1995

The Role of Follicular Dendritic Cells in Human Immunodeficiency Virus Pathogenesis

Sonya Lynn Heath

Virginia Commonwealth University
School of Medicine

This is to certify that the thesis prepared by Sonya Heath entitled "The Role of Follicular Dendritic Cells in Human Immunodeficiency Virus Pathogenesis" has been approved by her committee as satisfactory completion of the thesis requirement for the degree of Master of Science.

[redacted]

Gregory F. Burton, Ph.D., Director of Thesis

[redacted]

Eric H. Westin, M.D., School of Medicine

[redacted]

John G. Tew, Ph.D., School of Medicine

[redacted]

John G. Tew, Ph.D., Department Chairman(Acting)

[redacted]

Hermes A. Kontos, M.D., Ph.D., Dean School of Medicine

[redacted]

Jack L. Haar, Ph.D., Dean, School of Graduate Studies

12/8/95

Date

The Role of Follicular Dendritic Cells in Human Immunodeficiency Virus Pathogenesis

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

By

Sonya Lynn Heath
Bachelor of Science
Virginia Polytechnic Institute & State University
May 1993

Director: Gregory F. Burton Ph.D.
Assistant Professor
Microbiology/Immunology

Virginia Commonwealth University
Richmond, VA
December 1995

Acknowledgments

I extend my sincerest appreciation to Dr. Greg Burton for his patience, guidance, and insightful discussions throughout my graduate training. He has been an excellent mentor and friend. He opened my eyes to the beauty in science and opened a world in research that for me never before existed. I will always have the greatest respect for him.

I would also like to thank my committee members Drs. Eric Westin and John Tew whose generous time and wisdom allowed me to grow.

Finally I would like to thank my strongest supporters, my parents, Pat and Ken Heath, and my sister Jennifer Blackwell for their never ending support, encouragement, and faith in me. This is dedicated to my mother for teaching me to dream, believing that I would always make it, and encouraging me to never give up on myself.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Human Immunodeficiency Virus	HIV
Acquired Immunodeficiency Virus	AIDS
Follicular Dendritic Cells	FDC
Electron Microscopy	EM
Polymerase Chain Reaction	PCR
Long Terminal Repeat	LTR
Simian Immunodeficiency Virus	SIV
Interferon	INF
Reverse Transcriptase	RT
Nuclear Factor Kappa B	NFκB
Inhibitor Kappa B	IκB
Tumor Necrosis Factor-alpha	TNF-α
Interleukin 2- Receptor	IL-2R
Major Histocompatibility Complex	MHC
Syncytium Inducing	SI
Non-Syncytium Inducing	NSI
Granulocyte Macrophage Colony Stimulating Factor	GMSCF
Macrophage Colony Stimulating Factor	MCSF
T Cell Receptor	TCR
Antigen Transport Cell	ATC

Phyto-Hemagglutinin . . . . . PHA

Fluorescence Activated Cell Sorting . . . . . FACS

Magnetic Activated Cell Sorting . . . . . MACS

monoclonal Antibody . . . . . mAb

Subcutaneous . . . . . SC

Intraperitoneal . . . . . IP

Staphylococcal Enterotoxin E . . . . . SEE

Fetal Calf Serum . . . . . FCS

Cell Mediated Immunity . . . . . CMI

Abstract

THE ROLE OF FOLLICULAR DENDRITIC CELLS IN HUMAN IMMUNODEFICIENCY VIRUS PATHOGENESIS

Sonya Lynn Heath, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 1995.

Major Director: Dr. Gregory F. Burton Ph.D., Assistant Professor, Department of Microbiology and Immunology

Infection with Human Immunodeficiency Virus-1(HIV) results in a disease process characterized by three stages: an acute phase characterized by viremia, a clinically latent stage with little or no detection of virus in the blood, and the last stage, AIDS, which is characterized by marked immunodeficiency. During clinical latency, CD4+ T cells decline over a period lasting from a few to several years. Throughout this period, HIV is found trapped on the surface of follicular dendritic cells(FDC) in the germinal centers of secondary lymphoid tissues and this is the primary site of active viral replication. We hypothesize that FDC, and the unique microenvironment they help provide, play a critical role in HIV pathogenesis. The objective of these studies was to begin to characterize the role of FDC in HIV pathogenesis

by determining if HIV immune complexes trapped on FDC are infectious.

To begin to test this, HIV(IIIB) immune complexes were formed by incubating virus with serum from HIV infected individuals as a source of virus-specific antibody. Highly enriched tonsilar FDC (from non-infected individuals)were then incubated with these HIV-complexes to allow FDC trapping *in vitro*. HIV binding to FDC was confirmed by electron microscopy(EM). Unbound HIV was removed by washing the cells and FDC bearing HIV immune complexes or control FDC were cultured for four days with superantigen activated, FACS sorted, autologous CD4+ tonsilar T lymphocytes to determine if the T cells could be infected by the FDC trapped virus. HIV infection was detected using PCR amplification of proviral *gag* sequences that would be present in DNA isolated from the cultures. To ensure that our *in vitro* cultures were representative of *in vivo* events, we used a xenogeneic model where HIV immune complexes were formed and trapped on murine FDC *in vivo*. Immune complexes were formed by injection of virus-specific antibody followed by HIV (IIIB). Murine FDC bearing HIV trapped in vivo were isolated and incubated with activated human CD4+ T cells as the only source of virus for infection. Infection was assessed as before. HIV infection of

T cells was detected in cultures containing FDC bearing HIV immune complexes trapped *in vitro* whereas no infection was detected in controls. Furthermore, murine FDC bearing *in vivo* trapped HIV immune complexes also infected human CD4+ T cells.

In some *in vivo* experiments, HIV immune complexes were formed using a neutralizing antibody that could block infection. FDC bearing these neutralized HIV immune complexes also transmitted infection to T cells. This prompted the hypothesis that FDC may be able to negate the effects of neutralizing antibody. To test this, we formed HIV immune complexes with several doses (picogram to milligram) of neutralizing antibody and cultured these with T cells ± FDC. No infection was present in cultures of immune complexes and T cells without FDC, however, infection was clearly seen when FDC were added. Furthermore, neither macrophages, dendritic cells, nor FDC depleted populations of tonsilar cells could replace FDC in negating the effect of neutralizing antibody and this effect could be observed with different neutralizing antibodies and several strains of virus including a primary isolate.

These data indicate that FDC associated HIV is infectious and that FDC can negate the effects of high levels of neutralizing antibody thus permitting infection to occur. This finding may help explain why HIV infected individuals

with neutralizing antibody still have ongoing infection. In addition, this data may cause us to reshape our thinking about vaccination and treatment strategies. Finally, this work supports our hypothesis that FDC play an important role in HIV pathogenesis.

Background & Significance

Human Immunodeficiency Virus

Human immunodeficiency virus (HIV), is the etiologic agent of acquired immunodeficiency syndrome(AIDS)[1]. It is a member of the lentivirus subfamily of retroviruses. Retroviruses are characterized by their ability to convert their RNA genome into DNA and then integrate this DNA (referred to as the provirus) into the host cells' genome. Viruses within the lentivirus subfamily are associated with slow progressing, inflammatory and degenerative disorders. HIV is a blood borne and/or mucosal pathogen, that is transmitted by sexual contact, parenteral exposure to blood (mostly by intravenous drug users), and from mother to child during pregnancy and at parturition. There currently is no effective treatment or cure for AIDS[1].

Virus Life Cycle

The life cycle of HIV closely follows that of all other members of the lentivirus family. It begins by virus binding to host cell receptors. In the case of HIV this binding is mediated by gp120 on the surface of the viral envelope. Gp120

attaches to the CD4 molecule of the target cell, the T helper lymphocyte[2-4]. Following receptor binding, the virus penetrates the cell membrane and uncoating of the virus envelope and nucleocapsid occurs in the cytoplasm. After uncoating inside an activated host cell, virion associated reverse transcriptase reverse transcribes viral RNA into double stranded DNA and virally encoded integrase allows the integration of the provirus into the host's genome. Once integration has occurred, HIV can remain latent in the proviral integrated stage or alternatively be actively replicated[1]. In the latter instance, cellular transcription events cause expression of the viral regulatory proteins. HIV ends its life cycle by budding out of the host cell's plasma membrane where it acquires a lipid envelope.

Latency

Both clinical and virological latency play important roles in the persistence of HIV infection. Clinical latency is defined as the period of disease which is primarily asymptomatic (discussed under the heading "stages of disease")[1]. In contrast, virological latency refers to the state of integrated provirus within a cell that is not actively expressing viral proteins or replicating virus[1].

Evidence that a state of virological latency exists and that the majority of integrated proviruses are silent comes from two observations. Schmittman *et.al.* found that in the peripheral blood there is a 10 fold higher frequency of CD4+ cells with detectable HIV DNA compared to cells actually expressing HIV RNA or viral proteins[5]. Similar findings were also reported by Embretson *et.al.*[6]. She found a higher frequency of cells with HIV DNA than HIV RNA in lymphoid tissue. This state of viral latency provides a reservoir of latently infected cells which upon activation produce HIV. Once HIV has integrated into the host cell's genome, its expression depends mostly on host activation signals, host cell machinery, sequences within the HIV genome, and various viral proteins. To fully understand the mechanisms involved in viral replication, an understanding of the viral genome is needed.

