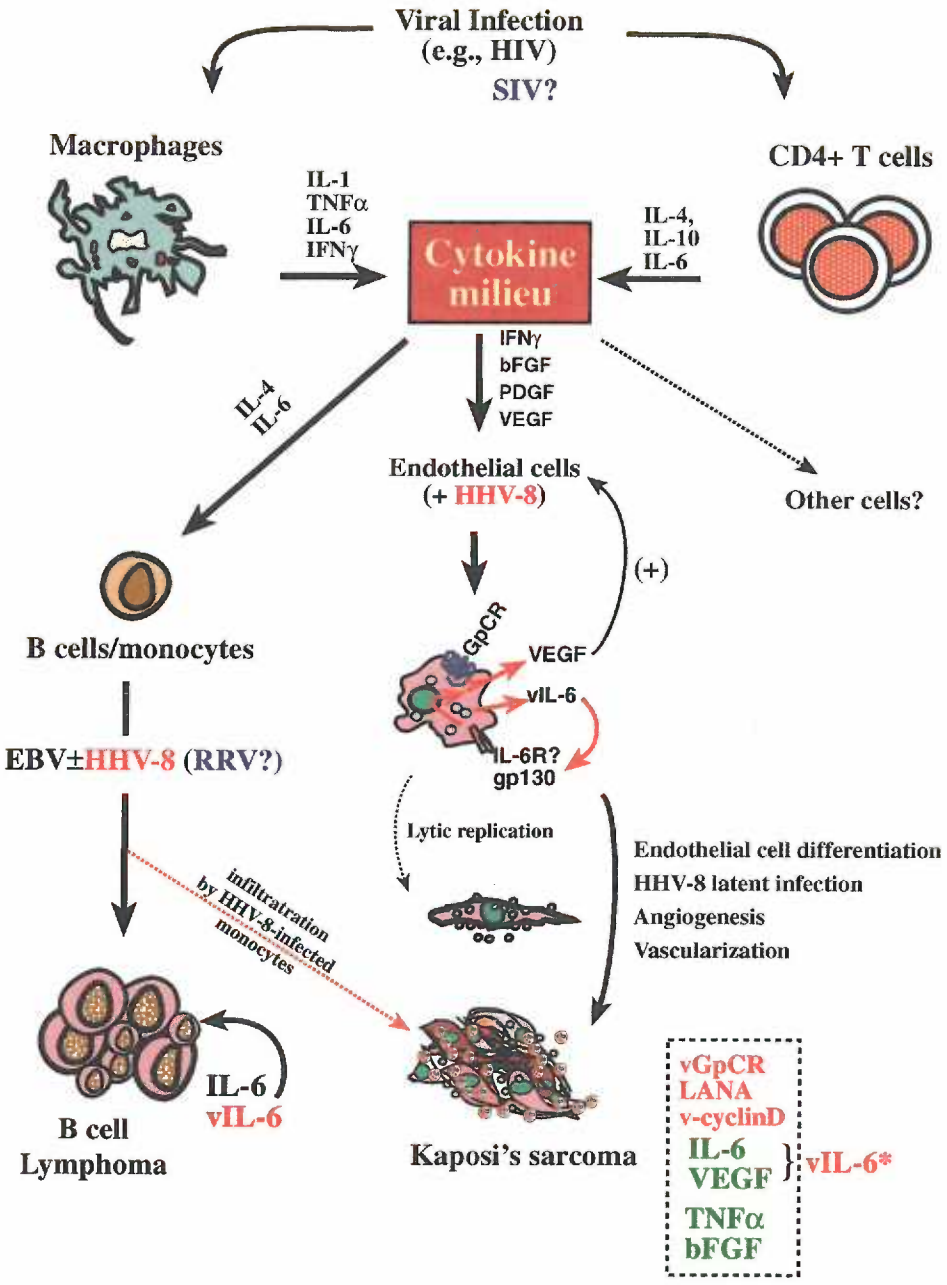
Controversy still exists regarding whether KS is a true neoplasm. Some argue that KS is merely a cytokine-mediated angio-proliferative disorder that, at least in the initial stages, is promoted by autocrine/paracrine cytokines and angiogenic growth factors (34, 141, 314, 315, 353). However, studies of the role of growth factors in KS have produced inconsistent findings, possibly because of use of different cell types. For example, basic fibroblast growth factor (bFGF), an angiogenic factor that also induces IL-6 synthesis (254) is a major stimulus for normal endothelial cell proliferation and is also secreted by KS cultures (141), suggesting that bFGF may promote growth of KS cells in vivo. In addition, platelet-derived growth factor (PDGF) and the PDGF receptor are expressed by normal endothelial cells and by short-term cultures of KS cells (141, 417), suggesting that PDGF too, may exert an autocrine effect on KS cells. Interleukin-1 (IL-1) has also been reported to be secreted by cultured KS cells of endothelial phenotype (141), although the stimulatory role of IL-1 for KS is much less clear than that ascribed to bFGF or PDGF. Oncostatin M (OSM), one of the members of the IL-6 cytokine family, has also been implicated in the proliferative function of KS cells, based on ability to support growth of KS-derived cell lines (314).

Of all the cytokines and/or growth factors that have been suggested to support KS cell growth, the evidence for the role of IL-6 is perhaps most compelling. Specific anti-sera to human IL-6 and other IL-6-inducible pro-inflammatory cytokines were shown to inhibit the in vitro growth of cultured AIDS-KS cells (141). In addition, cultured KS cells express mRNA for IL-6, secrete IL-6 protein, and proliferate in response to IL-6 (315).

Data supporting a strong link between IL-6 bioactivity and KS development and propagation are of particular interest in light of the fact that HHV-8 encodes a homologue of IL-6 (vIL-6). vIL-6 is rarely expressed in HHV-8-infected KS spindle cells but is highly expressed in HHV-8-infected monocytes and B cells that surround or infiltrate the tumor (326). Although vIL-6 may not be highly expressed in the lesion itself, vIL-6 secreted by HHV-8-infected monocytes and B cells could not only stimulate KS cell growth but also promote virus dissemination by propagating the infected cell reservoir. Moreover, the ability of vIL-6 to induce expression of endogenous IL-6 (330) may provide an additional mechanism for enhancing the overall IL-6 bioactivity in the vicinity of the lesion.

Fig. 1.3 — Virus-induced inflammation and tumorigenesis. Viral infection of macrophages, CD4+ T cells (or other cell types) induces production of pro-inflammatory cytokines that can stimulate proliferation of B cells, monocytes, endothelial cells, and other cell types. Enhanced cytokine-driven replication of these cells in the presence of oncogenic viruses like EBV (HHV-8 or RRV?) creates a setting for persistent infection and eventual development of virus-associated malignancies. Many B cell lymphoma histotypes are EBV^+ while others may be positive for both EBV and HHV-8. While the pathological consequences of HHV-8 infection of B cells and monocytes are not defined, HHV-8 infection of endothelial cells is thought to initiate a cascade of events that results in endothelial cell differentiation into spindle-shaped cells that form a major component of the KS lesion. Subsequent angiogenesis, vascularization, and infiltration of the lesion by HHV-8-infected monocytes all contribute to the complex cellularity of KS. In both B cell lymphomas and KS, ongoing production of cytokines and other growth factors by the tumor cells may be critical for maintenance of the malignant state. *This drawing was modified from the original (Fig. 12.11, p. 308) in ref.* (266).

Virus-induced inflammation and tumorigenesis



(d) In vitro culture of HHV-8

A major challenge in the field of HHV-8 research remains the lack of an animal model or a cell–culture system that allows high-titer propagation of HHV-8. Because of this limitation, the basic biology of HHV-8 has only been studied in vitro at the level of individual genes using heterologous expression systems, rodent fibroblast transformation assays, and HHV-8^+ KS-derived cell lines (49). However, many of these in vitro systems may not fully elucidate the infectious process of HHV-8 in vivo. With respect to in vitro cultivation, HHV-8 has been isolated and propagated in the human kidney cell line 293 (153, 505), but others have failed to repeat these findings (404). HHV-8 has also been cultivated in primary dermal micro-vascular endothelial cells (331, 376) and primary bone marrow endothelial cells (150). In addition, PEL-derived cell lines latently infected with HHV-8 have also been shown to support limited lytic replication after stimulation with phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (79, 405), and have therefore been invaluable in studying the molecular switch between latent and lytic infection in vitro. Based on induction of lytic gene expression in PEL-derived cell lines, three classes of HHV-8 transcripts have been characterized (430). Class I genes (e.g., v-FLIP or v-Cyclin D), are expressed constitutively without chemical induction, and are generally associated with latent infection. Class II genes (e.g., K7, or T1.1/nut) are constitutively expressed at low levels but their expression increases following induction, and they are associated with both latent and lytic infection. On the other hand, class III genes mainly represent viral structural genes, and can be detected only following appropriate stimulation; these are exclusively associated with lytic infection (539).

(e) HHV-8 open-reading frame K2 (viral IL-6)

HHV-8 open reading frame K2 encodes a viral homologue of IL-6 (vIL-6) that shares 24.7% amino-acid identity with human IL-6, and also displays significant conservation of residues in the receptor-binding domain (226, 326). vIL-6 can substitute for human IL-6 in supporting growth (and inhibition of apoptosis) of both murine and human IL-6-dependent cell lines (65, 326, 359). vIL-6-mediated stimulation of cells can be blocked by antibodies against IL-6R and gp130 subunits of the IL-6 receptor system, albeit with varying levels of sensitivity relative to human IL-6 (65, 359). Compared with cellular IL-6, however, 1000-to-4000-fold more vIL-6 protein is required to achieve the same level of activity, implying that the two proteins differ in receptor-binding properties that may account for the greater amounts of vIL-6 necessary for biologic activity in vitro.

(f) Interactions between vIL-6 and the IL-6R/gp130 receptor subunits

Because of the poor sensitivity of vIL-6 to the inhibitory effects of anti-IL-6R (359), participation of IL-6R in vIL-6 signaling has been questioned. In a study that was the first to provoke this debate, IL-6R-minus BaF/3 cells transfected with gp130 (BAF-130) responded to vIL-6 but not to human IL-6, supporting the notion that IL-6R may not be necessary for vIL-6 function (321). In another study by Burger et al (65), anti-IL-6R only blocked human IL-6 but not vIL-6, while a combination of anti-IL-6R and anti-gp130 antibodies blocked the proliferative effect of vIL-6 on the IL-6-dependent human myeloma INA-6 cell line; anti-IL-6R alone failed to block vIL-6 in this study. Recently, vIL-6 was suggested to have a lower binding affinity for IL-6R than cellular IL-6 (510),

perhaps due to amino-acid substitutions at positions that are critical for receptor binding, although the approach used by the authors did not directly address the role of IL-6R in vIL-6 signaling. A subsequent report showed that vIL-6 could bind soluble gp130 in vitro but failed to bind soluble IL-6R (336), supporting the conclusion by Molden et al. that vIL-6 may interact with membrane-bound gp130, but not with IL-6R (321).

Because vIL-6 may directly activate gp130 without the need for initial interaction with IL-6R, the viral protein may as such behave much like OSM (Fig. AIII.3), a member of the IL-6 cytokine family that is known to bind directly to, and stimulate heterodimerization of gp130 with LIF-R (171). However, OSM and vIL-6 do not share any significant homology, ruling out the implied possibility that LIF-R might form part of the receptor for vIL-6. Clearly, the minimal receptor requirements for vIL-6 are still poorly defined and additional studies are desperately needed before interactions between this cytokine with its receptor(s) can be fully understood.

(g) The role of vIL-6 in HHV-8 pathogenesis

The observation that HHV-8-associated diseases (i.e., KS, BCBL-PEL, MCD, and MM) can respond to, and/or require IL-6 for growth is a unifying feature among these syndromes, and supports a role for HHV-8-induced deregulation of IL-6 bioactivity in the disease process. In accordance with this view, nearly all HHV-8-infected cells make vIL-6 protein and both human and viral IL-6 proteins are expressed in some cells that infiltrate the KS lesion (326). Even more remarkable is the finding that vIL-6 can induce production of endogenous IL-6 (330), a setting that is expected to enhance the overall IL-

6 bioactivity. Although the relevance of vIL-6 for KS pathogenesis was initially questioned (as KS-resident spindle cells were shown to express only gp130, the second subunit of the IL-6 receptor) (341), vIL-6 was subsequently reported to elicit IL-6-like responses by direct activation of gp130 in the absence of IL-6R (202, 321). This property that may have profound implications for proliferative disease since almost all cells express gp130 and can therefore respond to vIL-6.

vIL-6 displays a distinct pattern of expression in the various HHV-8-associated abnormalities (126, 464), perhaps reflecting a relationship between vIL-6 expression and disease. Using in situ hybridization and immunohistochemistry, Cannon et al. found that vIL-6 was consistently expressed in PEL and in a case of HIV/AIDS-associated lymphadenopathy resembling MCD (68). In contrast, vIL-6 expression was much less demonstrable in most KS tissue specimens, except those from HIV/AIDS patients (326, 464). Predominantly positive for vIL-6 expression were the KS-infiltrating mononuclear cells, and some spindle-shaped cells that also showed high level of the T1.1 lytic transcript (464). Thus, patterns of vIL-6 expression appear to correlate with other measures of the lytic viral cycle, and could be an important parameter for defining the pathogenic process in HHV-8-associated diseases.

(h) Animal models of HHV-8 infection

HHV-8, the most recently described member of the gamma-herpesvirus subfamily, is not known to infect or cause disease in non-human animals. Two small-animal models for gamma-herpesvirus-associated lymphoproliferative syndromes have

been developed based on the pathogenesis of HVS (114, 115, 124, 394) and MHV-68 (467, 468). However, both HVS and MHV-68 may be limited as tools for defining viral determinants of HHV-8-associated disease because these two viruses do not encode a viral IL-6-like protein that may be critical in the pathogenesis of HHV-8-disease. However, recent descriptions of other, non-human primate herpesviruses with homology to HHV-8 (47, 116, 181, 414, 442) has elevated the potential for developing an in vivo infection model for HHV-8 disease in a genetically accessible fashion. Only one of these "new" viruses, rhesus monkey rhadinovirus (RRV) (116, 442), has been isolated and sequenced, and is the subject of this study.

Many rhesus monkeys in captivity were recently reported to naturally harbor a previously un-described herpesvirus named rhesus macaque rhadinovirus (RRV). RRV shares genomic homology with members of the lymphotropic *Rhadinoviridae* subfamily that includes HHV-8 (116, 442). RRV was first isolated and described at the New England Regional Primate Research Center as RRV strain H26-95 (116). Shortly afterwards, our laboratory at the Oregon Regional Primate Research Center independently sequenced another strain of RRV that was isolated from a bone-marrow aspirate of an SIV-infected rhesus macaque (animal I.D. # 17577) that developed widespread lymphoproliferative disease (LPD). The Oregon strain was therefore named RRV strain 17577 (442). Complete sequence analysis revealed that both RRV H26-95 and RRV 17577 are virtually identical in genomic organization, except for a few differences in the sequence and location of open reading frames encoding glycoprotein B (gB) and *dehydrofolate reductase* (DHFR), respectively (10, 442). Whether these differences translate into actual differences in pathogenesis of the two RRV strains remains to be determined; this may be the case, as generally more overt clinical manifestations have been recorded with RRV 17577 infection of rhesus macaques than with RRV H26-95, particularly in the context of SIV co-infection (285, 523).

Although all the data described in this thesis were generated using RRV 17577, "RRV" will be used throughout the thesis only in reference to this strain but should not be mistaken to imply that similar data have been obtained with RRV H26-95.

1. The RRV genome

RRV has been fully sequenced (442) and is classified as the newest member of the lymphotropic gamma-2-herpesvirus subfamily (Genus *Rhadinoviridae)* (see Fig. 1.1A). Phylogenetically, RRV appears to be genetically closer to HHV-8 than to other rhadinoviruses. Both HHV-8 and RRV encode positional analogues of cell growth control genes, as highlighted in Fig. 1.4, including functional homologues of IL-6 (i.e., K2 of HHV-8 and R2 of RRV, respectively). However, unlike HHV-8, RRV replicates to high titer in rhesus fetal fibroblasts, a property that should facilitate studies of the contribution of individual RRV gene products to viral pathogenesis via molecular genetic manipulations of the RRV genome, including genetic swaps with HHV-8-encoded genes. Ability to utilize RRV in this manner should therefore justify the use of this virus as an excellent model for HHV-8 pathogenesis in an accessible natural infection system.

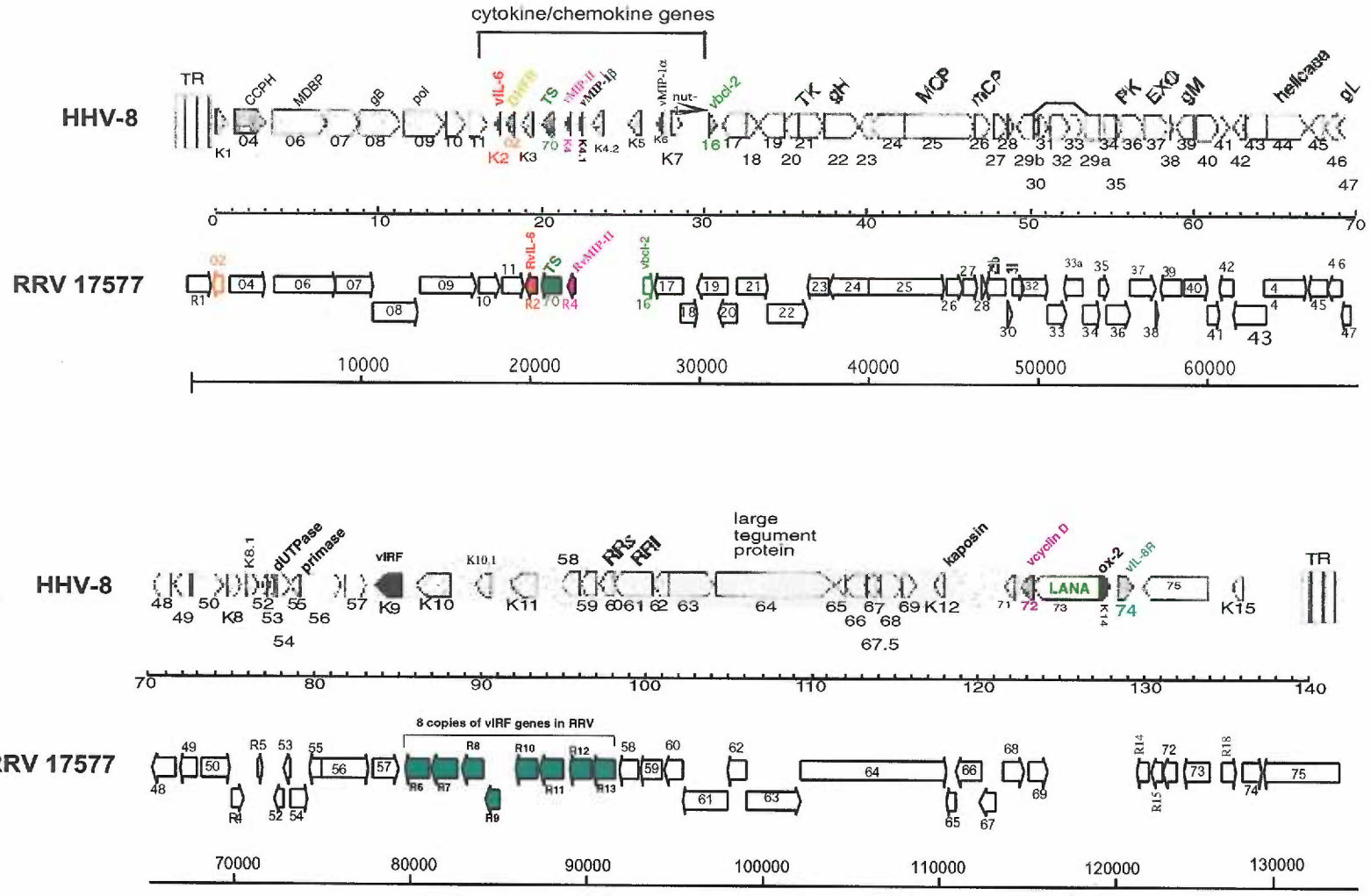
2. Seroprevalence and transmission of RRV.

Greater than 90% of the immuno-competent animals in captivity generate an antibody response to RRV, yet almost none of the RRV sero-positive animals develop overt RRV-associated disease (285, 523), implying that the host immune system is capable of controlling RRV dissemination. RRV may therefore be associated with disease only in the context of underlying immunodeficiency.

In spite of the high prevalence of RRV in the captive rhesus macaque population, the mode of transmission of RRV has not been defined, mainly owing to the fact that RRV was described only recently. Similarly, relatively little is known about the mode of

transmission of HHV-8, although the prevalence of KS in bisexual and homosexual individuals infected with HIV has led to a prevailing notion that HHV-8 might be sexually transmitted (290, 453, 488). However, non-sexual routes such as mother-to-child (53, 286, 304, 387) as well as transplant organ-mediated (75, 282, 401, 402) modes of transmission are also likely and could probably account for a high sero-prevalence of HHV-8 in parts of southern Europe and Africa where endemic and classical KS emerge even in the absence of detectable HIV infection.

Fig. 1.4 — Juxtaposition of HHV-8 and RRV genomes shows co-linearity between these two viruses. However, minor differences are also evident, most notable of which are the relative positions of ORF 2 that encodes a *dehydrofolate reductase* (DHFR) gene. Positional analogues encoding *thymidylate synthatase* (TS) and viral homologues of IL-6, MIP-1β, and bcl-2 are highlighted with similar colors to emphasize this point. Other aspects of comparative genomics between HHV-8 and RRV have been discussed by Searles et al. (442).


cytokine/chemokine genes
HHV-8
TR
CCPH
MDBP
gB
pol
vIL-6
DHFR
TS
vMIP-II
vMIP-Iβ
vMIP-Iα
nut-
vbcl-2
TK
gH
MCP
nCP
PK
EXO
gM
helicase
gL
RRV 17577
RvIL-6
RvMIP-II
HHV-8
dUTPase
primase
vIRF
large tegument protein
RRs
RFII
kaposin
cyclin D
ox-2
vIL-6R
LANA
TR
RRV 17577
8 copies of vIRF genes in RRV

3. RRV tropism

Although cell types that RRV infects in vivo are not defined, RRV appears to establish a persistent infection in the CD20+ B cell population (41, 285). One observation that may be related to RRV tropism is that animals that became persistently infected with RRV also exhibited CD20+ B cell hyperplasia beginning 4 weeks after infection, and lasting up to 36 weeks (523). The CD20 antigen is expressed on all circulating B cells and is involved in regulation of B cell activation (179, 446). Therefore, the fact that RRV appears to establish a persistent infection in CD20+ cells suggests that RRV may have evolved tropism for cells that could support persistent infection.

4. Diseases associated with RRV infection in rhesus monkeys.

Immunocompetent rhesus macaques infected with RRV generally appear normal, with no apparent virus-associated disease. However, Wong et al. showed that animals that are co-infected with both SIV and RRV develop AIDS-like symptoms accompanied by proliferative abnormalities (e.g., lymphadenopathy, splenomegaly, vascular hyperplasia with plasma cell infiltration, hypergammaglobulinemia, as well as autoimmune hemolytic anemia in some animals) (523). These syndromes, which mimic those commonly observed in humans infected with both HIV and HHV-8 (523), were not observed in animals infected with RRV or SIV alone. Therefore, SIV/RRV-infected animals share some clinical features with HIV/HHV-8-infected humans, suggesting that

both HHV-8 and RRV may be associated with disease only in the context of immunodeficiency that provides a replicative advantage to RRV or HHV-8.

The idea that RRV dissemination can be controlled by the immune system was affirmed by Mansfield et al. (285), who showed that inoculation of rhesus macaques (*Macaca mulatta*) and pig-tailed macaques (*Macaca nemestrina*) with RRV isolated from these species led to persistent infection with sustained viremia not observed in animals that were immune to RRV before inoculation. Therefore, the natural infection of RRV appears to mirror that of HHV-8 in humans. In HIV/HHV-8 co-infection individuals, for instance, HIV-induced immunodeficiency was previously thought to be responsible for what are now known as HHV-8-associated syndromes, as HIV was presumed to initiate an inflammatory cytokine response that in turn contributes to lymphoproliferation and/or lymphomagenesis (139) (Fig. 1.3). However, in light of the fact that HHV-8 encodes genes with potential angiogenic, oncogenic, and anti-apoptotic function, the lymphoproliferative process in HIV/HHV-8 co-infected AIDS patients is currently associated with HHV-8 rather than with HIV. A similar story is expected to emerge with SIV/RRV co-infection of rhesus macaques, since RRV encodes analogues of the same open reading frames encoded by HHV-8 that are suspected to have a pathogenic role.

Prior to the first description of RRV, rhesus macaques infected with SIV or simian type D retrovirus (SRV-2) were known to have an increased frequency of lymphoproliferative and mesechymo-proliferative disorders compared to immunocompetent animals, leading to the idea that SIV or SRV-2 might play a causative role in these disorders (29, 144, 493). However, the full spectrum of syndromes observed

in animals infected with SIV or SRV-2 alone could not be attributable to these retroviruses alone; at least one additional infectious agent might provide a potentiating effect for development of lymphoproliferative disease in the context of retrovirus-induced immune break down. In light of RRV seropositivity in most rhesus macaques in captivity, current evidence retrospectively points to RRV as the agent likely to play a potentiating role in lymphoproliferative syndromes previously attributed to SIV or SRV-2 infection — a claim that is supported by findings from our SIV/RRV co-infection system.

5. Retroperitoneal fibromatosis (RF)

RF is a highly vascular KS-like mesenchymal proliferative lesion originally associated with SRV-2 infection of rhesus macaques (177, 292, 491-493). However, in one study Rose et al. amplified two distinct herpesvirus-like DNA sequences from RF tissue of two macaque species *Macaca nemestrina (Mn)* and *M. mulatta (Mm)* (414). These DNA sequences (designated RFMn and RFMm) displayed significant homology to the conserved DNA polymerase region of HHV-8 and were therefore thought to represent actual homologues of HHV-8. Affected animals were also infected with SRV-2, displayed progressive fibrovascular proliferation, and suffered from retroperitoneal and subcutaneous fibrosis. Rose et al. therefore concluded that RFMn and/or RFMm might be involved in the etiology of RF. However, infectious isolates of these two putative HHV-8-like viruses have not been isolated or described, creating doubt as to whether the PCR-amplified sequences truly represent genomic portions of two distinct, HHV-8-related viruses or resulted from an experimental artifact.

On the other hand, one of the SIV/RRV co-infected animals in our longitudinal study at the Oregon Regional Primate Research Center also developed RF that was positive for the RRV-encoded cytokine-like genes Rv-MIP and RvIL-6 (our unpublished results). However, using probes specific to RFMm and RFMn fragments, we were unable to detect Rose et al's RFMn or RFMm sequences in RF tissue obtained from this animal. Although presence of RRV DNA in RF does not necessary imply a causal link between RRV and RF, detection of more than one RRV gene in affected tissue establishes such a relationship and also raises more questions as to which one of RFMn, RFMm, or RRV supplies the critical viral determinant for RF.

SIGNIFICANCE OF THESIS PROJECT

RRV and HHV-8 are genomically co-linear and encode analogous versions of cellular proteins with pro-inflammatory and pro-angiogenic potential. These two viruses therefore appear to share a number of biological features that imply some degree of similarity in the syndromes that are associated with each of them in the respective hosts.

Perhaps the most consistent disorder to which both RRV and HHV-8 are linked is B cell hyperplasia (or lymphoproliferative disease). B cell hyperplasia in hosts infected with HHV-8 and RRV is accompanied by follicular lymphadenopathy resembling the plasma-cell variant of MCD, a rare syndrome associated with excessive IL-6 bioactivity (216, 365, 441, 534). With respect to the critical role that IL-6 bioactivity plays in MCD, both HHV-8 and RRV encode distinct, yet structurally related functional homologues of cellular IL-6 (i.e., HHV-8 vIL-6 and RRV RvIL-6, respectively) that have been hypothesized to stimulate IL-6-dependent biological responses capable of triggering MCD or MCD-like syndromes. HHV-8 vIL-6 is expressed in the plasma cell variant of MCD coincident with clinical autoimmune hemolytic anemia and monoclonal gammopathy, supporting a role for vIL-6 in the immunoblastic hyperplasia characteristic of MCD (378). We similarly believe that RRV RvIL-6 may function as part of the mechanism for inducing an MCD-like lymphadenopathy in SIV/RRV co-infected animals. This thesis describes the first study of the biological function of RRV RvIL-6, and is part of our effort to develop RRV infection of rhesus macaques as a model for HHV-8 disease in a non-human primate system that can be manipulated at a genetic level in order to determine the viral determinants of MCD and other virus-associated diseases.

STATEMENT OF HYPOTHESIS

At the beginning of this thesis project, most of the RRV genome had been sequenced but no data was available on the biological function of any of the open-reading frames encoded by RRV, including the homologue of IL-6. Virus-encoded homologues of cellular genes are usually non-essential for virus replication but may instead function as modulators of host immune function in a manner that facilitates virus survival while at the same time causing untoward pathological syndromes. We therefore hypothesized that RRV-associated MCD-like hyperplasia in SIV/RRV-co-infected animals is induced by RvIL-6 alone or in concert with other RRV-encoded modulators of the host defense system during SIV-induced immune breakdown when there is limited immunological control. This hypothesis predicts that RvIL-6, a protein with B-cell stimulatory activity, may be the primary viral component responsible for B-cell proliferation and possibly other syndromes related to excessive IL-6 bioactivity. We therefore focussed on two main objectives: (a) determining the biological function of RvIL-6 in vitro (to establish a premise for potential function in vivo), and (b) construction of an RvIL-6-deletion mutant of RRV. The mutant virus would serve as an important reagent for defining the biological function of RvIL-6 in RRV-infected animals. Specific studies were designed to address the following hypothesis:

RvIL-6 stimulates B cell growth and contributes to lymphoproliferative disease (LPD) in SIV/RRV co-infected rhesus macaques.

SUMMARY OF THESIS SECTIONS

- **Chapter 1** (*Introduction*) — Literature review.
- **Chapter 2** (*Manuscript 1*) — We determined whether RvIL-6 is biologically functional using an IL-6-dependent bioassay.
- **Chapter 3** (*Manuscript 2*) —We examined differences in potency and receptor utilization between RvIL-6 and rhesus macaque IL-6 (MacIL-6) to establish the physiological significance of RvIL-6 in relation to host IL-6 function.
- **Chapter 4** (*Manuscript 3*) — We investigated systemic expression of RvIL-6 in biological fluid of SIV/RRV co-infected animals to determine whether RvIL-6 is present in bioactive form in animals that develop lymphoproliferative disease.
- **Chapter 5** (*Summary and Conclusions*)
- *Appendix I* — We initiated studies to determine whether RvIL-6 has growth-altering potential in RvIL-6-transduced NIH 3T3 cells.
- *Appendix II* — We constructed and generated an RvIL-6-deletion mutant of RRV that was additionally labeled with EGFP. The mutant virus was designed to serve as a reagent for separating the disease phenotype of RvIL-6-deletion mutants from that of wild type RRV in the context of SIV-induced immundeficiency.
- *Appendix III* — A brief overview of the basic biology of cytokines with emphasis on the function of IL-6 in lymphoproliferative disease.

CHAPTER 2

RvIL-6 is biologically functional

Data described in this chapter indicate the following:

1. RvIL-6 is biologically functional in a bioassay that relies on the biological response of the IL-6-dependent B9 murine plasmacytoma cell line.
2. RvIL-6 appears to utilize the gp130-mediated signaling pathway used by cellular IL-6 and other IL-6-related cytokines.
3. RvIL-6 produces an additive response when used in conjunction with human IL-6.

A Rhesus Macaque Rhadinovirus Related to Kaposi's sarcoma-associated Herpesvirus/Human Herpesvirus 8 Encodes a Functional Homologue of Interleukin-6

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Running title: IL-6 homologue encoded by rhesus rhadinovirus

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ABSTRACT

The rhesus rhadinovirus strain 17577 (RRV strain 17577) genome is essentially co-linear with human herpesvirus 8 (HHV8)/Kaposi's sarcoma-associated herpesvirus (KSHV), and encodes several analogous open reading frames (ORFs), including the homologue of cellular interleukin-6 (IL-6). To determine if the RRV IL-6-like ORF (RvIL-6) is biologically functional, it was expressed either transiently in COS-1 cells or purified from bacteria as a glutathione *S*-transferase (GST)-RvIL-6 fusion and analyzed in IL-6 bioassays. Utilizing the IL-6 dependent B9 cell line, we found that both forms of RvIL-6 supported cell proliferation in a dose-dependent manner. Moreover, antibodies specific to the IL-6 receptor (IL-6R) or the gp130 subunit were capable of blocking the stimulatory effects of RvIL-6. Reciprocal titrations of GST-RvIL-6 against human recombinant IL-6 produced a more than additive stimulatory effect, suggesting that RvIL-6 does not inhibit, but may instead potentiate normal cellular IL-6 signaling to B cells. These results demonstrate that RRV encodes an accessory protein with IL-6-like activity.

INTRODUCTION

Rhesus macaques are naturally infected with a herpesvirus, rhesus rhadinovirus (RRV), that is closely related to human herpesvirus 8 (HHV8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV) (116). HHV-8 is the etiological agent postulated to play a critical role in the development of all forms of Kaposi's sarcoma (KS) and in specific lymphoproliferative disorders such as primary effusion lymphoma and multicentric Castleman's disease (76, 209, 328, 457). The RRV strain 17577 genome has recently been sequenced, and is essentially co-linear with HHV-8, possessing several of the unique genes found in HHV-8 that distinguish it from other herpesviruses, including viral interleukin 6 (vIL-6), viral macrophage inflammatory proteins (vMIPs) and several viral interferon regulatory factors (vIRFs) (442). Although a role for these viral factors in HHV-8-associated disease has not been clearly established, several studies have shown that vIL-6 is functional in a number of IL-6-dependent bioassays and may as such elicit biological responses similar to those induced by cellular IL-6 (65, 326, 359).

The aim of this study was to investigate whether RRV vIL-6 (RvIL-6) possesses IL-6-like activity. The RvIL-6 open reading frame (ORF) encodes a polypeptide of 207 amino acids with overall amino acid sequence identity of 17.8% and 12.7% (35.6% and 27.4% similarity) with the genes encoded by rhesus macaque and HHV-8, respectively (Fig. 2.1). It is also 19.6% identical (41.2% similar) to human IL-6 (not shown). Its classification as an IL-6-like protein is illustrated by four conserved cysteines thought to facilitate disulfide bridging among the IL-6 family of cytokines (449). In human IL-6, the

disulfide bond formed by the second cysteine pair is critical for maintaining positional integrity of the so-called site I that binds the IL-6R (57, 133, 449, 455). This same cysteine pair aligns with RvIL-6 Cys93 and Cys103 which demarcate the topological equivalent of site I and also contains a conserved Phe98 whose aromatic character has been shown to be absolutely essential for human IL-6 interactions with its receptor (455).

RESULTS

Recombinant RvIL-6 is capable of supporting B9 cell growth. To determine whether RvIL-6 might elicit growth-stimulatory effects on IL-6-responsive cells, we cloned RvIL-6 into two different expression vectors. For expression in eukaryotic cells, we cloned full-length RvIL-6 into an expression vector utilizing the human cytomegalovirus (CMV) immediate-early promoter (138) and assayed supernatants from transfected COS-1 cells for IL-6-like activity in a bioassay using the IL-6-dependent B9 cell line essentially as described (1, 57). As shown in Fig. 2.2, RvIL-6-containing supernatant stimulated B9 cell proliferation in a dose-dependent manner. Maximal stimulation was three-fold greater than control supernatant, and also equivalent to about 65 pg/ml of hrIL-6 (used throughout the study as a positive control for B9 cell responsiveness to growth stimulus).

To express RvIL-6 in bacteria, the Rv-IL6 ORF minus the putative signal sequence (as determined by the SignalP program) (362), was cloned into the prokaryotic expression vector pGEX-2T (Pharmacia, Piscataway, NJ) to create a glutathione *S*-transferase-RvIL-6 (GST-RvIL-6) fusion (Fig. 2.3A). GST-RvIL-6 was purified from

lysates of bacteria transformed with the pGEX-GST-RvIL-6 plasmid by affinity over a Glutathione sepharose 4B matrix (Fig. 2.3B). Bacterially expressed fusion proteins were sterile-filtered and used directly in the bioassay. GST-RvIL-6 was capable of stimulating B9 cell growth in a dose-dependent manner, albeit less efficiently than hrIL-6, but at a rate statistically higher than purified GST, confirming the absence of bacterial lipopolysaccharide (LPS) in the GST-RvIL-6 preparation that could otherwise have a stimulatory effect on B9 cells (Fig. 2.3C) (379). Maximal GST-RvIL-6-mediated cell proliferation occurred with about 20 μg/ml of protein, equivalent to the effect of about 5 ng/ml of hrIL-6 on the same cell line. One explanation for this 4000-fold difference in bioactivity could be that GST-RvIL-6 is a weaker stimulator of B9 growth, perhaps owing to the GST-moiety (26 kDa) that could affect interactions between RvIL-6 (21 kDa) with its cognate receptor(s). However, our finding is similar to data from studies of HHV-8 vIL-6 function where, using different IL-6-dependent cell lines, others have independently reported a consistently similar magnitude of difference in potency between recombinant vIL-6 and hrIL-6 (65, 326, 359).

RvIL-6 utilizes the IL-6R/gp130 signaling pathway. The data above suggests that RvIL-6 may utilize the IL-6 signaling pathway (471, 514) reported to be the possible mechanism for HHV-8 vIL-6 function (65, 359). To determine whether IL-6R is required for RvIL-6 function, we tested the effect of an anti-mouse IL-6R monoclonal antibody (clone D7715A7, Pharmingen, San Diego, CA) on GST-RvIL-6 stimulation of B9 cells. B9 cells, maintained in the absence of hrIL-6, were incubated for 30 min with serial dilutions of anti-IL-6R prior to the addition of GST-RvIL-6 or hrIL-6 and then analyzed

for cell proliferation. Anti-IL-6R antibody was able to dose-dependently block growth signals from both GST-RvIL-6 and hrIL-6 (Fig. 2.4A). The inhibitory effect of the anti-IL-6R was evident only when cells were pre-treated with anti-IL-6R before addition of GST-RvIL-6 or hrIL-6 and not when added at the same time (data not shown), implying that the antibody was specifically preventing the initial binding reactions between IL-6R and GST-RvIL-6 or hrIL-6. It is also evident from the data that at lower concentrations of antibody, GST-RvIL-6 was slightly less sensitive than hrIL-6, suggesting that the viral protein may require a higher stoichiometric concentration of anti-IL-6R for effective neutralization of its cognate sites on IL-6R. This result is strikingly similar to previous reports of anti-human IL-6R inhibition of HHV-8 vIL-6 function on IL-6-responsive cell lines, relative to hrIL-6 (65, 359).

To test whether gp130 can also serve as the transducer of RvIL-6 signals, B9 cells were pre-treated with serial dilutions of monoclonal anti-human gp130 (generously provided by Dr. Beth Habecker, Oregon Health Sciences University). After 30 minutes, constant GST-RvIL-6 (10 μg/ml) or hrIL-6 (5 ng/ml) were added and cell proliferation analyzed. As shown in Fig. 2.4B, anti-gp130 antibody dose-dependently inhibited both GST-RvIL-6 and hrIL-6 growth signals. Unlike the result with anti-IL-6R, anti-gp130 had a comparable inhibitory effect on both GST-RvIL-6 and hrIL-6, with 50% inhibition of both signals occurring at about 700-800 ng/ml of antibody (Fig. 2.4B, inset). This suggests that RvIL-6 is capable of signaling through the shared gp130 subunit.