Viral Genome

The genome of HIV contains the three prototypic genes of lentiviruses, *gag, pol,* and *env* as well as additional genes encoding regulatory and accessory proteins (i.e., *tat, rev, nef, vpr, vpu and vif*). These genes are flanked on either side by long terminal repeats (LTR). HIV genes can be divided into:

1) early genes - *nef, tat, and rev*; and late genes - *gag, pol, vif, vpr, and vpu.*

Early genes:

NEF, first named negative factor because it was thought to down regulate replication[7-9], has recently been linked with increasing viral replication. *nef* mutants of Simian Immunodeficiency Virus (SIV) demonstrated that the gene product was essential for high viral replication and the development of Simian AIDS[10]. In addition, NEF down regulates CD4 expression on helper T cells[11,12]. This feature has been shown with SIV [13] and primary isolates of HIV[11]. The mechanism of downregulation of CD4 occurs at the post transcriptional level[14]. NEF functions by causing endocytosis and degradation of CD4. In addition, NEF causes displacement of $p56^{lyk}$(a tyrosine kinase) from CD4. When $p56^{lyk}$ is phosphorylated, it triggers T cell activation resulting in IL-2 production and cell proliferation. Down regulation of CD4 is important in that it prevents multiple infection of the same cell leading to a state of super-infection. Were this to occur, the resulting multiple copies of unintegrated DNA could be toxic. In this manner, NEF is thought to increase the life of virally infected cells.

TAT, the transactivator, is encoded by two exons that are separated from each other by much of the *env* gene. Since the gene is in a different reading frame than *env*, its expression requires both splicing of the *tat* transcript and a frameshift by the ribosome to be translated in the proper reading frame. Although the exact mechanism of its action is unknown, TAT activates transcriptional initiation of the viral genes. In the presence of TAT there is an increase in the number of full length viral mRNA transcripts. Without TAT, the HIV promoter produces many short viral transcripts that correspond to the Tar RNA stemloop structure that will be described later (see LTR)[15]. Taken together, this suggests that TAT may play a role as an anti-terminator of transcription[16,17].

REV, the regulator of expression of the virion, controls the temporal expression of the early and late genes. It increases expression of virion genes while decreasing expression of regulatory genes[18,19]. REV takes advantage of the fact that early gene products are translated from multiply spliced mRNA's while late gene products are the result of unspliced or singly spliced mRNA's. In the absence of REV, mRNA's remain in the nucleus. They are either degraded there or are multiply spliced before being transported to the cytoplasm to generate the early gene products[18]. On the

other hand, when enough REV has been generated, it will bind to the RRE, rev response element in the *env* sequence of the mRNA resulting in transport of the mRNA to the cytoplasm. This prevents further splicing and allows translation of the late genes[19]. REV provides additional mechanisms for maintaining latency. Latently infected cells, in some cases, may not have accumulated enough REV to begin production of infected virions[20]. There are also cellular proteins which can bind the RRE and block binding of REV. One of these is induced by interferon (INF) which is known to prevent HIV replication[21]. These mechanisms suggest that REV plays an important role in regulating HIV latency and activation.

Late genes:

Once sufficient REV has accumulated, the late genes of HIV are expressed and the virus can begin to make new virions. This requires the use of the three main late genes: *gag, pol,* and *env*.

gag, the group specific antigen, encodes the core protein or capsid which encloses the viral RNA. GAG, is translated as a polyprotein Pr55 which is cleaved into the matrix, capsid, and nucleocapsid proteins[22]. The matrix protein, or p17, is a component of the inner surface of the virion lipid bilayer

and the core structure. As such, it plays a role in virus assembly and virus entry [23]. The capsid protein, p24, is the major component of the cone shaped core structure which encloses the two strands of viral RNA. Finally the nucleocapsid proteins, p9 and p6, associate with the viral RNA and this is believed to be a recognition signal for packaging of the RNA into a new virion[24]. In addition to GAG, sometimes a GAG/POL fusion protein is produced during translation. This occurs when the ribosome "slips back" one nucleotide, changing to the *pol* reading frame. Since it takes many GAG precursors (approximately 1500) to make the core, which encloses the two strands of viral RNA, and only requires a few polymerase molecules for assembly, ribosomal frameshifting to produce POL only occurs at a frequency of 1 in 20 times.

pol encodes the virus specific enzymes: protease, reverse transcriptase (RT), and integrase. The function of virally encoded protease is to cleave the GAG and POL precursors into their various subunits. Protease is released by autoprocessing events and then is able to cleave the other subunits [25-28]. RT has both RNA and DNA dependent DNA polymerase activities. These activities allow the virus to reverse transcribe RNA into DNA and to copy DNA [29,30]. In addition, RT has RNase

H activity which degrades the RNA strand of the RNA-DNA hybrid[31,32]. This is necessary to produce the double stranded DNA provirus. Finally, the function of HIV integrase is to insert or integrate the proviral DNA into the host cells' genome[33,34]. Integrase binds to cellular DNA[35], knicks it using its endonuclease activity[35], integrates the provirus, and recombines the strands[29,36-38].

env encodes the viral envelope proteins comprising gp160. The product of *env* is brought to the surface of the virus particle where a cellular enzyme cleaves the major product, gp160, into its components gp120, the surface marker responsible for binding CD4 on host cells, and gp41, the transmembrane region which plays a major role in virus-host cell membrane fusion events.

The genes *gag, pol, env, tat,* and *rev*, are essential and the virus is unable to replicate unless these gene products are functional. In contrast, the gene products *nef, vif, vpr,* and *vpu* are not essential for replication but are crucial for maintaining viral spreading.

vif, the virion infectivity factor, encodes a protein which increases infectivity of the virus. *vif* mutants produce virus which infects cells a thousand times less efficiently than virus with wild type *vif*. Furthermore, *vif* mutants are

unable to completely synthesize proviral DNA[30]. It is believed that VIF plays a role in uncoating the virus and disrupting the core thus allowing cofactors of RT to enter.

The exact function of **VPR**, viral protein r, is not known. It has been implicated as: a weak transactivator of the HIV LTR; a transporter of the nucleocapsid to the nucleus; and an inducer of differentiation and growth arrest[39]. Recent data suggests that VIF induces growth arrest of infected cells at the G2 phase of the cell cycle which may play a role in modulating viral persistence[39]. G2 arrest is advantageous to the virus for several reasons. G2 arrest allows proviral integration (which occurs in the S phase of the cell cycle) and expression of the virus; it increases viral products; it prevents reentry of cells into the G0 phase of the cell cycle; and it may prevent apoptosis(oral presentation, Keystone Conference).

The function of the viral protein, **VPU** still remains largely unknown. VPU is associated with disrupting gp120 from CD4[40,41], and releasing HIV particles[42] but it is not incorporated into new virions. The genome of HIV is more complex than that of most lentiviruses and each protein plays a subtle yet important role in promoting and maintaining viral infection.

Long terminal repeat:

The LTR can be divided into three regions based on their regulatory function: 1)the core, 2) the modulatory region, and 3) the TAR elements. Within the LTR, sequences exists which initiate or suppress transcription. These sequences are the target of multiple cellular factors which are further influenced by cellular activation and differentiation of macrophages and lymphocytes, and by the presence of cytokines. In this way, HIV has linked its expression with its host cells regulation.

The core contains binding sites including SP1 and the TATA binding factor. SP1 acts as a strong transcriptional activator. The TATA box facilitate initiation of transcription when bound by cellular RNA polymerase II. The TATA box within the LTR may also bind LBP-1 (leader binding protein)[43]. This interaction actually blocks the binding of polymerase to the TATA box thereby preventing transcription initiation[44]. It is unknown whether other factors can override the effects of LBP-1 but nevertheless it contributes to virological latency.

The modulatory region dictates the rate at which transcription occurs. It also contains sequences which are targets for cellular factors. The binding sites of the

modulatory region include activator protein-1 (AP-1), chicken ovalbumin upstream promoter (COUP), nuclear factor of activated T cells(NF-AT), upstream stimulatory factor (USF),T cell factor-1 α, and nuclear factor kappa B (NF-κB). NF-AT and NF-κB are of particular importance because they are induced by activation of T helper cells. Resting T cells have an inhibitor kappa B protein (IκB) which is bound to cytoplasmic NF-κB. Upon activation of the T cell, by antigen or cytokines such as TNF-α, NF-κB dissociates from the inhibitor and NF-κB is transported to the nucleus. Subsequent binding of NF-kB to its binding site results in expression of cellular genes including IL-2R. NF-κB also binds to the region within the LTR of integrated proviral DNA and enhances transcription of the virus. The virus is able to rest in the cell until it becomes activated and begins replication. The variety of stimuli which induce Nf-κB also provide the signal required for reactivation of latent infection.

Alternatively, binding to the USF results in decreased HIV expression. This sequence resides in the negative regulatory binding factor binding site[45]. Deletions of this binding site result in increased expression of HIV further supporting its role as a down regulator of transcription[46].

Binding sites for AP-1, COUP, and T-cell factor-1 α are even further upstream. In a similar manner, however, deletion of this region results in increased expression suggesting once again that these areas of the LTR may be part of the negative regulatory sequence[47]. All of these sites probably contribute to maintaining proviral latency.

The last region of the HIV LTR is the TAR. Regulation in this region is dictated, not by cellular proteins, but by the viral protein TAT. Newly synthesized TAR RNA forms a stable stem loop structure which is then able to bind TAT. Expression of TAT and its binding to TAR results in an increase in viral expression[48]. Although the binding of TAT to TAR is essential, cellular proteins also aid in securing the TAR stem loop by binding to it. The absence of these proteins can decrease TAT induced transcription.

Along with transcriptional regulation, HIV has also developed a mechanism of post transcriptional regulation. This is imperative since the virus (which is less than 10 kb in length) expresses at least nine different genes resulting from overlapping reading frames and alternative splicing. Furthermore, the relative amounts of these gene products needed to produce a new virion are different for each product.

Structure:

The structure of HIV includes an inner nuclear core which contains two copies of positive polarity, single stranded, sense viral RNA which is enclosed by a capsid composed of *gag* encoded proteins. The inner nuclear core is encompassed by an envelope consisting of a lipid bilayer in which the *env* gene product gp41 is embedded. The other major *env* encoded glycoprotein, gp120, is noncovalently associated with gp41 and together they make up the major envelope glycoprotein, gp160. The significance of gp120 is multifold. It determines which cells become infected, plays a role in pathogenesis by inducing syncytium formation, and is the target of therapies attempting to intercept infection.