The finding that the RvIL-6 is capable of initiating a signal through IL-6R and gp130 implies that RvIL-6 can either compete with host IL-6 for the receptor system in

an inhibitory fashion or that it may function to enhance the underlying IL-6 response. We examined this issue by adding increasing amounts of GST-RvIL-6 to B9 cells in the presence of 2.5 ng/ml of hrIL-6, a concentration that is within the linear range of proliferation to this stimulus. We found that the corresponding stimulation index is consistently and increasingly higher with each additional concentration of GST-RvIL-6 (Fig. 2.5). Moreover, reciprocal addition of increasing amounts of hrIL-6 in conjunction with constant GST-RvIL-6 also caused a more than additive stimulatory effect (data not shown), suggesting that there may indeed be a synergistic integration of signals simultaneously delivered by both hrIL-6 and GST-RvIL-6. These findings are significant since they suggest that RvIL-6 may directly utilize the IL-6 receptor system without inhibiting the normal cellular IL-6 response.

The discovery that RvIL-6 is functional and capable of triggering the IL-6R/gp130 pathway is intriguing, especially since the protein has such limited homology with cellular IL-6. In contrast, viral interleukin-10 (vIL-10) encoded by Epstein-Barr Virus BCRFI displays 70% sequence similarity with its cellular counterpart (325). EBV vIL-10 also exhibits some of the known functions of IL-10 (337). It is therefore conceivable that only a limited number of conserved residues may be necessary for IL-6-like function, as has been found by mutational analysis of human IL-6 (449). As such, RvIL-6 may represent an ancestral host gene pirated by RRV 17577 during evolution, is keeping with a seemingly common theme among other known pathogenic herpesviruses (25).

The poor inhibitory effects of anti-IL-6R on RvIL-6 activity, relative to hrIL-6,

indicate that differences in IL-6R interactions exist between the viral protein and hrIL-6. Interestingly, HHV-8 vIL-6 was recently shown to stimulate STAT3-containing DNA binding activity in IL-6R-deficient cells, suggesting that HHV-8 vIL-6 may not require the IL-6R subunit (321). We contend that this finding is not contradictory to what we have observed with RvIL-6, since it is still possible that RvIL-6 and HHV-8 vIL-6 may indeed have binding activities for IL-6R when this subunit is available on the target cell. The apparent conservation of critical IL-6R-binding residues in both RvIL-6 and HHV-8 vIL-6 (Fig. 1) strongly supports this view. We predict that RvIL-6 may also bind and homodimerize gp130 in the absence of IL-6R, resulting in induction of DNA binding activity as observed for vIL-6. However, the signal generated from such an interaction would be qualitatively weaker than the one triggered in the presence of both gp130 and IL-6R. This concept is beyond the intended scope of this report, but could be evaluated in IL-6R- versus IL-6R+ cells upon exposure to either RvIL-6 or vIL-6.

The expression pattern, as well as the in vivo function of RvIL-6 in RRV-infected animals is still not known. However, because recombinant RvIL-6 can support B9 cell growth, we believe that this viral protein may function in a similar manner in vivo, and could as such contribute to B cell hyperplasia in immunocompromized animals infected with RRV. RvIL-6 may additionally be involved in RRV pathogenesis possibly by modulating some aspect of viral interaction with the immune system, especially since it may enhance, rather than inhibit, host IL-6 signaling. Moreover, RvIL-6 could exert a stimulatory effect on circulating lymphocytes and/or promote cell survival via the IL-6-inducible

interferon regulatory factor (IRF) (478) that can antagonize the interferon-mediated clearance of virus-infected cells. In accordance with this notion it is interesting that RRV strain 17577 has eight copies of a homologue of cellular IRF (442) which could function like the oncogenic HHV-8 v-IRF (K9) (167).

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Figure 2.1 — Amino-acid sequence alignment of RRV 17577-encoded RvIL-6 with rhesus IL-6 and KSHV vIL-6. The relatedness of RvIL-6 to rhesus IL-6, and KSHV vIL-6 was analyzed using the CLUSTAL method with PAM 250 residue weight table, and by BLAST search of GenBank sequences. The exact residue numbers for each of the three polypeptides are shown to the left of each sequence. Gaps have been introduced to account for the different lengths of the polypeptides, and to generate maximum alignment. Identical amino-acid residues are boxed accordingly for all three, or at least two sequences. The first N-terminal 21, 27 and 22 residues of RRV RvIL-6, Rhesus IL-6 and KSHV vIL-6, respectively, display characteristic features of a putative signal peptide sequence with a hydrophobic core that is followed by a typical signal peptidase cleavage site, as defined by the SignalP prediction (362). The four conserved cysteines (boxed and shaded) align with RvIL-6 residues 64, 70, 93 and 103, and the four putative domains (A1-2, B, C and D) that form the α-helical bundle structure typical of the long-chain cytokine family (335) are marked by solid black lines. Some conserved residues known to be critical for human IL-6 function are denoted as follows: *, residues that bind IL-6R (54, 57); ~, residues that facilitate human IL-6 interaction with gp130 (133); •, this conserved proline introduces the so-called "bent" effect that facilitates anti- parallel helical disorderedness characteristic of functional IL-6-like molecules (449). The KSHV vIL-6 and rhesus IL-6 sequences were adapted from references (326) and (507), respectively.

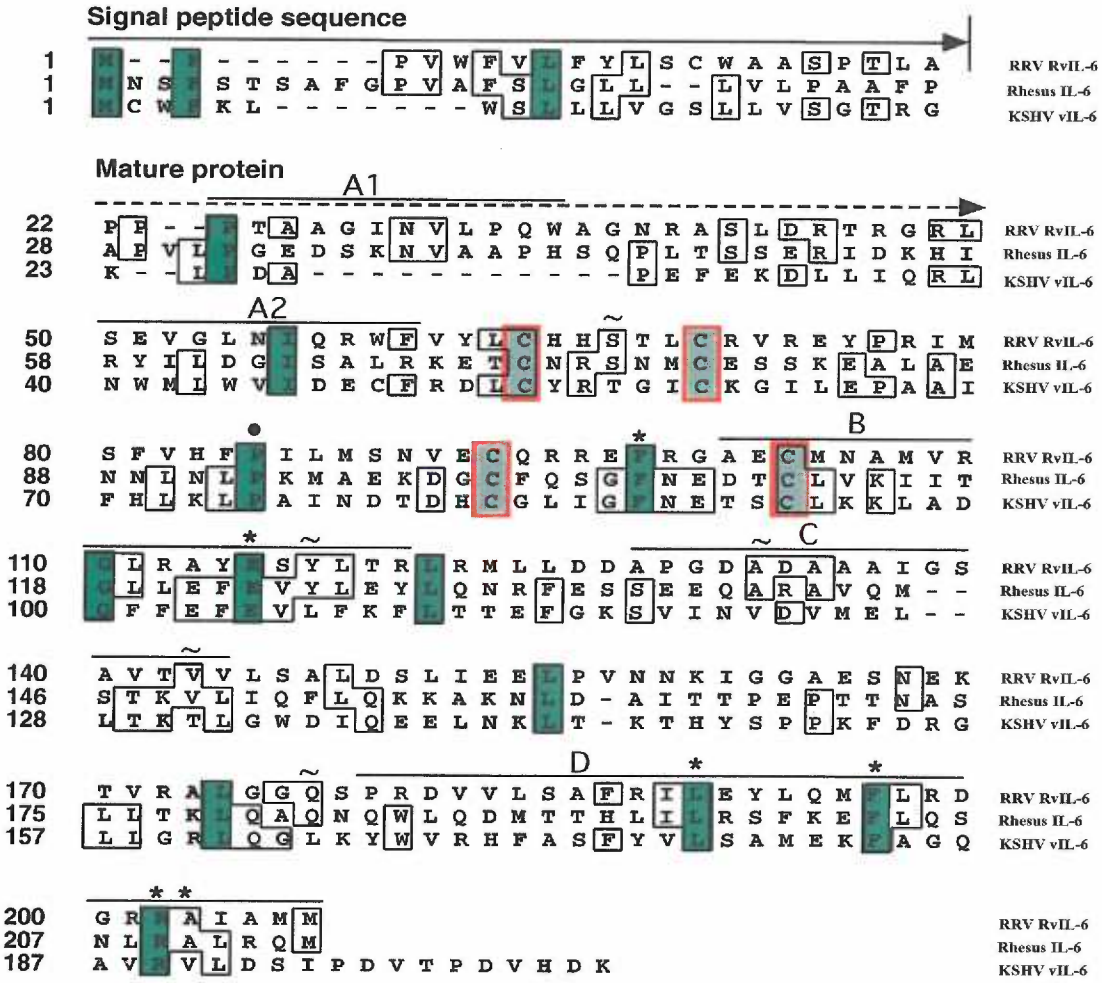

Signal peptide sequence
Mature protein
A1
A2
B
C
D
RRV RvIL-6
Rhesus IL-6
KSHV vIL-6

Figure 2.2: Assay for biological activity of recombinant RvIL-6. Serial dilutions of sterile-filtered culture supernatant from COS-1 cells transfected with either pCMV vector (red) or the pCMV-RvIL-6 construct (blue) were assayed for their ability to promote growth of the IL-6-dependent B9 cell line. A starting stock of 80 pg/ml of human recombinant IL-6 (black bar) was diluted in parallel with transfection supernatant and used as a positive control.

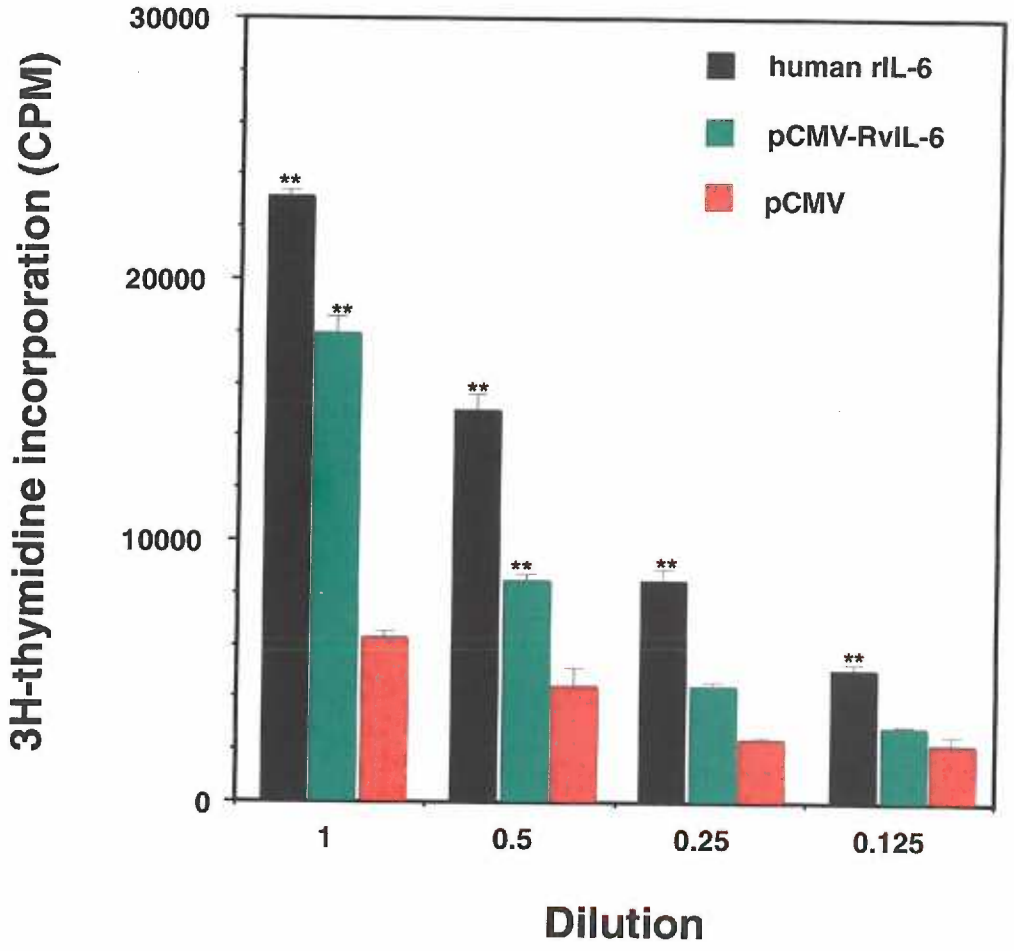
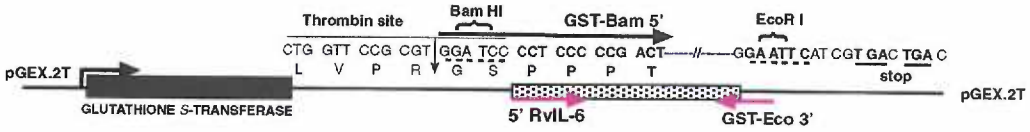
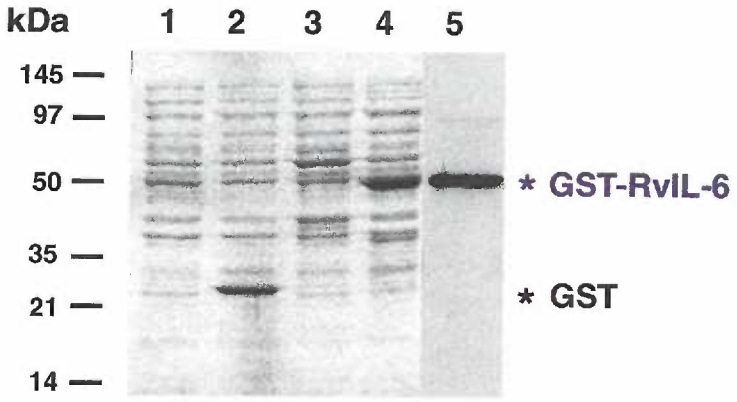

30000
20000
10000
0
3H-thymidine incorporation (CPM)
human rIL-6
pCMV-RvIL-6
pCMV
**
1
0.5
0.25
0.125
Dilution

Figure 2.3: Schematic representation of strategy for cloning RvIL-6 into the GST-fusion vector pGEX-2T (panel **A**), and purification of recombinant protein. **B**, 12% SDS-PAGE screening of GST-RvIL-6 expressed and purified from bacteria. An individual clone containing the pGEX.2T vector encoding GST alone, either uninduced (lane 1) or induced with 0.1 mM IPTG (lane 2), and lysates from a separate clone containing the pGEX.2T-RvIL-6 construct, either uninduced (lane 3) or induced with 0.1 mM IPTG (lane 4). Lane 5 was loaded with the affinity-purified GST-RvIL-6 fraction eluted from Glutathione Sepharose 4B beads following matrix binding with bacterial lysates containing GST-RvIL-6. Proteins were visualized by staining the gel with Coomasie brilliant blue, and the positions of GST and GST-RvIL-6 are indicated with asterisks. **C,** Increasing concentrations of purified GST (in μg/ml, open circles), GST-RvIL-6 (in μg/ml, closed circles), human recombinant IL-6 (in ng/ml, closed triangles) or corresponding volumes of starving media alone (open triangles) were assayed for IL-6 activity. IL-6 activity was determined by 3H-thymidine incorporation. The data are presented as means of triplicate values of CPM ± SEM. Analysis of data variance was performed using SuperAnova (Abacus Concepts, Inc., Berkeley, CA), and Tukey-Kramer was used for Post-hoc tests of significance; the data are presented as means of triplicate values of CPM ± SEM. $^{**}p < 0.01$.

A


Thrombin site
Bam HI
GST-Bam 5'
EcoR I
CTG GTT CCG CGT GGA TCC CCT CCC CCG ACT //---- GGA ATT CAT CGT GAC TGA C
L V P R G S P P P T
stop
pGEX.2T
GLUTATHIONE S-TRANSFERASE
5' RvIL-6
GST-Eco 3'
pGEX.2T

B


kDa
1
2
3
4
5
145
97
50
35
21
14
* GST-RvIL-6
* GST

C

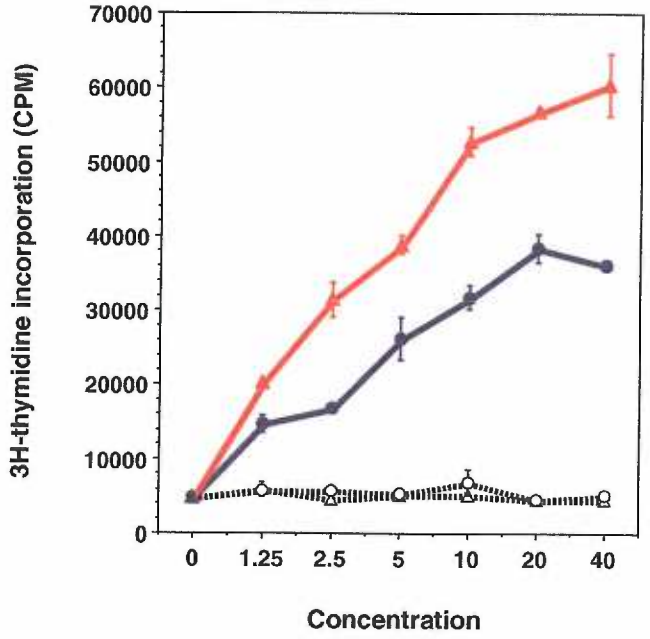

3H-thymidine incorporation (CPM)
70000
60000
50000
40000
30000
20000
10000
0
0
1.25
2.5
5
10
20
40
Concentration

Figure 2.4 — Antibodies to both IL-6R and gp130 receptor subunits inhibit GST-RvIL-6- mediated proliferation of B9 cells. B9 cells maintained in the absence of IL-6 were seeded in a 96-well plate containing increasing concentrations of either anti-IL-6R (**A**) or anti-gp130 (**B**). After 30 minutes of pre-incubation at room temperature, constant GST-RvIL-6 (10 μg/ml, for both panels A and B), or hrIL-6 (10 ng/ml for panel A, and 5 ng/ml for panel B) were added and cell proliferation analyzed. Data points represent the means of triplicate values of CPM ± SEM. The insets for each graph represent the calculated "percent neutralization", defined as: (CPM with antibody/maximum CPM without antibody) x 100%.

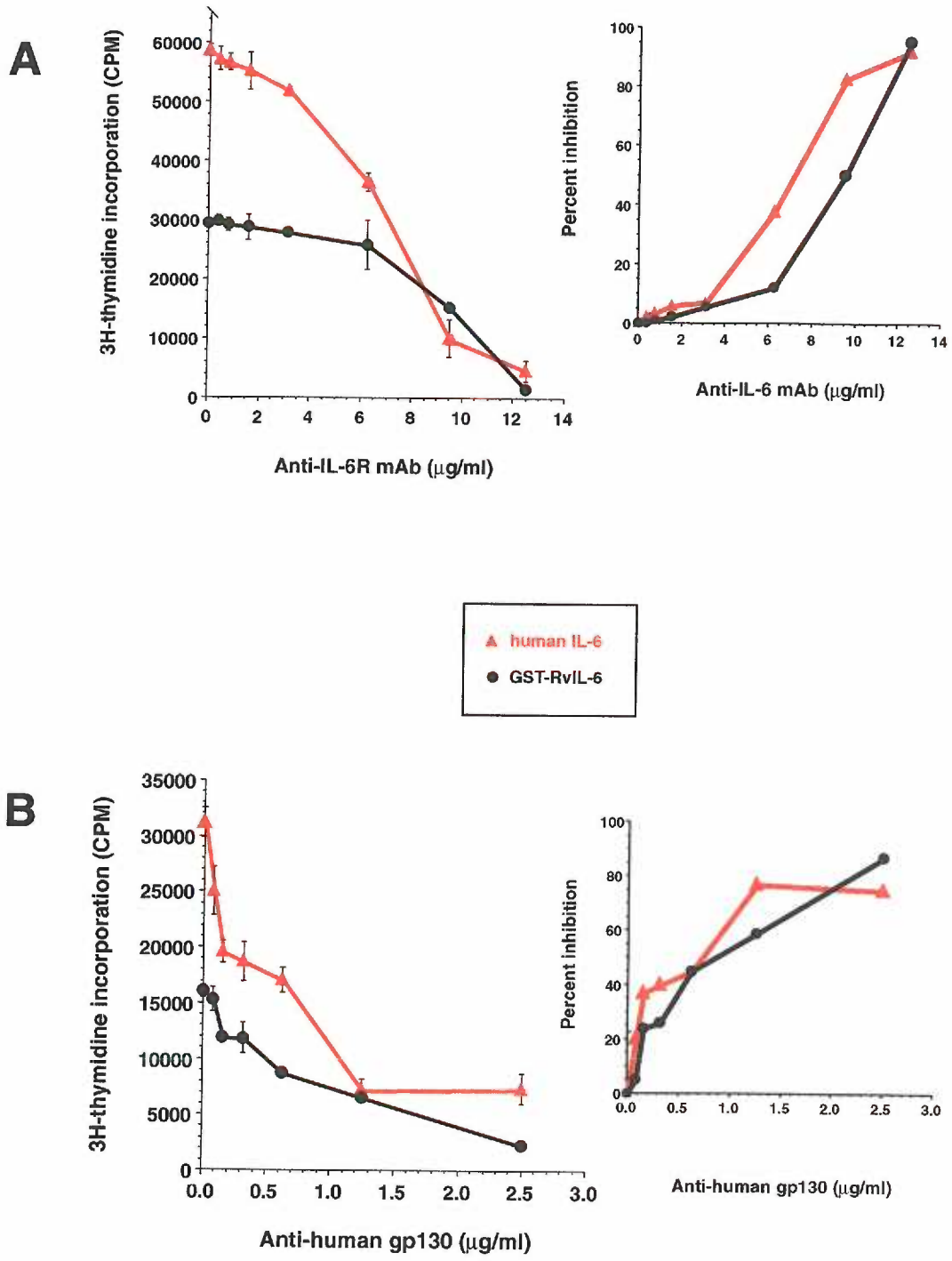
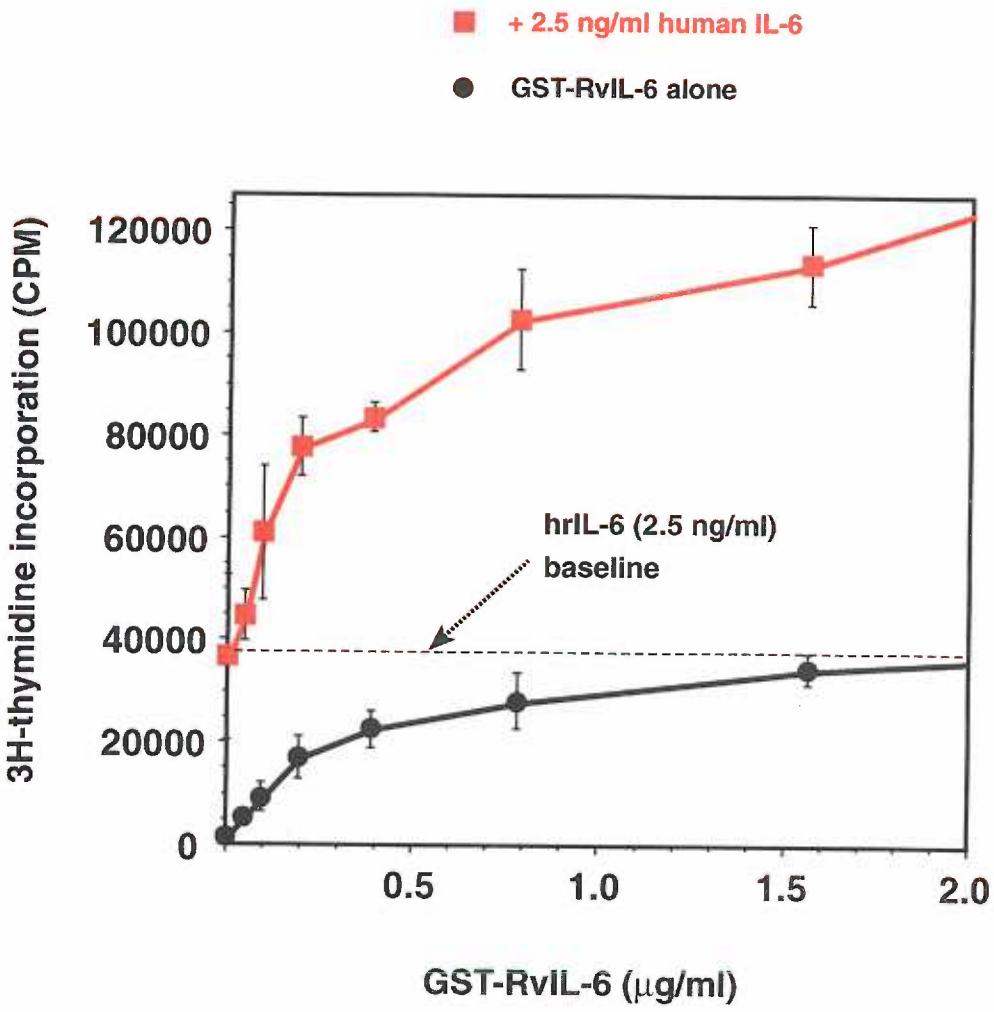

A
3H-thymidine incorporation (CPM)
60000
50000
40000
30000
20000
10000
0
0
2
4
6
8
10
12
14
Anti-IL-6R mAb (μg/ml)
Percent inhibition
100
80
60
40
20
0
Anti-IL-6 mAb (μg/ml)
human IL-6
GST-RvIL-6
B
3H-thymidine incorporation (CPM)
35000
30000
25000
20000
15000
10000
5000
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Anti-human gp130 (μg/ml)
Percent inhibition
Anti-human gp130 (μg/ml)

Figure 2.5 — GST-RvIL-6 does not inhibit hrIL-6-mediated growth of B9 cells. B9 cells maintained in the absence of IL-6 were incubated with increasing concentrations of GST-RvIL-6 alone or in the presence of a constant amount of hrIL-6 (2.5 ng/ml), and assayed for proliferation. The data are presented as means of triplicate values of CPM $\pm$ SEM. The horizontal dotted line indicates the average level of proliferation normally obtained with 2.5 ng/ml of hrIL-6.


+ 2.5 ng/ml human IL-6
GST-RvIL-6 alone
3H-thymidine incorporation (CPM)
120000
100000
80000
60000
40000
20000
0
hrIL-6 (2.5 ng/ml)
baseline
0.5
1.0
1.5
2.0
GST-RvIL-6 (μg/ml)

CHAPTER 3

We investigated differences in potency and receptor usage between recombinant RvIL-6 and its rhesus macaque counterpart (MacIL-6) The data suggest that RvIL-6 is 2-3-fold less potent than MacIL-6 and can activate gp130 in the absence of IL-6R. This mechanism of RvIL-6 signaling could have physiological significance with respect to the overall IL-6 bioactivity, particularly in SIV/RRV co-infected animals where RvIL-6 is likely to be expressed.

Biological Function of Viral Interleukin-6 (RvIL-6)[1] Encoded by a Rhesus Monkey Rhadinovirus (RRV) Genetically Related to Kaposi's Sarcoma-associated Herpesvirus (KSHV)*

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Running Title: Viral IL-6 Encoded by a Rhesus Monkey Herpesvirus

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Rhesus macaque rhadinovirus (RRV) is closely related to HHV-8, the human virus that is etiologically linked to Kaposi's sarcoma (KS) and other syndromes of B cell origin. Like HHV-8, RRV encodes a functional homologue of interleukin-6 (IL-6). We show here that the RRV-encoded IL-6-like protein (RvIL-6) supports growth of the IL-6-dependent B9 cell line, and is two-to-threefold less potent than its rhesus monkey counterpart (MacIL-6). RvIL-6 does not appear to inhibit MacIL-6 signaling, as a combination of RvIL-6 and MacIL-6 produced an additive stimulatory effect on B9 cells. Anti-gp130 inhibited both viral and host IL-6 proteins almost equally; however, anti-IL-6R was effective against MacIL-6 but failed to completely block RvIL-6, suggesting that the viral protein may be partially resistant to the neutralizing activity of anti-IL-6R. Polyclonal antibodies specific to RvIL-6 blocked RvIL-6 but not MacIL-6 bioactivity, whereas anti-human IL-6 antibody that cross-reacts with rhesus IL-6 blocked MacIL-6 but was ineffective against RvIL-6 bioactivity. Additionally, both MacIL-6 and RvIL-6 prevented apoptosis and induced neurite outgrowth in NGF-primed PC12 cells; and, as in the B9 bioassay, anti-IL-6R completely abrogated MacIL-6 but failed to block the anti-apoptotic effect of RvIL-6. Therefore, while RvIL-6 and MacIL-6 may display significant functional homology, these proteins may exhibit differences in the requirement for, and/or mechanism of interaction with IL-6R both on B9 cells and PC12 cells.

INTRODUCTION

Cytokines represent one of the ways that the immune system is activated to clear and prevent dissemination of infectious virus. Therefore, the ability of viruses to alter the normal function of cytokines constitutes a critical determinant of viral pathogenesis in vivo. Viruses achieve this via a number of strategies, including expression of genes that are homologous to host cytokines and/or cytokine receptors (106, 186, 307, 345, 445, 458, 459, 461). While a possible function of virus-encoded cellular homologues may be to promote virus survival, these viral proteins may, in settings of immunodeficiency, also cause a number of cell growth abnormalities that are often the cause of morbidity in the infected host.

HHV-8 encodes a number of homologues of cellular genes whose function may be targeted to the control of cell growth and survival (420). Accordingly, HHV-8 has been linked to a number of cell proliferative syndromes like Kaposi's sarcoma (KS) (50, 81, 82, 109, 259, 260, 370, 395, 396, 435, 516), body cavity-based lymphomas (BCBL), also known as primary effusion lymphomas (PEL) (14, 50, 81, 82, 109, 232, 259, 260, 370, 395, 396, 425, 435, 516), and multi-centric Castleman's disease (MCD) (126, 192, 239, 457). KS is a polyclonal and highly vascularized proliferative lesion characterized by prominent angiogenesis and monocytic infiltration (231). On the other hand, BCBL-PEL and MCD have phenotypic characteristics consistent with a B cell origin, perhaps reflecting the importance of the B cell compartment in the pathogenesis of HHV-8 (280, 311). Interactions between HHV-8 and the host are conceivably mediated by one or more

virus-encoded homologues of host genes, most notable of which is open reading frame K2 (vIL-6) that displays 46% amino-acid similarity with human interleukin-6 (IL-6) (326). vIL-6 possesses the two cysteine pairs that are necessary for conservation of secondary structure among members of the IL-6 cytokine family, and has also retained the B cell-stimulatory function of cellular IL-6 (65, 326, 357). In addition, vIL-6 is expressed in HHV-8^+ PEL cell lines and in MCD specimens (464, 481), and histochemical staining of lymph-node sections from HHV-8-infected patients with KS detected vIL-6 associated with cells of the haematopoietic lineage in areas containing abundant B cells (326). In addition, Aoki *et al.* (15) reported that vIL-6 causes tumorigenesis and angiogenesis in nude mice via direct induction of vascular endothelial growth factor (VEGF); human IL-6 has also been reported to induce VEGF (92). Because VEGF is an important mediator of angiogenesis (a defining feature of KS), the finding that vIL-6 induces VEGF expression implies a physiological link between vIL-6 and KS. Therefore, vIL-6 may play a critical role not only in PEL and MCD but also in KS. However, a complete scrutiny of vIL-6 function in HHV-8-associated syndromes can only be done using a physiologically relevant non-human primate system for HHV-8 infection — such as the rhesus macaque.

Rhesus macaque rhadinovirus (RRV), which is genomically colinear with HHV-8 (442), also contains analogues of the same genes encoded by HHV-8, including an IL-6-like gene (R2) that is situated at a similar position in the genome of RRV as vIL-6 in HHV-8 (116, 442). R2 encodes a 207 amino acid polypeptide (RvIL-6) that shares 18.6% and 12.7% identity (35.6% and 27.4% similarity) with rhesus macaque IL-6 (MacIL-6) and HHV-8 vIL-6, respectively (226). Previously, we showed that RvIL-6 can support

growth of the IL-6-dependent B9 cell line (226). In the present study, we have examined the biological function of RvIL-6 relative to rhesus macaque IL-6 (MacIL-6) using two distinct assays that are dependent on IL-6 bioactivity — B9 cell growth and neuronal differentiation of NGF-primed PC12 cells. In both these assays, recombinant RvIL-6 was two-to-threefold less potent than MacIL-6. RvIL-6 produced an additive stimulatory effect when used together with MacIL-6, but exhibited a distinct mode of gp130 activation relative to the host protein. Our finding that RvIL-6 can support B9 cell growth, as well as prevent apoptosis in a non-immune cell line (PC12) underscores the multifunctional nature of RvIL-6, including support of cell growth and survival.

EXPERIMENTAL PROCEDURES

Cells and cell culture conditions — The B9 murine IL-6-dependent hybridoma/plasmacytoma cell line was cultured as previously described (226). PC12 cells were maintained in DMEM media supplemented with 5% horse-serum, and 100 ng/ml of nerve-growth factor (NGF), essentially according to a published protocol (525). Hi-5 insect cells were cultured as monolayers in Excel 405 media (JRH Biosciences) supplemented with 2.5% Fetal Calf Serum. Sf9 insect cells were maintained either as suspension cultures in spinner flasks or as monolayers in Complete Grace's media (Gibco BRL).

B9 cell proliferation assay — Determination of B9 cell proliferation and evaluation of IL-6-like bioactivity of recombinant proteins was performed essentially as previously described (226).

Antibodies — Anti murine IL-6R antibody was purchased from Pharmingen (San Diego, CA). Polyclonal rabbit anti-human gp130 was a gift from Dr. Beth Habecker (Department of Physiology and Pharmacology, Oregon Health Sciences University, Portland, OR), and rat anti-murine gp130 was obtained from Dr. Tetsuya Taga (Osaka University). Rabbit polyclonal anti-RvIL-6 antibody (1011) was generated by immunizing two New Zealand white rabbits with 500 μg of purified GST-RvIL-6 fusion protein in PBS plus an equal volume of Freund's complete adjuvant (Sigma). After four weeks, each rabbit was alternately boosted with 150 or 250 μg of mature untagged RvIL-6 in a 50:50 mixture of RIBI and PBS/Freund's incomplete adjuvant (Sigma). Rabbits were bled ten days later, and the IgG fraction was prepared from whole rabbit serum by Hi-Trap protein A-affinity column (Biorad) as described by the manufacturer. The pool of GST-reactive antibody was pre-cleared from total rabbit IgG by three passages over a column of recombinant GST covalently linked to Sepharose beads. GST-selected anti-RvIL-6 IgG (1011) was sterile-filtered and stored in 1.0-ml aliquots at –20°C. Polyclonal anti-human IL-6 was purchased from R&D Systems (Minneapolis, MN), anti-rhesus CD3 (FN18) was a generous gift from Dr. M. Jonker (TNO), and anti-phosphotyrosine (4G10) was a generous gift from Dr. Brian J. Druker (Department of Hematology, Oregon Health Sciences University). Anti-phospho-ERK2 (mouse monoclonal IgG_{2a} clone E-4, cat. #: sc-7383) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PARP (c2-10) antibody was obtained as a generous gift from Dr. Guy Poirier (Laval University, Quebec, CANADA).

Expression and purification of recombinant proteins in E. coli — Complimentary

DNA clones of RvIL-6 (226) and MacIL-6 (generous gift from Dr. F. Villinger, Emory University, Atlanta) lacking the putative signal peptide sequences were separately subcloned into the pGEX.2T expression vector (Pharmacia, Piscataway, NJ). Recombinant pGST-RvIL-6 and pGST-Mac-IL-6 clones were fully sequenced, propagated in E. *coli* (XL-1 Blue strain), and GST-fusion proteins were affinity purified on a Glutathione Sepharose 4B matrix according to the instructions in Pharmacia's GST-fusion expression kit. His_6-tagged versions of RvIL-6 and Mac-IL-6 were constructed by subcloning respective cDNA sequences into the pET-16b expression vector (Novagen) and both pET-RvIL-6 and pET-Mac-IL-6 plasmids were propagated in the protease-deficient BL21 (ΔES) LysS strain of E. *coli*. Protein expression was induced by adding IPTG (0.2 mM final concentration) to log-phase bacterial culture (OD_{600} = 0.5) in LB supplemented with 50 μg/ml Ampicillin and Chloromphenical, 1 mM $MgSO_4$ and 0.002% Maltose. Highly expressed RvIL-6 and Mac-IL-6 were solubilized from bacterial pellets under native conditions and affinity purified on Ni^{2+}-NTA columns following instructions in Qiagen's protein expression kit.

Expression of RvIL-6 in insect cells — A cDNA fragment encoding mature RvIL-6 was sub-cloned into the baculovirus shuttle vector pBlueBacHis2B (Invitrogen), and the resulting construct (pBac-RvIL-6) was co-transfected into sub-confluent monolayers of Sf9 insect cells along with linearized Bac-N-Blue baculovirus DNA (Invitrogen) by cationic insect liposome-mediated fusion following instructions in Invitrogen's transfection module. Recombinant baculoviruses were plaque-purified three times in agarose media supplemented with Bluo-gal (GIBCO Life Sciences) and analyzed by PCR

to verify expression of RvIL-6 in proper context with the hexa-histidine (His_6) tag, driven by the polyhedrosis (P_H) promoter. For high level expression of recombinant proteins, Hi-5 cells were infected with recombinant baculoviruses at a multiplicity of infection (MOI) of ten virus particles per cell. Three days later, cells were pelleted and lysed by sonication in buffer containing 1% Triton X-100, 10 mM Tris-HCl, 100 mM Na_2HP0_4 and a 1X cocktail of mammalian protease inhibitors (Sigma). Recombinant Bac-RvIL-6 was purified by affinity chromatography following batch-wise incubation of clarified lysate with 10% (v/v) Ni^{2+}-NTA bead slurry equilibrated in lysis buffer, and eluted sequentially with graded concentrations of Imidazole (Qiagen).

Cold-binding of MacIL-6 and RvIL-6 to B9 cells — Pre-starved B9 cells were resuspended in starving media for 10 min. at 4°C. Cells were then incubated with excess RvIL-6 or MacIL-6 in a final volume of 500 µl ("excess concentration" was determined in prior titration assays as the concentration of either protein beyond which there was no additional stimulation of B9 cell growth). The B9/protein mixture was incubated on ice for various lengths of time ranging from 10-150 min. and then over-layed with RPMI supplemented with 20% FBS/100-µM 2-βme, after which cells were pelleted by centrifugation at 500 x g, 4°C. Cell pellets (with bound protein) were gently resuspended in starving media, seeded in a 96-well culture plate and directly assayed for proliferation. To assay the residual bioactivity of unbound protein in the wash fraction, pre-starved B9 cells were cultured with supernatant derived from the binding mixture and then analyzed in a proliferation assay.