Cell Tropism

Gp120 is the receptor which mediates infection. It binds the CD4 molecule on T helper lymphocytes, macrophages, and other cells (e.g. glial cells). The infection of monocytes/macrophages appears to serve as a reservoir of virus since these cells are usually not killed rather they persist[49]. Macrophages may become infected as a result of CD4 binding or by internalization of viral immune complexes which bind f_c receptors. Once inside, the macrophage acting as

an antigen presenting cell may process and present the virus in the context of MHC class II to CD4+ T helper cells. This cell to cell interaction between macrophages and T cells may facilitate transmission of the virus[50]. Anti-CD4 antibodies prevent infection of both macrophages and T cells.

Although the macrophage plays a role in HIV pathogenesis, the primary host cell infected by HIV is the CD4+ helper T lymphocyte. In addition, it is the destruction of the T lymphocyte, not the Mϕ, which dictates the course of disease caused. T helper lymphocytes play a crucial role in both cellular and humoral immune responses. They are able to interact with antigen presented in the context of MHC class II on B cells and other antigen presenting cells. Upon activation, they secrete cytokines which orchestrate the rest of the immune response. Therefore, their destruction throughout HIV infection has an impact on the entire immune system resulting in severe immunosuppression, AIDS, and death. The mechanisms of pathogenesis of AIDS will be discussed later in this section.

Syncytium formation

In addition to mediating infection, the envelope protein also has the ability to induce syncytium formation. The gp120

on the surface of infected cells can bind and fuse several uninfected cells leading to the formation of a giant multinucleated cell called a syncytia[51,52]. Some syncytium contain up to 500 nuclei. The cell usually dies within 48 hours after formation. This may account for the large decrease in T cells even though very few are actually infected. Although syncytium formation is rarely seen *in vitro*, HIV isolated during accelerated phases of infection in vivo have a greater capacity to induce syncytium formation *in vitro*. These syncytium inducing isolates (SI) are primarily T cell tropic whereas non-syncytium inducing (NSI) isolates, obtained earlier in the course of infection are believed to be monocyte/macrophage tropic[53,54].

Glycoprotein 120

The interaction of gp 120 with CD4 has been extensively studied. Both have been mapped to more clearly understand the interaction and more importantly how to interfere with it to prevent infection or pathogenic mechanism such as syncytium formation. Gp120 has five hypervariable domains, V1-V5, and five conserved domains, C1-C5. These regions are brought together by extensive disulfide bonds. The c-terminal region of gp120 contains the majority of residues which are important

for CD4 binding, including C2, V3, C3, V4, and C4[55-57]. Neutralizing antibodies are generated in response to HIV infection. The epitope which result in the most effective neutralization and inhibition of cell fusion is located within the third variable domain-V3[58].

Stages of disease:

The course of HIV infection consists of three stages experienced by most patients[59]. The first or acute stage occurs following primary infection. The immune system mounts a detectable cellular and humoral immune response which destroys the virus in the blood. During this period, patients experience a slight decrease in T cell numbers but they usually remain above 700 cells/mm^3. This is followed by the second stage of disease, clinical latency, the average length of which is ten years[60]. During this period, there is a gradual insidious decline in T cell number and function. T cell counts range from 700-200 cells/mm^3. As the cell number decreases, the patient becomes more susceptible to infection and begins to experience clinical symptoms. Finally, the third stage develops, AIDS, and death usually occurs within a period of two years after this stage begins. At this stage,

cell counts fall below 200 cells/mm^3. The loss of CD4+ T cells causes widespread immunosuppression. This leads to multiple opportunistic infections which the patient cannot clear.

The first stage of disease, termed acute HIV syndrome, is accompanied by characteristic flu like symptoms including fever, lethargy, malaise, sore throat, myalgias, and headache. This usually occurs within three to six weeks after infection with HIV although everyone does not experience it to the same degree[61-63]. There are high levels of viremia which elicit both a cellular and a humoral immune response within one week to three months[62,63]. An increase in virus specific cytotoxic T lymphocytes and complement binding antibody aid in clearing the virus from the blood. The decrease in viremia is associated with a loss of clinical symptoms although many patients continue to experience persistent generalized lymphadenopathy.

The second stage is termed "clinical latency" and lasts an average of ten years, shorter for the young and elderly[59]. Although most patients are clinically asymptomatic with no detectable viremia, the virus is actively replicating in lymphoid organs[64]. It is believed that HIV is carried to the lymph nodes in a similar way as other pathogens

and trapped on follicular dendritic cells in order to generate a specific humoral response. If so, this establishes a site of continual infection during clinical latency. Several lines of evidence support this theory and will be further discussed in the mechanisms of pathogenesis. At this time, the histology of the lymph node is characterized by follicular hyperplasia and increased cellular infiltrate. During the latter part of this stage, the small lymphocytic infiltrate forms aggregates which result in the disruption of follicles. The events that occur during clinical latency are aggressively being researched since this is the stage of progressive depletion of CD4+ T cells and it is believed that intervention at this time point would be most hopeful. Recent research by Lane *et.al.* (unpublished) suggests that once the T cell repertoire has been destroyed there is no way of retrieving it. However, existing T cell clones can be forced to proliferate when treated with IL-2. Such therapies may delay the onset of AIDS.

The latter stage of disease is associated with the reemergence of clinical symptoms and severe immunodeficiency. CD4+ T cells continue to decline usually averaging below 200 cells/mm^3. The lymph node is characterized by a decrease in FDC number[65] and an influx in CD8+ T cells, a cell normally

not found in the germinal center in high numbers. The lymph node architecture is disrupted as the follicle involutes and the FDC reticulum is lost. There is a subsequent decrease in viral RNA within the germinal center[66]. HIV is again able to circulate in the blood[62]. It is not known wether the increase in viral load is due to an increase in replication or a release of trapped virions from the lymph node. Patients in this stage of disease are in a state of severe immunosuppression as a result of the loss of T lymphocytes, a loss of germinal centers, and the continual state of high infection caused by circulating HIV. They experience multiple opportunistic infections and neoplasms which ultimately leads to death[59].

Mechanisms of pathology

The immunosuppression associated with HIV infection is directly correlated with CD4 + T lymphocyte depletion and dysfunction. The exact mechanism causing this, however, is unknown. Several potential mechanisms have been hypothesized, some directly related to HIV infection, others based on a deleterious immune response. Each of these will be discussed below.

HIV Related Mechanisms

HIV related cell killing can occur throughout the viral life cycle. After infection with HIV, the virus produces envelope proteins, gp41 and gp120, and inserts them into the target cell's plasma membrane. It has been proposed that this could result in ion fluxes leading to cell death. The envelope protein gp120 also contributes to mechanisms of direct killing by inducing syncytium formation. In vitro, syncytium formation has been correlated with the cytopathic effects of HIV on infected cells[67]. This mechanism discussed earlier involves fusion of infected cells with uninfected cells through the gp 120- CD4 interaction and LFA-1 molecules[67,68].

Another direct mechanism of target cell killing is related to unintegrated provirus. Accumulation of unintegrated proviral DNA is thought to kill the cell or prevent normal cellular protein synthesis thus rendering the cell nonfunctional[69]. A fourth proposed mechanism postulates that intracellular binding of CD4 to gp120 leads to these complexes becoming lodged in nuclear pores interrupting normal trafficking of mRNA from the nucleus to the cytoplasm. Finally, as the virus progeny buds from the host cell, the

membrane may be damaged ultimately killing the cell.

In addition to the quantitative loss of CD4 + T cells, HIV infection also causes loss of T cell function by a variety of mechanisms[70]. First, HIV infection results in abnormal responses to soluble antigen and defects in proliferation. Second, *in vitro* studies have demonstrated HIV induced T cell anergy. Exposure of cells to envelope proteins gp120 and gp41 and core protein p24 resulted in inhibition of T cell responses when challenged with another antigen. Finally, the binding of gp120 to CD4 may prevent normal antigen presentation through MHC class II and may prevent normal signaling required to elicit a response. Regardless of the cause, loss of T cell function contributes to immune suppression experienced in late HIV infection and AIDS.

Immunopathogenic Mechanisms

The remaining immunopathogenic mechanisms are not directly related to HIV infection, but to deleterious features of the normal immune response. Some of these features result in the maintenance and augmentation of HIV infection while others can be directly cytopathic to target cells.

Cytokines

The immune system is dependent on a network of cytokines to orchestrate immune responses. However, several cytokines have been implicated in activating HIV expression from latently infected cells. TNF-α, for example, has been shown to increase HIV replication when it induces NF-κB production[71]. In addition, IL-6 and granulocyte macrophage stimulating factor (GM-CSF) have been shown to increase viral replication in macrophages and this mechanism is primarily at the posttranscriptional level. Furthermore, TNF-α can act synergistically with either IL-6 or GM-CSF to increase HIV replication. Other cytokines implicated similarly include IL-1, IL-3, TNF-β, INF-γ, and M-CSF[71,72]. The network of cytokines may contribute to sustaining viral expression even in times of apparent latency.

Germinal Center Responses

In the late 1980's several groups made the observation that HIV in the lymph nodes during clinical latency was associated with histologic changes including follicular hyperplasia and expansion of the FDC network[73-76]. Recent studies have now focused on secondary lymphoid tissue and its

role in HIV immunopathogenesis. One current hypothesis suggests that the germinal center serves as a reservoir of virus and as such maintains viral infection throughout clinical latency for several years. After infection, the host mounts an immune response which results in immune elimination of virus from the blood. This response results in the generation of antibody which complexes with the virus which then is transported to the nearest lymph node where it will be trapped on FDC. Although this process is normal and important in generating and maintaining humoral immune responses, in HIV infection, it results in seeding multiple lymph nodes with infectious HIV immune complexes and also provides a reservoir of virus. Within the germinal center microenvironment, activated CD4+ T cells- essential for memory antibody responses- reside. Since these cells are activated they may be easily infected by virus trapped on the FDC. Furthermore, they may serve as a continual source of virus as they divide and replicate the integrated provirus. B cells in the environment may further propagate HIV infection by releasing TNF-α.

Several lines of evidence support the hypothesis that HIV in the lymphoid organs contributes to the pathogenicity of AIDS. For example, Panteleo *et. al.* compared the viral burden in CD4+ T cells in peripheral blood to that of lymphoid

organs[77]. He demonstrated that the frequency of infected cells was substantially higher in lymphoid tissue when assaying for proviral DNA. In addition, high levels of HIV RNA were detected in lymphoid tissue but not in peripheral blood samples. Later these high concentrations of virus were found to be actively replicating within the tissue[64]. This supports the hypothesis that the lymph node serves as a reservoir of virus and contributes to the depletion of CD4 + T lymphocytes.

Animal studies also support this hypothesis. Using the SIV model of acute infection Panteleo *et. al.* demonstrated localization of virus not only within germinal centers of secondary lymphoid tissue but trapped on the processes of FDC[78]. Lymph node biopsies were performed at three time points post infection with SIV. After 26 days, the virus was localized in the germinal centers. Furthermore, it was found to be complexed with complement and antibody and held extracellularly on the FDC. Using this same model, they examined the events leading up to HIV localization on FDC(figure 1)[48].

This correlates with that which is observed in the human response. Soon after infection, there is an increase in complement binding antibodies. This is associated with virus

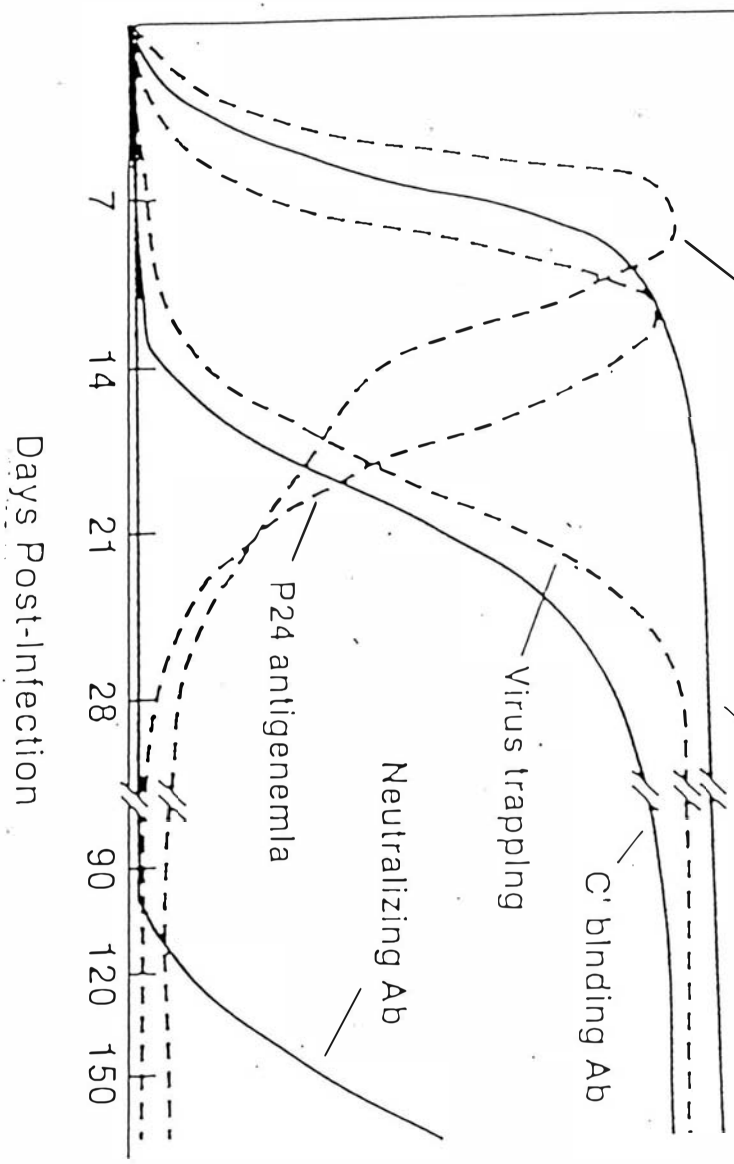
Virus expressing cells in LN
Virus specific CTL
C' binding Ab
Virus trapping
Neutralizing Ab
P24 antigenemia
7
14
21
28
90
120
150
Days Post-Infection

Figure 1

trapping in the FDC network[78]. Other humoral responses follow including the generation of neutralizing antibodies and these also contribute to immune complex formation and trapping. These normal immune responses which attempts to destroy the virus appears to be detrimental by localizing the virus to a site where it will not interact with all arms of the immune response which attempt to destroy it. Instead the virus has established a reservoir for further infection.

Cytopathic mechanisms:

The depletion of CD4+ T lymphocytes has also recently been linked with apoptosis, or programmed cell death[79-82]. CD4+ T cells normally interact with antigen in the context of MHC class II. This interaction results in one of two signals required for apoptosis. After receiving the second signal, the cell dies. This mechanism ensures that after a cell has been activated and performs its effector functions, it will be eliminated. In this manner, cell and tissue damage is tightly controlled. In HIV infected individuals, gp 120 on the surface of an infected cell or gp 120 anti-gp 120 immune complexes can bind to CD 4 on an uninfected cell. Crosslinking this receptor before the TCR is triggered provides a signal inducing apoptosis.

Another normal immune function which may prove to be both beneficial and deleterious is the production of HIV specific antibodies. Antibodies, some of which are neutralizing, are produced against several proteins including gp 120. Infected cells expressing gp 120 on its surface will bind antibody. Binding of this complex to natural killer cells will result in antibody dependent cellular cytotoxicity. In an attempt to rid the body of virus though, CD4 T cell depletion may also occur.

Another aspect of the immune response that may also be deleterious in HIV infection relates to the generation of CD8+ T cells. Although during the initial response these cells help to eliminate the plasma viral load, during clinical latency they are seen migrating into the germinal center, where they are normally not found in high levels. This may result in the destruction of other cells in the germinal center including helper T cells. Finally, destruction of the FDC network may result in immune suppression of many B cell responses including memory.

We believe that there is strong evidence that HIV in the lymph node is a major contributor to pathogenesis. This is the primary site of infection throughout clinical latency. Furthermore, full blown AIDS is accompanied by disruption of

the lymph node and the FDC reticulum. Therefore, the remainder of this background section will focus on the germinal centers in the lymph node, the follicular dendritic cell, and the unique microenvironment they provide which is conducive to HIV infection.

Germinal Centers and FDC

Lymphoid follicles are found in the B cell dependent areas of secondary lymphoid tissue, including lymph nodes, tonsils, and spleen. They are composed of B cells, T cells, macrophages, and follicular dendritic cells (FDC)7[83]. They can be further classified into primary or secondary follicles based on their state of activation. Primary follicles consist mostly of recirculating B lymphocytes with approximately 5% T lymphocytes, most of which are CD4+. These cells pass through a reticulum or cellular network formed by FDC. Primary follicles become secondary follicles when germinal centers form upon antigenic challenge. These sites are characterized by foci of rapidly proliferating B cells and some T cells surrounding antigen bearing FDC. The germinal center represents a microenvironment which facilitates the interaction of cells resulting in the production and

maintenance of memory humoral responses. These areas are associated with clonal expansion of B cells[84-86], somatic mutation[85-88], and selection of antigen specific B cell clones[89]. The germinal center is also associated with the maintenance of the humoral response by inducing the differentiation of memory B cells[90,91] and plasma cells[92-94].

Secondary lymphoid follicles can be divided into three different regions by light microscopy. The first of these, the follicular mantle forms on the outer edge of the follicle. This region consists primarily of recirculating B cells. The second and third regions are the light and dark zones. The germinal center, which is located beneath the follicular mantle, is found in these two regions. The light region, which is adjacent to the mantle, is heavily infiltrated by the FDC network. Most of the cells in this area are not proliferating and do not stain intensely, thus the name light zone. Adjacent to the light zone is the dark zone, named because it has a much denser population of highly activated, rapidly proliferating lymphocytes which stain intensely. Furthermore, tingible body macrophages are found here. This area, being the farthest from the follicular mantle, is infiltrated only with the fine dendritic processes of the FDC network.

Follicular Dendritic Cells

Follicular dendritic cells were discovered by Nossal *et. al.* [95] and Szakal & Hanna [96] in 1968. This cell, which is fundamental to germinal center development and the immune responses that occur there, has three cardinal features distinguishing it from other dendritic cells. First, FDC are normally found only within the follicles of secondary lymphoid tissue although in pathological states they can be found elsewhere (e.g. rheumatoid nodules). Second, they have a characteristic dendritic morphology with long, highly convoluted, dendritic processes emanating from the cell body and finally, their most important feature is their ability to trap and retain antigen in the form of immune complexes on their surface for several months to several years[97]. Along with the cardinal features, the literature is highly consistent on the lack of phagocytic activity or any other internalization process, irregularly shaped euchromatic nucleus, complement receptors, and adhesion molecules including ICAM-1 and VCAM-1 [92,97-105]. Further analysis of surface markers has shown that FDC lack the classical markers for T cells: CD8 and Thy-1; B cells: B220; macrophages: Mac 1,2,3; and NK cells: NKH-1[98,99,103,106-110]. Researchers are divided on the expression or lack of expression for other

surface markers including MHC class II, F_c gamma receptors, F_c epsilon receptors, leucocyte common antigen, S-100 protein, CD4, CD14, CD19, CD20, CD21, and CD45 [98,99,102-112]. This inconsistency may be due to the heterogeneity of FDC and may be further complicated by the state of activation of the cell[99,105].