Western blot analysis —PC12 cells were centrifuged and lysed in buffer containing

20 mM Tris, pH 7.5, 1% SDS, 2 mM EDTA, 2 mM EGTA, 6 mM 2β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate (Na_3VO_4), and 10 μg/ml each of pepstatin A, leupeptin, and aprotinin. After a 10-min incubation on ice, cell suspensions were sonicated, clarified, and protein concentration was determined by the Bradford assay (Bio-Rad). Equal amounts (50 μg) of total cellular protein were boiled in SDS sample buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), and subjected to 8% SDS-PAGE. Resolved proteins were then transferred onto nitrocellulose membrane in transfer buffer (39 mM glycine, 48 mM Tris, pH 8.3, 0.03% SDS, and 15% methanol) and subsequently stained with Ponceau Red to verify complete transfer. Membranes were blocked with 5% nonfat milk in 10 mM Tris, pH 8.0, 0.15 M NaCl, and 0.05% Tween-20 (TBS-T) for 1 hour before incubation with respective primary antibodies. To probe for cleavage of poly-(ADP)-ribose) polymerase (PARP), membranes were incubated with a 1:4000 dilution of rabbit anti-PARP antibody (c2-10), followed by goat anti-rabbit secondary antibody at a 1:20,000 dilution in TBS-T, and developed by chemiluminescence (ECL, Amersham Life Science). The level of activated ERK was determined by stripping the blot in Tris-HCl buffer containing 67.5 mM β-me and 2% SDS at 50°C, and re-blotting with anti-phospho-ERK (Santa Cruz Biotechnology) as primary antibody. To assay phosphorylated gp130, RvIL-6-treated PC12 cells were scraped and re-suspended in lysis buffer (100 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40 (NP-40)/deoxycholate, 100 μg/ml BSA, 1 mM sodium orthovanadate, plus 1X protease inhibitors) for 30 min on ice. After centrifugation, the supernatant was incubated with anti-human gp130 conjugated to protein-A sepharose

beads for 1 hour at 4 °C. Beads were washed three times with lysis buffer, boiled in 1X SDS sample buffer and bound proteins were resolved in 8% SDS-PAGE gel followed by western blotting using the 4G10 anti-phospho-tyrosine antibody.

MTT assay and Cell Counts — PC12 cell death was quantified by determination of viable cell numbers with the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay as previously described (113). The percentage of cell survival was calculated by taking the optical density of treated cells, dividing that number by the optical density for untreated, control cells, and then multiplying by 100. Alternatively, cell death was determined by manual counts of the numbers of trypan blue-excluding cells following trypsinization. Percent cell survival was calculated by taking the number of trypan blue-excluding cells in the treated category, dividing by the number of untreated control cells, and multiplying by 100.

Statistical Analysis — All numerical results are presented as means of triplicate values from three independent assays +/- standard errors of the means. Where indicated, data variance was analyzed using SuperAnova (Abacus Concepts, Inc., Berkeley, CA), and Tukey-Kramer was used for post-hoc tests of significance.

RESULTS

RvIL-6 and MacIL-6 share limited sequence homology (18.6% identity, 46% similarity) (Fig. 3.1A). However, as revealed in the consensus sequences ranging from C72 to C78, C111 to R/Q130, and L/M192 to M215, a number of dissimilar positions in RvIL-6 are replaced by residues with comparable charge and/or side-chain character in

what represents conservative substitutions. The above region also contains conserved residues that, in human IL-6, are critical not only for maintenance of secondary structure but also for IL-6R binding (56, 132, 133). In addition, RvIL-6 and MacIL-6 are predicted to assume similar structural tendencies, as illustrated by the seemingly analogous hydrophilicity and amphiphilic-sheet profiles (Fig. 3.1B).

Recombinant RvIL-6 is two-to-threefold less potent than MacIL-6 — We previously reported that GST-RvIL-6 was almost 4000-fold less potent than recombinant human IL-6 (226). To achieve a more accurate comparison of the specific activities of viral and host IL-6, we expressed both RvIL-6 and MacIL-6 under the same conditions in E. coli, and then tested the purified proteins in parallel. As shown in Fig. 3.2, recombinant RvIL-6 and MacIL-6 proteins expressed without GST showed a 2-3-fold difference in potency (Fig 3.2B). Remarkably, GST-MacIL-6 was also twice potent as GST-RvIL-6 in this assay (data not shown), indicating that the GST moiety interferes with the function of both proteins equally. Therefore, when expressed under similar conditions, the individual bioactivities of RvIL-6 and MacIL-6 are separated by a much smaller magnitude than the 4000-fold difference in potency that we had previously reported between GST-RvIL-6 and human IL-6 (226).

Effect of various antibodies on the function of RvIL-6 and MacIL-6 — In a previous study, we suggested that the mechanism of signal transduction by RvIL-6 might be analogous to that of host IL-6, since RvIL-6 was completely blocked by anti-gp130, and partially by anti-IL-6R (226). We have confirmed these previous results in the current study, and additionally found that polyclonal rabbit anti-RvIL-6 (#1011) neutralized RvIL-

6 (Fig. 3.3A, IC_{50} ~ 2 mg/ml), but not MacIL-6 (Fig 3.3B). As expected, pre-immune rabbit IgG, as well as anti-CD3 (FN18) each had no effect on either protein (Fig. 3A, and B). The differential neutralizing effects of anti-RvIL-6 against RvIL-6 relative to MacIL-6 was not unique to the protein preparations used in Figs. 3A and B, as anti-IL-6R had a similar effect against two other preparations of RvIL-6 and MacIL-6 (Fig. 3.3C). We further found that polyclonal anti-human IL-6 effectively blocked MacIL-6 (IC_{50} ~ 10 ng/ml) but did not have any effect on RvIL-6 (Fig. 3.3D), indicating that anti-human IL-6 cross-reacts with MacIL-6 but does not block RvIL-6, while anti-RvIL-6 recognizes RvIL-6 but not MacIL-6.

Monoclonal anti-IL-6R effectively blocked MacIL-6; however, as shown in Fig. 3.3E, at least five times as much anti-IL-6R was required to achieve a comparable level of neutralization of RvIL-6 relative to MacIL-6 (i.e., IC_{50} against MacIL-6 ~ 1.75 μg/ml, while IC_{50} against RvIL-6 ~ 9 μg/ml). Moreover, while MacIL-6 could be completely neutralized by about 12.5 μg/ml of anti-IL-6R, no more than 50-60% of the RvIL-6 signal could be blocked even in the presence of up to 30 μg/ml of anti-IL-6R. Therefore, anti-IL-6R is less effective against RvIL-6 compared to MacIL-6. Coupled with the fact that RvIL-6 is at-least threefold *less* potent than MacIL-6 (see Fig. 2 above), the data suggest that MacIL-6 and RvIL-6 indeed exhibit differences both in the requirement for, and/or mechanism of interaction with the receptor(s).

RvIL-6 and MacIL-6 proliferative effects on B9 cells are additive — One of the implications for potential differences in receptor usage between RvIL-6 and MacIL-6 is that the viral protein may have some effect on normal host IL-6 function when both

proteins are used together. To the contrary, we found that when MacIL-6 and RvIL-6 were pre-mixed and then added to B9 cells, the proteins produced an additive signal (Fig. 3.4A). In addition, when increasing concentrations of MacIL-6 were mixed with a fixed concentration of RvIL-6 and then immediately added to B9 cells, the underlying RvIL-6 signal was enhanced according to the dose of added MacIL-6 (Fig. 3.4B, closed circles). However, when cells were pre-incubated with a fixed concentration of RvIL-6 for 30 min. before adding increasing concentrations of MacIL-6 (Fig. 3.4B, closed triangles), the additive signal due to added MacIL-6 was considerably less than when the same concentrations of the proteins were added at the same time.

Interestingly, the reciprocal mixing experiment revealed a slightly different picture. For instance, adding increasing amounts of RvIL-6 to cells that were pre-incubated with MacIL-6 (Fig. 3.3C, open triangles) produced virtually the same level of additive signal as when the proteins were added at the same time (Fig. 3.4C, closed squares). This result is in contrast to data in Fig. 3.3B above, where pre-incubation of RvIL-6 with B9 cells appeared to attenuate the additive signal resulting from added MacIL-6. Together, these results indicate that prior binding of RvIL-6 to B9 cells may partially prevent optimal interactions between MacIL-6 and its receptor, but MacIL-6 does not have the same effect on RvIL-6. The mechanism by which prior binding by RvIL-6 to B cells would affect the quality of a subsequent MacIL-6 signal is not clear. One possibility is that RvIL-6 may directly bind a component of the receptor used by MacIL-6, possibly gp130, in a manner that prevents gp130 from fully transducing a signal triggered by the MacIL-6/IL-6R complex.

RvIL-6 binds to the surface of B9 cells more tightly than MacIL-6 — The finding that prior binding of RvIL-6 to target cells may have an attenuating effect on subsequent binding by MacIL-6 compelled us to examine whether RvIL-6 might bind to B9 cells more tightly than MacIL-6. To do this, B9 cells were incubated with RvIL-6 or MacIL-6 for 30 min. on ice, and the proliferative activities of cell-bound as well as unbound protein recovered in the wash were determined. In this experiment, most of RvIL-6 remained bound to cells, while most of MacIL-6 was recovered in the wash (Fig. 3.4D), suggesting either that RvIL-6, unlike MacIL-6, interacts more tightly with its receptor on B9 cells, or that the two proteins display distinct affinities for the same receptor.

RvIL-6 and MacIL-6 function independent of each other — The signaling complex for human IL-6 consists of two molecules each of IL-6, IL-6R and gp130. To determine whether both MacIL-6 and RvIL-6 can be recruited into the same signaling complex, the proteins were pre-mixed and then added to B9 cells in the presence of either anti-RvIL-6 or anti-human IL-6. These antibodies are specific to RvIL-6 and MacIL-6 respectively (see Fig. 3.3C&D), so that if RvIL-6 and MacIL-6 form a stimulatory hetero-complex, each antibody would neutralize only a portion of the additive signal. However, we found that anti-RvIL-6 and anti-human IL-6 were separately able to block up to 50% of the response; and together, anti-RvIL-6 and anti-human IL-6 completely blocked the additive signal (Fig. 3.5A). The partial block by each individual antibody was not due to limiting antibody because the same range of antibody concentrations used in Fig. 3.5A was sufficient to completely neutralize the same amount of protein (Fig. 3.5B). Interestingly, each antibody when used in excess still blocked only about 50% of the

additive response, while a combination of both antibodies removed the entire additive activity (Fig. 3.5C). These results suggest that RvIL-6 and MacIL-6 do not associate but may instead function independently, perhaps via interaction with distinct receptor subunits.

RvIL-6 prevents apoptosis and induces neurite outgrowth in NGF-primed PC12 cells — PC12 cells depend on trophic factors such as nerve growth factor (NGF) for survival and differentiation; however, in the absence of NGF, PC12 cells lose their capacity to differentiate and instead undergo programmed cell death (270, 318) Because PC12 cells express both IL-6R and gp130 (294), cellular IL-6 can rescue NGF-starved PC12 cells (497) and further stimulate these cells to differentiate and acquire a neuronal phenotype independently and synergistically with NGF (525, 527). To determine whether RvIL-6 shares this function with cellular IL-6, we incubated NGF-primed PC12 cells with various combinations of RvIL-6 and NGF, and then scored the cultures for neurite outgrowth. In the presence of sub-optimal concentrations of NGF (0 to 10 ng/ml), RvIL-6 induced neurite outgrowth by at least two cell-body lengths in almost 55% of treated cells and also exhibited a synergistic interaction with NGF (Fig. 3.6). Notably, a slight reduction in the percentage of cells with neurites was observed in cells that were cultured with 10 ng/ml of NGF and 100 ng/ml of RvIL-6; whether this reduction is due to technical error or systemic feed-back regulation is not clear.

We also investigated the ability of both RvIL-6 and MacIL-6 to rescue NGF-primed PC12 cells from apoptotic death using both MTT conversion (113) as well as cleavage of activated poly-(ADP-ribose) polymerase (PARP) (176) as independent indicators of

apoptosis; data from both these assays are correlatively presented in Fig. 3.7. Both MacIL-6 (Fig. 3.7A) and RvIL-6 (Fig. 3.7B) were each able to rescue PC12 cells from apoptosis, although some striking differences were notable. In MacIL-6-treated cells, over 95% of cells were protected (Fig. 3.7A, lane 2). Both anti-murine and anti-human gp130 each blocked the MacIL-6-mediated rescue by 30% and 50% respectively (Fig. 3.7A, lanes 3 and 4), while anti-IL-6R blocked the MacIL-6 signal by about 40% (Fig. 3.7A, lane 5). Similarly, over 97% of NGF-primed cells were protected from apoptosis by RvIL-6 (Fig. 3.7B, lane 1); anti-RvIL-6 (lane 2), as well as anti-human gp130 (lane 4) were able to neutralize RvIL-6, while control anti-CD3 (lane 3) had no effect. Remarkably, anti-IL-6R that was able to block MacIL-6 (see Fig. 3.7A, lane 5) failed to block RvIL-6, consistent with the apparent contrast in the relative effects of anti-IL-6R against RvIL-6 and MacIL-6 (compare with Fig. 3.3E). Therefore, data from the B9 assay and the PC12 system are in agreement with respect to anti-IL-6R effects on RvIL-6 function.

The anti-apoptotic effect of RvIL-6 was also found to be dose-dependent, as depicted by the progressive stability of PARP in the presence of increasing amounts of RvIL-6 (Fig. 3.7C). The data also suggest that the mechanism for the anti-apoptotic effects of RvIL-6 may be analogous to that of human IL-6, involving Jak/Stat-mediated gp130 activation (52, 463, 515). In fact, the dose-dependent protection by RvIL-6 correlated with the level of gp130 phosphorylation (Fig. 3.7C), as anti-gp130 immuno-complexes from lysates of RvIL-6-treated cells contained hyper-phosphorylated gp130 molecules (Fig. 3.7C). In contrast, the level of activated (phosphorylated) ERK remained unchanged over the same range of RvIL-6 concentrations, suggesting that, like human IL-6, the effects of

RvIL-6 on NGF-primed PC12 cells likel occur via the classical JAK/STAT pathway rather than the Ras/Erk pathway that is normally stimulated by NGF (211, 524, 525).

DISCUSSION

We recently reported that GST-RvIL-6 was almost 4000-fold less potent than human IL-6 (226). In that study, we suggested that the extraneous GST moiety on RvIL-6 hindered interaction between RvIL-6 with the receptor, resulting in such a considerable reduction in activity. Here, we have attempted to carry out a more accurate comparative analysis of the bioactivity of RvIL-6 relative to the appropriate host protein encoded by the rhesus macaque (MacIL-6). We now present evidence that RvIL-6 and MacIL-6 expressed under the same conditions are functionally separated by a less-than-threefold difference in potency. This result was initially surprising, since RvIL-6 shares limited homology with MacIL-6 (see Fig. 3.1A) but probably reflects a characteristic feature of the IL-6 family of cytokines that are structurally distinct but exhibit overlapping functions (87).

Poor sensitivity to anti-IL-6R antibody has been demonstrated not only for HHV-8 vIL-6 (65, 359) but also for RRV RvIL-6 (226) (and this study), raising questions as to the degree to which IL-6R plays a role in the signaling mechanisms of these viral proteins. Elsewhere, studies have shown that while HHV-8 vIL-6 may induce signaling events in IL-6R^{-} cells (202, 321), the IL-6R subunit could still play a role in vIL-6 signaling if available on the target cell surface (65, 359), indicating that interactions between the viral protein(s) and the cellular receptor(s) may be complex.

Our current data show that in spite of differences in sensitivity to anti-IL-6R, RvIL-

6 and MacIL-6 produce an additive signal when used in conjunction (Fig. 3.4); these proteins may therefore function independently of each other. Interestingly, we also found that prior binding of RvIL-6 to B9 cells may prevent optimal formation of a signaling complex between MacIL-6, IL-6R, and gp130, while prior binding of MacIL-6 to B9 cells does not appear to have an attenuating effect on subsequent RvIL-6 signaling (Fig. 3.4B&C). These data are consistent with a model where RvIL-6 possibly binds a receptor subunit other than the primary receptor for MacIL-6, so that prior RvIL-6 binding to this subunit somehow affects MacIL-6 signaling. This model presupposes direct interaction between RvIL-6 and gp130, an interaction that would conceivably interfere with participation of gp130 in the transduction of a signal initiated by the MacIL-6/IL-6R complex. The possibility that RvIL-6 can utilize a receptor subunit that is separate from the primary receptor of MacIL-6 could also explain our finding that RvIL-6 and MacIL-6 produce an additive signal rather than compete for the same receptor.

We have additionally shown that RvIL-6 is capable of preventing apoptosis in NGF-primed PC12 cells, indicating that RvIL-6 shares this neuro-regulatory function of cellular IL-6. We currently have no evidence for the significance of such a function for RvIL-6 in the neuronal compartment of SIV/RRV-infected animals. One possibility is that in the event of SIV-induced neuronal damage, an anti-apoptotic role for RvIL-6 expressed locally or systemically delivered to the CNS could reverse such damage. Interestingly, a number of hypotheses have been posed with respect to the potential protective effect of HHV-8 from AIDS dementia in individuals infected with both HIV and HHV-8 (22, 48, 122, 127, 248, 269, 407), although there is no direct evidence for the HHV-8-encoded vIL-

6 as a correlate of such a protective advantage from HHV-8. In this respect, our SIV/RRV infection model should provide a framework for investigation of the pathogenetic function of viral IL-6 proteins as well as other viral gene products suspected to play a role in viral pathogenesis.

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FIG. 3.1. **Amino acid sequence alignment and predicted structural features of RvIL-6 and MacIL-6.** *A,* Sequences were analyzed using the ClustalW algorithm (486) formatted for pair-wise alignment with the BLOSUM similarity matrix. Identical residues (18.6%) are highlighted and boxed while conservative changes (18.1%) representing amino acids with similar charge or side-chain character are also boxed and marked with an asterisk ("*"). *B,* The Goldman/Engelman/Steiz algorithm in the MacVector protein analysis package (Oxford Molecular) was used to analyze hydrophilicity and amphiphilic-sheet profiles of RvIL-6 and MacIL-6. Amino acid residues are numbered beginning with the first Methionine in each sequence.

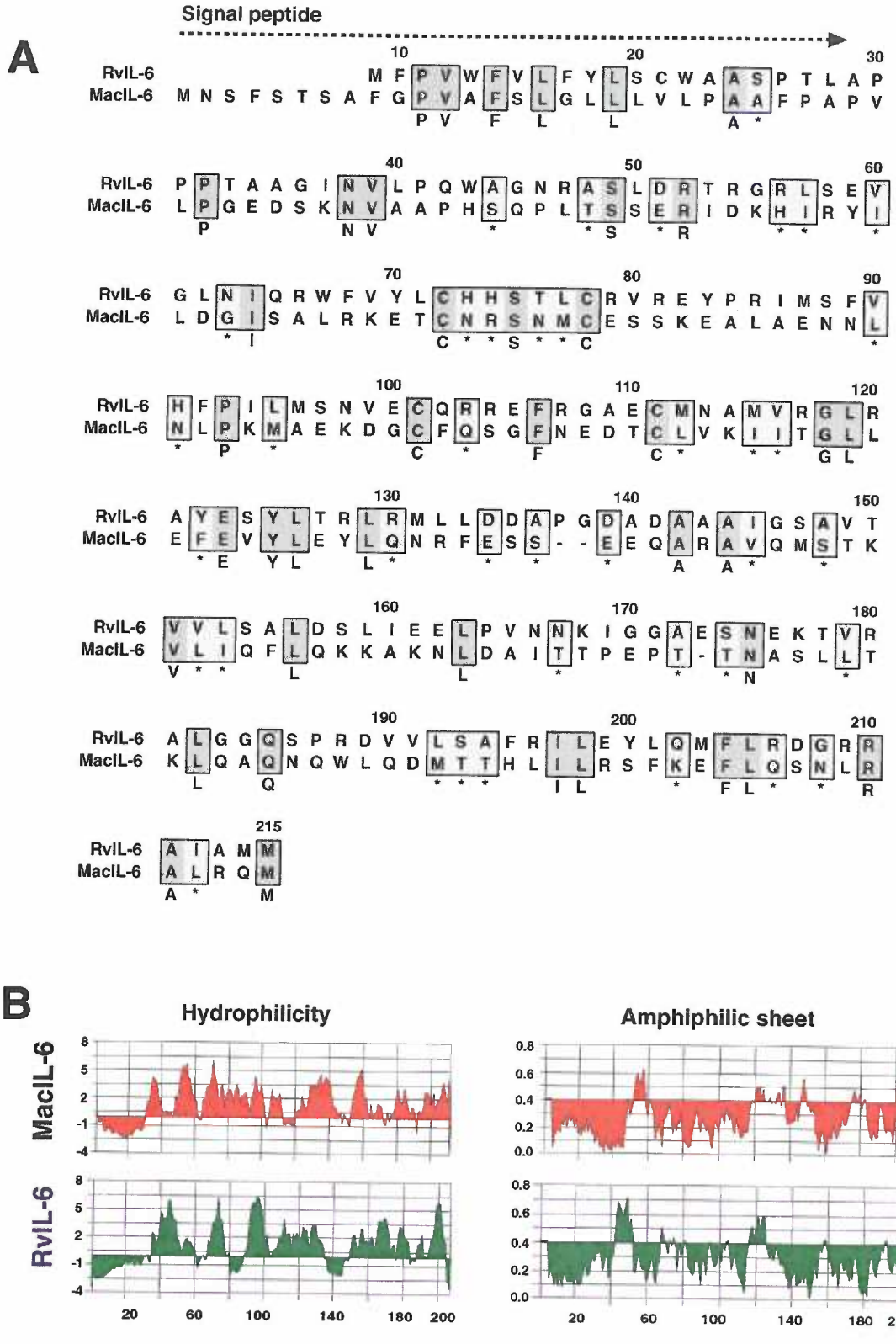

A
Signal peptide
RvIL-6
MacIL-6
B
Hydrophilicity
Amphiphilic sheet
MacIL-6
RvIL-6

FIG. 3.2. **Bioactivity of recombinant RvIL-6 relative to rhesus monkey IL-6.** Pre-starved B9 cells were cultured at $5x10^4$ cells/well in the presence of serial dilutions of RvIL-6 or MacIL-6 proteins expressed either in E. *coli* (RvIL-6 and MacIL-6) or insect cells (RvIL-6 only), and cell proliferation was analyzed by 3H-thymidine uptake as previously described (226). (Statistical analysis: *, $p<0.05$; **, $p<0.001$ compared to "no-Ab" control)

Relative potency between RvIL-6 and rhesus monkey IL-6 (MacIL-6)

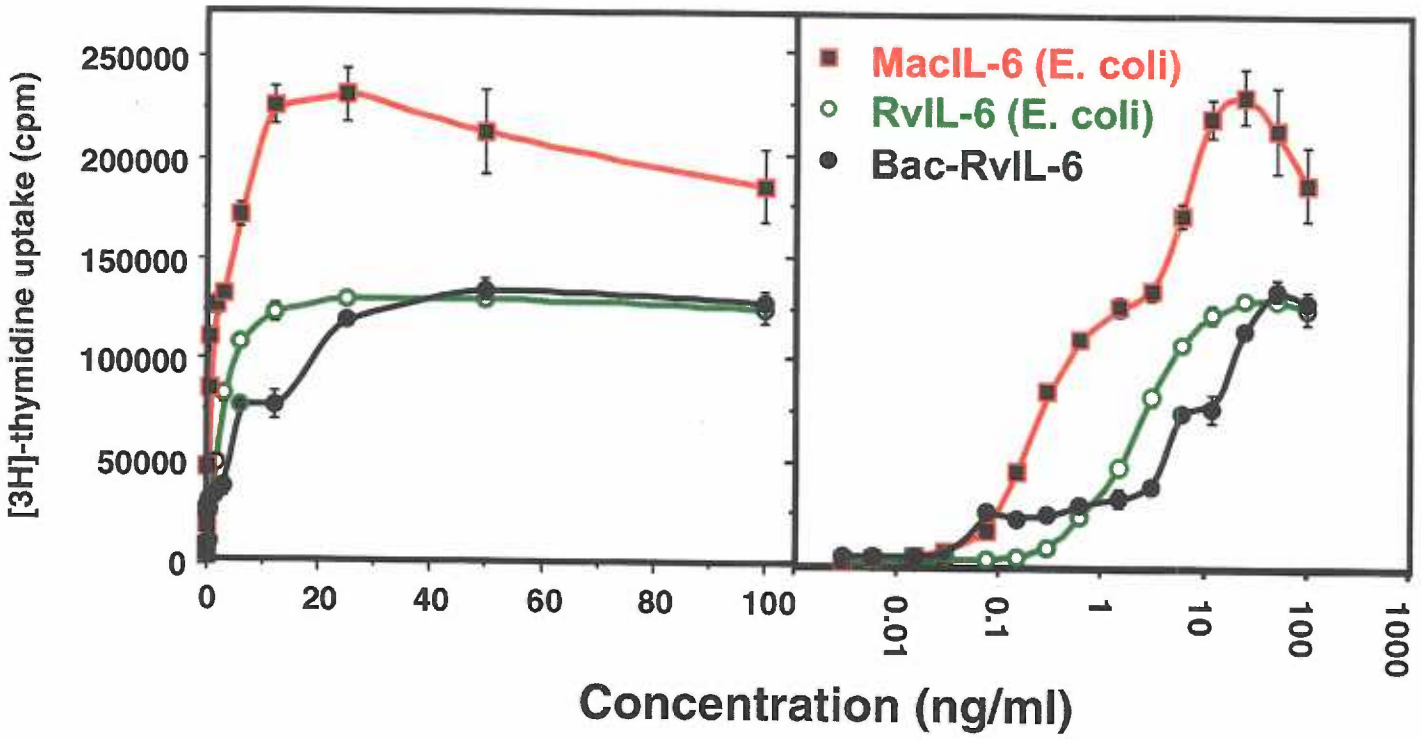


FIG. 3.3. **Effect of neutralizing antibodies on RvIL-6 and MacIL-6 function**. The bioactivity of ***A***, Mature RvIL-6 (60 ng/ml) or ***B***, Mature MacIL-6 (20 ng/ml) was examined in the presence of increasing amounts of anti-rhesus CD3 (FN18, closed circles), rabbit pre-immune IgG (open circles), or rabbit anti-RvIL-6 (1011, closed squares). ***C***, Two independent preparations of RvIL-6 (squares) and MacIL-6 (triangles) were tested on B9 cells in the presence of anti-RvIL-6. ***D***, RvIL-6 (60 ng/ml, closed circles) or MacIL-6 (20 ng/ml, open circles) were added at the same time to B9 cells in the presence of anti-huIL-6 antibody. ***E***, B9 cells were pre-incubated with increasing concentrations of anti-IL-6R on ice for 15 min., and either RvIL-6 (20 ng/ml, closed circles) or MacIL-6 (20 ng/ml, closed squares) was added to each triplicate set. Cell proliferation was analyzed by 3H-thymidine incorporation as described above. (Statistical analysis: *, $p<0.005$; **, $p<0.0001$ compared to control cells without antibody).

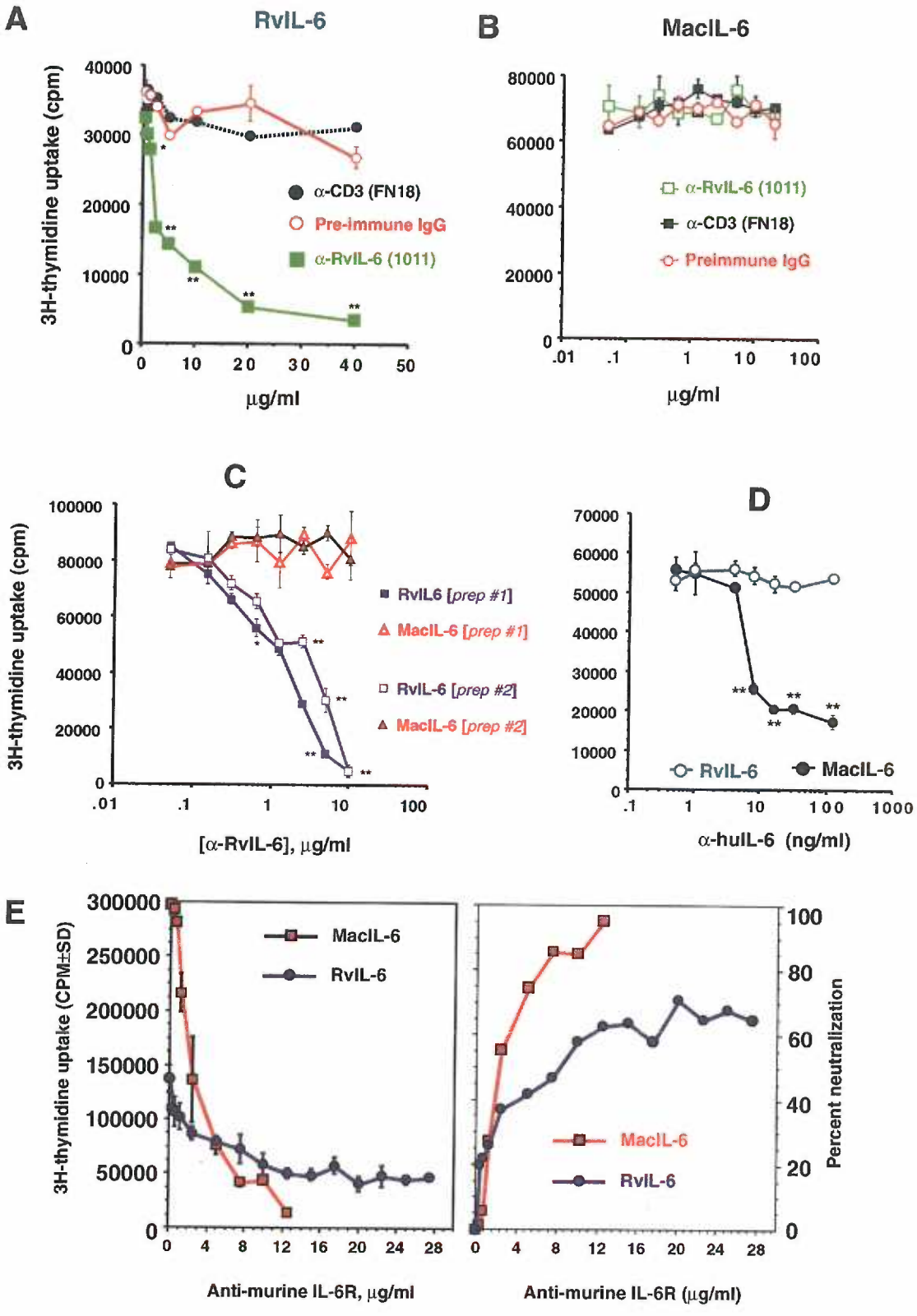

A
RvIL-6
3H-thymidine uptake (cpm)
40000
30000
20000
10000
0
0 10 20 30 40 50
µg/ml
α-CD3 (FN18)
Pre-immune IgG
α-RvIL-6 (1011)
B
MacIL-6
80000
60000
40000
20000
0
.01 .1 1 10 100
µg/ml
α-RvIL-6 (1011)
α-CD3 (FN18)
Preimmune IgG
C
3H-thymidine uptake (cpm)
100000
80000
60000
40000
20000
0
.01 .1 1 10 100
[α-RvIL-6], µg/ml
RvIL6 [prep #1]
MacIL-6 [prep #1]
RvIL-6 [prep #2]
MacIL-6 [prep #2]
D
70000
60000
50000
40000
30000
20000
10000
0
.1 1 10 100 1000
α-huIL-6 (ng/ml)
RvIL-6
MacIL-6
E
3H-thymidine uptake (CPM±SD)
300000
250000
200000
150000
100000
50000
0
0 4 8 12 16 20 24 28
Anti-murine IL-6R, µg/ml
MacIL-6
RvIL-6
100
80
60
40
20
0
Percent neutralization
0 4 8 12 16 20 24 28
Anti-murine IL-6R (µg/ml)
MacIL-6
RvIL-6

FIG. 3.4. **A combination RvIL-6 and MacIL-6 proteins produces an additive stimulatory effect on B9 cells.** *A,* B9 cells were cultured in the presence of serial dilutions of either RvIL-6 alone (striped bars), MacIL-6 alone (checkered bars), or pre-mixed dilutions of both RvIL-6 and MacIL-6 (solid black bars). *B,* RvIL-6 was either titrated alone (RvIL-6, open circles), or a constant amount of RvIL-6 within its sub-linear range of stimulation (2 ng/ml) was first pre-mixed with increasing concentrations of MacIL-6 and then added to B9 cells (RvIL-6 + MacIL-6, closed circles). In a parallel assay, B9 cells were initially pre-incubated with RvIL-6 (2 ng/ml) on ice for 30 min. followed by addition of increasing amounts of MacIL-6 (pre-RvIL-6 + MacIL-6, closed triangles). *C,* B9 cells were either incubated with serial dilutions of MacIL-6 alone (MacIL-6, open squares), or a constant amount of MacIL-6 (2 ng/ml) was first pre-mixed with increasing concentrations of RvIL-6 and then added to B9 cells (RvIL-6 + MacIL-6, closed squares). Additionally, B9 cells were initially pre-incubated with MacIL-6 (2 ng/ml) on ice for 30 min. followed by addition of increasing concentrations of RvIL-6 (open triangles). *D*, B9 cells were incubated with excess MacIL-6 and RvIL-6 at 4°C, pelleted, and both "bound" and "unbound" protein were assayed by 3H-thymidine uptake.

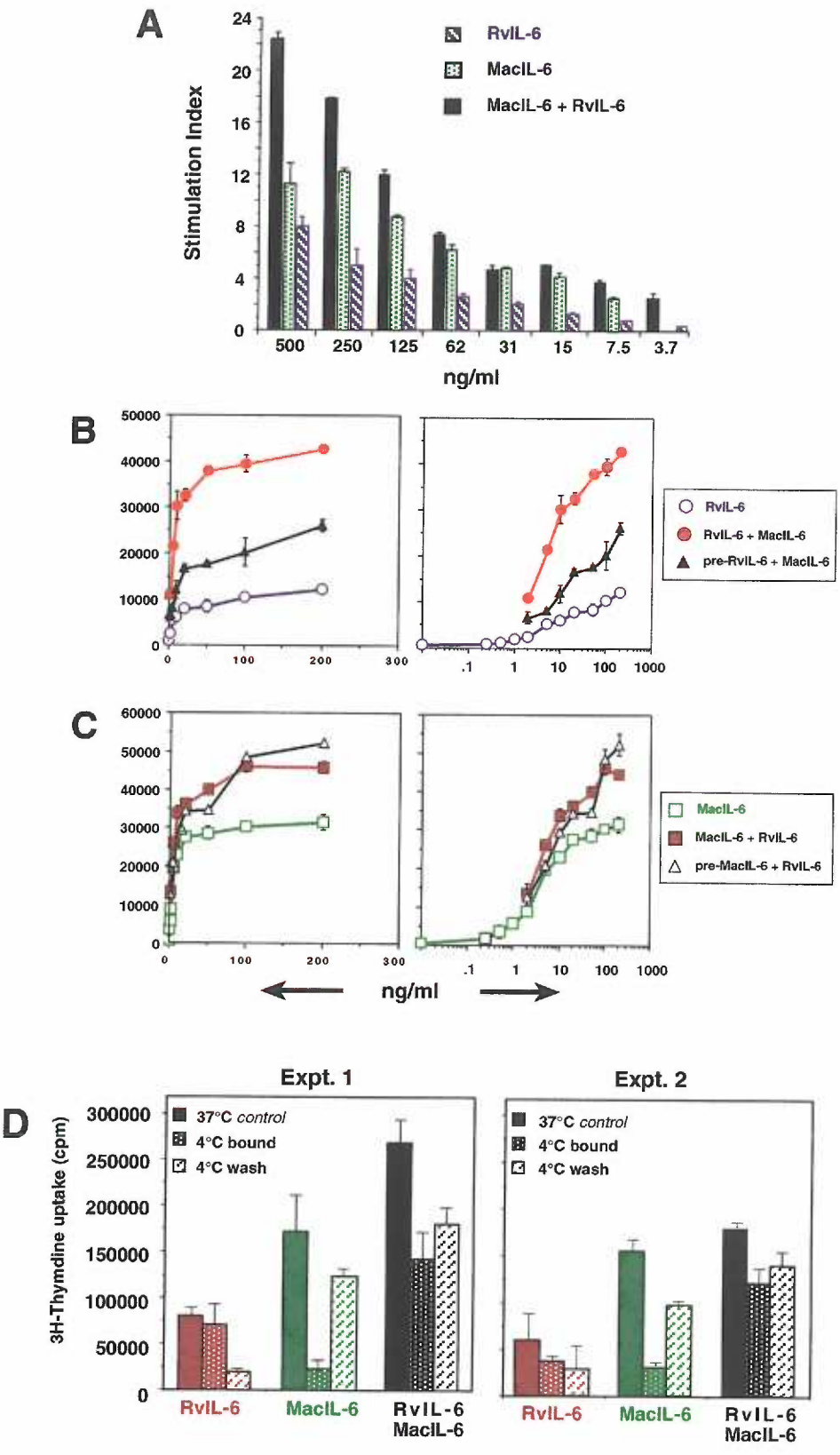

A
Stimulation Index
24
20
16
12
8
4
0
500
250
125
62
31
15
7.5
3.7
ng/ml
RvIL-6
MacIL-6
MacIL-6 + RvIL-6
B
50000
40000
30000
20000
10000
0
0
100
200
300
.1
1
10
100
1000
RvIL-6
RvIL-6 + MacIL-6
pre-RvIL-6 + MacIL-6
C
60000
50000
40000
30000
20000
10000
0
0
100
200
300
.1
1
10
100
1000
ng/ml
MacIL-6
MacIL-6 + RvIL-6
pre-MacIL-6 + RvIL-6
D
Expt. 1
Expt. 2
3H-Thymdine uptake (cpm)
300000
250000
200000
150000
100000
50000
0
37°C control
4°C bound
4°C wash
RvIL-6
MacIL-6
RvIL-6
MacIL-6

FIG. 3.5. **The stimulatory effects RvIL-6 and MacIL-6 on B9 cells are independent.** ***A***, A combination of MacIL-6 (20 ng/ml) and RvIL-6 (60 ng/ml) was added to B9 cells in the presence of increasing amounts of anti-CD3 (crossed squares), anti-huIL-6 (closed squares), anti-RvIL-6 (open squares), anti-gp130 (open triangles), or a combination of both anti-huIL-6 and anti-RvIL-6 (closed triangles). ***B***, B9 cells were separately stimulated with MacIL-6 (20 ng/ml) or RvIL-6 (60 ng/ml of) in the presence of anti-huIL-6 (solid black bars), or anti-RvIL-6 (hatched bars), respectively, to test the neutralizing effects of these antibodies on the same concentration of proteins as that used in panel *A*.. *C,* MacIL-6 (20 ng/ml) or RvIL-6 (60 ng/ml) were added to B9 cells in the presence of excess amounts of anti-RvIL-6 (40 μg/ml), anti-huIL-6 (10 μg/ml), anti-gp130 (5 μg/ml), or a combination of anti-huIL-6 (10 μg/ml) and anti-RvIL-6 (40 μg/ml). Cell proliferation was assayed by 3H-thymidine uptake. (Statistical analysis: *, $p<0.01$; **, $p<10^{-4}$).