The expression of various molecules on the surface of FDC facilitates their interaction with B and T lymphocytes. FDC which express ICAM-1 and VCAM-1 interact with adhesion molecules LFA-1 and VLA-4, respectively on B and T cells. These adhesive interactions aid in securing the cells and play an important role in the responses that occur in the germinal center. Monoclonal antibodies YN1/1.7[113,114] or MK1 [115,116] which block intracellular adhesion molecules, prevent FDC-B cell clustering and B cell proliferation. ICAM on FDC may provide a second signal to activate B cells within the germinal center. T lymphocytes have similar adhesion molecules and are also believed to interact with FDC.

FDC are also believed to have interactions which aid in sparing cells from apoptosis[99]. FDC express CD 23 which is known to stimulate B cells[117]. In addition, FDC express CD 40 which interacts with its ligand on T cells. B cells also express CD 40. These interactions may contribute to a

microenvironment which decreases apoptosis and facilitates germinal center B cell responses[99].

FDC and the Germinal Center Response

The immunologic events that occur in the germinal center are not found at any other site in the body. It usually takes a few micrograms of antigen to elicit a secondary response yet FDC retain only 100-500 picograms/ node and this maintains potent memory[118]. The ability to generate this type of response with only low levels of antigen depends on all of the cells that contribute to the dynamic microenvironment of the germinal center. FDC are believed to play a critical role in the germinal center reaction and when they are decreased in number or aberrant, marked alterations in germinal center size and number occur[119].

The events leading to a secondary response and the role of the germinal center have been characterized. After injection of antigen, immune complexes are rapidly formed and most are cleared by macrophages within 24-72 hours. We refer to this method of antigen handling as the classical antigen pathway[120]. Some immune complexes, however, enter the alternate transport pathway and are transported by a variety of non-phagocytic cells with dendritic morphology to the

lymphoid follicles[120]. These antigen transport cells (ATC) are similar to FDC[120] and may even be FDC precursors. As these ATC near the light zone of the germinal center they become continuous with the FDC reticulum.

Within the germinal center, immature filiform FDC retain immune complexes on their convoluted dendritic processes[121]. As they mature, some FDC appear to have beaded dendrites. These beaded dendrites interact with the processes of other FDC to form immune complex coated bodies, or ICCOSOMES[121]. ICCOSOMES can be released into the germinal center microenvironment and are therefore thought to play an important role in the alternative transport pathway. These highly immunogenic iccosomes are easily endocytosed by neighboring B cells. The antigen is then processed by the B cells and presented in the context of MHC class II to antigen specific T cells. This now allows a T cell specific response in the form of cytokine secretions[122]. Thus, the alternative transport pathway is believed to play a role in germinal center formation, B cell proliferation, and memory B production[122,123].

The consequences of the germinal center reaction can be divided into two phases- antibody production and memory B production[99]. Analysis by light microscopy and EM shows B

cells differentiating into antibody forming cells, or plasma cells[120,124,125]. Three to five days after antigenic challenge, these cells can be seen migrating from the germinal center to medullary cords where plasma cell maturation occurs. Iccosomes are also formed and released during this time period. It is believed that the release of iccosomes and the signals within the germinal center microenvironment are related to the induction of plasma cell formation[99].

The second phase of the germinal center reaction occurs within 6-14 days after antigenic challenge. At this time, antigen is retained on the FDC for long term retention and iccosome formation and release is not observed. Germinal center B cells during this period of time become memory B cells and plasma cell formation ceases[90,93].

Memory B cells are essential in the maintenance of the humoral response. As previously stated, FDC can retain unprocessed antigen in the form of immune complexes for several months to several years. This retained antigen is believed to play a critical role in the maintenance of antibody responses. One existing model states that the concentration of FDC retained antigen, free antibody, and antigen-antibody complexes dictates the events that maintain these antibody levels[126,127]. Exposed antigen on the

surface of the FDC will result in memory B cell stimulation and antibody production. When sufficient amounts have been produced, the antibody will again bind and cover any epitopes exposed on the FDC retained antigen. The cycle repeats to maintain and regulate serum antibody levels. The requirements for FDC retained antigen to maintain antibody responses was further supported by Gray & Skarvall in lymphocyte transfer studies[128]. Lymphocytes from the thoracic ducts of immunized rats were transferred to naive, antigen free rats. The naive rats were then challenged at various time points to determine if B cell memory was present. The response declined over the time course and by day twelve was virtually non-existent. Although the rats had the memory B cell population, they lacked the second signal of antigen and were unable to maintain the humoral immune response. Therefore, the maintenance and survival of memory B cells is believed to depend on the FDC and its retained antigen.

Although the literature on T cells within the germinal center is not as extensive as that seen with FDC and B cells, T cells are believed to play an essential role in germinal center formation as evidenced by the observation that without them, germinal centers do not form and the associated immunological events of the germinal center do not occur[129].

Although their exact role is not known, it is believed that T cells interact physically with B cells and provide help for T dependent B cell responses by secreting various cytokines. In support of this, recent evidence demonstrates that germinal center T cells are antigen specific[130]. Normally, antigen stimulates a subset of T cells presenting the requisite antigen receptor. Upon antigenic challenge that T cell subset becomes activated and proliferates. Nahm *et.al* showed that these two events occur in germinal centers. Germinal centers that were induced with a specific antigen recruited a subset of T cells that were specific to that antigen[130]. Furthermore, these cells proliferated in response to the antigen. The percentage of specific T cells with the expected TCR was greater in the germinal center than that found in the T cell associated paracortical region of the lymph node.

Among its functions, FDC have recently been shown to be potent costimulatory cells to both B and T lymphocytes [99,131]. Costimulation promotes cellular proliferation within this microenvironment. When provided with a primary signal such as LPS or pokeweed, murine FDC provide costimulation resulting in enhancement 19-60% over mitogen induced B cell proliferation[99,132]. Addition of other cell types including macrophages and nude mouse spleen cells had no

effect on proliferation. However, FDC from draining lymph nodes showed even greater enhancement of B cell proliferation. We reasoned that antigen recently trapped on FDC in draining lymph nodes provided additional activation signals. These cells, which had recently been loaded with antigen *in vivo*, were the most potent costimulators. In 1988, Heinen *et.al* reported a similar costimulatory function in human FDC populations[133].

To determine if cell to cell contact was required to achieve FDC accessory cell function, FDC were replaced with supernatants from FDC cultures. Supernatants alone did not enhance LPS induced B cell proliferation suggesting that the cell to cell contact between B cells and FDC is required for costimulation of LPS stimulated B cells[99].

In addition to the costimulatory signaling of FDC, the antigen provided by FDC both on its surface and through iccosomes contributes to a microenvironment conducive to generating and maintaining humeral responses. The interaction of FDC with B and T cells and the interaction of B and T cells with each other also contributes to this microenvironment.

During clinical latency, HIV is confined to the germinal centers of secondary lymphoid tissue. Furthermore, HIV immune complexes are trapped on the surface of FDC[66]. Since FDC

are known to trap and retain antigen for many months and possibly several years, and this is the site of active infection during clinical latency, we believe that the microenvironment of the germinal center and the features of FDC contribute to the maintenance of active infection and to the overall pathogenesis of AIDS. As trapped antigen, HIV is in an area where susceptible CD4+ T cells continually circulate. These T cells should be highly activated. This activation can occur as a result of several events including FDC costimulation and presentation of antigen in the context of MHC class II by B cells through the alternate antigen pathway. Activated T cells are required for active HIV replication and integration[134]. Once activated a variety of events can facilitate the binding of gp120 to CD4 thus allowing infection. The FDC reticulum is a dense network through which cells circulate thus optimizing the opportunity for cell to cell contact. In addition, adhesion molecules such as ICAM-1 and VCAM-1 on FDC can interact with LFA-1 and VLA-4 on T and B cells. These adhesion molecules probably aid in FDC- lymphocyte interactions. We believe that this plays a critical role in maintaining infection within the germinal center, especially in light of the low concentration of CD4+ T cells present in this microenvironment. We reason that

adhesion molecules would stabilize the FDC-T interaction in a manner that would allow the gp 120 of the trapped virus on FDC to come in close proximity to CD 4 molecules on the T cell. In addition to adhesion molecules, the interaction between CD40 on FDC and CD40 ligand on T cells may also play a role in facilitating infection through signaling, activation, or securing the cells. Finally within the germinal center, FDC costimulation exists as well as cytokines produced by T cells in response to B cell interactions thus causing increased proliferation and activation. Taken together, the micro-environment of the germinal center represents a site where permissive host cells, FDC retained infectious virus, and potent costimulatory signaling coexists. It is our hypothesis that this microenvironment and FDC play a critical role in the pathogenesis of HIV. The aim of my work was to investigate the role of FDC in HIV pathogenesis by determining if FDC trapped HIV was infectious. My results indicated that it was highly infectious and that FDC could negate the effects of high levels of neutralizing antibodies.

METHODS

Animals

Female, 5-8 week old BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were housed and maintained in the university animal facilities in standard shoebox cages under laminar flow air. The mice were given food and water *ad libitum*.

Human tissue and blood

Human tonsilar tissue was obtained from HIV negative patients undergoing surgery for diagnostic and/or pathologic reasons. The material was provided by the MCV/VCU Massey Cancer Center Tissue Collection Core Facility or Department of Pathology. Blood samples from HIV infected individuals were supplied by Drs. Thomas Kerkering & Vivian Bruzzese of the Richmond AIDS Consortium and HIV/AIDS Clinic, respectively. Blood samples from HIV non-infected individuals were obtained from healthy volunteers by G.Burton. All patient samples were labeled to maintain confidentiality.

HIV Preparations

HIV-1 lab passaged strains, IIIB, MN and SF2 were selected for initial testing due to the relative ease of growing and maintaining infectious virus and to the availability of antibodies and molecular reagents for these strains. The strains IIIB and MN were cultured in the H-9 T cell line. SF2 was grown in the Hut-78 cell line. In addition, the primary HIV-1 isolate, 301714, was used to confirm major findings. It was grown in PHA/IL-2 stimulated peripheral blood T cell blasts obtained from HIV non-infected individuals(see isolation of human T cells). Culture supernatants containing the lab or primary isolate of virus were harvested from infected cell cultures at peak p24 activity. They were pooled, filtered through a 0.22 mm membrane, aliquoted, and frozen in liquid nitrogen to provide a uniform stock of infectious virus. Infectious titers of virus ($TCID_{50}$) were performed on thawed samples and typically yielded titers of 10^4-10^5 $TCID_{50}$/ml. The ACH-2 T cell line was selected as a positive control for all experiments because it contains a single copy of HIV proviral DNA in its genome. All reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (ARRRP) as follows: strains IIIB and MN, and the cell line H-9 from Dr. Robert

Gallo[135-138]; strain SF2 from Dr. Jay Levy[139]; and cell line ACH-2 from Dr. Thomas Folkes[140,141].

Antibodies

The HIV specific antibodies selected for these studies included: H-902, F105, Chessie 8, and b12. The characteristics of each antibody are as follows:

	Species	Isotype	Epitope recognized	Strain Reactivity	Neutra-lizing
H-902	murine	IgG1	gp120	IIIB	yes
F105	human	IgG1	gp120	IIIB,MN,SF2	yes
Chessie8	murine	IgG1	gp41	IIIB	no
b12	human	IgG1	gp120	Clade B	yes

The following reagents were obtained through the ARRRP: Hybridoma 902 from Dr. Bruce Chesebro[142,143], F105 from Dr. Marshall Posner[144,145], and Chessie 8 from Dr. George Lewis. b12 was graciously provided by Drs. Dennis R. Burton and Paul Parren, LaJolla, CA[146].

Anti-CD4 (Leu 3a + 3b; Becton Dickinson) was also used as a control antibody in some experiments to block infection of helper T cells through the CD4 receptor.

Continuous Percoll Gradients

Continuous density gradients were prepared by centrifuging 25 ml of 50% Percoll solution (Percoll, dH_2O, Hepes Buffer, 1.5M NaCl) in 1 x 3.5 inch cellulose nitrate ultra-centrifuge tubes. Gradients were centrifuged at 30,000 x g for 20 minutes at 4 degrees C in a Beckman 50.2 Ti rotor. Density marker beads (Pharmacia) were added to one gradient prior to centrifugation to identify specific densities of interest.

Isolation of Human cells (FDC, T cells, Mϕ, and FDC depleted populations)

Human cells were obtained from tonsils of non-HIV infected individuals using a modification of the procedure used to isolate murine FDC from lymph nodes[131,147]. The modification consisted of increased incubation times and volumes of enzymes to accommodate the larger mass of tissue present in the tonsils. Briefly, fresh human tonsils were carefully dissected into 3 mm squares and incubated for two hours at 37 deg C in medium containing collagenase (10mg/ml) and DNASE I(1% v/v). Following incubation, the cells were collected and placed in medium containing 10% heat

inactivated, fetal calf serum (FCS). The remaining tissue was again incubated in fresh medium containing enzymes and the cells collected as before. After the final incubation of tissue, medium without enzymes was added to the culture dish and the tissue pipetted up and down gently in a 10 ml pipet to free cells trapped in the tissue. Following repeated pipetting, the cells were pooled, washed, resuspended in fresh medium and layered onto 50% continuous Percoll gradients. The gradients were centrifuged at 500 x g for 30 min. The low density band (1.050-1.060 g/ml) containing FDC and macrophages was collected as was the high density band(1.075-1.085g/ml) containing T cells. Both fractions were washed free of Percoll and resuspended in media containing FCS prior to additional manipulations.

In some experiments, FDC were further enriched using florescence activated cell sorting (FACS)[131,147]. FDC were stained using two, murine IgM, anti-human FDC monoclonal antibodies: HJ2 (provided by Dr. M. Nahm)[148,149] and DRC-1 (Dako). After washing to remove unbound antibody, the preparations were stained with biotin conjugated donkey anti-mouse IgM (Jackson Immuno Research) followed by a final wash and incubation with strepavidin fluorochrome (FITC or PE; Tago). The cells were then sorted based on anti-FDC

florescence and forward light scatter using a Coulter Epics 753.

For all other studies FDC were further enriched using positive selection on a magnetic activated cell sorter (MACS) instead of FACS scanning. After incubation with HJ2, the preparation was washed to remove unbound antibody and then incubated with rat, anti-mouse IgM magnetic microbeads(Microbeads; Miltenyi Biotec, GmbH). Cell suspensions were layered over a column attached to a magnet. Microbeads bound to cells were attracted to the magnet and thus remained in the column while other cells without bound beads passed through. The column was then removed from the magnet and washed with medium to collect the desired cells.

Resulting preparations consisted of FDC with a purity of >85% based on flow cytometry. The FDC showed typical dendritic morphology, structure and function. FDC preparation was γ-irradiated (3000R) prior to incubation with HIV immune complexes to block proliferation and thus minimize the ability of cells in this preparation to support HIV infection.

MACS was also used to prepare tonsilar macrophages (positively selected using anti-CD14 Microbeads), tonsilar T cells (positively selected using anti-CD3 and anti-CD4 microbeads) and FDC deficient cell populations (negatively

selected i.e. those cells not binding to the magnetic column when reacted with anti-FDC mAbs).

For some experiments, peripheral blood CD4+ T cells were substituted for tonsilar T cells. In addition, peripheral blood T cells were used to culture the primary HIV-1 isolate, 301714. Blood was obtained from normal donors and peripheral blood mononuclear cells (PBMC) were enriched on Lymphocyte Separation Medium (LSM;Organon Technika). Briefly, blood was diluted in half with medium and layered over 7ml LSM in 15ml conical centrifugation tubes. The tubes were centrifuged at 400 x g for 20 minutes at 25 degrees C. Lymphocytes were collected form the tissue culture medium-LSM interface and further purified by sorting using positive selection with anti-CD3 and anti-CD4 magnetic microbeads.

FDC trapping of HIV-1 *in vitro*

Human tonsilar FDC were incubated overnight (4°C) with HIV-immune complexes formed by incubating (2 hr, 37°C) 100µl fresh frozen serum from an HIV infected individual (as a source of specific antibody and complement) with 100 µl HIV_{III-B} cell free supernatant (5000 $TCID_{50}$). This dose of HIV provided sufficient virus for immune complex formation and subsequent trapping by FDC. HIV-immune complexes not bound to FDC were

removed by washing. Binding of virions to FDC was confirmed by electron microscopy.

FDC trapping of HIV-1 *in vivo*

To form and trap HIV immune complexes onto FDC *in vivo* mice were injected with specific antibody and antigen. For our system, we performed this by first injecting (sc or ip) 1.2 mg, murine anti-gp120 mAb into mice. Two hours later, the animals were exposed to 600R γ-irradiation to eliminate radiation sensitive lymphocytes and thus enrich for radiation resistant FDC[116,147]. One day later mice received injections of HIV_{IIIB} to allow immune complex formation and FDC trapping *in vivo*. $5000TCID_{50}$/site of HIV (five sites total) was injected in the feet and behind the neck to distribute the virus to FDC in several draining lymph nodes. This dose of HIV resulted in consistent binding of HIV on FDC in multiple draining lymph nodes. Five days later, when immune complexes or antigens are confined to murine FDC[127], the mice were sacrificed by ether anesthesia followed by cervical dislocation and the lymph nodes excised (including the popliteal, brachial, axial and submandibular). FDC were isolated as described [147]. These preparations contained 25-45% FDC with the remaining cells being equal numbers of T and

B lymphocytes.

Neutralized HIV immune complexes

HIV immune complexes were formed by mixing picogram, microgram, or milligram doses of neutralizing murine anti-HIV-1_{IIIB} gp120, H-902, with 5000$TCID_{50}$ HIV_{IIIB}. HIV-immune complexes were also formed using HIV-1 strains (IIIB, MN and SF-2) mixed with 1 µg human anti-HIV-1 gp120, F105, and the primary isolate 301714 mixed with 20mg monoclonal antibody b12. Control cultures included the use of a non-neutralizing antibody, Chessie 8, mixed with 5000$TCID_{50}$ HIV_{IIIB}. This dose of HIV was used to provide a high level of virus-anti-virus immune complexes such as could potentially exist on FDC *in vivo*.

Experimental Tests

To determine if FDC trapped HIV was infectious, we cultured FDC bearing HIV immune complexes (as the only source of virus) with human CD4+ T cells which had been activated overnight with Staphylococcal Enterotoxin E (SEE) (Toxin Technology, Sarasota, FL). Human tonsilar FDC($6x10^4$) bearing HIV trapped *in vitro* or control FDC were cocultured with $1x10^5$, SEE activated (100 pg/ml), autologous CD4+ T cells.

Murine FDC ($1x10^5$) bearing *in vivo* trapped HIV immune complexes were cocultured with $1x10^5$ SEE (5 ng/ml) activated T cells, obtained from peripheral blood.

To determine if FDC could negate the effect of neutralizing antibody, neutralized immune complexes were first formed at 37°C for 1 hr and then incubated with SEE activated tonsilar T cells ($5x10^4$) or the H-9 T cell line. MACS purified human tonsilar FDC($1x10^4$) were also added to some of the cultures. To prove that it was FDC and not contaminating cells that were responsible for negating the effect of the antibody, we also cultured neutralized immune complexes and T cells with MACS purified macrophages and FDC depleted populations at a concentration of 1×10^4 cells per well. In addition, blocking anti-CD4 was added to some cultures at a dose of 1 µg to confirm that infection was occurring via gp120-CD4 receptor binding.

All cultures were performed in triplicate in 48 well plates with media containing 10% FCS. Plates were incubated for 4 days at 37 degrees prior to analysis of infection.