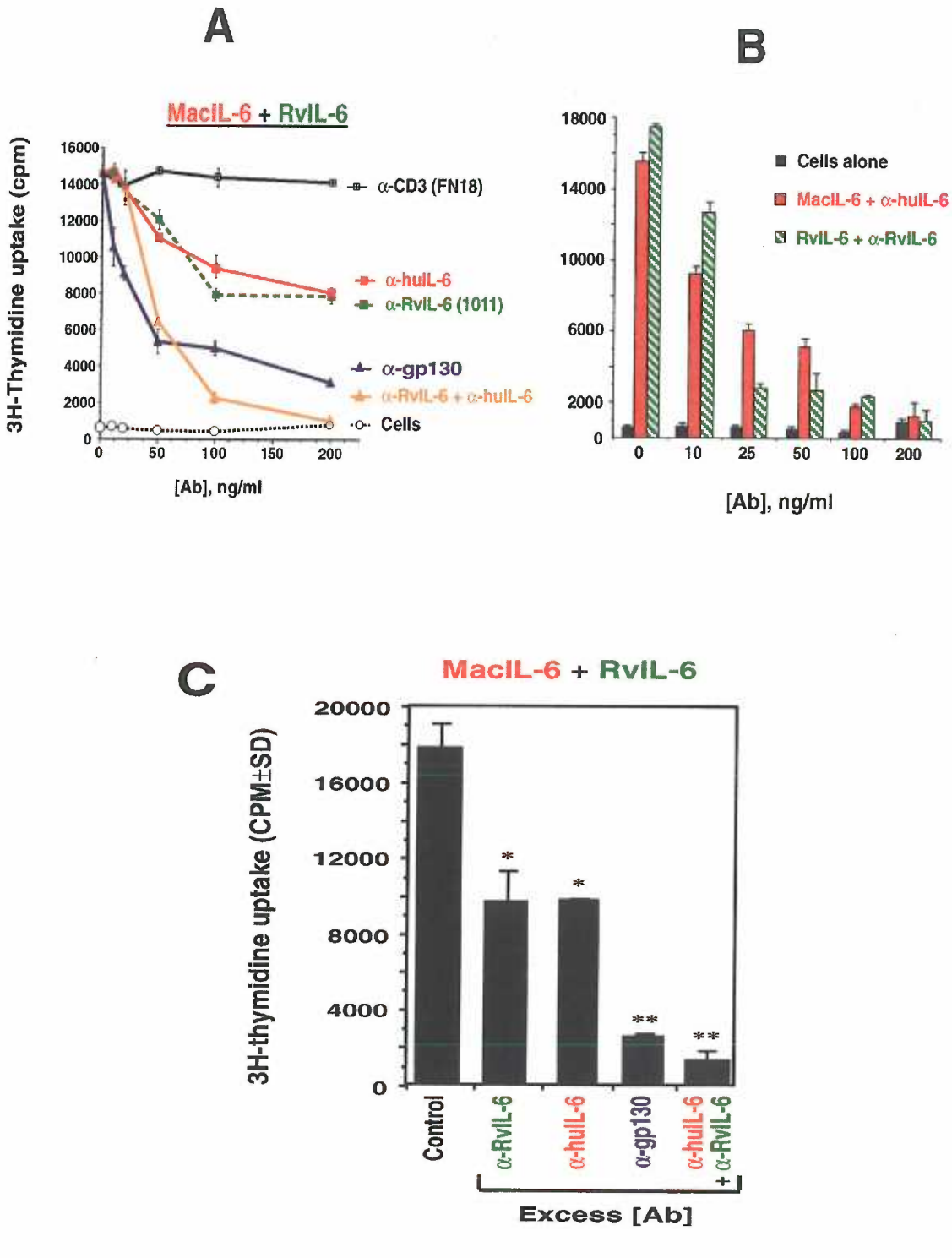

A
MacIL-6 + RvIL-6
3H-Thymidine uptake (cpm)
16000
14000
12000
10000
8000
6000
4000
2000
0
0
50
100
150
200
[Ab], ng/ml
α-CD3 (FN18)
α-huIL-6
α-RvIL-6 (1011)
α-gp130
α-RvIL-6 + α-huIL-6
Cells
B
18000
14000
10000
6000
2000
0
0
10
25
50
100
200
[Ab], ng/ml
Cells alone
MacIL-6 + α-huIL-6
RvIL-6 + α-RvIL-6
C
MacIL-6 + RvIL-6
3H-thymidine uptake (CPM±SD)
20000
16000
12000
8000
4000
0
*
*
**
**
Control
α-RvIL-6
α-huIL-6
α-gp130
α-huIL-6 + α-RvIL-6
Excess [Ab]

Fig. 3.6 — **Molecular cross talk between RvIL-6 and nerve growth factor (NGF).** NGF-starved PC12 cells were re-plated in the presence of indicated combinations of RvIL-6 and NGF, and the cultures were scored for neurite outgrowth by counting cells showing at least 2 cell-body lengths of new neurites. Cells were counted in five separate fields per well. Data are presented as a percentage of cells in each category that had at least 2 cell body lengths of new neurites, compared to control cells that were re-plated in the absence of stimulus.

Synergistic cross talk between RvIL-6 and NGF signaling pathways

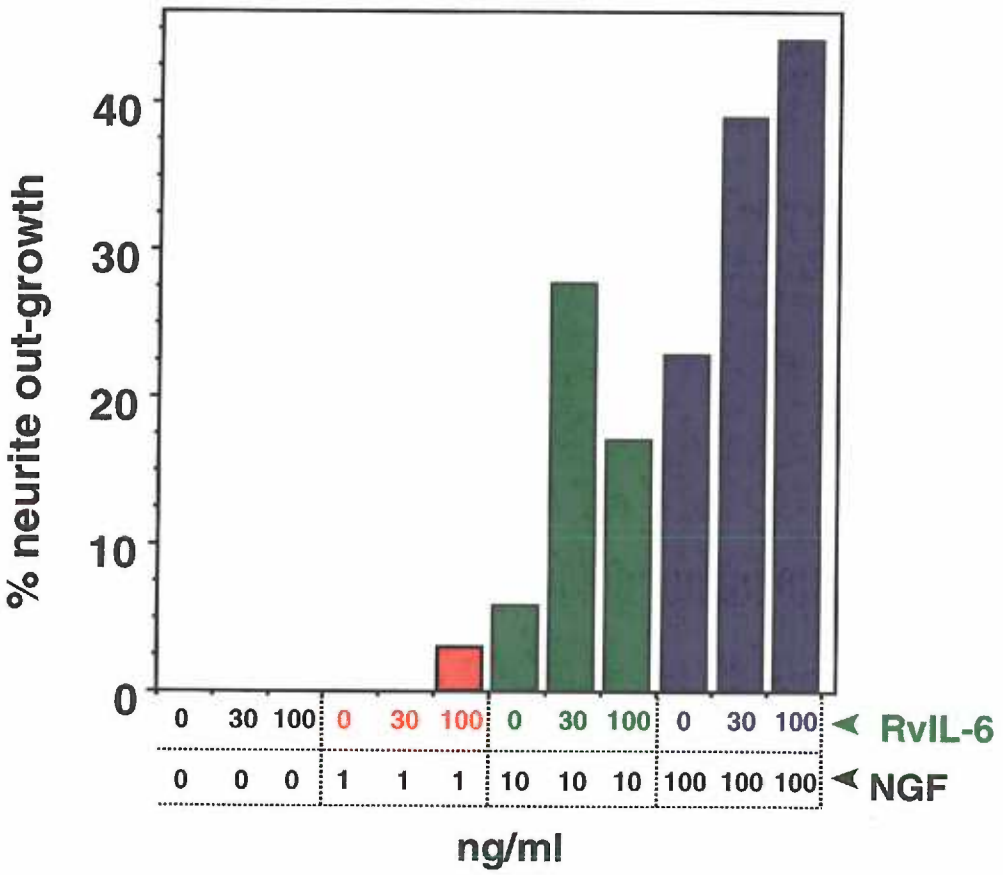
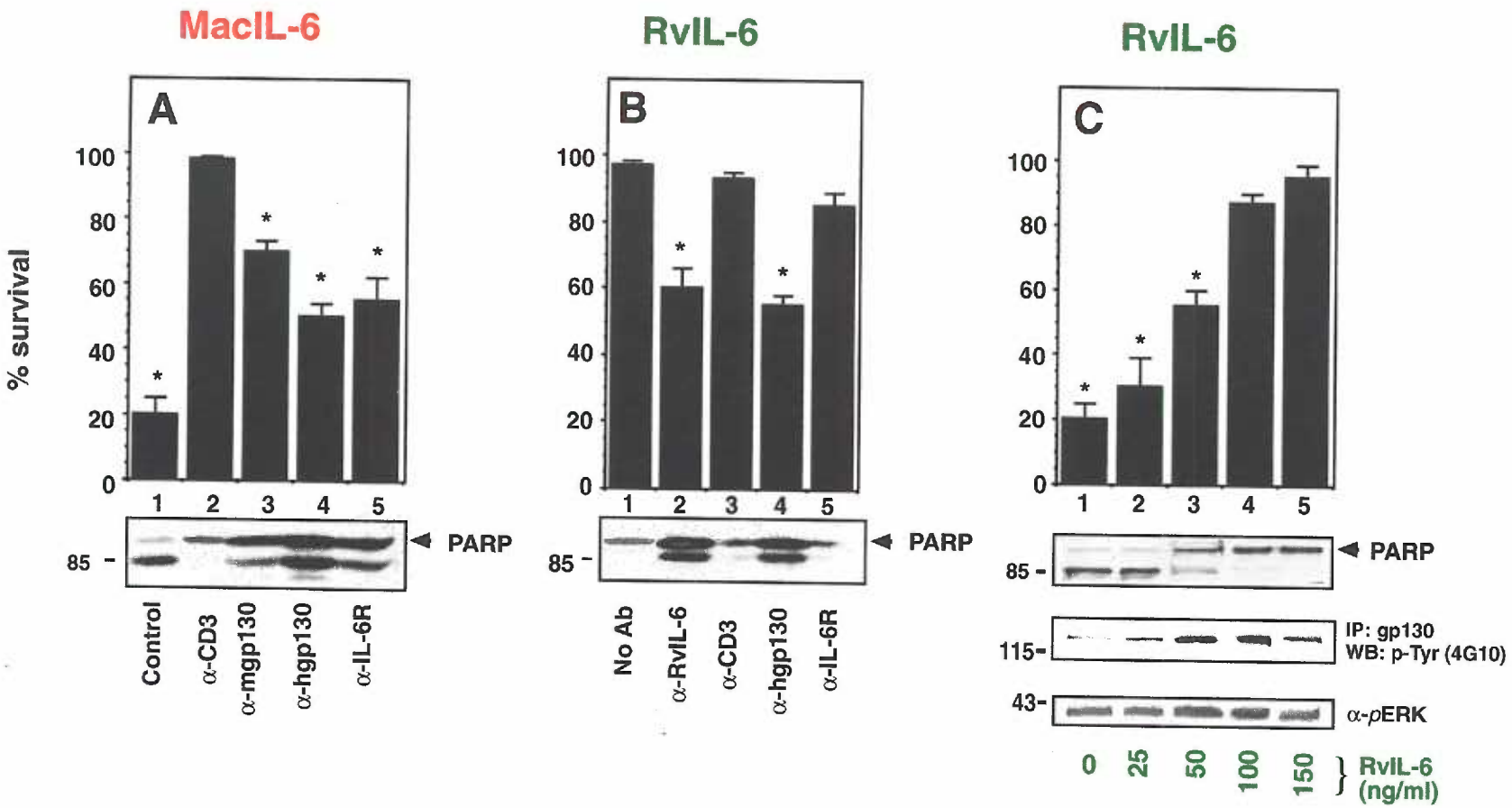


Fig. 3.7 — **RvIL-6 can rescue the PC12 cells from apoptosis following NGF withdrawal.** *A,* NGF-primed PC12 cells were re-plated alone (control, lane 1), or with MacIL-6 (50 ng/ml, lanes 1-4) in the presence of anti-CD3 (10 μg/ml, lane 2), anti-murine gp130 (10 μg/ml, lane 3), anti-human gp130 (5 μg/ml, lane 4), or anti-IL-6R (2 μg/ml). *B,* NGF-primed PC12 cells were incubated with either RvIL-6 (150 ng/ml) alone (lane 1), or in the presence of anti-RvIL-6 (10 μg/ml, lane 2), anti-CD3 (10 μg/ml, lane 3), anti-human gp130 (5 μg/ml, lane 4) or anti-IL-6R (10 μg/ml). After three days, treated cells were analyzed for apoptotic death either by MTT conversion (bars) or by PARP cleavage, as described in *EXPERIMENTAL PROCEDURES.* In panel *C*, NGF-primed PC12 cells were incubated with an increasing dose of RvIL-6 (1-150 ng/ml, lanes 1-5), and assayed for survival both by MTT assay or by anti-PARP immuno-blots as described above. In a separate experiment, cell lysates from each RvIL-6 treatment were subjected to SDS-PAGE and transferred to nitrocellulose membranes, and blots were incubated with anti-phospho-ERK (E-4, Santa Cruz Biotechnology). Alternatively, lysates were immunoprecipitated with anti-gp130, and the immuno-complexes were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the anti-phosphotyrosine 4G10 antibody.


MacIL-6
RvIL-6
RvIL-6
A
B
C
% survival
100
80
60
40
20
0
1 2 3 4 5
85 -
PARP
Control
α-CD3
α-mgp130
α-hgp130
α-IL-6R
No Ab
α-RvIL-6
α-CD3
α-hgp130
α-IL-6R
115-
IP: gp130
WB: p-Tyr (4G10)
43-
α-pERK
0 25 50 100 150
RvIL-6 (ng/ml)

CHAPTER 4

The aim of this study was to determine whether RvIL-6 is present in biological fluid of SIV/RRV co-infected animals that develop lymphoproliferative disease (LPD). Presence of bioactive RvIL-6 in plasma of these animals would suggest a possible link between RvIL-6 and induction of LPD.

Viral Interleukin-6 (RvIL-6) of RRV is present in plasma of SIV/RRV co-infected rhesus macaques that develop B cell hyperplasia.

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Running Title: Viral IL-6 and B cell hyperplasia in rhesus macaques

Key Words:

Rhesus macaques, Simian Immunodeficiency virus, lymphoproliferative disease, rhesus macaque rhadinovirus.

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RRV infection of SIV-seropositive rhesus macaques is associated with hyperplastic B-cell expansion as well as other cell proliferative disorders similar to those that result from interleukin-6 (IL-6) deregulation in individuals infected with both HIV and HHV-8. Like HHV-8, RRV encodes a homologue of interleukin-6 (RvIL-6) with B cell growth-stimulatory activity. We therefore investigated whether biological fluid of SIV/RRV co-infected animals that develop LPD also contain RvIL-6 with IL-6-like activity. We show that plasma from SIV/RRV co-infected animals with LPD, unlike plasma from animals infected with SIV or RRV alone, supported growth of B9 cells but did not contain rhesus macaque IL-6 protein, suggesting that plasma IL-6-like activity was due to a factor other than host IL-6. Plasma activity could be blocked by anti-gp130, and partially by anti-IL-6R, suggesting that the factor in LPD plasma may utilize a component of the host IL-6 signaling pathway that is anchored by membrane-bound gp130. In addition, plasma activity could be blocked and also depleted by affinity precipitation with anti-RvIL-6, but not with anti-human IL-6 that cross-reacts with rhesus IL-6. In western blots, plasma from LPD animals contained RvIL-6 protein (~ 5000 pg/ml), along with an elevated concentration of another protein that could be detected with anti-human IL-6R. Together, these results suggest that RvIL-6 is present and circulates systemically in bioactive form either as a monomer or in a complex with sIL-6R, and may be available to stimulate B cell growth in vivo.

INTRODUCTION

The recent identification of human herpesvirus 8 (HHV-8) as the opportunistic agent etiologically linked to a variety of syndromes in HIV patients (149, 374) has provided new research frameworks for understanding some of the defining clinical features of HIV disease. HHV-8 belongs to the lymphotropic γ_2-herpesvirus subfamily whose members are associated with lymphoproliferation and oncogenesis in experimental hosts. Compelling evidence suggests that infection with HHV-8 is a risk factor for development of all clinical-epidemiological forms of Kaposi's sarcoma (KS) (81, 231, 370, 395, 435), the most common neoplastic complication in HIV-infected individuals. HHV-8 is also implicated in the etiology of other immune cell-growth syndromes such as body cavity-based lymphomas, also known as primary effusion lymphomas (PEL) (14, 232, 425), and a subset of multicentric Castleman's disease (MCD) (126, 457). Both PEL and MCD are generally rare in the general population but may occur more frequently in individuals that are undergoing generalized immunosuppression, often in the presence of deregulated expression of IL-6.

The preponderance, in HIV/HHV-8-infected individuals, of syndromes associated with IL-6 function has led to increasing speculation that the IL-6 homologue (vIL-6) encoded by HHV-8 may contribute to the pathogenesis of some of these disorders (16, 68, 378, 464). This view is supported by the fact that during HIV infection IL-6 receptor expression is markedly increased on monocytes and B cells, the potential reservoirs of HHV-8 (20, 204, 311, 501) that are also the likely targets of vIL-6 action in vivo. However, studies of the pathogenic contribution of individual viral gene-products as well as interactions between HIV and HHV-8 have been hampered by lack of a physiologically

relevant natural infection model for HHV-8, largely because HHV-8 is not known to infect or cause pathology in non-human animals.

Simian immunodeficiency virus (SIV) is a non-human primate lentivirus that is both genetically and biologically similar to HIV and induces a sequelae of debilitating syndromes in rhesus macaques similar to those caused by HIV in humans (305). This laboratory previously showed that experimental co-infection of SIV+ animals with the recently described rhesus monkey rhadinovirus (RRV) (116, 442) induces several of the same syndromes commonly observed in HIV+ individuals simultaneously infected with HHV-8 (523). A major manifestation in SIV/RRV co-infected animals is angiofollicular lymphadenopathy and CD20+ B cell hyperactivation that correlates with RRV viremia in peripheral blood mononuclear cells (PBMC) (41, 523). RRV is phylogenetically closest to HHV-8 than to other known members of the γ_2-herpesvirus subfamily (10, 442), with nearly all open reading frames in RRV as co-linearly represented by corresponding analogues in the HHV-8 genome. HHV-8 and RRV encode a number of homologous versions of host proteins normally involved in the control of cell growth and survival, including distinct homologues of IL-6 encoded by K2 of HHV-8 (vIL-6) (420) and R2 of RRV (RvIL-6) (10, 442). Like HHV-8, RRV is associated with disease only in the context of immune breakdown. Therefore, based on our hypothesis that RRV RvIL-6 plays a role in B cell hyperplasia in SIV/RRV co-infected animals, we now present evidence that RvIL-6 is indeed expressed and circulates as a functional protein in biological fluid of SIV/RRV co-infected animals that develop LPD. Our data also suggest that induction of LPD may be related to the level of bioactive RvIL-6 in animal plasma, providing a basis for exploration

of therapeutic modalities targeted towards expression and/or function of this viral cytokine.

Materials and Methods

Cell culture conditions — The murine IL-6-dependent hybridoma/plasmacytoma B9 cell line was obtained from Dr. L. Aarden (Central Laboratory, Red Cross Blood Transfusion, Amsterdam, The Netherlands) and maintained as previously described (226). Evaluation of IL-6-like bioactivity in biological fluids of test animals was performed using a bioassay based on the response of the IL-6-dependent murine plasmacytoma B9 cell line according to a published procedure (226).

Reagents — Anti-human IL-6 was purchased from R&D Systems (Minneapolis, MN). Anti-murine IL-6R antibody was purchased from Pharmingen (San Diego, CA). Rabbit anti-human gp130 was a gift from Dr. Beth Habecker (Oregon Health Sciences University, Portland, OR), and anti-rhesus CD3 (FN18) was generously provided by Dr. M. Jonker (TNO). Generation of rabbit polyclonal anti-RvIL-6 antibody (1011), as well as expression and purification of recombinant RvIL-6 and MacIL-6 have been described in the *Experimental Procedures* section of Chapter 3. Rhesus amniotic fluid (source of physiological rhesus IL-6) was provided by Drs. Miles Novy and George Haluska at the Oregon Regional Primate Research Center).

Collection and analysis of biological fluid — Blood plasma samples were collected at various times either from normal animals or from animals that were infected with (i) RRV alone, (ii) SIV alone, or (iii) both SIV and RRV, and stored at –70°C. Before use, plasma samples were thawed on ice, heat-inactivated at 56°C for 10-15 minutes to de-

activate complement proteins, centrifuged at 500 x g for 5 min., and filter-sterilized.

Enzyme-linked immunosorbent Assay (ELISA) — The Quantikine High Sensitivity (HS) ELISA kit (R&D Systems, Minneapolis, MN) developed for human IL-6 was used to detect rhesus IL-6 in rhesus monkey plasma, taking advantage of the fact that rhesus and human IL-6 proteins share at-least 98% identity at amino-acid level. The detection limit for the ELISA kit is approximately 0.1 picograms per milliliter of sample. Heat-inactivated plasma samples were either added to the ELISA plate in undiluted form, or in the plasma diluent provided with the kit. The assay was performed according to the manufacturer's instructions. Rhesus amniotic fluid was used as a source of physiological rhesus IL-6 for generating a standard curve.

Affinity-depletion of IL-6 bioactivity from animal fluid — Plasma from animal 18483 (infected with SIV*mac239* and RRV) was diluted 1:1 (v/v) in 2X B9 cell-starving media (RPMI plus 20% calf serum, 100 μM 2β-mercaptoethanol, and 2% penicillin/streptomycin). Diluted plasma samples were then added to a matrix of protein A-Sepharose beads (binding capacity ~3 mg protein A/20 mg IgG) pre-loaded with various antibodies (i.e., anti-CD3, anti-human IL-6, or anti-RvIL-6) and incubated for 90 min. at 4°C. The mixture was spun at 500 x g to exclude protein A-Sepharose beads, and the unbound fraction was sterilized through a 0.2-μm filter before use in a B9 cell proliferation assay, as previously described (226).

Western blot analysis — Animal plasma was diluted 1:5 (v/v) with PBS supplemented with 50 mM dithiothreitol (DTT) and incubated end-over-end for one hour at 4°C, and then transferred to 37°C for an additional 30 minutes. Plasma samples were then

boiled in sample buffer (20 mM Tris-HCl, pH 6.8, 20% glycerol, 200 mM 2β-mercaptoethanol, 0.02% bromophenol blue) and separated in a 10% continuous SDS-PAGE gel. Resolved proteins were transferred onto nitrocellulose membrane and incubated in blocking buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 5% non-fat milk) overnight at 4°C. After two washes in TBS-T (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.05% Tween-20), the membranes were incubated for one hour in blocking buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 1% non-fat milk) containing primary antibody (either polyclonal rabbit anti-RvIL-6 or polyclonal goat anti-sIL-6R). The membranes were subsequently incubated with respective horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence (ECL, Amersham, IL) according to standard procedures.

Analysis of associations between RvIL-6 and soluble IL-6R — To examine whether RvIL-6 associates with IL-6R, a constant amount of recombinant soluble IL-6R (R&D Systems, Minneapolis, MN) was incubated with increasing amounts (0, 5, 10, 25, 50, 100 and 200 ng) of recombinant RvIL-6 for 90 min. at 4°C. The mixture was then immunoprecipitated with polyclonal goat anti-sIL-6R, and analyzed by western blot using a cocktail of polyclonal goat anti-sIL-6R and polyclonal rabbit anti-RvIL-6 as primary antibodies, followed by incubation in a second cocktail of appropriate horseradish peroxidase-conjugated anti-goat and anti-rabbit secondary antibodies.

Statistical Analysis — All numerical results are presented as means of triplicate values +/- "standard errors" of the means. Where necessary, data variance was analyzed using SuperAnova (Abacus Concepts, Inc., Berkeley, CA), and Tukey-Kramer was used for post-hoc tests of significance.

Results

RvIL-6 can support growth of the IL-6-dependent B9 cell line (10, 226), suggesting that RvIL-6 may also exhibit B cell stimulatory activity in vivo. To determine whether bioactive RvIL-6 is present in biological fluid of animals that develop B cell hyperplasia (or LPD), we first tested plasma samples from animal 18483 that developed persistent LPD subsequent to SIV/RRV co-infection. As shown in Fig. 4.1A, 18483 plasma supported B9 cell growth in a dose-dependent manner, suggesting that this plasma sample contained IL-6-like activity. Within the linear range of dilutions, terminal plasma contained at least two-fold more activity than pre-terminal plasma. Because animal 18483 displayed marked lymphadenopathy, splenomegaly, and diffuse lymphoid hyperplasia at necropsy (523), a relatively higher level of IL-6 bioactivity in terminal plasma would be consistent with a direct relationship between plasma IL-6-like bioactivity and disease.

Plasma from uninfected animal 18563 or SIV-infected animal 18540 had minimal activity compared to 18483 (Fig. 4.1B), indicating that plasma IL-6-like bioactivity may be an attribute of SIV/RRV co-infection. However, a possible reason for absence of IL-6-like activity in plasma from animals 18563 (-) or 18540 (SIV+) could be that these samples contain an endogenous inhibitor of rhesus IL-6 that is not present in the plasma of the co-

infected animal. To test this idea, we added recombinant MacIL-6 to plasma samples and then incubated B9 cells in this mixture. Here, recombinant MacIL-6 was not inhibited by any of the plasma samples collected from animals 19563 (-), 18503 (SIV+), or animal 19286 (RRV+) (Fig. 4.1C), ruling out presence of an inhibitor of endogenous IL-6 bioactivity in the plasma of animals 18563 (-) or 18540 (SIV+) that failed to stimulate B9 cell growth.

Although the above data indicate that a factor or factors with B9 cell stimulatory potential are present in SIV/RRV co-infected animals, the data do not clarify whether this IL-6-like activity is due to RvIL-6 or rhesus IL-6. A distinction between these two proteins is critical especially since host IL-6 is elevated in serum of individuals infected with HIV (43, 60, 291) and in rhesus macaques infected with SIV(38, 189). To address this issue, we measured rhesus macaque IL-6 levels in animal plasma using a commercially available ELISA kit. The polyclonal anti-human IL-6 antibody provided with the kit cross-reacts with rhesus IL-6 and can detect up to 0.094 pg/ml of the monkey protein in an ELISA format. We used rhesus amniotic fluid as positive control for generation of a standard curve because IL-6 is highly expressed in amniotic fluid as part of the angiogenic response that accompanies folliculogenesis and formation of maternal decidua (334). As shown in Table 4.1, rhesus IL-6 was not detected in plasma samples from animals 18540 (uninfected), 18503 (SIV alone), or the two SIV/RRV co-infected animals 18483 and 18570. These data suggest that a factor other than rhesus IL-6 is responsible for the B9 stimulatory activity in plasma of SIV/RRV-infected animals.

To verify that IL-6-like bioactivity was not merely an animal-specific phenomenon but is peculiar to plasma from SIV/RRV co-infected animals, we tested a panel of plasma

samples from a cohort of animals for ability to support B9 cell growth. Table 4.2 is a tabulation of the maximal level of B9 cell stimulation due to each indicated sample, and is based on reproducible data from at least three independent assays. We found that plasma from co-infected animals (n=6) displayed a consistently higher level of activity compared to plasma from animals infected with either SIV (n=4) or RRV (n=4) alone. Plasma activity from SIV/RRV co-infected animals was almost twenty-fold that of control cells cultured without plasma, while the stimulation index for plasma from SIV- or RRV-infected animals was not significantly different from controls (2.7- and 3.5-fold, respectively).

Besides IL-6, B9 cells can also respond to the stimulatory effects of other cytokines including interleukin-11 (IL-11) (64) and Oncostatin M (OSM) (28) that have unique ligand-specific receptors but share gp130 as the common signal transducing subunit. Therefore, to determine whether the B9 stimulatory effect in plasma is mediated through the IL-6 receptor pathway, B9 cells were cultured with plasma in the presence of antibodies against IL-6R and gp130, the two subunits involved in IL-6 signaling. As shown in Fig. 4.2, anti-gp130 effectively blocked the activity of plasma from co-infected animals 18483 and 18570 (Fig. 4.2A&C). On the other hand, anti-IL-6R blocked only up to 50% of the plasma activity, perhaps indicating that IL-6R may not be absolutely required for signaling by the plasma factor. Interestingly, the partial block by anti-IL-6R is reminiscent of the poor effect of anti-IL-6R on recombinant RvIL-6 (226) (also see Fig. 3.3E), leading to speculation that RvIL-6 may be the B9 stimulatory factor in plasma. In accordance with this speculation, polyclonal anti-RvIL-6 inhibited almost 80% of plasma activity while anti-human IL-6 that can also inhibit rhesus IL-6 (see Fig. 3.3) failed to block plasma IL-6-like bioactivity (Fig.

4.2B&D). As expected, neither anti-CD3 (Fig. 4.2A&C) nor rabbit pre-immune IgG (Fig. 4.2B&D) had any effect on plasma activity. Together, these results indicate that the B9 cell stimulatory activity in plasma is sensitive to antibodies that block IL-6 signaling. Moreover, the fact that anti-RvIL-6 had such a dramatic effect on plasma activity, coupled with the observation that anti-IL-6R was as poorly effective on plasma IL-6-like activity as on recombinant RvIL-6 in the B9 bioassay (see Fig, 2.4 and Fig. 3.3E), the B9 stimulatory factor in plasma may indeed be RvIL-6.

To unequivocally identify the IL-6-like factor in plasma, we incubated samples from LPD animal 18483 with various antibodies that had been conjugated onto Protein A Sepharose CL-4B beads (Pharmacia) and then used pre-absorbed plasma samples to stimulate B9 cell growth. Using this approach, IL-6-like bioactivity was significantly reduced when plasma was pre-absorbed with anti-RvIL-6, but not with anti-CD3, anti-gp130, or anti-human IL-6 (Fig. 4.3A). To determine whether depletion of IL-6 bioactivity from plasma was due to specific binding of plasma RvIL-6 by anti-RvIL-6, we first incubated protein A/anti-RvIL-6 complexes with either recombinant RvIL-6 or MacIL-6 and then applied animal plasma to these columns under gravity flow. The plasma flow-through fraction was then sterile filtered and used in a B9 bioassay. As shown in Fig. 4.3B, pre-absorption of RvIL-6 onto the anti-RvIL-6 column abrogated the ability of the anti-RvIL-6 to remove the activity from plasma. On the other hand, pre-incubation of the anti-RvIL-6 column with MacIL-6 before absorption of plasma did not affect ability of the column to deplete plasma bioactivity. These results suggest that anti-RvIL-6 specifically removed plasma IL-6-like activity by binding bioactive RvIL-6 in plasma.

To confirm presence of RvIL-6 protein in plasma that contained IL-6-like activity, we subjected 18483 animal plasma to SDS-PAGE followed by western blot immuno-detection with anti-RvIL-6. As shown in Fig. 4.4, greater than 5 ng of RvIL-6 was detected only in plasma from LPD animals 18483 and 18570 (Fig. 4.4A); the level of RvIL-6 in both animals was highest in plasma that was collected at necropsy (Fig. 4.4A, 18570 lane 7, and 18483 lane 7). However, RvIL-6 was not detected in plasma from non-LPD animals infected with only RRV (Fig. 4.4, lane 3) or SIV alone (lane 4). Moreover, RvIL-6 was not detected in plasma of SIV+ animals 18483 and 18570 prior to RRV inoculation (lanes 5 and 8), implying that induction of LPD is related to presence of RvIL-6 only in plasma of co-infected animals. Interestingly, polyclonal anti-human sIL-6R antibody detected a protein corresponding to the soluble IL-6R (Fig. 4.4B). This anti-human sIL-6R-reactive protein was detected in SIV+ animals even prior to RRV co-infection (Fig. 4.4B, lanes 4 and 8). The plasma levels of sIL-6R in the two SIV/RRV co-infected animals 18483 and 18570 steadily increased until necropsy (Fig. 4.4B, lanes 5-11), perhaps indicating that secretion of this protein is related to increased inflammatory reactivity during the viral infection process.

Presence of sIL-6R in plasma of infected animals appears to be in keeping with previous findings of elevated levels of sIL-6R in biological fluid of individuals with plasma-cell dyscrasias (454) and other proliferative syndromes associated with inflammatory responses (384). Therefore, plasma sIL-6R in SIV/RRV co-infected animals could account not only for aberrant cell growth abnormalities (e.g., B cell hyperplasia) but also for other proliferative syndromes resembling sIL-6R-mediated KS cell growth in AIDS patients (342). However, the biological relevance of sIL-6R with respect to B cell hyperplasia in

SIV/RRV co-infected animals is not obvious from these data because the level of sIL-6R in plasma from LPD animal 18483 remained fairly unchanged over time while RvIL-6 appeared to increase until necropsy (Fig. 4.4C, lanes 1-5).

Nonetheless, based on the stimulatory potential of sIL-6R and human IL-6 on gp130+ cells (123), we believe that simultaneous presence of RvIL-6 and sIL-6R in plasma of animals that develop LPD could have implications for the overall plasma IL-6-like bioactivity, particularly if RvIL-6 and sIL-6R associate to stimulate gp130+ cells. In order to test the ability of recombinant RvIL-6 to associate with recombinant sIL-6R, a constant amount of sIL-6R (5 ng) was incubated with increasing amounts of recombinant RvIL-6. The mixture was then immuno-precipitated with anti-sIL-6R and blotted with a cocktail of both anti-sIL-6R and anti-RvIL-6 as primary antibody so that the total sIL-6R-associated RvIL-6 could be detected on the same blot. This approach facilitated a simple side-by-side quantification, on the same gel, of the maximum amount of RvIL-6 required to saturate 5 ng of sIL-6R. As shown in Fig. 4.5, we found that recombinant RvIL-6 (~21 kDa) indeed associated with recombinant human sIL-6R (~50 kDa), as illustrated by ability of anti-sIL-6R to co-precipitate both sIL-6R and RvIL-6 from a mixture of both these proteins (Fig. 4.5, lanes 1-7). A comparison of the RvIL-6 signal from the complex with sIL-6R (Fig. 4.5, lanes 2-7) to the same amount of free RvIL-6 (lanes 8-11) reveals that, regardless of additional amounts of RvIL-6 incubated with sIL-6R, only about 5 ng of RvIL-6 could be co-precipitated with 5 ng of sIL-6R. This experiment provides the first evidence that RvIL-6 can indeed associate with sIL-6R in vitro. Moreover, preliminary studies have also indicated that sIL-6R in plasma can bind recombinant RvIL-6 (data not shown), suggesting that the

viral protein could also associate with physiological sIL-6R. The functional implication of such an association is intriguing with respect to the overall in vivo stimulatory function of a complex between sIL-6R and RvIL-6, and therefore warrants further investigation.

DISCUSSION

Our laboratory previously reported that SIV/RRV co-infected animals develop B cell hyperplasia accompanied by angiofollicular lymphadenopathy that resembles the plasma-cell variant of MCD (523). MCD is an otherwise rare condition that is more commonly observed both in AIDS patients as well as otherwise immunocompromized individuals infected with HHV-8 (58, 352, 365, 534). Therefore, as a first attempt to define the virological basis of RRV-associated B cell hyperplasia in SIV/RRV co-infected animals, we determined whether biological fluid of animals that develop disease also contain bioactive RvIL-6 encoded by RRV. Data presented in this chapter show for the first time, that biological fluid from SIV/RRV co-infected animals that develop disease also contain IL-6-like B-cell growth-promoting activity. However, the same plasma samples lack rhesus macaque IL-6, suggesting that host IL-6 may not contribute to aberrant B cell growth in LPD animals.

The fact that only plasma from SIV/RRV co-infected animals has IL-6-like bioactivity indicates that RRV gene expression during SIV-induced immune-breakdown may be critical for the emergence of the B cell proliferative phenotype. This notion is supported by data from a related study, where RRV viremia was found to coincide with B cell hyperplasia (41, 523). We favor RvIL-6 as the factor potentially responsible for plasma

IL-6-like bioactivity in plasma, based on a number of pivotal observations: (i) absence of rhesus IL-6 in bioactive plasma (Table 4.1), (ii) ability of the plasma IL-6-like activity to be neutralized by anti-RvIL-6 but not by anti-human IL-6 (Fig. 4.2), (iii) specific depletion of the activity by anti-RvIL-6 and detection of RvIL-6 in plasma immuno-complexes (Fig. 4.3), (iv) poor sensitivity of plasma activity to anti-IL-6R antibody (Fig. 4.2, since anti-IL-6R is also partially capable of blocking recombinant RvIL-6 (226), and (v) ability to detect RvIL-6 protein in plasma by western blot (Fig. 4.4). Together, these data provide the first evidence of a potential link between a secreted viral gene product, RvIL-6, and the lymphoproliferative process in SIV/RRV-infected animals.

Systemic expression of RvIL-6 in SIV/RRV-infected macaques as a strategy for viral pathogenesis may not be unique to RRV, as examples of other secreted viral gene products have been described elsewhere, whose functions are thought to impact virus/host interactions (257, 306, 308, 445). In these cases, the viral protein circulates freely in the infected host, usually in the absence of a competent immune system that might otherwise affect the bioavailability of the viral protein. In keeping with the requirement for underlying immunodeficiency, evidence for bioactive RvIL-6 in biological fluid is strong only in the context of SIV-induced immunodeficiency, conceivably because this setting permits RRV gene expression (523).

A role for RRV in B-cell hyperplasia is consistent with classification of this virus among members of the gamma-herpesvirus subfamily (e.g., HHV-8, EBV, HVS) that are associated with lymphoproliferative disorders. Some diseases associated gamma-herpesvirus infections may be mediated by a virus-encoded protein (e.g., LMP1 of EBV)

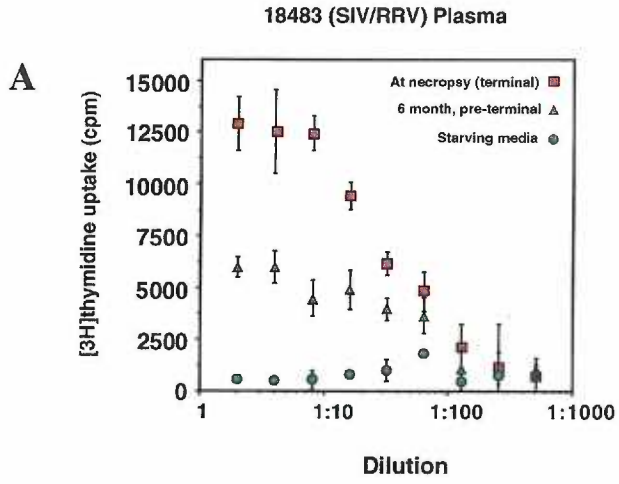
whose actions may target an important host-signaling pathway involved in regulation of cell growth and survival (97). Others (e.g., BCRF1 of EBV) are related to the function(s) of virus-encoded homologue of a cytokine or a cytokine receptor (27, 306, 458). BCRF1 shares significant structural and functional similarity with human IL-10 and serves as an autocrine growth factor for EBV-infected B cells as well as cell lines derived from EBV^+ B-cell lymphomas (240, 332, 479). In patients with EBV-associated non-Hodgkin's lymphoma, secreted viral IL-10 contributes to elevation of total serum "IL-10" levels that correlate with poor prognosis, suggesting that systemic viral IL-10 plays a pathogenic role in this class of cancer (96, 230). We believe that secreted RRV RvIL-6 may function in a similar manner, although a determination of the relative contributions of RvIL-6 and host IL-6 towards the overall IL-6 bioactivity in SIV/RRV co-infected animals will await further investigation.