Detection of HIV infection by PCR & Southern blot analysis

Samples were centrifuged and the DNA isolated using lysis buffer and proteinase K as described[150]. DNA was stored at

-20°C until tested. PCR analysis for HIV proviral *gag* and β-globin sequences was performed in the same reaction vessel using primer pairs SK38/39[151] and GH20/PC04[152] respectively. Control cultures of ACH-2 cells were isolated, and diluted as indicated with uninfected H9 cells to provide a constant amount of cellular DNA prior to isolation for PCR analysis. PCR amplification was performed for 35 cycle(94 °Cx2 min 1st cycle, 92 °Cx1 min all other cycles; 55° Cx1.5 min; 72 °Cx 2 min; after cycle 35, an additional 72° Cx 7 min incubation was performed). Amplified products (10μl) were analyzed by electrophoresis (2% agarose), stained with Ethidium Bromide for β-globin detection, and blotted onto Nytran. Blots were probed using ^{32}P labeled SK19[151]. Autoradiography was performed for 3 days at -80°C. Autoradiographs and Polaroid prints of ethidium stained gels were scanned at 400 dpi, aligned using Microsoft Power Point, transferred to film and printed on Kodak F5 resin coated paper.

Electron Microscopy

Cultures containing T cells, FDC, and HIV immune complexes (formed with HIV-IIIB and the neutralizing antibody-H902) were analyzed by electron microscopy. Three to 5 days

after culturing, germinal center clusters were isolated and gently fixed by 2.5% glutaraldehyde in sodium cacodylate buffer for 2 hours[153]. This was followed by fixation in .5% Osmium Tetra Oxide for 90 minutes. They were next post fixed with 1% Tannic Acid AR (an Allepo nut derivative; TA Mallinckrodt, NO.1764, St. Louis, MO) in 0.05% sodium cacodylate buffer at ambient temperature for 20 minutes. Fixation was followed by routine embedding in 812 epoxy resin. Samples were sectioned on an Ultra-Microtone. Sections were then stained in uranyl acetate and lead citrate and examined on a Zeiss 10 CA Electron Microscope[153].

HIV Quantitation

In an attempt to quantitate integrated proviral DNA, we designed a competitive PCR assay(Clonetech). Competitive fragments of neutral DNA, Verb, were designed in a manner that would allow them to be amplified by our primers SK38/39, but would generate a product that would be 200 base pairs longer than the *gag* amplification product. Briefly, primers were generated which would amplify the neutral piece of DNA, but were also flanked at their 5' end with SK38 and SK39 sequences. After amplification with these primers, the product generated contained Verb DNA flanked by *gag* DNA SK38

and SK39 sequences. This product was amplified again with SK38/SK39 primers in a secondary PCR. The secondary product verified that amplification could occur with SK38/SK39. This product then serves as a competitive fragment in PCR reactions.

To quantitate, experimental samples are "spiked" with varying, yet known concentrations of the competitive fragment. PCR amplification is performed with SK38/SK39 primers which will amplify both HIV proviral DNA and the competitive fragment. Products are run on agarose gels and stained with ethidium bromide. The intensity of the mimic fragment is compared to that of the unknown sample. Where the intensities match, the two DNA samples equally competed for the primers, therefore the concentration of the competitive fragment equals the concentration of the sample. The sensitivity of the competitive PCR generated here is at the attomolar range.

RESULTS

In this report, we describe an *in vitro* model of the germinal center, designed to mimic events during clinical latency, to determine whether or not FDC retained HIV could cause infection. We obtained FDC from the tonsils of HIV non-infected individuals and incubated these with HIV-immune complexes *in vitro*. Viral immune complexes not bound to FDC were removed by washing and FDC were cultured with activated, autologous CD4+ T cells. Infection was monitored by PCR analysis of DNA from the cultures. Infection was demonstrated by the presence of proviral HIV-1 DNA isolated from cultures of T cells and FDC bearing HIV-immune complexes but not in cultures containing control FDC (Fig 2). Syncytium formation was also observed in cultures of T cells and FDC bearing HIV-immune complexes. HIV binding to FDC was confirmed by electron microscopy (not shown). Portions of FDC dendritic processes bore 5-10 virus particles extracellularly which is consistent with observations *in vivo* [64,154,155]. The distribution of HIV virions on FDC processes suggested that a T cell could interact with several virus particles on a single portion of an FDC process.

Figure 2: Infection of T cells by FDC trapped HIV-1. CD4+ T cells and autologous FDC bearing HIV immune complexes trapped *in vitro* were cultured and infection monitored using PCR. Proviral HIV -1 *gag* (115bp) DNA was detected where FDC bearing HIV-1 were used as the only source of virus for infection of the T cells. Lysates of decreasing numbers of ACH-2 cells, a chronically infected T cell clone containing one copy of proviral DNA per cell, were run in parallel for comparison. The intensity of the signal from T cells infected by FDC bearing HIV-immune complexes appeared between the intensity obtained from 10^3 to 10^4 ACH-2 cells. As a control, β-globin DNA was amplified simultaneously (bottom). T+SEE = T cells activated with Staphylococcal Enterotoxin-E; FDC~HIV (IC) = FDC incubated with HIV-1_{IIIB} immune complexes; FDC = control FDC not incubated with HIV.

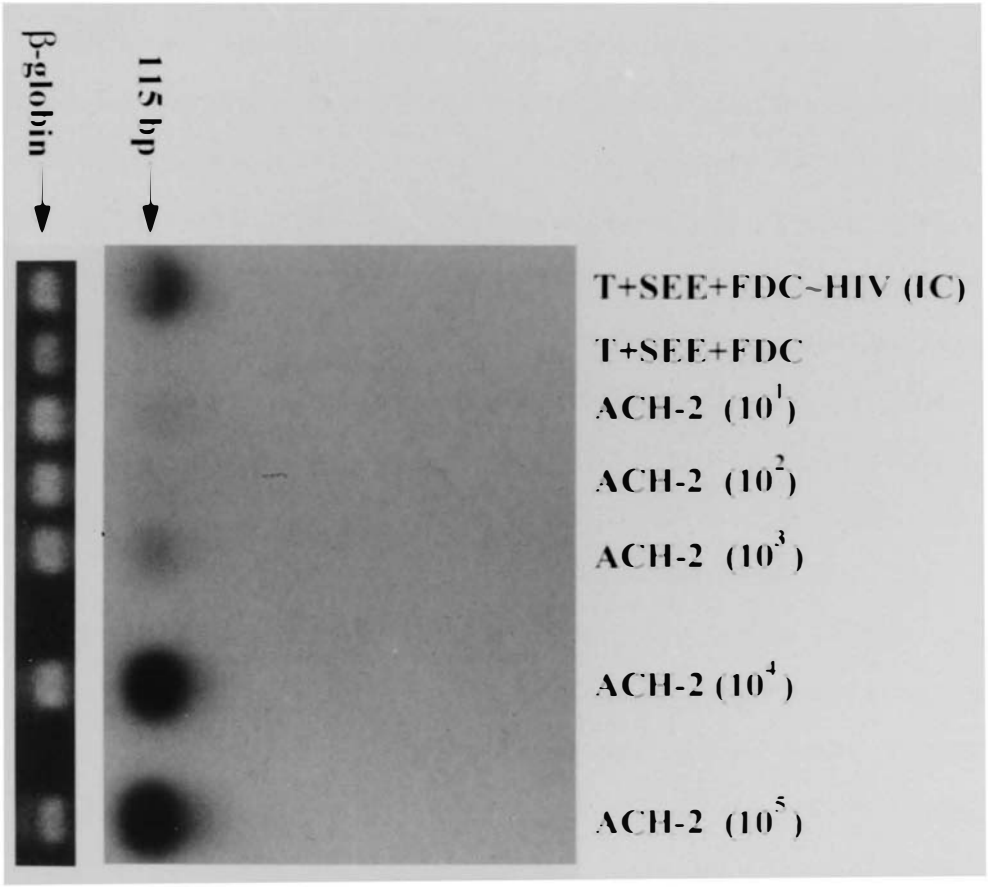
β-globin
115 bp
T+SEE+FDC~HIV (IC)
T+SEE+FDC
ACH-2 (10^1)
ACH-2 (10^2)
ACH-2 (10^3)
ACH-2 (10^4)
ACH-2 (10^5)

Figure 2

HIV-immune complex trapping on FDC *in vitro* may differ from trapping *in vivo*. We therefore examined the infectivity of HIV trapped on FDC *in vivo* using a murine model. Previous work has shown that FDC can provide accessory function to lymphocytes across species (Burton unpublished) and we reasoned that HIV-1 gp160 on FDC-trapped virus could interact with human CD4 on T cells regardless of FDC species. Antigens, including virus, can be localized on murine FDC *in vivo* by passive immunization followed by antigen challenge[120,156]. FDC trapping of HIV was accomplished by passively immunizing mice with HIV-1_{IIIB} specific anti-gp120 and one day later, injecting 5000 $TCID_{50}$ HIV_{IIIB} in several sites to allow trapping of HIV immune complexes on FDC in multiple lymph nodes. Five days later draining lymph nodes were obtained and the FDC isolated[147]. The murine FDC bearing HIV trapped *in vivo* were cocultured with activated human T cells obtained from the blood of a normal donor (Fig 3). Infection of human T cells was detected when HIV immune complexes on murine FDC were used as the only source of virus. Transfer of infection by murine FDC also indicated that HIV infection of FDC was not needed because the mouse is a non-permissive host. This model also excluded the possibility that human CD4+ cells in the FDC preparation were needed for transfer of infection.