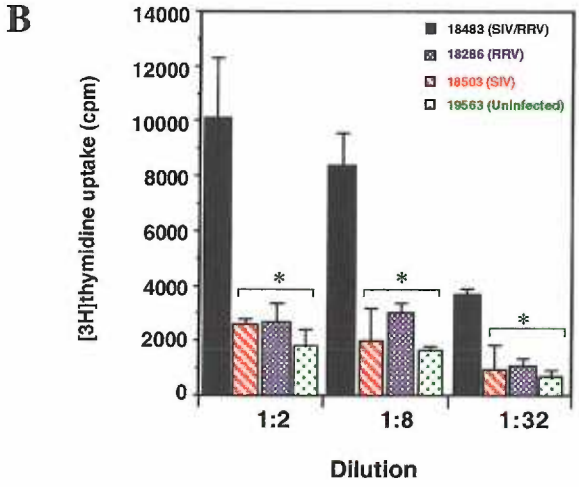
We used the proliferative response of the IL-6-dependent murine plasmacytoma B9 cell line to measure IL-6-like bioactivity in biological fluids of rhesus macaques. However, this assay is sensitive to interference by plasma components that may impact the bioavailability of plasma-borne IL-6-like factors. For example, human IL-6 exists in plasma both in macromolecular complexes with carrier proteins such as sIL-6R (522), and in bioactive high molecular weight oligomers in plasma (300-302). We have similarly observed that RvIL-6 may exist not only as a monomer, but also potentially in a complex with another component, putatively sIL-6R (Figs. 4.4 &4.5). However, we cannot rule out other serum components that may associate with RvIL-6, since our assay was designed to detect only those proteins that were associated with RvIL-6 following incubation of

recombinant RvIL-6 with plasma. Nonetheless, the possibility that RvIL-6 might associate with a soluble component implies that our evaluation of RvIL-6 bioactivity in plasma may not reflect the full extent of its bioavailability.

In conclusion, we have shown that RvIL-6 circulates in the plasma of SIV/RRV-infected animals with LPD. Because RvIL-6 was found only in SIV/RRV co-infected animals, and not in animals infected with SIV or RRV alone, we propose that SIV infection is important for establishment of an environment for RRV replication and subsequent expression of RRV genes, including RvIL-6. Our ultimate understanding of the pathogenic role of RvIL-6 as part of local RRV-induced influences on cell growth will be facilitated by analysis of expression of this viral protein in vivo.

Fig. 4.1— SIV/RRV-infected animal plasma supports B9 cell growth. *A*, $5x10^4$ B9 cells were seeded in triplicate in a 96-well plate and incubated in a total volume of 200 µl in the presence of serial dilutions of plasma from SIV/RRV-infected animal 18483 collected either six months before necropsy (pre-terminal, closed triangles) or at necropsy (terminal, closed squares). *B*, Plasma samples from animal 18483 (SIV/RRV, terminal), 18286 (RRV), 18540 (SIV) or 19563 (uninfected) were each diluted as indicated and added to $5x10^4$ B9 cells, and plasma IL-6 bioactivity was assayed by 3H-thymidine uptake. *C*, $5x10^4$ cells were incubated with a single 1:2 dilution of the indicated animal plasma samples alone or in the presence of 10 ng/ml of recombinant MacIL-6 and B9 cell proliferation was assayed as described above. (Data are presented as means of triplicate values (CPM) ± standard deviation (SD); *, $p<0.001$ compared to 18483 plasma; #, $p<0.001$ compared to other plasma samples used alone).


18483 (SIV/RRV) Plasma
A
At necropsy (terminal)
6 month, pre-terminal
Starving media
[3H]thymidine uptake (cpm)
15000
12500
10000
7500
5000
2500
0
1
1:10
1:100
1:1000
Dilution


B
18483 (SIV/RRV)
18286 (RRV)
18503 (SIV)
19563 (Uninfected)
[3H]thymidine uptake (cpm)
14000
12000
10000
8000
6000
4000
2000
0
*
*
*
1:2
1:8
1:32
Dilution

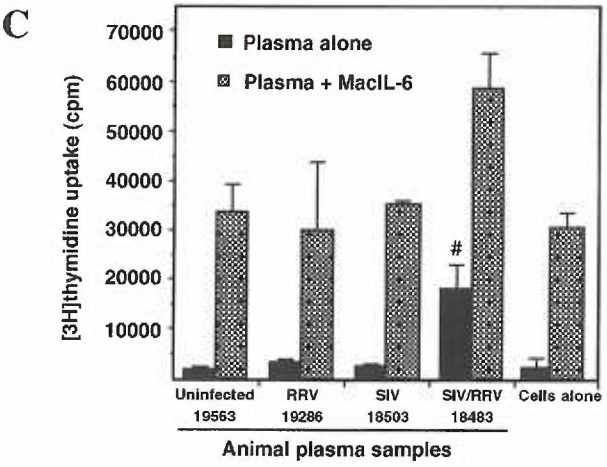

C
Plasma alone
Plasma + MacIL-6
[3H]thymidine uptake (cpm)
70000
60000
50000
40000
30000
20000
10000
#
Uninfected 19563
RRV 19286
SIV 18503
SIV/RRV 18483
Cells alone
Animal plasma samples

Table 4.1 — The human IL-6 Quantikine High Sensitivity ELISA kit (R&D Systems, MN) was used to detect rhesus IL-6 in rhesus monkey plasma. Heat-inactivated plasma samples were added to the ELISA plate either in undiluted form or in the plasma diluent provided with the kit, and the assay was performed according to the manufacturer's instructions. The detection limit for the kit is ~0.1 pg/ml of IL-6.

Table 4.1 — Concentration of rhesus macaque IL-6* in infected animal plasma

	Animal I.D #, and Virological status			
Weeks post infection	18483 (SIV/RRV)	18570 (SIV/RRV)	18503 SIV	18540 -
0	0.440	0.40	< 0.156	0.340
4	< 0.156	0.45	< 0.156	0.240
8	0.200	0.33	< 0.156	< 0.156
26	< 0.156	0.33	< 0.156	0.600
36	< 0.156	0.58	0.600	0.340
Rhesus macaque amniotic fluid (AF) § ·····➤				6765

* IL-6 was measured by ELISA and is expressed in pg/ml based on human IL-6 standards supplied with the ELISA kit (R&D Systems, Minneapolis, MN).
§ Rhesus macaque amniotic fluid (AF) was obtained from Dr. M. Novy (Oregon Regional Primate Research Center) and used here as a source of physiological rhesus IL-6 (Motro, 1990).

Table 4.2 —***Evaluation of IL-6 bioactivity in animal plasma.*** B9 cells were incubated in the presence of a single 1:4 dilution of plasma from a cohort of animals infected with SIV (n=4), RRV (n=4), and SIV/RRV (n=6) as indicated, and cell proliferation was analyzed as described above.

Table 4.2 — Measurement of IL-6 bioactivity in infected animal plasma.

Patho-status	Animal I.D.	CPM[a]	mean CPM (± SEM[b])	S.I.
SIVmac239	19185	2,881 ± 512	2,982±687	**2.7**
	18540	4,168 ± 994		
	18503	3,792 ± 1070		
	19316	1,089 ± 1346		
RRV17577	19286	5,103 ± 1861	4,064±464	**3.7**
	19182	2,919 ± 853		
	19092	4,421 ± 701		
	19218	3,816 ± 1064		
SIVmac239 + RRV17577	18483	36,721 ± 2466	19,953±4398	**19**
	18570	29,402 ± 1006		
	19105[§]	17,910 ± 792		
	19182[#]	14,338 ± 1911		
	19185[§]	11,641 ± 468		
	19218[#]	9,709 ± 1160		

[#]These animals were injected with SIV 6 months post-RRV infection, and plasma was collected 4 months after SIV infection.

[§]These animals were injected with RRV 6 months post-SIV infection, and plasma was collected 4 months post RRV infection

[a]CPM (counts per minute) of triplicate values from three independent assays

[b]SEM was calculated as follows: CPM for "*n*" values ± SD.$[n^{1/2}]^{-1}$

S.I, Stimulation Index is the mean CPM for each animal category divided by 1073 (i.e., the mean CPM for "cells-alone" assay)

*, $p<0.001$ compared to the stimulation index corresponding to the mean of "SIVmac239" or "RRV 17577" CPM values.

Fig. 4.2 — ***IL-6 bioactivity in plasma of SIV/RRV-infected animals can be blocked by polyclonal anti-RvIL-6 and by anti-gp130, but not by anti-human IL-6.*** B9 cells were incubated in triplicate with a single 1:2 dilution of plasma from animal 18483 (A and B) or 18570 (C and D) in the presence of increasing concentrations of anti-IL-6R, anti-gp130, anti-CD3 (panel A and C), or rabbit pre-immune IgG, anti-RvIL-6, and anti-human IL-6 (panels B and D). B9 cell proliferation was then assayed by 3H-thymidine uptake. (Data are presented as means of triplicate values of counts-per-minute (CPM) ± standard deviation (SD).

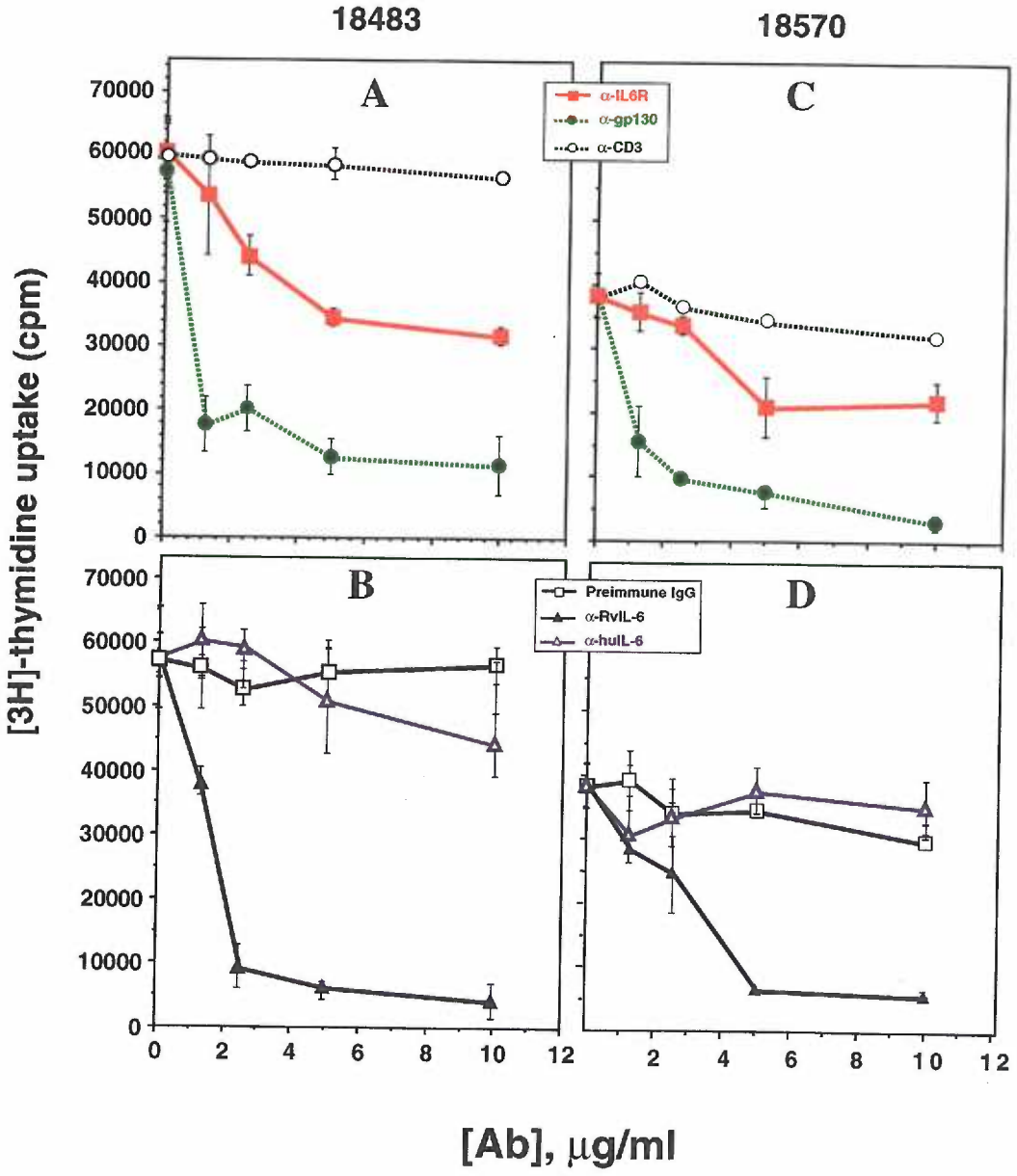
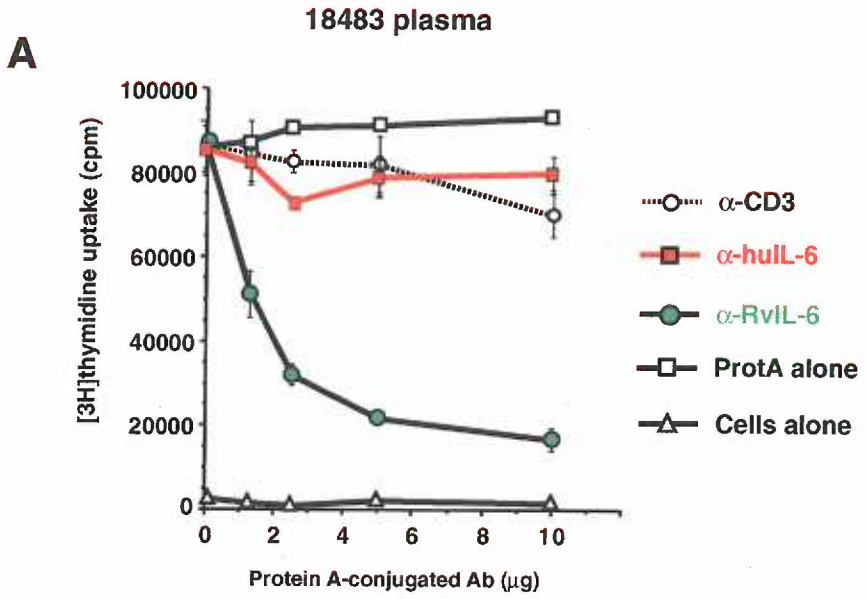

18483
18570
A
B
C
D
α-IL6R
α-gp130
α-CD3
Preimmune IgG
α-RvIL-6
α-huIL-6
[3H]-thymidine uptake (cpm)
[Ab], μg/ml
0
10000
20000
30000
40000
50000
60000
70000
0
2
4
6
8
10
12

Fig. 4.3 — ***Anti-RvIL-6 depletes the B9 cell-stimulatory activity in plasma of SIV/RRV-infected animals.*** Plasma from animal 18483 (SIV/RRV) was incubated with protein A Sepharose beads alone (open squares), or with beads that were pre-loaded with increasing amounts of anti-CD3 (open circles), anti-human IL-6 (closed squares), or anti-RvIL-6 (closed circles); the protein A beads have a specific binding capacity of ~3 mg of protein A per 20 mg of IgG. The unbound plasma fraction in each case was then used to stimulate B9 cell growth. *B,* Plasma from animal 18483 was pre-cleared with (i) nothing, (ii) protein A beads alone, (iii) a protein A/anti-RvIL-6 complex, (iv) a protein A/anti-RvIL-6 complex that had been pre-incubated with excess recombinant RvIL-6 exceeding the binding capacity of beads, or (v) protein A/anti-RvIL-6 complex that had been pre-incubated with excess recombinant MacIL-6. Following this step, pre-cleared plasma was directly used to stimulate B9 cells in a proliferation assay.

Anti-RvIL-6 specifically depletes IL-6 bioactivity from plasma



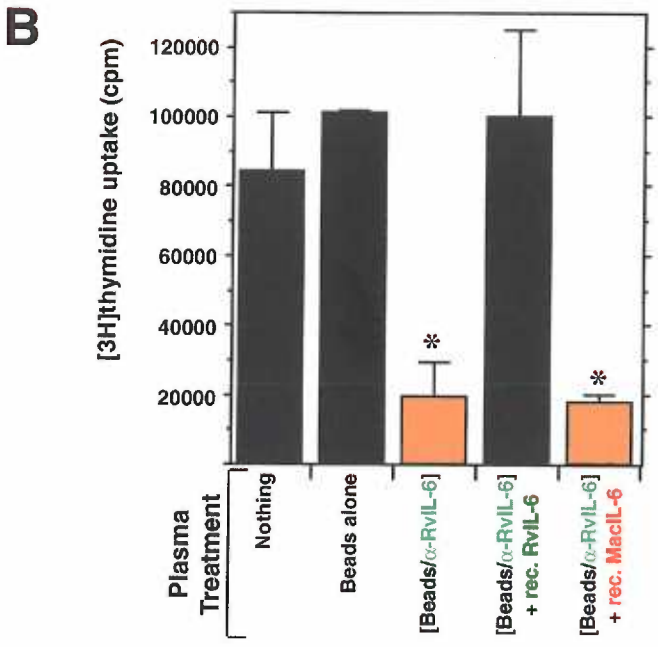
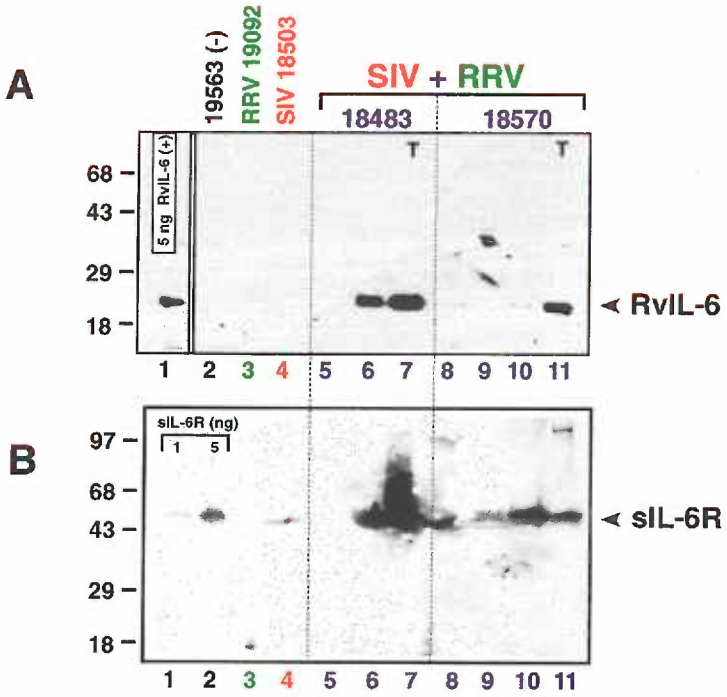


Fig. 4.4 — ***Plasma from SIV/RRV co-infected animals with LPD contain RvIL-6 and sIL-6R.*** Western blot analysis was used to detect RvIL-6 (panel A) or sIL-6R (panel B) in plasma from uninfected animal 19563 (panel A lane 2), or from animals infected with RRV (19092, A&B lane 3), SIV (18503, A&B lane 4), SIV/RRV (18483; A&B lanes 5-7), and animal 18570 (A&B lanes 8-11). Lanes 5 and 8 were loaded with samples collected from SIV+ 18483 or 18570 prior to RRV inoculation.

For both panels A & B, lanes 5 (18483) and 8 (18570) were loaded with plasma collected just before RRV inoculation, at 6 months post SIV infection; lanes 6 and 9 (4 months post-RRV inoculation) while lanes 7 and 11 are terminal (T) plasma collected at necropsy. Lanes 9 and 10 (collected 4 months apart prior to necropsy of 18570). As positive controls, lane 1 of ***panel A*** was loaded with 5 ng of RvIL-6, while lanes 1 and 2 (*panel B*) were loaded with 1 and 5 ng of recombinant sIL-6R, respectively.

In ***panel C***, plasma samples from SIV/RRV co-infected animal 18483, starting at 4 months post RRV infection (lanes 1 and 6) and then at 12-week intervals following RRV infection until necropsy was subjected to SDS-PAGE and analyzed by western blot with either anti-human sIL-6R (lanes 1-5) or anti-RvIL-6 (lanes 6-10).

Both RvIL-6 and sIL-6R are present in plasma of SIV/RRV co-infected animals with LPD



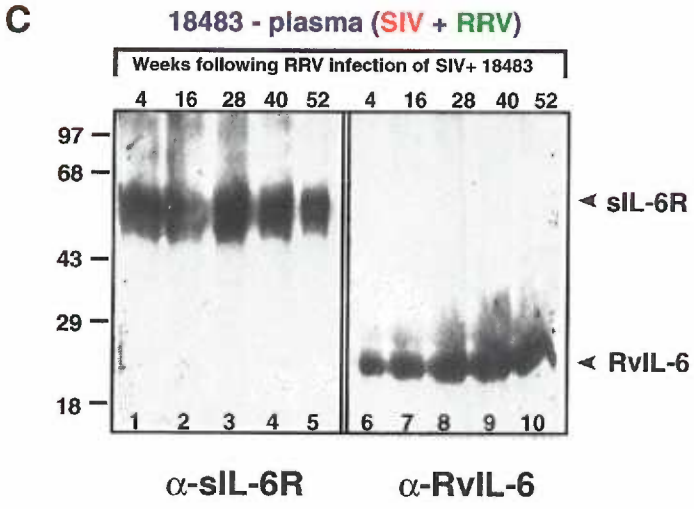
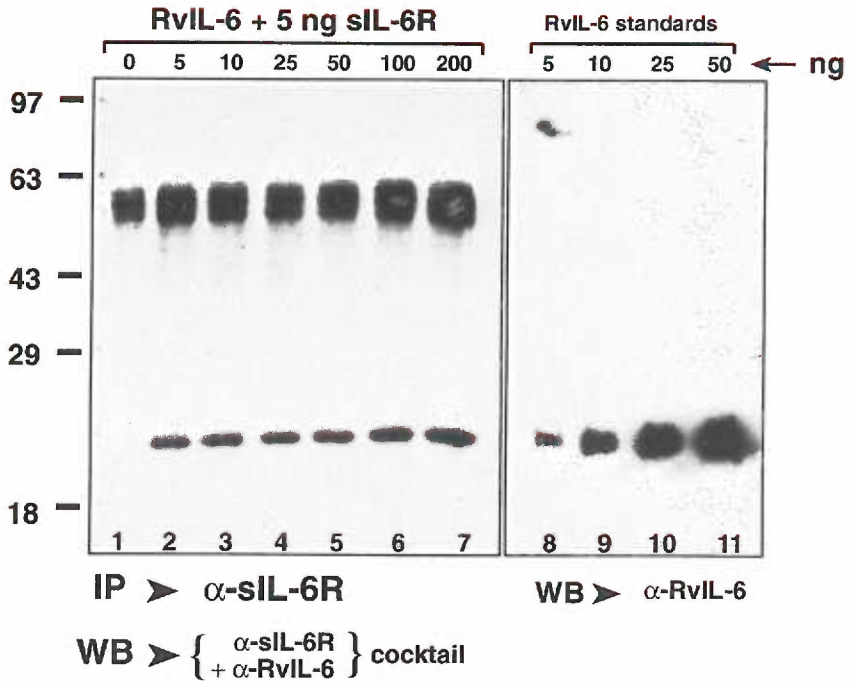


Fig. 4.5 — ***RvIL-6 associates with recombinant human sIL-6R.*** sIL-6R (5 ng) was incubated with increasing amounts (0 – 200 ng) of recombinant RvIL-6 for one hour at 4°C. The mixture was then immunoprecipitated with polyclonal anti-sIL-6R, and analyzed by western blot with a cocktail of both polyclonal goat anti-sIL-6R and polyclonal rabbit anti-RvIL-6 as primary antibody. For comparative standards, lanes 8-11 were loaded with 5, 10, 25, and 50 ng of RvIL-6 and immuno-blotted with anti-RvIL-6.

RvIL-6 associates with recombinant sIL-6R



CHAPTER 5

SUMMARY AND CONCLUSIONS

Over the past decade, studies of the basic biology of large DNA viruses have revealed several strategies that some viruses have evolved to shut down host anti-viral immunity while at the same time re-directing the host cell machinery to either support production of virus progeny or viral persistence. The herpesviruses, in particular, have co-evolved with hosts for untold millennia to ensure a state of persistent parasitism, often accompanied by overt virus-associated disease syndromes. The ability of herpesviruses to maintain replicative genomes in the infected host for long periods of time presupposes viral mechanisms for avoidance and/or circumvention of immune surveillance and anti-viral defenses. Herpesviruses achieve these mechanisms via a number of strategies that include, but are not limited to the following:

1. Establishment of a state of *latency* in which the virus is partially or even completely hidden from the immune system (412).
2. Expression of viral proteins that bind the Fc-domains of Ig. This mechanism not only protects against Fc-mediated phagocytosis of Ig-coated virions but may also prevent complement-mediated lysis of infected cells (31, 120).
3. Interference with cellular processes of antigen processing, including the biosynthetic pathway of cell-surface recognition structures known as the major histocompatibility complex (MHC) (389)
4. Mimicry and exploitation of host proteins that regulate immunity (458, 459, 461).
5. Expression of immunosuppressive molecules (327) as well as antagonistic cytokine receptor homologues (27, 230, 275, 306, 308, 445).

Several examples of these virus/host interactions have been described not only for

herpesviruses but also for other virus subfamilies (4, 25, 102, 148, 345, 460). The increasing discovery of viral factors that mediate specific aspects of virus/host interactions provided compelling evidence that in vitro actions of protein products of genes encoded by complex viruses can be exploited not only as therapeutic targets (253) but also as probes into mammalian immune function (40, 195). From these studies, a prevailing notion is that virus infections may in fact reveal fundamental features of our own biology (462, 540).

HHV-8 encodes several accessory proteins that might have been acquired from the host during evolution (420) and therefore provides one of the more elaborate examples of how a virus can co-evolve with the host. Because many of the HHV-8-encoded genes represent homologues of host genes normally involved in regulation of cellular function, the activities of these viral proteins in the context of viral infection may contribute to HHV-8-associated abnormalities. However, the actual viral and host factors underlying induction of these abnormalities are still unknown, largely because the basic biology of HHV-8 has only been studied using recombinant gene expression assays in vitro. Such studies, by nature, are performed out of the context of natural infection and have so far provided limited insight into mechanisms by which HHV-8 contributes to syndromes such as KS, MCD or BCBL-PEL to which the virus has been etiologically linked.

On the other hand, isolation of stable, infectious forms of previously unknown non-human primate herpesviruses that are genomically related to, or genetically homologous with HHV-8 (46, 47, 116, 181, 414, 442) is likely to elevate the potential for development of a model for HHV-8 pathogenesis. Only one of these "new" viruses, RRV, has been isolated from rhesus macaques. Because RRV shares a number of unique features with HHV-8 (see

below), this monkey virus may serve as the best available model agent for analysis of HHV-8 pathogenesis in a physiologically relevant non-human primate system.

1. RRV infection of rhesus macaques as a suitable model for HHV-8 pathogenesis.

A number of observations support the view that RRV pathogenesis in rhesus macaques can be exploited as a model system for HHV-8 infection in humans. First, both RRV and HHV-8 are associated with lymphoproliferative disease that is highly favored in immunocompromized hosts, such as those co-infected with SIV or HIV, respectively. RRV and HHV-8 therefore appear to share a common property as opportunistic agents that can be controlled by the immune system. Second, the RRV genome shares considerable genomic co-linearity with HHV-8 (Fig. 1.4); the unique "K" genes in HHV-8, as well as the analogous "R" genes in RRV are not represented in the genomes of other gamma-herpesviruses (420, 442), suggesting that HHV-8 and RRV may have undergone evolutionary divergence from other gamma-herpesviruses. Third, HHV-8 and RRV encode analogous copies of cellular proteins with known functions in mitogenesis, angiogenesis, and tumorigenesis (which are the main biological responses underlying many of the abnormalities associated with HHV-8 and RRV) underscoring a potential overlap in the pathogenetic strategies of these two viruses (Fig. 5.1). Based on these and other factors, we believe that RRV and HHV-8 may share several mechanisms of interaction with the host possibly mediated by similar viral gene products, all aimed at modulating growth, differentiation and/or turnover of specific cell types vital to virus survival.

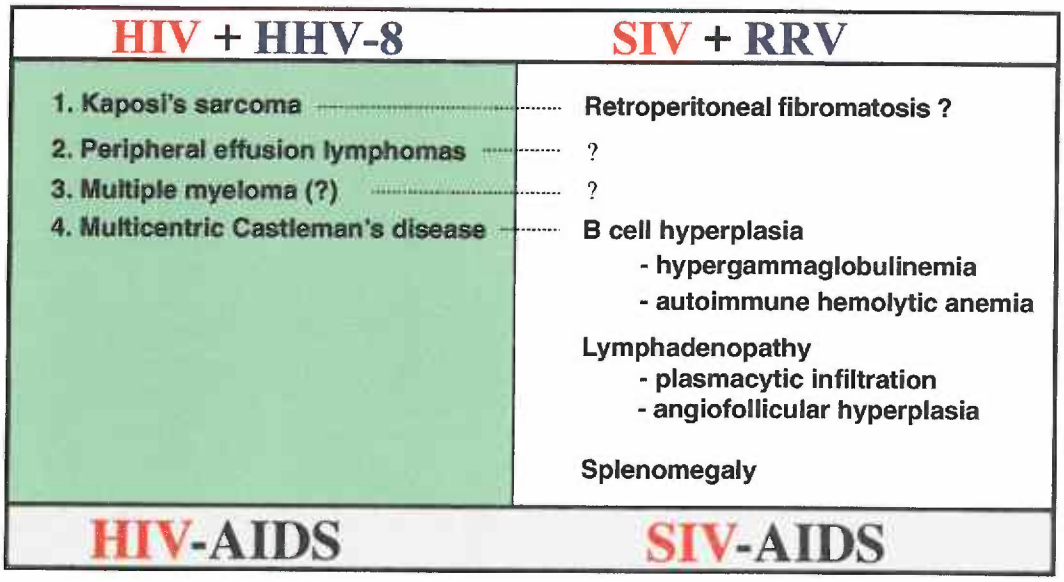
The nature of RRV- and HHV-8-associated syndromes suggests that the IL-6

homologues encoded by these two viruses (i.e., RRV RvIL-6 and HHV-8 vIL-6) may play a critical role in viral pathogenesis. We have noted, for example, that RRV-associated abnormalities in SIV co-infected rhesus macaques (i.e., B cell hyperplasia, splenomegaly, lymphadenopathy, hypergammaglobulinemia) (523) resemble the IL-6-associated syndromes that emerge in HIV/HHV-8 co-infected individuals (20, 68, 91, 110, 136, 137, 250, 281, 482, 501). RRV-associated B cell lymphoproliferation, which persists for several months post-infection, is also accompanied by angiofollicular lymphadenopathy with characteristics resembling those of the plasma-cell variant of MCD. This variant of MCD is known to be propagated by IL-6 over-expression (6, 8, 210, 242, 244, 349, 443, 532, 534), and is also the form of MCD that develops in HIV/HHV-8 co-infected individuals (26, 159, 333, 363). Together, these observations suggest that deregulation of IL-6 bioactivity plays a role in the pathogenesis of RRV and HHV-8. Therefore, SIV/RRV co-infection of rhesus macaques may be uniquely suitable as a model for IL-6-associated syndromes that emerge during HIV/HHV-8 co-infection in humans.

The primary goal of this thesis project was therefore to study RvIL-6 function because data derived from these studies would represent an important contribution to our understanding of RRV pathogenesis in rhesus macaques and by extension, HHV-8 pathogenesis in humans.

Fig. 5.1 — Comparison of diseases potentially associated with RRV and HHV-8. In individuals with weakened immune systems, such as HIV-infected humans, HHV-8 infection is associated with Kaposi's sarcoma, peripheral effusion lymphomas, multi-centric Castleman's disease, and possibly with multiple myeloma. Similarly, in SIV-infected rhesus macaques, RRV infection is associated with a number of syndromes that may resemble those listed above that are etiologically linked to HHV-8. Thus, retroperitoneal fibromatosis in SIV/RRV co-infected rhesus macaques may display histopathological properties of HHV-8-associated Kaposi's sarcoma. The disease correlates of peripheral effusion lymphoma and multiple myeloma have not been documented in the SIV/RRV co-infection system. However, RRV-associated B cell hyperplasia, with attendant lymphadenopathy and splenomegaly are manifestations that resemble the plasma-cell variant of human multi-centric Castleman's disease and can therefore be exploited as a model for the human disease.

Potential pathogenetic overlap between HHV-8 and RRV*



* In HIV or SIV-infected hosts with HIV-AIDS or SIV-AIDS, HHV-8 and RRV appear to be associated with proliferative syndromes that normally result from excessive IL-6 bioactivity, leading to the hypothesis that vIL-6 (of HHV-8) or RvIL-6 (of RRV) may play a significant role in virus-associated abnormalities.

2. RvIL-6 is biologically functional

Prior to our studies of RvIL-6 function, Moore et al. had reported for the first time that HHV-8 vIL-6 could substitute for cellular IL-6 in stimulating growth of the IL-6-dependent B9 cell line in spite of a low (~24 %) level of sequence identity between the two proteins (326). vIL-6 bioactivity has also been demonstrated in other settings, all showing that this viral cytokine has retained many of the functions of its cellular counterpart albeit with a seemingly atypical mechanism of action with respect both to IL-6 receptor usage and the downstream signaling pathways involved (16, 194, 202, 330, 336, 364).

Like vIL-6, RvIL-6 shares limited sequence identity with cellular IL-6 and vIL-6 (226). One notable fact, however, is that amino-acid residues that are critical for preserving cellular IL-6 structure and function are conserved not only in all known IL-6-like polypeptides (449) but also in vIL-6 and RvIL-6 (226). We therefore hypothesized that RvIL-6 might exhibit IL-6-like activity on B cells, like host IL-6 and vIL-6; we tested this hypothesis in two phases. First, we answered the question of whether RvIL-6 is functional, and second, we evaluated the biological activity of RvIL-6 relative to host IL-6. Both phases were designed to address conceptually related questions but they differ in scope. As such, results from each study have been reported in this thesis as separate Chapters 2 & 3 and have been discussed thematically under three main questions of (i) RvIL-6 function, (ii) potency, and (iii) mechanism of signaling relative to host IL-6.

(a) RvIL-6 stimulates B cell growth in vitro.

In Chapter 2, we showed that both recombinant RvIL-6 expressed in COS-1 cells

(Fig. 2.2), as well as a GST-RvIL-6 fusion (Fig. 2.3) could support B9 cell growth in a dose-dependent manner (226). Purified RvIL-6 lacking a GST moiety also supported B9 cell growth in a dose-dependent manner, and the protein was equally active regardless of expression in bacteria or insect cells (Fig. 3.2), indicating that RvIL-6 function may not be affected by absence of post-translational modifications. Cellular IL-6 was also found to retain activity regardless of expression in bacteria or insect cells (298, 476, 538). Another group using RvIL-6 encoded by RRV H26-95 has also repeated our basic observation of RvIL-6 bioactivity on B9 cells (10); therefore, RvIL-6 action as a B cell-growth factor is the first evidence of shared function between cellular IL-6, vIL-6 and RvIL-6. We believe that demonstration of RvIL-6 function in this assay indicates a potential in vivo function for RvIL-6 as B-cell stimulation, consistent with our hypothesis that RvIL-6 may contribute to B cell proliferative disorders in SIV/RRV co-infected animals.

An emerging theme is that positionally analogous proteins in the genomes of related viruses often play similar functions (97, 100, 101). Based on this observation, we believe that vIL-6 and RvIL-6 that are not only situated at analogous positions in HHV-8 and RRV, respectively, but are also functional homologues, may mediate similar aspects of viral pathogenesis. This claim will be substantiated by future determinations of whether RvIL-6 also exhibits those functions that have been demonstrated for vIL-6 (16, 65, 161, 202, 321, 330, 372).

(b) RvIL-6 is capable of inducing neuronal differentiation in PC12 cells.

Cellular IL-6 plays an important role in the development, differentiation and

survival of neurons in the CNS (293-295, 297, 434, 485, 499). A neuroregulatory function for IL-6 has been demonstrated in the PC12 neuronal cell differentiation model, where IL-6 can reverse programmed cell death induced by nerve growth factor (NGF) withdrawal (211, 296, 348, 524, 525). We similarly found that RvIL-6 could prevent apoptosis and was also able to induce significant neurite outgrowth in NGF-primed PC12 cells (Fig. 3.7), indicating that RvIL-6 can substitute for host IL-6 in the PC12 system. Interestingly, HHV-8 vIL-6 was also recently shown to exhibit a stimulatory signal in PC12 cells (202), suggesting that the biological function(s) of both RvIL-6 and vIL-6 are not limited to B cell growth but may, in fact, include other systems in which host IL-6 is active.

Although PC12 cells express both IL-6R and gp130 subunits of the IL-6 receptor (294), these cells are generally unresponsive to IL-6 except after pre-exposure to NGF. In accordance with this paradigm, the anti-apoptotic effects of RvIL-6 were realized only in NGF-primed, but not in untreated native PC12 cells, suggesting that RvIL-6, like host IL-6, can also participate in a synergistic relationship with NGF in this system. In PC12 cells, NGF stimulates the Ras/Erk pathway, while IL-6 stimulates the Jak/Stat pathway (524, 525). In the present study we found that increasing concentrations of RvIL-6 did not affect the level of phosphorylated Erk (*p*Erk) in NGF-primed PC12 cells, while Jak-mediated phosphorylation of gp130 in the same cells appeared to increase to saturable levels concomitant with increasing concentrations of RvIL-6 (Fig. 3.7). These results indicate that in PC12 cells, RvIL-6 activates the Jak/Stat pathway (160, 351, 528) but not the Ras/Erk pathway that is activated by NGF. These two pathways may converge down-stream of receptor activation in a synergistic fashion (see Fig. 3.6) similar to what has been proposed

for human IL-6 and NGF (524, 525). One question with regard to RvIL-6 function in this system is whether, like IL-6, the viral protein can also exert direct differentiation effects on PC12-E2 cells (526). PC12-E2 cells can robustly respond to host IL-6 in the absence of NGF priming (524, 525), ostensibly because this variant of PC12 cells constitutively express an NGF-dependent survival/differentiation-associated factor whose effects are potentiated by the IL-6 signal (524, 525).

The anti-apoptotic function of RvIL-6 in PC12 cells suggests a neuroregulatory role for this protein but does not answer the question of whether RvIL-6 can also prevent neuronal cell death in vivo, especially during viral infections that are likely to cause cytopathogenic effects in the central nervous system (CNS). In one murine model of virus-induced multiple sclerosis (MS)-like encephalomyelitis, recombinant IL-6 was found to alleviate or curtail the de-myelination process in the CNS (408), although the precise mechanism for this effect was not clear. Other studies have also shown that host IL-6 mediates B cell immunity and also induces local secretion of neurotrophic factors in the CNS as part of the repair mechanisms initiated during the course of virus-induced tissue damage (152, 156, 164, 265), suggesting that IL-6 may indeed have a neuroregulatory role in the CNS. Although not directly implied by our data, we cannot rule out the possibility that RvIL-6 may have a role in the CNS similar to that of host IL-6 particularly if RvIL-6 is expressed in, or systemically delivered to the CNS of SIV/RRV infected animals.

We have not measured levels of RvIL-6 protein in cerebral spinal fluid, nor have we investigated the expression patterns of RvIL-6 protein and/or mRNA in brain specimens of SIV/RRV co-infected animals. In fact, the neuro-pathogenic potential of RRV is not known.

As such, our data on the anti-apoptotic effects of RvIL-6 in PC12 cells provide no information as to whether RvIL-6 function in this manner would impact RRV pathogenesis in the neuronal compartment of infected animals. However, one interesting observation is that although SIV/RRV co-infected animals develop overt lymphoproliferative abnormalities in the periphery, these animals show no detectable signs of neurological dysfunction that tends to manifest in more than 50% of SIV-infected animals (386, 431, 456). With the suggestion that RRV may have a slight attenuating effect on SIV-induced disease and/or dissemination (523), absence of signs of brain disease in SIV/RRV co-infected animals could be related to the regulatory activities of one or more RRV gene products. One of these genes, RvIL-6, could function as a survival factor in the face of SIV-induced neuronal damage as a consequence of local infection, by SIV, of cells of the monocyte-macrophage lineage in the brain.

In vivo factors underlying the cross-viral inter-relationship between SIV and RRV in the CNS (or other sites) are unknown. However, because of the apparent pathogenic parallelism between RRV and HHV-8, some published views concerning HIV/HHV-8 co-infection in humans are worth mentioning. For instance, HHV-8 viral DNA sequences have been detected in clinical specimens of AIDS-positive, as well as AIDS-negative individuals with KS (95, 424), suggesting that HHV-8 may infect the brain. Although primary CNS disease in HIV-AIDS patients is often thought to be related to HIV infection of brain tissue itself, the function of viral proteins encoded by other opportunistic agents like HHV8 may influence the overall neurological crisis. In support of this view is the finding that vIL-6 of HHV-8 not only induces secretion of endogenous IL-6 in vitro (330), but can also trigger

biological responses in the PC12 neuronal cell line (202), although a similar function for vIL-6 in vivo remains to be demonstrated.

Interestingly, arguments for the protective effect of HHV-8 on HIV-associated neurological dysfunction, also known as the AIDS dementia complex (ADC), are not centered on the potential neuro-regulatory function of vIL-6 but are instead focussed on the function of HHV-8-encoded vMIP proteins that can bind and potentially inhibit chemokine receptor-mediated HIV entry into target cells (22, 48, 122, 127, 248, 269, 407). These arguments notwithstanding, increasing evidence of viral IL-6 activity at the level of neuronal cell survival will provide new insights into the potential contribution of HHV-8 and its gene products on the pathogenesis of HIV. Therefore, the molecular correlates of HHV-8-mediated protection from ADC-like symptoms in hosts co-infected with HIV and HHV-8 are likely to include not only vMIP but also vIL-6 proteins. As a model for HIV/HHV-8 co-infection in humans, our SIV/RRV co-infection protocol should be useful for elucidation of these fascinating cross-viral effects since we can now generate recombinant RRV lacking various combinations of genes potentially involved in the pathogenetic relationship between SIV and RRV.

3. Comparisons of potency between RvIL-6 and host IL-6.

In our initial studies of RvIL-6 function (Chapter 2), the activity of GST-RvIL-6 in the B9 bioassay was almost 4000-fold (4000X) less potent than recombinant human IL-6 (226). This difference was remarkably reproducible and could have been due to: (i) limited homology between RvIL-6 and human IL-6, (ii) the fact that RvIL-6 was fused to a GST

moiety that might interfere with interactions between RvIL-6 and its cognate receptor(s) on B9 cells, (iii) mis-folding of the GST-RvIL-6 fusion in bacteria, or (iv) differences in purification and/or source of human IL-6 and GST-RvIL-6. Reasoning that efforts aimed at eliminating or minimizing some of these factors might permit a more accurate estimation of relative potency between RvIL-6 and host IL-6, we expressed both RvIL-6 and MacIL-6 under the same conditions and analyzed them in parallel bioassays (Chapter 3). Here, we used rhesus IL-6 (MacIL-6) and not human IL-6 as the suitable "host" protein for comparative studies because the rhesus macaque is currently considered to be the natural host species for RRV (ability of RRV to infect humans is not known).

In contrast to the 4000X difference that we initially observed between GST-RvIL-6 and human IL-6, recombinant RvIL-6 lacking GST was only 2-to-3-fold (2-3X) less potent than MacIL-6 (Fig. 3.2). GST-RvIL-6 was also 2-3-fold less potent than GST-MacIL-6, although the respective maximal activity of the GST-fusion material was generally lower than that of mature proteins without GST (data not shown). This result supported our original interpretation that GST might indeed interfere with the overall activity of the fusion proteins. A 2-3-fold difference in potency was also realized in the PC12 neuronal cell survival assay, where at least twice as much RvIL-6 was required to rescue the same percentage of PC12 cells from apoptosis compared to MacIL-6 (Fig. 3.7). Therefore, in two distinct assays — the B9 and PC12 cell assays — we observed a consistent difference in potency that we believe represents a more accurate estimation of the activities of RvIL-6 relative to MacIL-6.

At the protein level, differences in potency between RvIL-6 and MacIL-6 may be

related to the specific activity of each protein based on the secondary structure that is assumed at purification. For instance, both RvIL-6 and MacIL-6 possess 4 (four) conserved cysteines that are thought to facilitate intra-molecular disulfide bridges. At the re-naturation step of protein purification, these cysteines are likely to direct random formation of up to 4! (4x3x2x1) potential conformations of recombinant proteins, each with a unique specific activity, so that the overall activity of purified proteins would be the sum total of the individual activities of a pool of these re-folded species. However, we did not perform circular dichroism (CD) spectroscopy (44, 288)) on the re-folded material, and so we could not determine the conformational integrity of the material we used in the bioassay. Nonetheless, the reproducibility of our data, and the fact that similar antibody blocking results were obtained using two independent preparations of each protein (Fig. 3.3C) suggests that the tendency of the purified proteins is to predominantly re-fold into one conformation with a single specific activity. One issue that arises from this discussion is whether recombinant RvIL-6 and MacIL-6 that are uniformly re-folded into the same conformational state would also display similar specific activity. We believe this is highly unlikely, however, since other structural constraints (mainly dissimilar residues) may still lower the overall activity of RvIL-6 relative to MacIL-6. However, given the limited homology between RvIL-6 and MacIL-6, we found the 2-3X difference to be more impressive than would have been predicted from the amino-acid sequence alone; therefore, the secondary structure, rather than the primary sequence of RvIL-6, may determine the extent to which RvIL-6 has retained IL-6-like bioactivity. Fig. 3.1 reveals evidence of *conservative substitution* at critical positions that may allow the viral and host proteins to

assume remarkably similar structural tendencies.

Conservative substitution may be a vital evolutionary strategy for RvIL-6, for two reasons. First, by maintaining the biochemical or side-chain character at specific positions, RvIL-6 may acquire a (helical) conformation similar to that of host IL-6 and therefore retain IL-6 bioactivity, as our data clearly indicate. A similar argument has been used elsewhere to explain the functional overlap by members of the helical IL-6 cytokine family that share limited sequence homology but display similar secondary structure (87, 197, 199, 354). Second, conservative substitution may create RvIL-6 with a different (tighter or looser) affinity for the IL-6 receptor(s) and may therefore antagonize or augment the host IL-6 signal, depending on the nature of interaction with the receptor. We found that RvIL-6 actually augments host IL-6 (Fig. 2.5, and Fig. 3.4A; see below). In light of these possibilities, evaluation of the functionally critical residues in RvIL-6 will be necessary in order to elucidate the biological activity of this protein. Since such residues in host IL-6 have been mapped (54, 56, 131-133), future efforts to define structure-function relationships for RvIL-6 may be facilitated by "mutagenic swaps" with host IL-6.

4. RvIL-6 does not inhibit cellular IL-6 signaling.

As discussed above, RvIL-6 produced an additive signal when used together with either human or MacIL-6 in the B9 bioassay (Fig. 2.5, and Fig. 3.4A). Worth mentioning is that comparative studies on the relative potency of HHV-8 vIL-6 and host IL-6 have not been done in a fashion similar to what we have done here with RvIL-6 and MacIL-6. Since host IL-6 is normally secreted as part of the host inflammatory response to viral infection

(37, 135, 229, 287, 408), the finding that RvIL-6 augments host IL-6 function may have physiological significance with respect to the overall IL-6 bioactivity, particularly in the face of viral infection. For instance, both HIV and SIV have been linked to increased production of host IL-6 (43, 60, 151, 155, 189, 350, 444, 501) that may in turn contribute to some of the virus-associated disease processes. RvIL-6-mediated B cell growth in a manner that does not inhibit the stimulatory activities of host IL-6 would not only enhance the overall IL-6 bioactivity in the B cell compartment but also exacerbate and therefore contribute to B-cell abnormalities.

Mechanisms underlying the augmenting effect(s) of RvIL-6 on the function of host IL-6 are unknown. However, we predict that these mechanisms may be part of a pathogenic strategy whereby RvIL-6 has evolved to retain *and* promote IL-6 bioactivity without exerting inhibitory effects on the signaling pathway(s) of the host ligand. One of the attractive models to illustrate this strategy would be where the viral protein does not compete for the primary receptor of host IL-6 but that the signals respectively initiated by the host and viral proteins converge at a level downstream of receptor binding. An important aspect of this model may already have been demonstrated for vIL-6; this protein induces IL-6-like responses independent of the ligand-specific IL-6R used by host IL-6 (202, 321). A similar story appears to be emerging with regard to receptor usage by RvIL-6 (see below).

5. The role of IL-6R and gp130 in RvIL-6 signaling

The IL-6 receptor system consists of the ligand-binding IL-6R subunit, and a signal-transducing component, gp130. Neither IL-6 nor IL-6R can independently interact with

gp130 except in the context of a pre-formed IL-6/IL-6R complex. Gp130 is subsequently activated by homodimerization following interaction with the IL-6R/IL-6 complex, forming a signaling unit that consists of two molecules each of IL-6, IL-6R and gp130 (513, 514). Antibodies to anti-IL-6R or gp130 can neutralize IL-6 function by preventing formation of this signaling unit. In our studies, anti-gp130 was able to neutralize human IL-6 (Fig. 2.4B), as well as MacIL-6 and RvIL-6 (Fig. 3.2A), suggesting that gp130 can transduce signals initiated not only by host (human and rhesus macaque) IL-6, but also by RvIL-6. In contrast, anti-IL-6R effectively blocked both human IL-6 (Fig. 2.4A) and MacIL-6 (Fig. 2.4A and 3.3E) but was less effective at neutralizing RvIL-6, requiring almost ten times as much antibody to achieve a similar level of inhibition of the host protein(s). One interesting distinction is that anti-IL-6R blocked MacIL-6 regardless of whether the antibody was added to B9 cells before or at the same time as MacIL-6. In contrast, the partial inhibitory effect of anti-IL-6R on RvIL-6 was evident only when cells were pre-treated with the antibody before addition of RvIL-6, but not when antibody and RvIL-6 were added at the same time. Together, these results suggest that RvIL-6 may be able to access the cognate receptor(s) even in the presence of anti-IL-6R, while MacIL-6 cannot. Furthermore, the data indicate that anti-IL-6R is able to block the initial interaction between MacIL-6 and IL-6R but not between RvIL-6 and its receptor(s). Therefore, RvIL-6 either bypasses IL-6R to signal directly through gp130, or utilizes epitopes on IL-6R that are not targets of anti-IL-6R. Interestingly, in studies of HHV-8 vIL-6 function, anti-IL-6R antibody only partially blocked vIL-6 signaling both in B9 cells (359) and in a myeloma cell line (65). These findings, along with our present data, have led to the conclusion that RvIL-6 and vIL-6 may

function in a mechanistically analogous manner but that the viral IL-6 proteins may utilize a slightly distinct mode of activation of gp130 compared to host IL-6 — perhaps one that is independent of IL-6R.

To begin to answer the question of whether RvIL-6 requires IL-6R for signaling, we have considered published data on vIL-6 interactions with IL-6R and gp130. Recently, a pivotal study by Molden et al (321) showed that vIL-6 could indeed initiate biological responses in BAF-130 cells, a mouse pro-B-cell line that lacks membrane IL-6R but is transfected with human gp130 (321). Subsequent reports have now confirmed that vIL-6 can activate gp130 directly without the need for prior interaction with IL-6R (202, 336, 510), indicating that vIL-6 may be able to signal to $gp130^+$ cells in the absence of IL-6R on the cell surface. This mode of signaling is clearly distinct from that of host IL-6, where initial formation of IL-6/IL-6R complex is requisite for IL-6-induced activation of gp130 (513, 514). In spite of the above indications, however, anti-IL-6R blocked as much as 50% of the vIL-6 signal (359) on B9 cells that express both IL-6R and gp130. While this finding does not necessarily contradict data supporting direct use of gp130 by vIL-6, a partial block of vIL-6 by anti-IL-6R supports an additional model for vIL-6 function. That is, vIL-6 may activate gp130 directly particularly in the absence of IL-6R, as clearly demonstrated by Molden et al (321), but IL-6R could still participate in the signaling reactions of vIL-6 on $gp130^+$/$IL\text{-}6R^+$ cells. On such cells, anti-IL-6R would still be able to partially neutralize participation of IL-6R in vIL-6 signaling, thus explaining the 50% block of vIL-6 signaling by this antibody. Alternatively, or in addition, vIL-6 may strictly signal via gp130 alone, but that anti-IL-6R binding to membrane IL-6R on the surface of target cells perhaps interferes

with direct interactions between vIL-6 and gp130. This would also account for the partial block of vIL-6 by anti-IL-6R in the two systems where this antibody effect has been documented (65, 359).

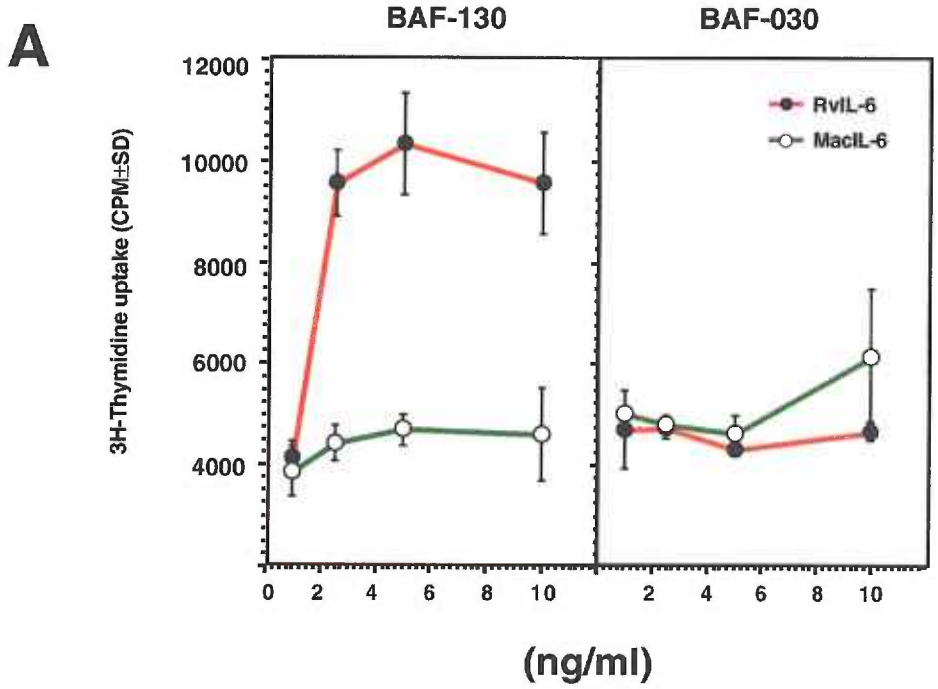
The picture for RvIL-6 function with respect to IL-6R usage is less clear but may be similar to that of vIL-6. This assertion is based on the finding that, as with vIL-6, anti-IL-6R only partially blocked RvIL-6 in the B9 bioassay (Fig. 2.4A and Fig. 3.3E). Moreover, in the PC12 cells that express IL-6R (294), anti-gp130 as well as anti-RvIL-6 antibodies each effectively blocked RvIL-6-mediated cell survival, but anti-IL-6R had no effect on the anti-apoptotic function of RvIL-6 in this system. In contrast, anti-IL-6R completely blocked MacIL-6 in PC12 cells, suggesting that unlike MacIL-6, RvIL-6 may not require IL-6R for function in this system. Therefore, in B9 cells and the PC12 neuronal differentiation assay — two systems that rely on distinct biological responses — our data are corroborative with respect to the neutralizing effects of anti-IL-6R on RvIL-6. Our conclusion from these results is that RvIL-6 exerts stimulatory effects on target cells either (a) independent of IL-6R, as has been reported for HHV-8 vIL-6 (194, 202, 321, 510), or (b) via epitopes on IL-6R that are not targeted by anti-IL-6R. The first possibility assumes that when both gp130 and IL-6R are present on the surface of a target cell, only gp130 is strictly utilized, as proposed above for vIL-6. The second possibility implies that RvIL-6 still requires IL-6R but that the mode of interaction between RvIL-6 and IL-6R is slightly different from that of host IL-6.

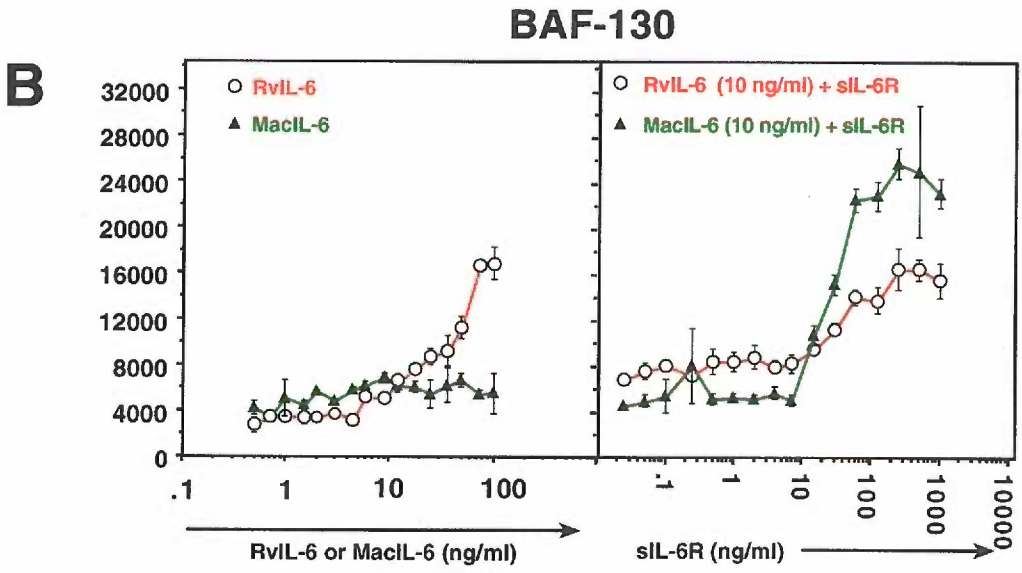
We have began testing both these possibilities using various cell lines including (a) BAF-030 cells that do not express IL-6R or gp130, (b) BAF-130 cells that express human

gp130 but do not express IL-6R, as well as (c) B9 cells that express both IL-6R and gp130. Based on preliminary data from these studies, we have found that (i) both MacIL-6 and RvIL-6 were unable to stimulate $gp130^{-}$/$IL\text{-}6R^{-}$ BAF-030 cells, and that (ii) RvIL-6 was indeed able to activate gp130 on BAF-130 cells in the absence of IL-6R (Fig. 5.2A). Interestingly, the RvIL-6 signal on $gp130^{+}$ BAF-130 cells was significantly enhanced in the presence of recombinant sIL-6R (Fig. 5.2B), perhaps indicating that in addition to direct activation of gp130, RvIL-6 may also interact with sIL-6R to activate gp130 to a level qualitatively greater than that elicited by direct interaction with gp130. Data presented in Fig. 4.5 shows that RvIL-6 can indeed associate with human sIL-6R. Human sIL-6R been shown to enhance the sensitivity of B9 cells to human IL-6 (119), possibly by mediating additional activation of gp130 via formation of a complex between IL-6 and sIL-6R.

Fig. 5.2 — RvIL-6 is capable of activating gp130 on IL-6R$^-$ BAF-130 cells. ***Panel A***, BAF-130 or BAF-030 cells were cultured in a 96-well plate at $5x10^4$ cells/well (200 µl total volume) in the presence of increasing concentrations of either RvIL-6 or MacIL-6. Panel B, in a separate assay, $5x10^4$ BAF-130 cells were seeded with constant 10 ng/ml o f either RvIL-6 (open squares) or MacIL-6 (closed triangles) and cultured in the presence of increasing amounts of human sIL-6R. Cell proliferation was analyzed by 3H-thymidine uptake as previously described (226).

RvIL-6 is capable of activating gp130 in the absence of IL-6R





Because of the absolute requirement for IL-6R by host IL-6, IL-6 responsiveness is normally confined to hepatocytes and leukocyte sub-populations (e.g., monocytes, neutrophils, T cells, and B cells) where IL-6R is predominantly expressed. On the other hand, gp130 is ubiquitously expressed in all organs including heart, kidney, spleen, liver, lung, placenta, and brain (426). Therefore, the ability of vIL-6 and RvIL-6 to signal through gp130 in the absence of IL-6R suggests that these viral proteins have evolved a mode of signaling that circumvents the regulatory limitation of IL-6R expression by exploiting the widely expressed gp130, thus stimulating cells that would not traditionally respond to IL-6. The over-riding consequence of this mode of signaling by vIL-6 and RvIL-6 may be in vivo sensitization of several cell types in a manner that could contribute to the complex cellularity that defines the disease states associated with RRV and HHV-8. In addition, the current study also shows that, besides binding membrane gp130, RvIL-6 can also form a complex with the soluble form of IL-6R, thereby taking advantage of the stimulatory potential of such a complex. A case can therefore be made for RvIL-6-mediated proliferation initiated by RvIL-6/sIL-6R complexes in vivo.

6. RvIL-6 is present in biological fluid of animals with lymphoproliferative disease.

The main observation in Chapter 4 is that plasma from SIV/RRV co-infected animals, but not plasma from animals infected with SIV or RRV alone, contains a potent B9 cell growth-promoting activity. This activity was due to a factor other than secreted rhesus IL-6 protein, because we could not detect rhesus IL-6 protein in any of the animal samples including those with IL-6-like bioactivity. The finding that only SIV/RRV co-infected

animals developed B cell hyperplasia/LPD implies a relationship between induction of disease and RRV gene expression during SIV-induced immunodeficiency. One RRV accessory protein, RvIL-6, was found to circulate as a bioactive protein only in plasma of SIV/RRV co-infected animals but not animals infected with RRV or SIV alone, suggesting that SIV-induced immunodeficiency may provide a suitable setting for expression and circulation of the viral protein. In this setting, RvIL-6 may function as part of a mechanism for induction of B cell hyperplasia and possibly other immune cell syndromes normally associated with aberrant IL-6 bioactivity.

Some features of LPD in SIV/RRV co-infected animals resemble syndromes in HIV/HHV-8 co-infected individuals that are related to excessive IL-6 bioactivity (see Fig. 5.1) leading to our view that RvIL-6 is a potential determinant for LPD. This view is based on a number of observations. First, we could not detect rhesus IL-6 protein in the plasma of LPD animals, ruling out host IL-6 as a contributor to the disease process. Second, plasma IL-6-like bioactivity was readily neutralized by anti-RvIL-6, but not by a polyclonal anti-human IL-6 antibody that cross-reacts with rhesus IL-6. Third, plasma IL-6-like bioactivity could be specifically depleted by anti-RvIL-6, suggesting that plasma RvIL-6 may be responsible for the B9 stimulatory activity. Fourth, plasma IL-6-like bioactivity was poorly sensitive to the neutralizing effects of anti-IL-6R. This particular result is similar to blocking data we obtained with anti-IL-6R against recombinant RvIL-6 in the B9 bioassay (145, 146), indicating that the B9 stimulatory factor in plasma is either antigenically related to RvIL-6, or is RvIL-6 itself. Fifth, using western blot analysis, RvIL-6 was detected in plasma collected from SIV/RRV co-infected animals that also developed LPD, but not in plasma

from SIV- or RRV-infected animals (Fig. 4.4B&C). Together, these findings point to a correlation between B cell hyperplasia and bioactive RvIL-6 found in plasma of dually infected animals.

RvIL-6 may exist in plasma of LPD animals both as a monomer and potentially in a complex with a soluble protein that was detected by anti-human IL-6R on western blots (Figs. 4.4B&C). Interestingly, human IL-6 also exists physiologically not only as a monomer and dimer, but also in complexes with other factors such as antibodies and sIL-6R (299-303). Levels of sIL-6R and RvIL-6 in plasma of SIV/RRV co-infected animals were elevated compared to animals infected with SIV or RRV alone. However, the plasma concentration of sIL-6R in dually infected animals remained relatively unchanged over time (Fig. 4.5C, lanes 1-5), while that of RvIL-6 appeared to increase slightly until necropsy (see Figs. 4.5A & C, lanes 6-10). Simultaneous presence of RvIL-6 and sIL-6R in biological fluid of animals with LPD may have physiological significance because human sIL-6R (112, 157, 203) can interact with IL-6 to form a highly agonistic sIL-6R/IL-6 complex capable of activating gp130 on cells that do not traditionally express membrane IL-6R (383, 385). This sIL-6R-mediated IL-6 sensitization has been referred to as "trans-signaling" (72, 340, 342, 437, 473). Incidentally, signals initiated by the IL-6-sIL-6R complex are qualitatively more potent than those initiated by interactions of IL-6 with membrane-bound IL-6R (295, 384, 437), an observation that led to the creation of the highly bioactive "hyper-IL-6" consisting of mature IL-6 fused to the extracellular region of IL-6R (147, 434). In fact, a high serum concentration of sIL-6R has been linked to the pathogenesis of proliferative syndromes associated with plasma-cell dyscrasias (454) and other inflammatory states (384), indicating

that sIL-6R, together with serum IL-6 can induce the proliferative state characteristic of these syndromes. In light of the proliferative potential of the complex between sIL-6R and human IL-6 (72, 340, 342, 437, 473), systemic circulation of both sIL-6R and RvIL-6 in plasma of LPD animals could be significant with respect to the potential contribution of these proteins to the overall lymphoproliferative process.

A proliferative function for the sIL-6R/RvIL-6 complex in vivo, while not directly tested here is supported by two observations: (i) sIL-6R enhances RvIL-6-mediated growth of IL-6R$^-$/gp130$^+$ BAF-130 cells (Fig. 5.2), and (ii) recombinant RvIL-6 can associate with recombinant human sIL-6R in vitro (Fig. 4.5), and with physiological sIL-6 in vivo (not shown). Based on the precedent from human IL-6 and sIL-6R, association of RvIL-6 with sIL-6R may (a) prolong the half-life of RvIL-6, as previously described for other cytokines (50, 169) (101, 102), (b) provide a mechanism for chaperoning RvIL-6 around the body, and (c) maintain RvIL-6 in a stable conformation displaying high-affinity binding sites for gp130, similar to TNFα and soluble TNF receptors (3).

Many important questions remain to be answered in this system. For instance, (i) in what form does RvIL-6 predominantly circulate in vivo — as a monomer, dimer, or as a complex with carrier proteins? We have shown that anti-RvIL-6 can neutralize and actually deplete RvIL-6 from plasma; whether this finding indicates that RvIL-6 exists predominantly in a monomeric form that is accessible to anti-RvIL-6 is not clear. To what extent is RvIL-6 expressed in tissues of RRV-infected animals, and to what degree do RRV-infected animals generate an antibody response to RvIL-6? (ii) Is the stimulatory signal initiated by the sIL-6R/**RvIL-6** complex more potent than that elicited by RvIL-6 alone on

gp130^{+}/IL-6R^{-} BAF-130 cells or gp130^{+}/IL-6R^{+} B9 cells? (iii) From the preliminary findings in Fig. 5.2, what are the molecular determinants underlying differences in signals from the sIL-6R/**RvIL-6** complex relative to the sIL-6R/**MacIL-6** complex on gp130^{+}/IL-6R^{-} BAF-130 cells? Finally, (iv) what is the patho-physiological significance of the relative concentrations of sIL-6R and RvIL-6 in RRV-infected, compared to SIV/RRV co-infected rhesus monkey plasma with respect to the proliferative process?

7. What is B cell hyperplasia — is it lymphomagenesis or B cell hyper-activation?

During B cell lymphomagenesis, an accumulation of genetic changes usually leads to progression from a hyper-activated state to an overtly malignant manifestation, driven by expansion of a variant clonal sub-population with a growth advantage over normal B cells (66, 111). We have not examined the level of clonality or the extent of genetic alterations within the hyperplastic CD20+ B cell population in SIV/RRV co-infected animals. However, the expansion of B cells in these animals appears to have features unlike those of malignant B cells (70, 71, 79, 347). Thus, CD20+ B cell hyperplasia tends to persist for sometime and then wanes (523) in a manner coincident with RRV viral load in peripheral blood mononuclear cells (PBMC) (41). Because of the apparent correlation between hyperplasia and RRV viremia, we believe B cell hyperplasia in SIV/RRV co-infected animals results from unspecific virus-induced hyper-activation rather than from proto-oncogenic induction of a B cell lymphoma. We propose that a viral factor, possibly RvIL-6, may be responsible for the unspecific B cell expansion in SIV/RRV co-infected animals. Based on this model, we argue that at the core of RvIL-6-induced B cell hyperplasia is the

ability of RvIL-6 to stimulate aberrant growth of either a normal or RRV-infected circulating B cell sub-population (most likely a CD20+ cell) that subsequently expands in response to autocrine or paracrine effects of RvIL-6 secreted from infected cells. Direct proof of this model using RvIL-6-deletion mutants of RRV (see Appendix II) will be critical in future patho-biological studies of RvIL-6 function

What other viral factors besides or in addition to RvIL-6 could support a potential relationship between RRV infection and induction of B cell hyperplasia in SIV/RRV co-infected animals? One argument is that instead of a direct stimulatory role of RvIL-6, RRV may supply antigens that can chronically stimulate B cell growth (see step "v" of Fig. 5.3). Viral antigenic stimulation as a basis for B cell hyper-activation would be supported by the fact that most B-cell lymphoproliferative disorders derive from mature Ig^+ B lymphocytes that are capable of responding to antigen (277). The problem with this model, however, is that SIV-induced immunodeficiency during SIV/RRV co-infection may impair the fundamental elements of immune recognition and activation that are necessary to engender a response to RRV antigens. Alternatively, B cell hyperplasia in SIV/RRV co-infected animals could result from increased virus-induced production of one more host inflammatory cytokines such as IL-6 (see Fig. 1.3) with a direct stimulatory effect on B cell growth and differentiation (207), although this model too may be unlikely for two reasons. First, we could not detect host IL-6 in biological fluid of SIV/RRV co-infected animals (Table 4.1), indicating that host IL-6 is either not secreted in this setting, or is not induced by RRV in this system. Second, cytokine production normally relies on activation of a cytokine-secreting subset of effector cells, a process

that may also be impaired by SIV-induced immune debilitation. The above situations collectively point to RvIL-6 as the factor likely to play a stimulatory role for B cells under these circumstances.

8. Some postulates of RvIL-6 function in the B cell compartment

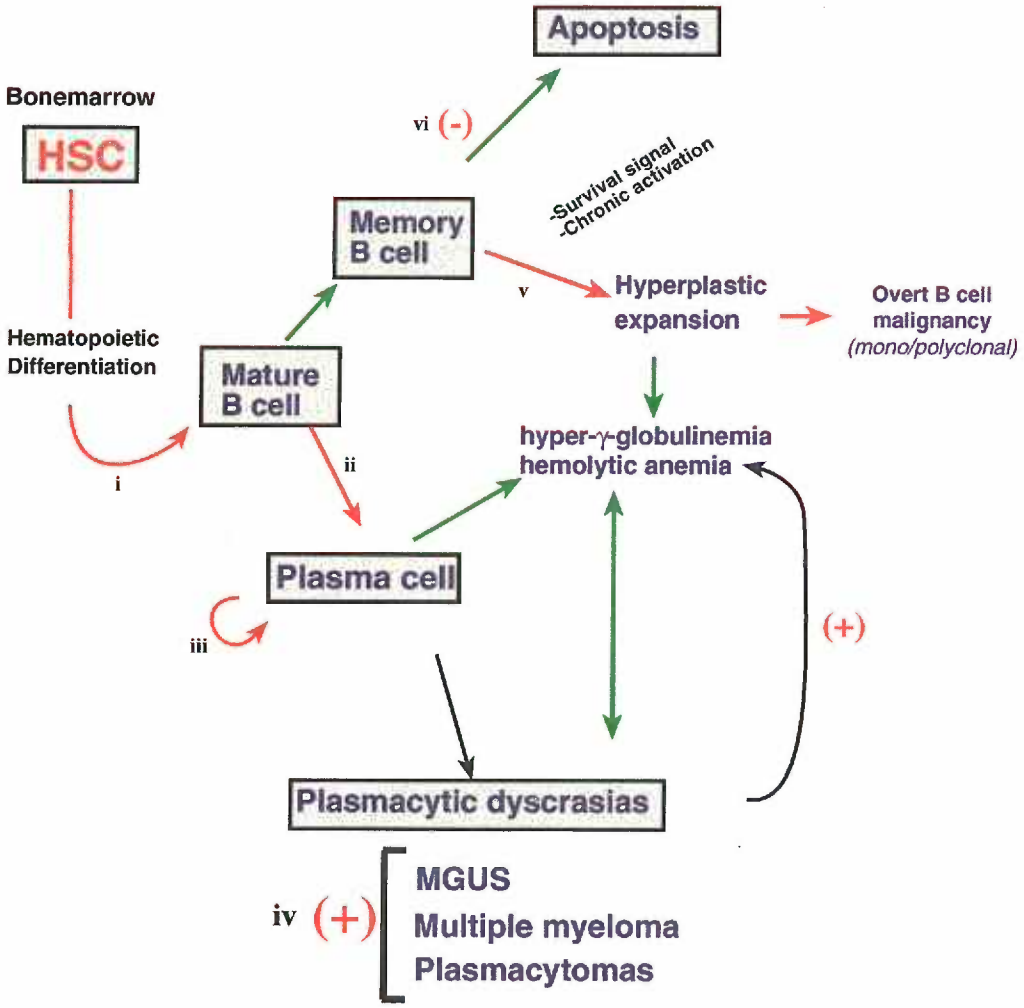
Ideas regarding the potential function of RvIL-6 in the B cell compartment may be developed from considerations of regulatory processes within the peripheral B cell repertoire. The size of this repertoire is generally determined by an intricate balance between development and differentiation in the bone marrow, as well as activation and proliferation in the periphery, all regulated by a complex interplay of growth factors (89, 276, 279, 419). Therefore, one correlate of B cell hyperplasia within the B cell compartment is likely to be deregulated expression of one or more growth factors including IL-4 for activation of resting B cells (177, 248), IL-5 for differentiation as well as growth of activated B cells (160), and IL-6 for the final differentiation of activated B cells into high-rate Ig-secreting plasma cells (419, 477).

IL-6 bioactivity has been implicated not only in polyclonal B cell abnormalities (534), but also in some proliferative syndromes and late-stage B-cell dyscrasias such as plasmacytomagenesis (366, 367) and myeloma (234). A viral protein such as RvIL-6 with IL-6-like activity might also display some of the same functions of the cellular counterpart. Thus, although the patho-physiological impact of RvIL-6 function within the B cell compartment is far from clear, we have shown that RvIL-6 stimulates B cell growth and can also prevent apoptosis — two processes that are likely to be critical in the B cell

differentiation and survival pathways. Moreover, based on the postulate that RvIL-6 and host IL-6 share several functional features, the IL-6-like bioactivity of RvIL-6 is likely to be most relevant at stages within the B cell compartment that are also responsive to IL-6 action. Fig. 5.3 attempts to construct a hypothetical scheme of in vivo processes within the B cell compartment that could be targets of RvIL-6 bioactivity. Future studies will be centered on establishing concrete evidence for RvIL-6 activity both in vitro and in many of the highlighted processes in vivo in order to elucidate the importance of RvIL-6 to virus survival in the infected host.

Fig. 5.3 — Potential sites of RvIL-6 action in the B cell compartment — This scheme represents processes where host IL-6, but not RvIL-6, has been shown to have biological function. Thus, RvIL-6 could behave like host IL-6 and (i) promote hematopoiesis by inducing maturation of progenitor B cells (215, 241, 243), or (ii) support differentiation of mature B cells to plasma cells in the periphery (419, 477). RvIL-6 could also (iii) support and sustain plasma cell growth and plasmacytomagenesis (200), or (iv) propagate IL-6-dependent plasmacytic dyscrasias and myeloproliferative syndromes (e.g., multicentric Castleman's disease, monoclonal gammopathy of undetermined significance, multiple myeloma, and development of plasmacytoma cells), resulting in clinical hyper-gamma-globulinemia, hemolytic anemia and autoimmunity (6, 8). Like human IL-6, RvIL-6 could either (v) provide a survival signal, and/or reverse apoptosis of short-lived memory B cells, resulting in non-specific expansion, accumulation of random genetic changes in B cell populations and eventually into monoclonal or polyclonal malignancy (6). Additional research will be necessary to determine whether RvIL-6 also exhibits these functions. The red arrows represent processes at which RvIL-6 is hypothesized to operate, while "(+)" or "(-)" respectively denote the potential RvIL-6-mediated promotion or reversal of the indicated processes.

Potential (hypothetical) sites of RvIL-6 action in the B cell compartment



Appendix I

Studies of RvIL-6-mediated alteration of cell growth in NIH 3T3 cells retrovirally transduced with RvIL-6

The main observations from this preliminary study are the following:

1. RvIL-6-transduced NIH 3T3 cells secrete bioactive RvIL-6 into the supernatant.
2. NIH 3T3-RvIL-6 clones display an altered growth phenotype characterized by focus formation and serum-independence.
3. NIH 3T3-RvIL-6 clones are more responsive to exogenous RvIL-6 than are native NIH 3T3 cells.

Background

Aoki et al. recently reported that vIL-6 encoded by HHV-8 can transform murine NIH 3T3 cells and was also able to induce tumorigenesis in athymic mice injected with NIH 3T3-vIL-6 cells (15). In that study, vIL-6 expression was associated with (i) increased hematopoiesis, (ii) increase in peripheral blood lymphocytes, (iii) plasmacytic infiltration of the lymphoid organs, and (iv) hyper-gammaglobulinemia (15). In addition, NIH 3T3-vIL-6 clones induced progressively growing tumors displaying neovascularization and marked angiogenesis related to vIL-6-induced expression of vascular endothelial growth factor (VEGF). These findings led to the conclusion that vIL-6 might play a direct role in hematopoiesis and angiogenesis — two of the main physiological processes that are critical for development of nearly all the known HHV-8-associated diseases. Using retroviral transduction of RRV RvIL-6, we have initiated studies to determine whether RvIL-6 might also induce a transformed phenotype in NIH 3T3 cells; preliminary data are consistent with a potential role for RvIL-6 in alteration of cell growth.

Retroviral transduction of NIH 3T3 cells

RvIL-6 was subcloned into the Cla I and Hind III sites of the pLNCX retroviral vector (317) between the two viral long terminal repeats (LTR), to create pLNCX-RvIL-6 that was then transfected into the amphotropic retroviral packaging cell line PA317 (316) using Lipofectin transfection reagent (Gibco BRL). Infectious retroviral particles were harvested and immediately used to transduce murine NIH 3T3 fibroblasts by "single-hit" infection in the presence of 4 μg/ml polybrene. One day later, transduced cells were split

and re-seeded in the presence of 1 mg/ml of the neomycin derivative G418 (Gibco BRL). G418-resistant clones were selected and propagated under increasing drug pressure for analytic studies (see below).

Focus-forming assay in soft agar —We studied the ability of RvIL-6-transduced NIH 3T3 cells to form foci in soft agar. To do this, sub-confluent clones were trypsinized and resuspended in a mixture of 0.3% agar and complete DMEM, and seeded in 35 mm^2 dishes at a density of $5x10^3$ per well over a 6% base-agar support. The following cell clones were used: native NIH 3T3, NIH-3T3/pLNCX, NIH-3T3/pLNCX-RvIL-6 clones 2 and 3, as well as NIH-3T3/v-12ras as a positive control.

Results and Discussion

Supernatant from RvIL-6-transduced NIH 3T3 clones supported B9 cell growth (Fig. AI.1), while supernatant from "naïve" NIH 3T3 cells, or from vector-transduced NIH 3T3 cells did not stimulate B9 cell growth. The individual stimulatory effects of NIH 3T3-RvIL-6 clones could be serially diluted out, indicating that B9 cell proliferation was dependent on an endogenous factor secreted by RvIL-6-transduced NIH 3T3 cells (data not shown).

The IL-6 bioactivity of supernatant from RvIL-6-transduced clones was blocked by polyclonal rabbit anti-RvIL-6, but not by polyclonal anti-human IL-6 (Fig. AI.2). NIH 3T3-RvIL-6 supernatant also contained a protein that co-migrated with recombinant RvIL-6 (Fig. AI.3A, lanes 1, 4 and 5). The RvIL-6 co-migrating protein was determined to be RvIL-6 based on immuno-reactivity with polyclonal anti-RvIL-6 (Fig. AI.3B). In addition, NIH 3T3-RvIL-6 clones grew continuously in low (1%) fetal calf serum (FCS) for almost two

weeks and formed several isolated foci in soft-agar cultures but cultures of NIH 3T3 cells or the pLNCX vector clones failed to produce foci in this assay (data not shown).

To determine whether RvIL-6-mediated alteration of cell growth was autocrine or paracrine in nature, cells were trypsinized, washed extensively and then re-plated in minimal serum (1% FCS) in the presence of increasing concentrations of exogenous RvIL-6 or MacIL-6. After two days, cells were pulsed with 3H-thymidiine and analyzed for proliferation. In this experiment, exogenous MacIL-6 did not affect growth of a pLNCX clone (Fig. AI.4*d*), or NIH 3T3-RvIL-6 clones (Fig. AI.4*e* and *f*). In contrast, RvIL-6 dramatically enhanced growth of RvIL-6-expressing clone 2 by nearly five-fold (Fig. AI.4*b*), and that of clone 3 by at least two-fold (Fig. AI.4*c*); interestingly, a slight proliferative effect was evident in pLNCX cells (Fig. AI.4*a)*. These data indicate that NIH 3T3 cells generally do not respond to exogenous RvIL-6 or MacIL-6. On the other hand, endogenous expression of RvIL-6 may itself be able to induce responsiveness to exogenous RvIL-6, perhaps via upregulation of a surface receptor(s).

Most cells ubiquitously express gp130, while IL-6R expression is more restricted. Therefore, the fact that MacIL-6 did not affect NIH 3T3 cell growth suggests either that NIH 3T3 cells do not express IL-6R or that these cells express very low levels of this ligand-specific receptor that is absolutely required for MacIL-6 function. In contrast, RvIL-6 stimulated a modest level of proliferation even in pLNCX vector-transduced cells (Fig. AI.4a), with a more pronounced effect in RvIL-6-transduced clones (Fig. AI.4*b*&*c*). Therefore, RvIL-6 may signal in NIH 3T3 via direct binding to the ubiquitous gp130 subunit, as suggested by our data in Fig. 5.2. Alternatively, or in addition, endogenous

expression of RvIL-6 by NIH 3T3-RvIL-6 clones may induce or up-regulate surface expression of gp130 or any other yet-to-be-discovered receptor for RvIL-6.

A paracrine function of RvIL-6 reveals an important function for this protein in vivo especially in light of our finding that RvIL-6 circulates as a functional protein in biological fluid of animals that develop lymphoproliferative disease (Chapter 4). In this serum-borne form, RvIL-6 may be available to directly exert paracrine effects on RRV-infected B cells (those that presumably secrete bioactive RvIL-6), a model consistent with a role for RvIL-6 in induction of B cell hyperplasia.

FUTURE STUDIES AND TECHNICAL NOTES

(a) We intend to inoculate NIH 3T3-RvIL-6 clones into nude mice to determine whether RvIL-6 also has the ability to induce tumorigenesis in vivo.

(b) We plan to study the physiological function of RvIL-6 in B cells by transducing primary rhesus B cells as well as established pre-B cell lines with RvIL-6 to determine whether RvIL-6 can regulate the transition of mature B cells to a plasmacytic phenotype. Moreover, since we know that exogenous RvIL-6 can actually prevent apoptosis in PC12 cells (Chapter 3), we should also be able to determine whether RvIL-6 can confer resistance to apoptosis in retrovirally transduced pre-B or fully differentiated B cell lines. With respect to the latter, we intend to use the WEHI-231 cell line, one of the most well characterized models for studies of signals governing Fas- or surface-Ig-induced cell death (36, 180). RvIL-6-transduced WEHI-231 cells will facilitate a determination of whether RvIL-6 can

influence "life and death" signals.

(c) Some viral proteins may function in concert, a phenomenon perhaps best exemplified by HCMV genes US2, US3, and US11 that are known to interfere with the biosynthetic pathway for major histocompatibility complex (MHC) class I antigen-recognition structures that facilitate immune detection of virus-infected cells (389). US3 retains class I molecules in the endoplasmic reticulum (223, 263), while US2 (222) and US11 (520) mediate translocation of retained class I molecules to the cytosol where these molecules are degraded by the proteosome. Should RvIL-6 function together with other RRV-encoded proteins in a proliferative or angiogenic response, RvIL-6-transduced clones will provide a good background for co-expression of additional genes on a second plasmid by a single transfection, obviating the need to co-transfect two separate plasmids.

Fig. AI.1 — IL-6 bioactivity of supernatant fluid from RvIL-6-transduced NIH 3T3 cells. Panel A, Supernatant from native NIH 3T3 cells, or from G418-resistant clones (pLNCX, pLNCX-RvIL-6 #2, or pLNCX-RvIL-6 #3) grown in the absence of the drug was diluted 1:4 (v/v) in B9 cell starving media and used to stimulate B9 cell growth. Cell proliferation was determined by 3H-thymidine uptake in a bioassay as previously described (226), and the data is presented as the mean of "counts per minute" (CPM) for three independent experiments, each performed in triplicate.

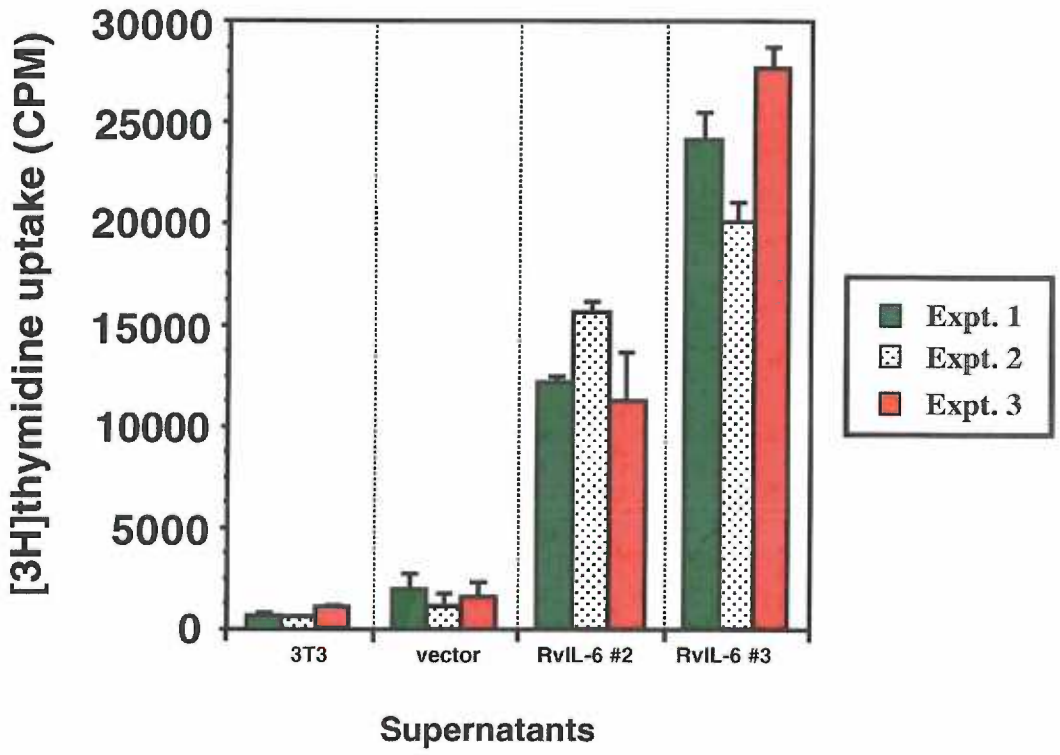

[3H]thymidine uptake (CPM)
30000
25000
20000
15000
10000
5000
0
3T3
vector
RvIL-6 #2
RvIL-6 #3
Supernatants
Expt. 1
Expt. 2
Expt. 3

Fig. AI.2 — IL-6 bioactivity of supernatant fluid from RvIL-6-transduced NIH 3T3 cells is blocked by anti-RvIL-6 but not by anti-human IL-6. G418-free supernatant from NIH 3T3-RvIL-6 cultures was used at a 1:4 dilution and added to pre-starved B9 cells in the presence of increasing amounts of either anti-RvIL-6 or anti-human IL-6 that does not cross-react with RvIL-6. Cell proliferation was determined in a bioassay as previously described (226). The data is presented as average "counts per minute" (CPM) for triplicate assays.

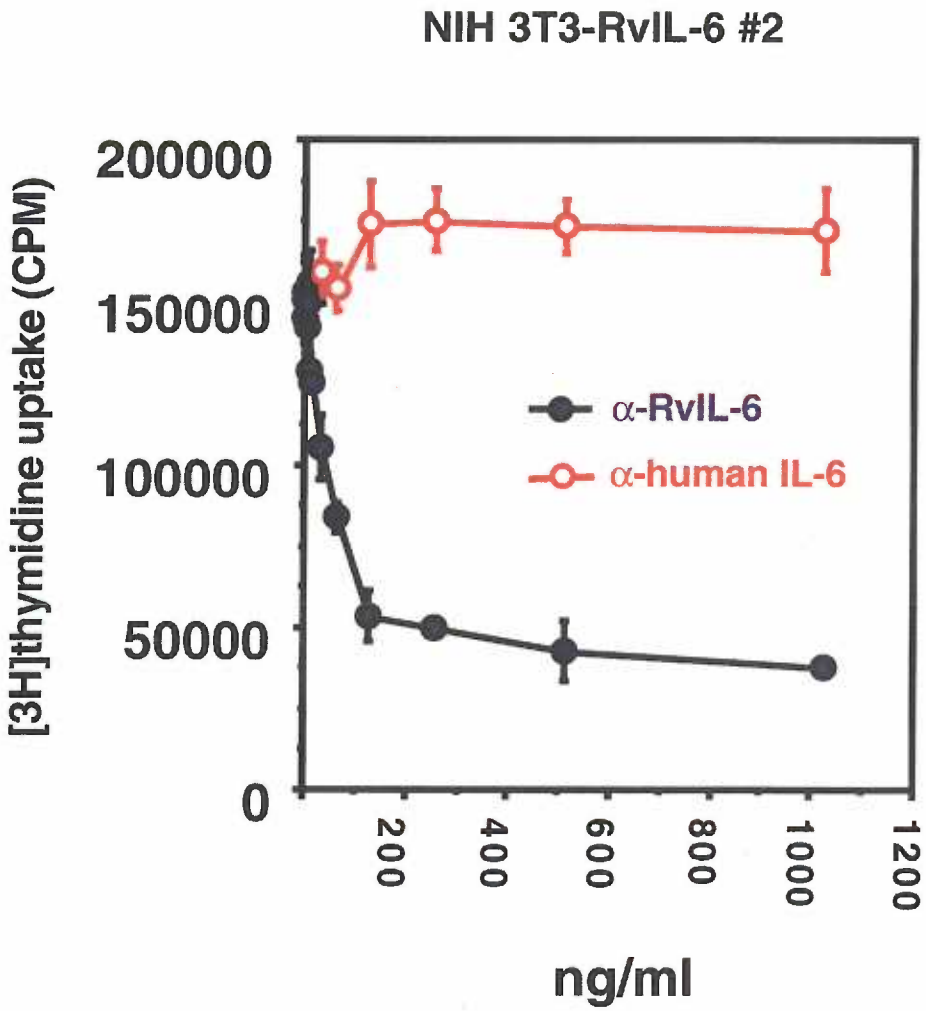
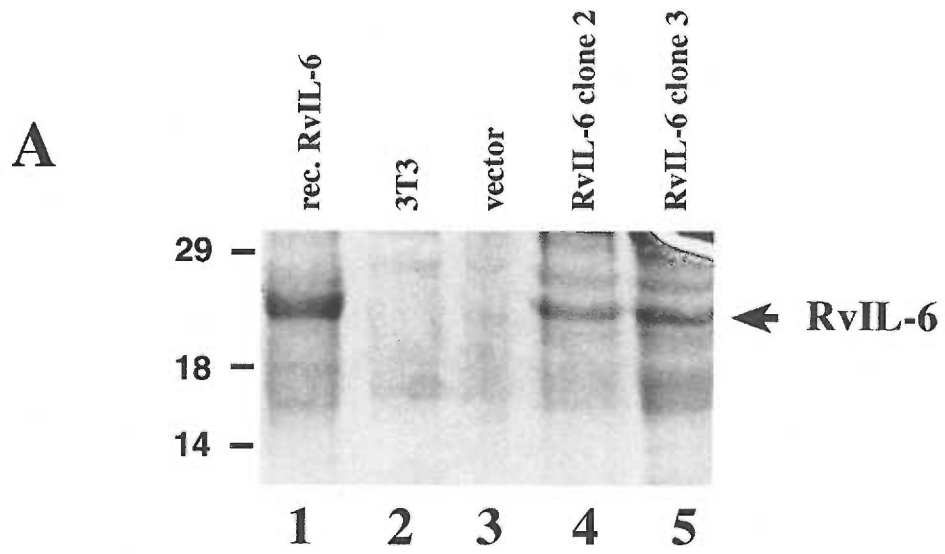

NIH 3T3-RvIL-6 #2
[3H]thymidine uptake (CPM)
200000
150000
100000
50000
0
200
400
600
800
1000
1200
ng/ml
α-RvIL-6
α-human IL-6

Fig. AI.3 —Supernatant from RvIL-6-transduced NIH 3T3 cells contain secreted RvIL-6. Crude supernatant from clone #2 or 3.1 was boiled in 4X protein sample buffer (227) and subjected to SDS-PAGE on duplicate 12 % acrylamide gels as previously described (227). One gel was stained with Coomasie blue R250 (panel A), and the other was transferred onto nitrocellulose membrane and probed with anti-RvIL-6 in a western blot analysis (panel B). Recombinant RvIL-6 (10 μg) or supernatant from NIH 3T3 and pLNCX clones were used as controls.


A
rec. RvIL-6
3T3
vector
RvIL-6 clone 2
RvIL-6 clone 3
29
18
14
RvIL-6
1
2
3
4
5

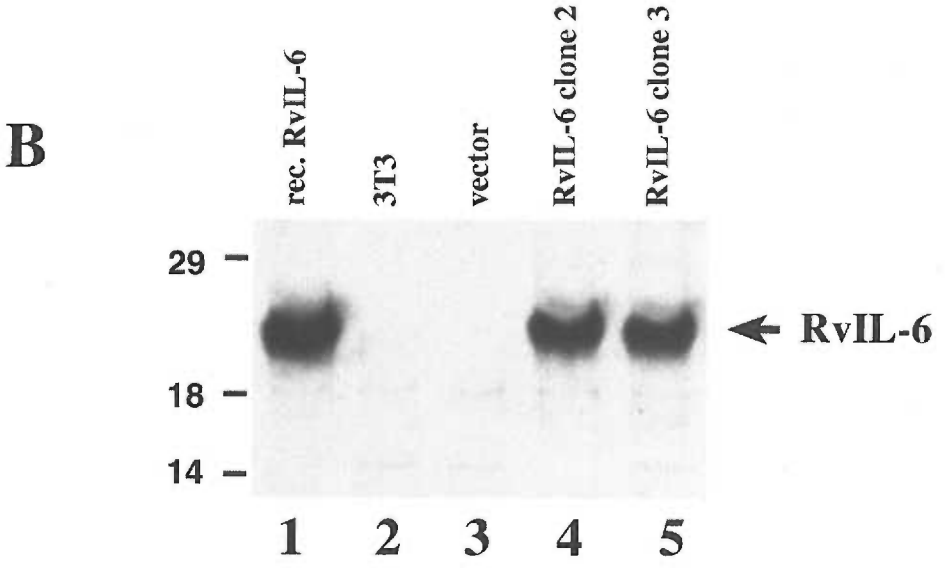
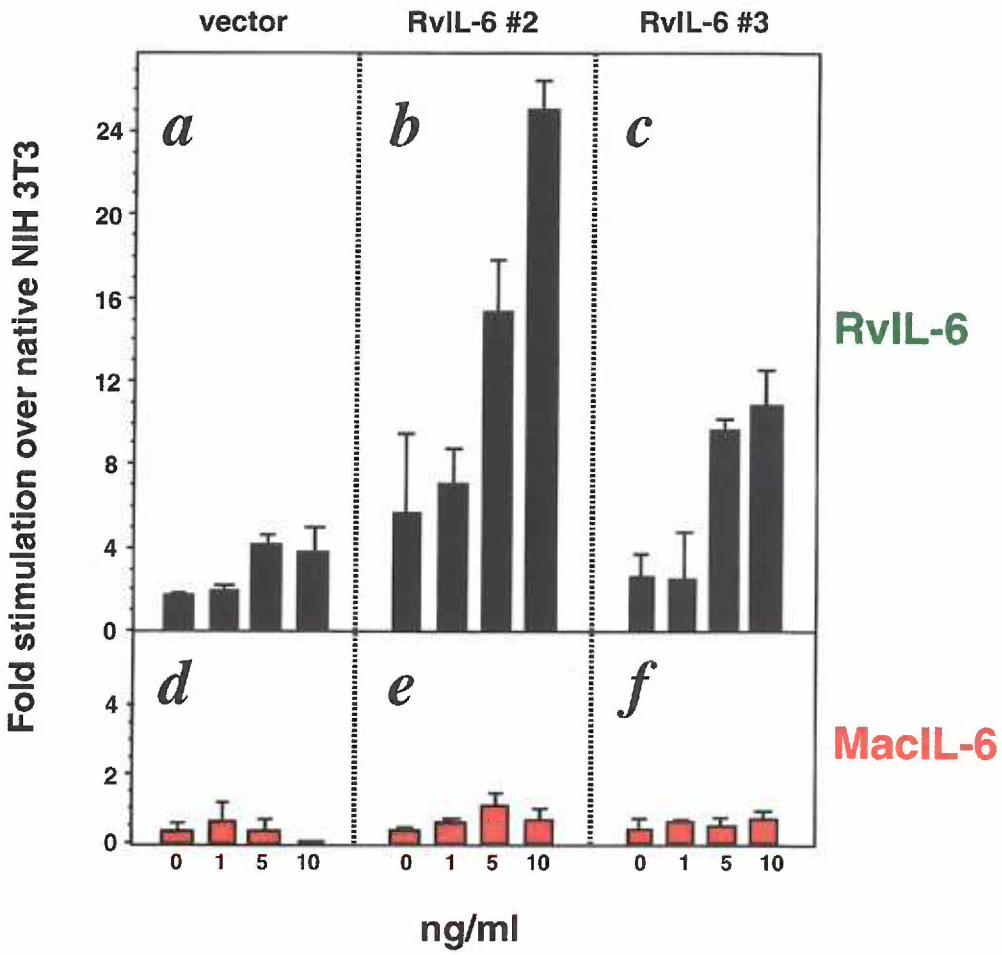

B
rec. RvIL-6
3T3
vector
RvIL-6 clone 2
RvIL-6 clone 3
29
18
14
RvIL-6
1
2
3
4
5

Fig. AI.4 — Endogenous expression of RvIL-6 in retrovirally transduced NIH 3T3 cells induces cellular responsiveness to the paracrine effects of exogenous RvIL-6. Sub-confluent native NIH 3T3, NIH 3T3-pLNCX vector, or NIH 3T3-RvIL-6 clones 2 and 3 were re-seeded in 24-well plates at $2x10^5$ cells/well in complete DMEM media without G418. The next day, increasing concentrations of either RvIL-6 or MacIL-6 were added; after 18 hours, cells were pulsed with 1 μCi of 3H-thymidine for 14 additional hours, and 3H-thymidine uptake was counted in a gamma-scintillation counter. Data are presented as fold-stimulation due to recombinant RvIL-6 or MacIL-6 compared to unstimulated NIH 3T3 cells.


vector
RvIL-6 #2
RvIL-6 #3
a
b
c
d
e
f
Fold stimulation over native NIH 3T3
24
20
16
12
8
4
0
4
2
0
0 1 5 10
0 1 5 10
0 1 5 10
ng/ml
RvIL-6
MacIL-6

Appendix II

Generation of recombinant RRV with a deletion of RvIL-6 and carrying enhanced green fluorescent protein (EGFP) inserted into the RvIL-6 locus.

EXPERIMENTAL APPROACH

We developed a strategy for generation of RvIL-6-deletion mutants of RRV as a reagent for determining whether RvIL-6 plays a role in B cell hyperplasia. The strategy is based on co-transfection of rhesus fetal fibroblasts with wildtype viral DNA along with a linearized recombinant plasmid where most of the RvIL-6 gene was deleted and replaced with a cDNA cassette encoding enhanced green-fluorescent protein (EGFP). Wildtype RvIL-6 sequences in the viral genome would then be replaced with the modified sequences following homologous recombination between RRV and the modified plasmid, and recombinant progeny viruses isolated based on EGFP fluorescence.

Transcriptional analysis of RRV gene expression

Because of the complex organization of ORFs in the cytokine/chemokine gene region of RRV that contains RvIL-6 (Fig. AII-1), we analyzed mRNA transcripts that derive from this region before designing a scheme for deleting RvIL-6. We performed Northern-blot analysis on total RNA isolated from RRV-infected rhesus fetal fibroblasts at various times following infection, and probed the RNA with ^{32}P-random-primed cDNA oligonucleotides derived from (i) major-capsid protein (MCP), (ii) open reading frame 57 (ORF57), (iii) single-stranded DNA-binding protein (ssDBP), (iv) thymidylate synthatase (ORF70, or TS), (v) viral IL-6 (RvIL-6), and (vi) viral macrophage inflammatory protein (vMIP).

As shown in Fig. AII-2, Rv-MIP appears abundantly as a single transcript (~ 1.4-kb) at 48 hpi, indicating that the Rv-MIP transcript may be made monocistronically and may not

have coding potential for proteins other than Rv-MIP. On the other hand, the TS probe hybridized to two transcripts approximately 2.4-kb and 3.9-kb that appeared abundantly at 48 hpi. The RvIL-6 probe hybridized to two transcripts (~2.4-kb and 3.9-kb) that were expressed as early as 24 hpi and appeared to overlap in size with the two transcripts detected by the TS probe at 48 hpi. Therefore, messages for TS and RvIL-6 are either co-transcriptionally synthesized or co-terminally processed. In future studies, the identity of the two transcripts detected by RvIL-6 and TS probes will be established using ribonucleotide probes specific to the (-) and (+) strands of both these open reading frames.

ORF 57 appeared within 12 hours post-infection, while ssDBP was detected 24 hpi. MCP appeared 48 hpi and became increasingly abundant. Together, these transcriptional data show that during lytic replication of RRV, temporal expression of six RRV genes (i.e., Rv-MIPβ, RvIL-6, TS, ORF57, ssDBP, and MCP) may follow a coordinately regulated profile similar to that of other herpesviruses (412), and with timing similar to that of their respective counterparts encoded by HHV-8 (219, 466). However, the coding potential of each of these genes, as well as the relationship between viral gene expression and pathogenesis are currently unknown.

Deletion of RvIL-6 and insertion of EGFP into the RvIL-6 deletion site

ORF R2 that encodes RvIL-6 is contained on plasmid 29-2 (Fig. AII-3) and has three unique Nar I sites. Taking advantage of the fact that the ef-1α/EGFP cassette in the pQ100 vector [ref. (500), and Jeff Vieira, personal communication] is flanked by two Cla I sites (AT*CGAT) that can be ligated to the GG*CGCC over-hangs of Nar I, the Cla I fragment

of ef-1α/EGFP derived from pQ100 was directly ligated into the Nar I site of 29-2, creating 29-2ΔRvIL-6::EGFP (the "::" denotes EGFP insertion "into" the RvIL-6 locus). The ef-1α/EGFP cassette also contains sequences for the *E. coli gpt* gene that encodes *Xanthine-Guanine Phosphoribosyl Transferase* (XGPRT) for selection (338, 339); therefore, besides EGFP fluorescence, expression of XGPRT in HAT media can be used as one additional strategy for screening recombinants.

Mutagenesis of RvIL-6 ATG^{Met} to a TS-polyA site (AATAAA)

Rationale: Northern analysis of transcripts within the "RvIL-6 — TS — Rv-MIP" gene block in RRV shows that transcripts that contain RvIL-6 may also contain TS, and vice versa (see Fig. AII.2). Interestingly, there is no canonical poly-A (AATAAA) site, or the functionally related sequence AATATA at the 3' end of TS, or in the intergenic region between the TS stop codon and the initiation codon of RvIL-6, yet there are two tandem poly-adenylation sequences at the 3' end of RvIL-6 (Fig. AII.1). Thus, the two transcripts detected by TS and RvIL-6 probes may not be specific to either gene, since one or both of the two tandem poly-A sites at the 3' end of RvIL-6 (designated "b'b" in Fig. AII-1) could be used to process transcripts encoding TS, RvIL-6, or both. The two transcripts may therefore result from read-through processing of the TS transcript whose processing may be directed by one of these two 3' poly-A sequences. In light of this possibility, a deletion of RvIL-6 by insertion mutagenesis could affect other messages potentially expressed on the same transcript as RvIL-6. We believe that the gene most likely to be abrogated by this approach is TS, since ORF 11 that is 3' RvIL-6 may be transcribed on the (-) strand,

opposite RvIL-6 (see Fig. AII-1).

Strategy: To ensure that a deletion of RvIL-6 would not interfere with proper processing of TS, we used a PCR-based approach to disable RvIL-6 by modifying the initiation codon of RvIL-6 (RvIL-6 ATG^{Met}) to a poly-A sequence (AATAAA) that would in effect direct post-transcriptional processing of the TS message. Fig. AII-3 depicts how RvIL-6 deletion and EGFP insertion into the RvIL-6 deletion site were carried out, while Fig. AII-4 is a scheme of how the ATG > AATAAA mutagenesis was accomplished.

Screening for orientation of the ef-1/EGFP cassette in the mutagenic plasmids.

The ef-1α/EGFP cassette was inserted into the RvIL-6 deletion site (Nar I) without regard to orientation (see Fig. AII-5A). Although orientation of ef-1/EGFP in the recombinant viral genome would not affect EGFP expression (EGFP has its own ef-1α promoter), we still determined orientation and presence of single or tandem insertions of ef-1α/EGFP relative to RvIL-6 by restriction mapping of modified derivatives with Pst 1 or Sph 1. Fig. AII-5B shows the correct Pst I and Sph I maps for constructs 29-2Δ-GFP (Fig. AII3C) and 29-2Δ-A-GFP (Fig. AII-3D). All constructs were fully sequenced in both directions using oligonucleotide primers that span the modified sequences. The correct maps for each digestion ("**a**") appear similar because they are only distinguished by a 3-bp change represented by the ATG > AATAAA mutation.

Summary of generated plasmids

(a) 29-2Δ, Fig. AII-3A

(b) RRV-ΔRvIL-6-polyA-TS (from 29-2Δ-A, Fig. AII-3B)

(c) RRV-ΔRvIL-6::EGFP (from 29-2Δ-GFP, Fig. AII-6.3C)

(d) RRV-ΔRvIL-6::EGFP-polyA-TS (from 29-2Δ-A-GFP, Fig. AII-3D).

Analysis of generated plasmids for EGFP expression

To verify that EGFP could be expressed from the above plasmids in vitro, approximately 10 μg of plasmid DNA was transfected into rhesus fetal fibroblasts and cells were periodically visualized by fluorescent microscopy for up to six days following transfection.

Table AII.1: EGFP fluorescence in cells transfected with mutagenic plasmids.

Plasmid	Plasmid description	% fluorescence*
1	RRV-ΔRvIL-6	ND
2	RRV-ΔRvIL-6-polyA-TS	ND
3	RRV-ΔRvIL-6::ef-1/EGFP	0.03
4	RRV-ΔRvIL-6::ef-1/EGFP-polyA-TS	0.09
5*	pQ100 (source of ef-1/EGFP cassette)	0.07
6#	pMel-R/EGFP-n (Melatonin receptor fusion)	0.15

*Number of EGFP+ cells per field of 10^4 cells.

#PQ100 and pMel-R/EGFP-n were used as positive controls for EGFP expression.

As shown in the above table, the level of fluorescence from all plasmids was extremely low (average ~ 0.06%), indicating that expression of EGFP under these

circumstances is quite limited. Generation of "RvIL-6-deletion/EGFP-insertion" mutants of RRV will be achieved by co-transfection of infectious RRV DNA along with modified plasmids, and recombinant progeny viruses can then be purified based on EGFP expression.

Discussion and future studies

As a model for HIV/HHV-8 infection in humans, the SIV/RRV infection system has several practical advantages over the two available small-animal models of gamma-herpesvirus pathogenesis that are based on experimental infection of New World monkeys with HVS (2) and of mice with MHV-68 (130, 467). First, RRV encodes positional analogues of the same accessory genes encoded by HHV-8 (such as viral MIP-I/II and viral IL-6) that are not represented in the genomes of HVS or MHV-68. Second, RRV naturally infects rhesus macaques, a species that is arguably more physiologically related to humans than are New World monkeys or mice. Third, unlike HHV-8, RRV can be propagated to high titer in rhesus fetal fibroblasts, thus facilitating fast generation and analysis of genetically modified recombinant RRV lacking genes suspected to play a role in pathogenesis. Finally, in light of evidence that HHV-8-associated clinical manifestations in humans are preponderant in individuals that are also undergoing immunodeficiency, such as during HIV infection, we believe that the best model for HHV-8 pathogenesis in such individuals is one that also seeks to re-create the contribution of immunodeficiency. In this regard, no other available in vivo model for gamma-herpesvirus pathogenesis is more suitable for modeling the HIV/HHV-8 co-infection of humans than our SIV/RRV co-

infection of rhesus macaques.

We believe that recombinant RvIL-6-minus RRV carrying EGFP as an insertion into the deleted RvIL-6 locus will facilitate definitive studies of whether RvIL-6 contributes to B cell hyperplasia. One of the assumptions we have made in this study is that RvIL-6 is dispensable (i.e., not required) for viral replication. However, as we are just in the process of generating recombinant virus, we still cannot rule out the possibility that lack of RvIL-6 might attenuate some aspect of viral replication. With this possibility, the retroviral vector pLNCX-RvIL-6 (Fig. AI-1) that was generated for other purposes (see Appendix I) will be transduced into rhesus fetal fibroblasts to generate a complementing cell line that would supply RvIL-6 *in trans*. Other studies are also underway to generate "wildtype" RRV-EGFP which, unlike unlabelled RRV, should provide a more suitable control background for the recombinant RvIL-6-minus, EGFP-expressing virus, since both can be tracked by EGFP fluorescence in infected tissues.

With both EGFP-expressing "wildtype" and recombinant viruses in hand, ongoing and future studies will include (i) characterization of in vitro growth, (ii) verification that RvIL-6 is not synthesized by the "mutant", EGFP-expressing virus (by Southern analysis of viral DNA, and/or Northern-blot analysis of viral RNA from infected cells), and (iii) evaluation of the integrity of the TS gene in the RvIL-6-deleted virus. Finally, we will also compare pathogenesis of the recombinant virus in parallel with wildtype RRV based on a number of diagnostic and clinical parameters:

1. Viral load either by PCR and co-culture of infected peripheral blood mononuclear cells

with rhesus fetal fibroblasts

2. Measurement of anti-RRV antibody titers by ELISA
3. Analysis of changes in lymphocyte populations by flow cytometry, and evaluation of clinical manifestations that depend on IL-6 bioactivity (i.e., lymphadenopathy, splenomegaly, hypergammaglobulinemia and hemolytic anemia.
4. Determination of both lymphoid and non-myeloid tissue-specific expression of RvIL-6 by RT-PCR and in situ hybridization using gene-specific ribonucleotide probes. In addition, polyclonal rabbit or chicken antibodies already generated against this protein (unpublished results) will be used in immunohistochemical assays to determine protein expression both in infected cells and frozen tissue sections.

Technical note:

Successful generation of recombinant RRV by homologous recombination in a setting where a segment of viral DNA is replaced with plasmid DNA occurs at very low frequency, usually to the order of 0.1%. Therefore, other methodologies could be used to disable the RvIL-6 gene in RRV. These include:

(a) Insertion of nonsense mutations in the RvIL-6 coding region using a linker oligonucleotide containing one or more stop codons in all reading frames. A critical feature of such a synthetic linker is that it contains a unique restriction site that can be used for screening of mutagenic plasmids, and for identifying "mutant" viral genomes in the absence of a selection marker. In fact, functional domains of HSV-1 ICP-4 were defined using this approach (111, 391). However, although this

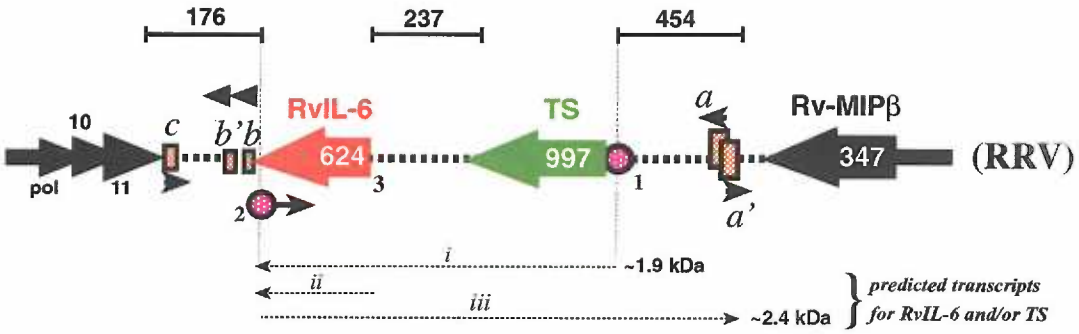
methodology involves introduction of the least amount of extraneous foreign DNA into the virus genome, the need to perform molecular biological analysis of recombinants either by PCR or by restriction mapping makes it much less attractive than our EGFP-insertion approach that relies on fluorescence.

(b) Alternatively, recombinant RvIL-6-minus RRV could be generated by co-transfecting rhesus fetal fibroblasts with a full complement of cosmid clones representing the entire genome of RRV, where one or more cosmids carry the desired modifications. Recombinant clones of HSV-1 (251, 487) and CMV (236, 320) have been generated in this manner, so we have reason to believe that a cosmid-based construction of deletion mutants of RRV should be successful. One advantage of this approach is that, in the absence of contaminating wildtype viral DNA, any infectious progeny virus derived from the transfection is likely to contain the desired modifications, thus obviating the need for additional screening steps.

In conclusion, it is clear that RRV, a virus that grows well in culture, is amenable to genetic manipulation. Molecular biological modification of RRV is therefore anticipated to provide tremendous opportunity for defining viral gene function. Moreover, in a step-wise fashion, the effect of specific mutations introduced in specific areas of the viral genome can first be studied in cell culture to define their dispensability for viral replication, followed by studies of the pathogenic role of the modified gene products by experimental infection of rhesus macaques. These efforts will be the basis of long-term goals to define the basic biology of RRV.

Fig. AII.1 — Organization and orientation of open reading frames encoding RvIL-6, Rv-MIPβ, and TS — The cytokine/chemokine gene block contains MIP (347 bp), TS (997 bp) and RvIL-6 (624 bp). Base-pair lengths of the intergenic, untranslated sequences between these open-reading frames are also indicated. "1" and "2" are positions of potential TATA sequences on the (+) and (-) strands, respectively, while "3" is the position of the 5' ATG initiation codon of RvIL-6. Letters "a", "b" and "c" are positions and orientations of the canonical poly-adenylation (poly-A) site **5'AATAAA-3'**. Thus, each MIP and TS ORFs possess TATA boxes at their respective 5' ends, while RvIL-6 does not. However, RvIL-6 possesses two tandem poly-A sites located 10 bp apart on the (+) strand at the 3' end of the gene (denoted "b"), while MIP contains one ("a"). In addition, there exists a 5'-TATA-3' box "2" on the (-) strand of RvIL-6 starting at the 3' end of the gene, with a poly-A site "a" located 2312 bp downstream of this TATA site. Whether TATA box "2" and the polyA site "a'" are on the same transcript is not clear. Based on presence of the above subsequences known to direct transcription and/or processing of mRNA, dashed arrows indicate the potential unprocessed mRNA transcripts *i, ii* and *iii*, with their predicted lengths and orientation.

Organization of ORFs around the RvIL-6 locus in RRV (with predicted transcripts and their orientation)



poly A sequences (i.e., 5'—AATAAA—3')

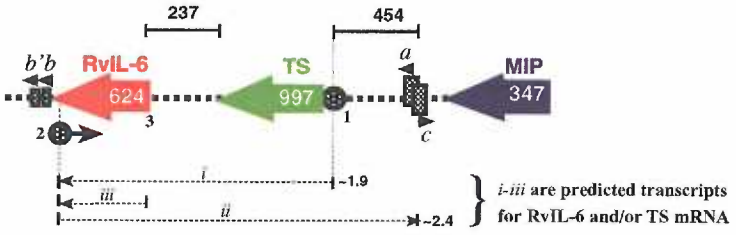
TATA sequence

Intergenic untranslated sequences

Orientation of ORF or mRNA transcript(s)

Fig. AII.2: Northern blot analysis of total cellular RNA from RRV-infected rhesus fetal fibroblasts — Cells were infected with RRV at a multiplicity of infection (MOI) of five particles per cell. At 12, 24, 48, 72 and 96 hours post-infection, total RNA was extracted, resolved by electrophoresis in a formaldehyde/agarose gel (10 μg/lane), transferred to nitrocellulose, and hybridized with ^{32}P-labelled DNA probes specific to the indicated ORFs.

Northern blot analysis of RRV gene expression



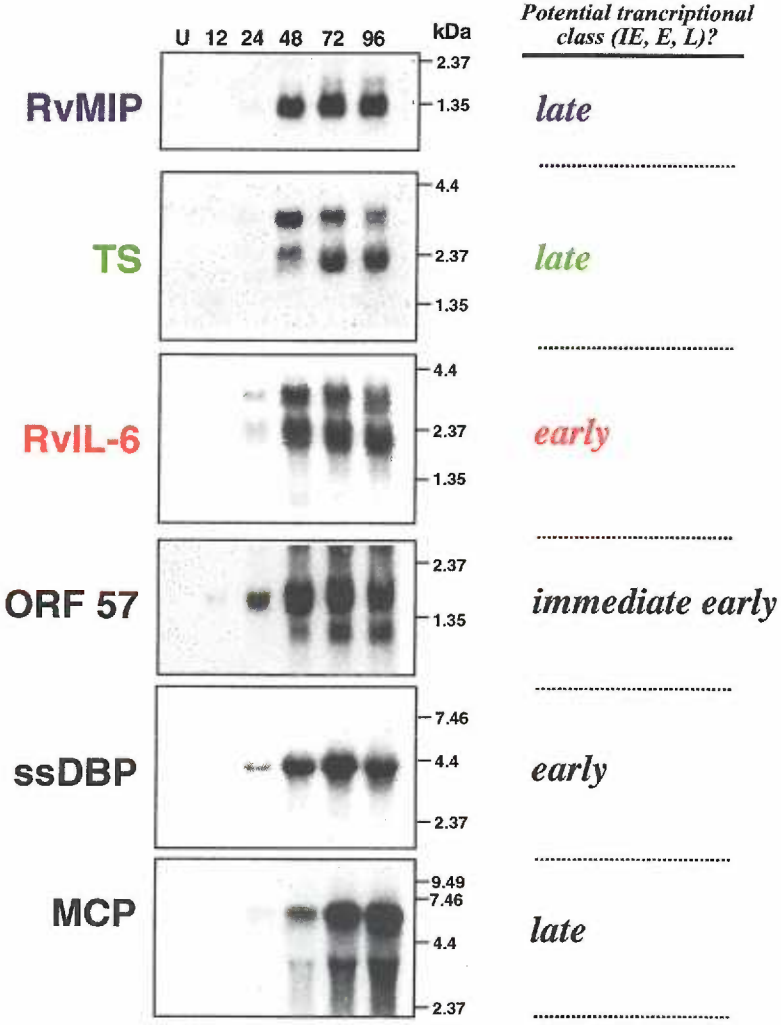


Fig. AII.3: Deletion and EGFP-insertion mutagenesis of RvIL-6 — Plasmid 29-2 was digested with Nar I and then either re-ligated upon itself to generate construct A: 29-2Δ or ligated to a Cla I fragment containing Ef-1α/EGFP cassette with the *gpt* selection gene, to generate construct C: 29-2Δ-GFP. In addition, construct A: 29-2Δ was subjected to PCR mutagenesis designed to change the ATG initiation codon of RvIL-6 to AATAAA polyA sequence as shown in construct B: 29-2-A (the details of this procedure are illustrated in Fig. AII-6.4 below). Furthermore, plasmid B: 29-2-pA was also linearized with Nar I and ligated to the Cla I fragment containing Ef-1α/EGFP, to obtain the construct D: 29-2D-pA-GFP. The final constructs C and D were subsequently screened by restriction endonuclease digestion to determine orientation of the Ef-1α/EGFP fragment.

Construction of recombinant plasmids (A, B, C&D) for modification of the RvIL-6 locus by homologous recombination with wildtype RRV DNA

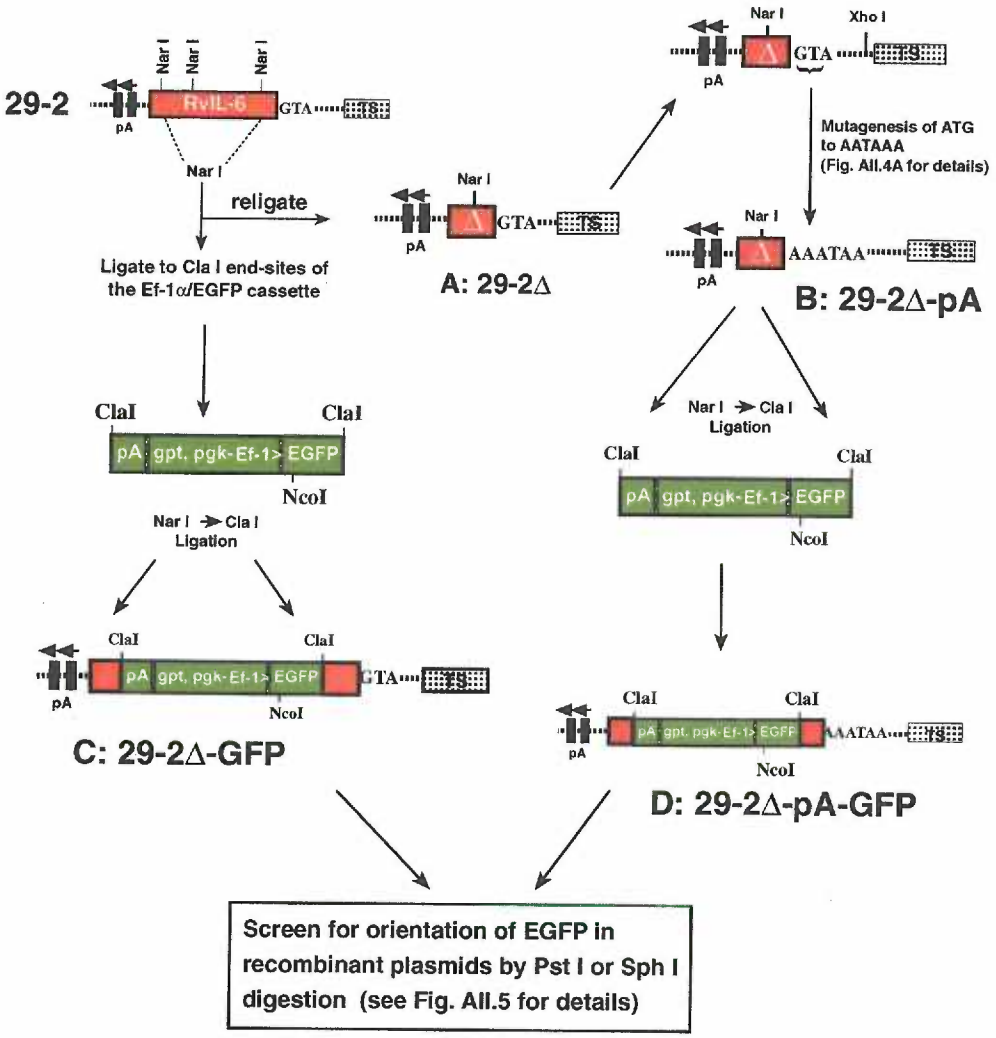
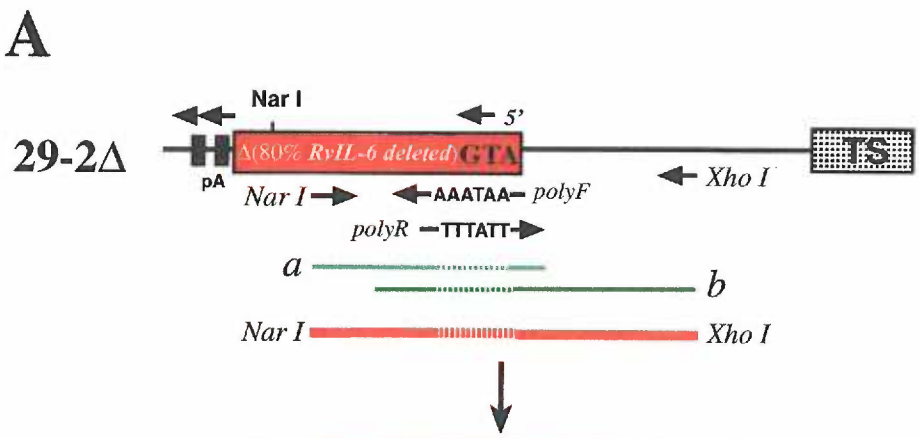


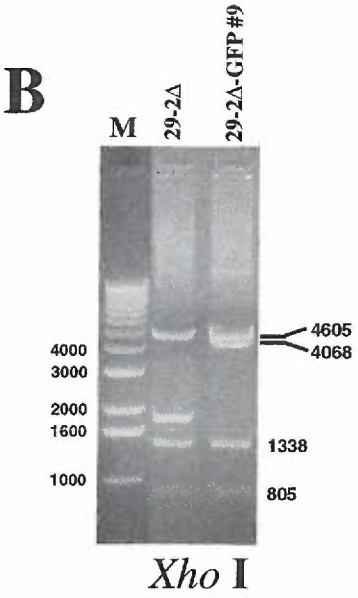
Fig. AII.4: PCR-based mutagenesis of 5'RvIL-6 ATG^{Met} initiation codon to the canonical poly-adenylation sequence AATAAA — A, Modification of the ATG initiation codon to a poly-A sequence was done in three steps. First, a reverse ***3'-Nar I-R*** primer 5'-CGG GGG A GG CGC C AG CGT AGG GCT-3' and a second, mutagenic ***5'-poly-F*** primer 5'-TAA CCG GTC **AAT AAA** TTC CCT GTC-3'with a forward (+) AATAAA subsequence were used to amplify a 290-bp product "**a**" that spans the ATG of RvIL-6. In a separate reaction, a second pair of primers ***Xho I-F***: 5'-CTG AGT CTC GAG GGC TAC GAC CCC-3' and a reverse ***3'-poly-R*** mutagenic primer 5'-GAC AGG GAA **TTT ATT** GAC CGG TTA-3' were used to amplify a short, 70-bp product "**b**", which also spans the ATG of RvIL-6. Both PCR products "**a**" and "**b**" were purified, mixed in equimolar ratios in a single PCR reaction, and used as template in a final reaction that was ran to amplify a final **Nar I-Xho I** product that was then purified, cleaved with Nar I and Xho I, and ligated back into the original plasmid 29-2Δ. The new construct, 29-2Δ-pA was sequenced to verify the ATG > AATAAA change.

B, Constructs 29-2Δ (control) and one EGFP-positive clone 29-2Δ-GFP #9 were digested with Xho I to screen for successful insertion of EGFP into the RvIL-6 deletion site. Notice the shift of the 1.9-kb fragment, representing insertion of the EGFP cassette.

C, Constructs 29-2Δ (control) and two EGFP-positive poly-A clones 29-2D-pA-GFP #s 5* and 9 were digested with Xho I to verufy successful insertion of EGFP into the RvIL-6 deletion site. Clone #5* contains two tandem inserts of the EGFP cassette in the same orientation as RvIL-6, while clone #9 contains a single insert (see Pst I or Sph I maps "a" and "c" in Fig. AII.5A)

PCR-based mutagenesis of 5' RvIL-6 "ATG" to a polyA subsequence





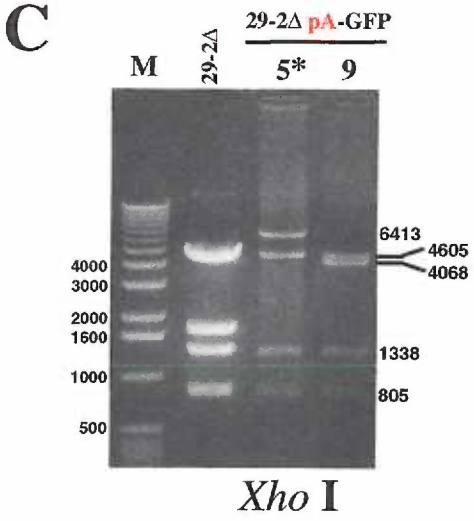
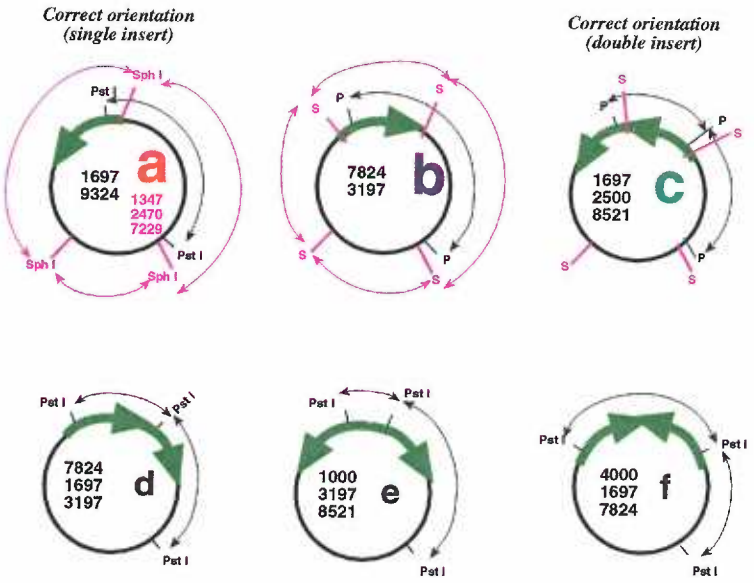


Fig. AII.5: Restriction maps of EGFP clones to screen for orientation of Cla I insert(s) — *Panel A*, Predicted plasmid maps (and fragment sizes) of possible recombinants "a" through "f" containing the Ef-1α/EGFP insert in 29-2Δ or 29-2Δ-pA. The heavy green arrow(s) represent single (a and b) or tandem (c-f) inserts of the Ef-1α/EGFP cassette.

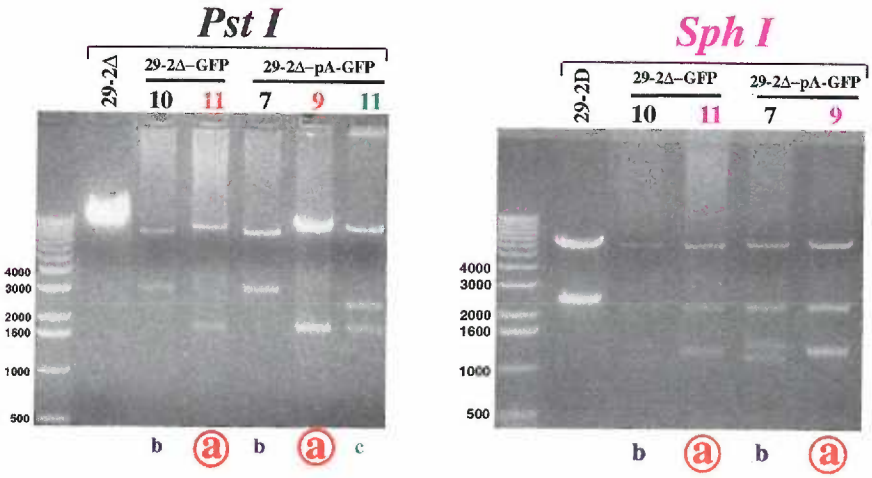
Panel B, Pst I or Sph I restriction maps of clones 29-2Δ-GFP (#s 10 and 11) and 29-2Δ-pA-GFP (#s 7, 9 and 11). For both Pst I and Sph I maps, the encircled letter "a" is the correct map for the recombinant plasmids.

Restriction mapping of recombinant clones to determine orientation of EGFP insert

A



B



Appendix III

The biology of cytokines: a brief overview

This section is a brief overview of the basic biology of cytokines, with some emphasis on the biological function of interleukin-6. We believe this information on the fundamental aspects of IL-6 bioactivity provides a foundation for formulation of hypotheses about the potential biological activities of RvIL-6.

The evolution of an effective immune response depends on communication between various cells of the immune system, and is mediated by a group of secreted as well as membrane-bound signaling proteins called cytokines (17). Because cytokines transmit regulatory signals between various cell types in a stimulatory and/or inhibitory fashion, the complexity of cytokine-mediated intercellular communication has been described as a "network" (484).

Cytokines exert their effects on target cells by binding to specific high-affinity cell surface receptor(s) (7). When bound by cytokines, cytokine receptors may initiate a signal that ultimately leads to one or more biological responses. The effects of a given cytokine may be *autocrine* (affecting the cytokine-secreting cell itself), *paracrine* (targeting a nearby cell), or *endocrine* (where the cytokine circulates systemically and exerts its effects on responsive cells some distance from the secreting cell or tissue) (129). The endocrine nature of some cytokines defines their bioavailability and extent of function, which is why cytokine detection in biological fluids of patients can have clinical significance in disease (8). For example, in healthy individuals, serum IL-6 is usually <10 pg/ml (8), but elevation in the serum concentration of IL-6 correlates with poor prognosis in a number of proliferative syndromes, including multiple myeloma (278, 403), renal cell carcinoma (45), and IgA nephropathy (121). In addition, some cytokines are *pleiotropic* (i.e., they are capable of stimulating multiple biological effects on more than one cell type). Functional pleiotropy is a common feature of most cytokines and growth factors that have a role in the immuno-hematopoietic system (312, 361) and may result either from cytokine receptor expression on more than one cell type, and/or from

molecular cross talk with other cytokines or growth factors (529). Cytokine function can also be *redundant*, whereby different cytokines mediate similar or overlapping functions (246, 312). In addition, a pair of cytokines can also be *synergistic*, such that the combined activity of the two cytokines on a given cell is greater than the additive effects of the individual cytokine alone (67, 206). In some cases, cytokines may display an *antagonistic* relationship as well, where the effects of one cytokine on a particular cell are inhibitory of those of another as exemplified by the Th1/Th2 cytokine paradigm (103). Taken together, functional pleiotropy, redundancy, synergism and antagonism all constitute the complex nature of interactions within the "cytokine network" (23).

INTERLEUKIN IL-6

Interleukin-6 (IL-6) is a pro-inflammatory cytokine that is secreted by a variety of cell types, including T lymphocytes, B lymphocytes, monocytes/macrophages, fibroblasts, bone marrow stromal cells, mesengial cells, hepatocytes, keratinocytes, astrocytes, vascular endothelial cells, and various tumor cells (8, 502). Bioactive IL-6 is secreted as a monomeric protein with molecular weight ranging from 21 to 29 kDa, depending on the degree of glycosylation and phosphorylation. IL-6 gene transcription is controlled by at least three factors: activator protein-1 (AP-1) complex, nuclear factor IL-6 (NF IL-6), and NFκB (5, 502). IL-6 expression is upregulated by bacterial products (e.g., endotoxin and lipopolysaccharide) (439, 496), by viruses such as HTLV-1 (506) and HIV (51, 60, 350).

The multifunctional nature of IL-6

The protein now known as IL-6 was originally referred to by a number of diverse names, each reflecting a different biological activity controlled by the same molecule, as shown in Table AIII.1 below.

Table AIII.1 — The pleiotropic functions of IL-6

Name	Associated function	Ref(s).
IFNβ	Anti-IFNβ-sensitive factor	(517)
26K	26 kDa IL-1-induced protein	(93, 185)
BSF-2	B cell stimulatory factor-2	(201)
PCT-GF	Plasmacytoma growth factor	(367, 368)
HEP	Hematopoietic factor	(213)
HP-GF	Hybridoma growth factor	(55, 503)
HSF	Hepatocyte (acute phase)-stimulating factor	(169)
CDF	Cytotoxic T cell differentiation factor	(475)

IL-6 is now known to play a central role in many host defense mechanisms such as immune response, hematopoiesis and acute-phase reactions (8, 355, 502); (see also Fig. AIII.1). Perhaps the biological function of IL-6 was best demonstrated by in vivo studies using IL-6-deficient mice that developed normally but displayed impaired immune and acute-phase responses (143, 252, 390, 398). Within the B cell compartment, IL-6 acts mainly at the late phase of the B cell differentiation pathway and induces the final maturation of B cells into plasma cells (502), consistent with IL-6 receptor expression on activated B cells but not on resting B cells (472). In addition, IL-6 acts as an essential accessory factor for proliferation as well as differentiation of effector cytotoxic T lymphocytes (CTL) (475), and has also been shown to promote inflammation by induction of chemokines (413).

Both IL-6 and the two IL-6 receptor subunits (IL-6R and gp130) are expressed in the central nervous system (CNS) in response to inflammatory and other environmental stimuli (418). IL-6 prevents neuronal cell death and promotes survival in neurons (293, 296), indicating that this cytokine may play a regulatory role in the neuronal compartment. IL-6 is also capable of inducing morphological differentiation in NGF-primed PC12 neuronal cells, suggesting that there may be molecular cross talk between NGF and IL-6 signaling in PC12 cells (527). Thus, along with other cytokines (67), IL-6 represents an important frontline component of the body's armory against infection and tissue damage (8, 360). Fig. AIII.1 below depicts some of the known functions of IL-6.

Fig. AIII.1: Pleiotropic functions of interleukin-6

1. Induces B cell differentiation (243).

2. Stimulates production of acute-phase proteins in liver cells (170).

3. Promotes growth of myeloma/plasmacytoma/hybridoma cells (1, 368)

4. Induces IL-2 and IL-2R expression and promotes T lymphocyte proliferation or differentiation into effector cytotoxic T cells (475)

5. Induces expansion of hematopoietic progenitors (470)

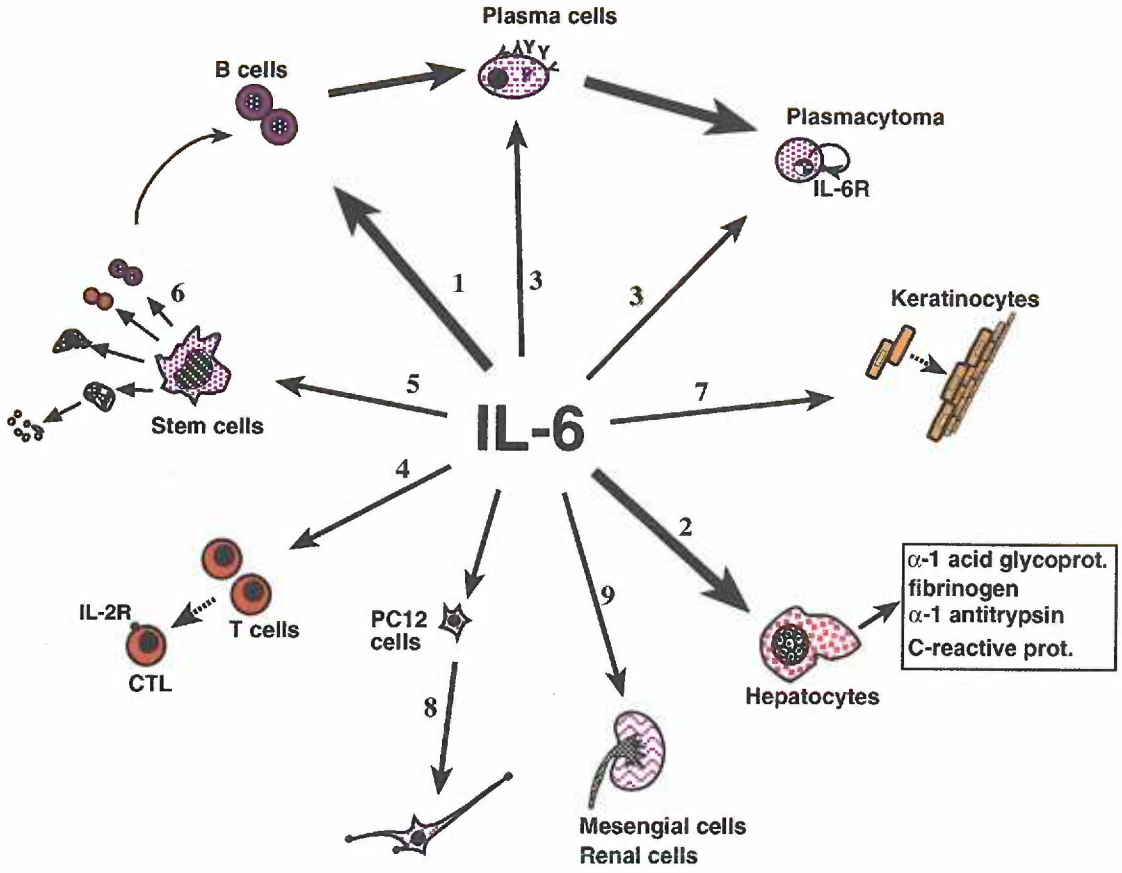
6. Stimulates proliferation and differentiation of multi-lineage megakaryocytic progenitors (213)

7. Stimulates keratinocyte differentiation (182)

8. Induces neurite outgrowth and neuronal differentiation of the pheochromocytoma PC12 cell line (432).

9. Induces mesengial and renal cell growth (205)

The pleiotropic function of Interleukin-6 (IL-6)



In accordance with functional pleiotropy, abnormal expression of IL-6 has been implicated in the pathogenesis of numerous myeloproliferative syndromes (6, 8) including MM (234), RA (198); MCD (534), AIDS (350), mesengial proliferative glomerulonephritis (205), psoriasis (315), KS (315), (315, 489), sepsis (509), and osteoporosis (221). Given the association of abnormal production of IL-6 and development of clinical disorders, intense research has been focussed on understanding the biochemical mechanisms controlled by IL-6 as well as development of potential therapeutic agents in the treatment of IL-6-associated diseases.

Mechanism of IL-6 function

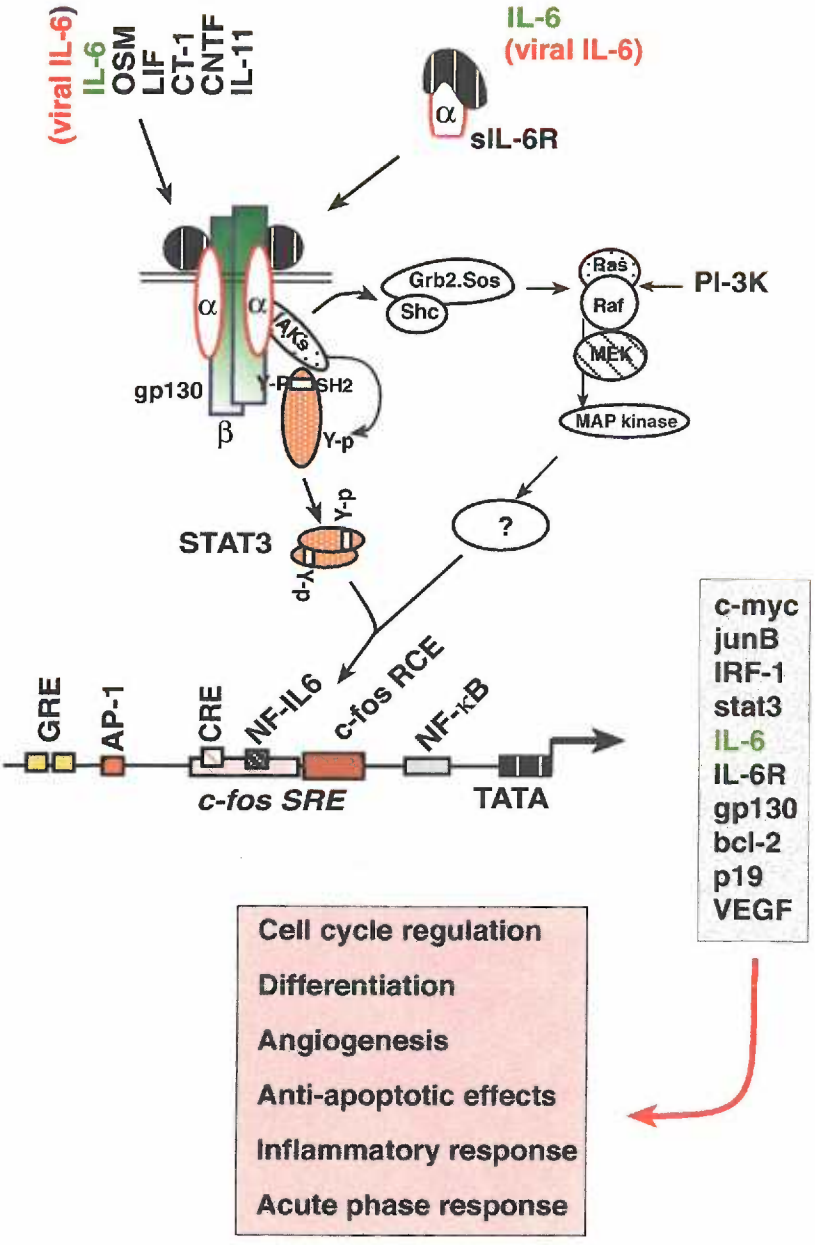
IL-6 signals through a receptor system consisting of an alpha subunit, IL-6R (or gp80), and a beta subunit, also known as gp130 (474). IL-6R is a low-affinity ligand-binding subunit expressed on the surface of a variety of cell types including macrophages, myelomonocytic cell lines, hepatocytes, resting T cells, plasma cells and activated or Epstein-Barr virus (EBV)-infected cells. IL-6R has no intrinsic signaling activity. In contrast, gp130 is expressed ubiquitously but does not bind IL-6 directly. However, gp130 has a cytoplasmic domain containing a series of tyrosine residues whose phosphorylation upon gp130 activation is critical in transduction of signals initiated by the IL-6R/IL-6 complex.

Mechanistically, IL-6 must first bind IL-6R, and the IL-6/IL-6R complex then recruits and induces homodimerization of gp130, forming a hexameric unit consisting of two molecules each of IL-6, IL-6R, and gp130 (377, 514). Formation of this hexameric

unit induces auto-activation of gp130-associated Janus activated tyrosine kinases (JAK) that subsequently phosphorylate proximal tyrosine residues on gp130 (340, 473). Phospho-tyrosine residues then serve as docking sites for SH2 domains in cytosolic signal transducers and activators of transcription (STATs) (104) that are then phosphorylated by JAK. Phosphorylated STAT molecules subsequently form homo- or heterodimers with each other via interactions of phosphorylated residues on one STAT molecule with the SH2 domain of another. Pairs of STAT molecules then translocate to the nucleus where they bind and activate specific transcriptional control elements in the promoter regions of cytokine-dependent genes (104, 212). This hierarchy of events typifies the so-called "JAK/STAT"-mediated induction of cytokine-dependent responses (436) (Fig. AIII.2).

Fig. AIII.2 — Mechanisms for signal transduction by IL-6 and related cytokines. Members of the IL-6 cytokine family form initial interactions with a receptor alpha subunit, and subsequently activate gp130 (the shared signal-transducing beta-subunit) either by homo- or hetero-dimerization, depending on the cytokine (see text for details). After phosphorylation of tyrosine residues in the cytoplasmic domain of gp130 by receptor-associated tyrosine kinases (e.g., Jak), phosphotyrosine residues serve as docking sites for cytosolic transcription factors which, upon phosphorylation, translocate to the nucleus. In the nucleus, STAT molecules bind and activate transcriptional control elements in the promoters of various cytokine-dependent genes.

Mechanisms of signaling in the IL-6 cytokine family

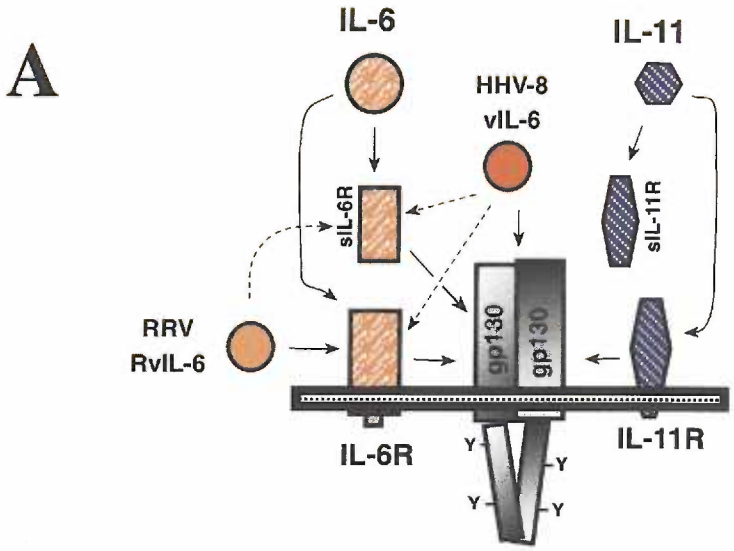


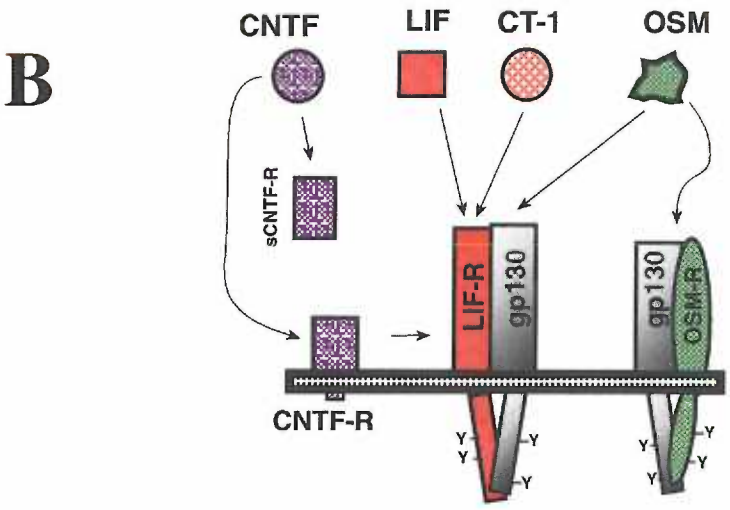
Gp130 also functions as the signaling component in several additional receptor complexes for other members of the IL-6 cytokine family that include interleukin-11 (IL-11), ciliary neurotrophic factor (CNTF), cardiotropin-1 (CT-1), oncostatin M (OSM), and leukemia inhibitory factor (LIF) (449). Each of these cytokines may act through its own specific ligand-binding receptor alpha subunit but the mechanism for activation of gp130 can be unique to each cytokine, as depicted in Fig. AIII.3.

IL-6 and IL-11 bind to IL-6R and IL-11R subunits, respectively, and the IL-6/ IL-6R or IL-11/ IL-11R complexes then associate with a homodimer of gp130. On the other hand, the complex between CNTF and CNTF-R associates with a heterodimer of gp130 and LIF-R that is structurally similar to gp130 (105, 172, 214). In spite of high homology between LIF-R and gp130, however, LIF does not bind gp130 directly but instead interacts with LIF-R before formation of a heterodimeric complex with gp130 (173). CT-1 is also thought to trigger gp130/LIF-R formation by binding to LIF-R first, and then inducing formation of gp130/LIF-R heterodimers. This notion is supported by the observation that binding of CT-1 to recombinant LIF-R protein is enhanced by addition of gp130 (382). However, LIF-R might not be the exclusive dimerizing partner of gp130 in the functional receptor for CT-1 because the phenotype in cardiac development in gp130-deficient mice was not observed in LIF-R deficient animals (533). Oncostatin M (OSM) can either interact with OSM-R to form a heterodimeric complex with gp130, or with gp130 directly followed by formation of gp130/LIF-R heterocomplexes (272-274, 341).

Fig. AIII.3 — Components of receptor complexes for the IL-6 family of cytokines. All signals initiated by members of the IL-6-cytokine family are transduced via the shared gp130 subunit. However, the mechanism of gp130 activation may be distinct. As shown in (panel A), one mechanism involves gp130 homodimerization, in the case of IL-6, IL-11 and potentially, the two viral IL-6-like proteins HHV-8 vIL-6 and RRV RvIL-6 (65, 226, 359); RvIL-6 can also function in the presence of sIL-6R (Fig. 5.2, this work). A second mechanism involves heterodimerization of gp130 with another ligand-binding subunit, in the case of CNTF, LIF, CT-1, and OSM (panel B). Notice that the receptors for IL-6, IL-11 and CNTF also exist in soluble form and can interact with ligand in an agonistic fashion (297, 415). Solid arrows denote known mechanisms of ligand-to-receptor associations, while dotted arrows represent interactions that are either still hypothetical or under investigation.

Cytokine/receptor pairs in the IL-6 cytokine family





Although a shared gp130-mediated signaling mechanism might explain the functional redundancy among the IL-6 cytokine family (246), quantitative and qualitative differences in cellular responses to these cytokines have also been noted (256). Specificity of cytokine-dependent responses may therefore be determined either by the regulated expression of cytokine ligand-specific receptors or by events that occur at a step downstream of ligand binding (87, 256). The latter is especially reasonable considering that several JAK and STAT molecules are activated by a number of diverse signals to mediate a variety of specific as well as overlapping cytokine-induced responses (436). Based on differences in receptor binding as well as the downstream events following ligand binding, cytokine responses in the IL-6 cytokine family may be mechanistically determined by: (i) affinity of interactions between ligand and receptor (245, 247), (ii) the proximity or disposition of JAK substrates (usually tyrosine residues in the cytoplasmic domain of gp130) (463), (iii) the class of JAK (e.g., Jak1, 2) that is associated with gp130 following ligand binding, and (iv) the type of STAT that is recruited to activated gp130 (515). A combination of these parameters can have diverse influences on the overall response to a particular cytokine. Moreover, presence of a series of cytokine-responsive elements in the promoter sequences of many growth control genes may provide one additional molecular explanation for functional pleiotropy ascribed to these cytokines (see Fig AIII.2).

SELECTED ABBREVIATIONS

Ab — Antibody

AIDS — Acquired immunodeficiency syndromes

APC — Antigen presenting cell

BAF — B cell activating factor

BCB-L — Body cavity-based lymphoma

Bcl-2 — B cell lymphoma proto-oncogene 2

BHV — Bovine herpesvirus

BL — Burkitt's lymphoma

BSA — Bovine serum albumin

BSF-1 — B cell stimulatory factor –1

CD — Cluster of differentiation

cDNA — Complementary DNA

CHX — Cyclohexamide

CNS — central nervous system.

CNTF(R) — ciliary neuro-tropic factor (receptor)

CPM — counts per minute

CT-1 — cardiotrophin-1

DNA — de-oxyribonucleic acid

dNTP — 2'-deoxynucleoside 5'-triphosphate

E — Early

EBV — Epstein-Barr virus

EGFP — Enhanced green fluorescent protein

EGFP — Enhanced green fluorescent protein

EHV — Equine herpesvirus

ELISA — Enzyme-linked immunosorbent assay

ERK — extracellular signal-regulated kinase

FBS — Fetal bovine serum

FCS — Fetal calf serum

gp — glycoprotein

GST — glutathione-S-transferase

HAART — Highly active anti-retroviral therapy

half-maximal stimulation due to a specific growth stimulus

HCMV — Human cytomegalovirus

HD — Hodgkin's disease

HHV8 — human herpesvirus 8

HIV — Human immunodeficiency virus

HPV — Human papilloma virus

HSC — Hematopoietic stem cells

HSV — Herpes simplex virus

HVEM — Herpesvirus entry mediator

HVS — Herpesvirus saimiri

IC_{50} — "Inhibitory Concentration" to neutralize half of the maximal response

IE — Immediate early

Ig — Immunoglobulin

IL-11 — interleukin-11

IL-6(R) — interleukin-6 (receptor)

IM — Infectious mononucleosis

IFNγ — Interferon-gamma

IPTG — Isopropyl-β-D-thiogalactopyranoside

JAK — Janus receptor-associated tyrosine kinase

kDa — kilo-Dalton

KS — Kaposi's sarcoma

KSHV — Kaposi's sarcoma-associated herpesvirus

L — Late

LCMV — lymphocytic choriomeningitis virus

LIF (R) — Leukocyte inhibitory factor (receptor)

LMP — Latency-associated membrane protein

LPD — Lymphoproliferative disease

LPS — lipopolysaccharide

LTR — left terminal repeat

MacIL-6 — rhesus macaque IL-6

MCD — Multicentric Castleman's disease

MCMV — Murine cytomegalovirus

MGUS — monoclonal gammopathy of undetermined significance

MHC — Major histocompatibility complex

MHV-68 — Murine herpesvirus 68

MM — Multiple myeloma

MTT — 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NGF — nerve growth factor

NP-40 — Nonidet p40

NPC — nasopharyngeal carcinoma

ORF — Open reading frame

OSM(R) — Oncostatin M (receptor)

PAA — Phosphonoacetic acid

PARP — poly-(ADP-ribose) polymerase

PBMC — peripheral blood mononuclear cells

PC12 — pheochromocytoma cell line

PEL — Peripheral effusion lymphoma

PRV — Pseudorabies virus

RAG — Recombination activating genes

RFMm — Retroperitoneal fibromatosis herpesvirus *Macaca mulatta*

RFMn — Retroperitoneal fibromatosis herpesvirus *Macaca nemestrina*

RNA — ribonucleic acid

RRV — Rhesus macaque rhadinovirus

RTR — right terminal repeat

RvIL-6 — RRV-encoded homologue of IL-6 (ORF R2)

SDS/PAGE — Sodium dodecyl sulfate/poly-acrylamide gen electrophoresis

SH-2 — src-homology-2 domain

SIV — Simian immunodeficiency virus

SRV — simian retrovirus

STAT — Signal transducers and activators of transcription

STP — Saimiri transforming protein

TNF — tumor necrosis factor

TPA — 12-*O*-tetradecanoylphorbol-13-acetate

VEGF — vascular endothelial growth factor

vIL-6 — viral IL-6 encoded by ORF K2 of HHV-8

VZV — Varicella zoster virus

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