Figure 3: T cell infection by HIV-1 trapped on murine FDC *in vivo*. Xenogeneic (murine) FDC bearing HIV-immune complexes trapped *in vivo* were cultured with human CD4+ T cells and infection monitored using PCR. Infection was clearly evident where human T cells were cultured with FDC bearing *in vivo* trapped HIV were used as the only source of virus for infection [T+SEE+FDC-HIV (trapped *in vivo*)]. Infection was also detected where control, murine FDC (no HIV trapped) were incubated with human T cells in the presence of exogenous HIV (5000 $TCID_{50}$) and this signal appeared slightly more intense than that obtained from the FDC with *in vivo* trapped HIV. In contrast, no proviral DNA signal was obtained when the same FDC bearing virus were cultured with resting human T cells [T+FDC-HIV(trapped *in vivo*)] indicating the need for T cell activation to support infection by the trapped virus. No proviral DNA signal was observed in control cultures of either the human T cells alone or the murine FDC bearing HIV trapped *in vivo*. ACH-2 ($5x10^4$) were included as a positive control. The ability of HIV on xenogeneic FDC to infect human T cells was not unexpected since there is no *a priori* reason that gp120 on FDC trapped virus could not interact with CD4 present on the human T cells. Furthermore, murine dendritic cells (DC, not to be confused with FDC) have also successfully infected human T cells *in vitro* [150].

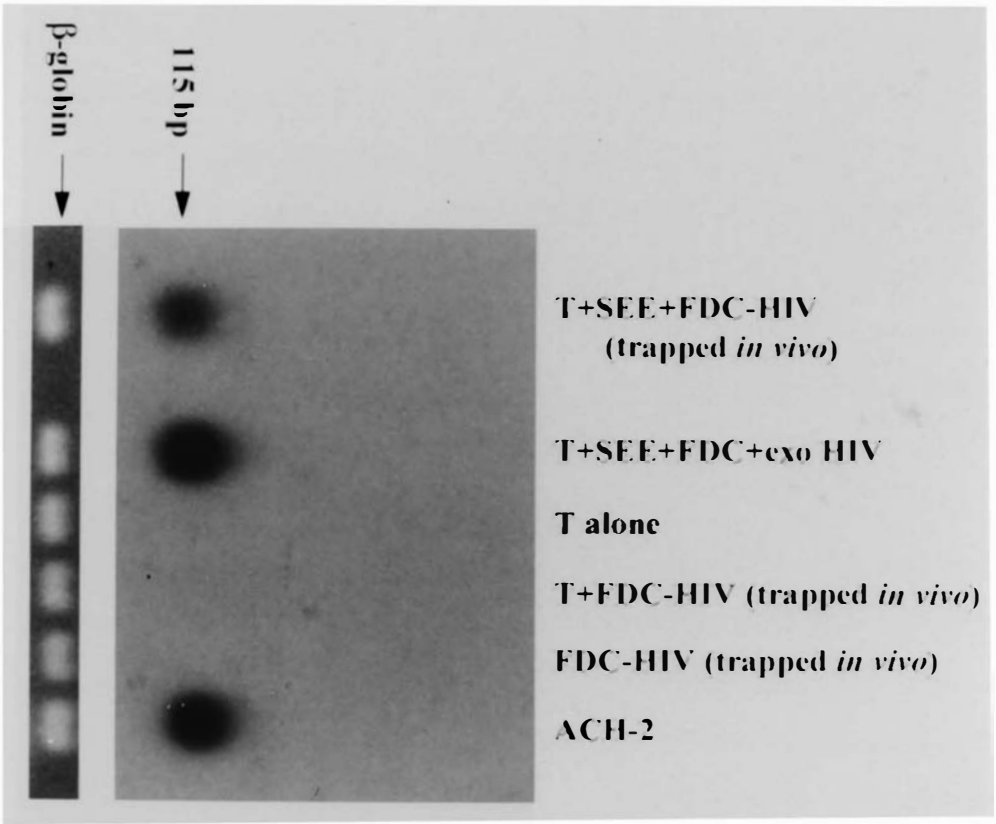
β-globin
115 bp
T+SEE+FDC-HIV
(trapped *in vivo*)
T+SEE+FDC+exo HIV
T alone
T+FDC-HIV (trapped *in vivo*)
FDC-HIV (trapped *in vivo*)
ACH-2

Figure 3

The monoclonal antibody (H902) used to trap HIV on the murine FDC has been used to neutralize HIV-1_{IIIB} [142]. The HIV complexes trapped on FDC *in vivo* were infectious; therefore, we reasoned that FDC may be able to reverse the effect of neutralizing antibody. To test this, neutralized HIV-1_{IIIB} immune complexes were formed *in vitro* using increasing doses of neutralizing antibody and cultured with the H-9 T cell line(data not shown) or activated T cells in the presence or absence of FDC (Fig 4). One nanogram of antibody consistently neutralized 5000 $TCID_{50}$ HIV-1_{IIIB} (Fig 4a). Infection occurred when FDC were present at doses of neutralizing antibody ranging from 1 thousand to 1 million fold above the 1 nanogram needed to prevent infection in the absence of FDC (Fig 4b&c). Tonsilar macrophages and tonsilar cells specifically depleted of FDC failed to promote infection by neutralized HIV-1 immune complexes (Fig 4c). Anti-CD4 in cultures of HIV immune complexes, T cells and FDC blocked infection confirming the importance of the surface marker CD4. As expected, incubation of the virus with Chessie 8, a nonneutralizing antibody, resulted in infection of T cells in the presence and absence of FDC (Data not shown). Increasing amounts of neutralizing antibody reduced the amount of proviral HIV-1 *gag* detected indicating that even though antibody did not block HIV on FDC,

Figure 4: FDC mediate HIV infection of T cells in the presence of neutralizing antibody. PCR amplification of HIV proviral DNA (top panels) and control β-globin DNA (bottom) from cultures of SEE activated CD4+ T cells (T+SEE) and HIV-immune complexes(HIV-IC) formed with various doses of neutralizing antibody in the absence (a) or presence of human FDC (b&c). a) Infection of SEE activated CD4+ T cells by HIV-1_{IIIB} immune complexes formed with 1 picogram of neutralizing anti-gp120 (H902) was evident whereas infection was blocked when viral complexes were formed using 1 nanogram, 1 microgram and 1 miligram of antibody. Signal intensity is compared with 50,000 ACH-2 cells. (b & c-left panel) Addition of FDC to cultures reverses the effect of neutralizing antibody even in vast excess of the 1 nanogram quantity needed to block infection when FDC were not present. (c-right panel) Tonsilar macrophages (Mϕ) and tonsilar cells depleted of FDC (FDC dep) could not substitute for FDC in promoting infection by HIV-IC formed with 1 nanogram of neutralizing antibody. Addition of anti-CD4 blocked infection even when FDC were present in the cultures (+αCD4).

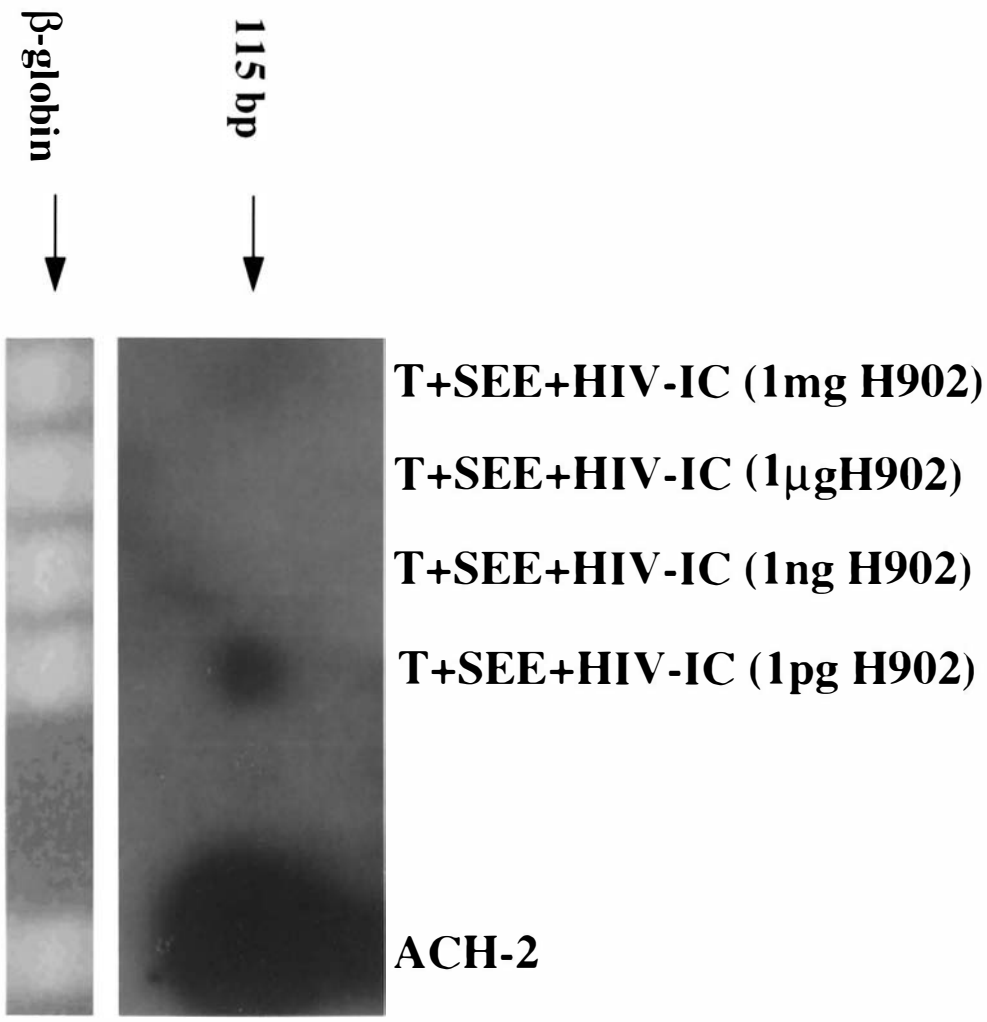
β-globin
115 bp
T+SEE+HIV-IC (1mg H902)
T+SEE+HIV-IC (1μgH902)
T+SEE+HIV-IC (1ng H902)
T+SEE+HIV-IC (1pg H902)
ACH-2

Figure 4 a

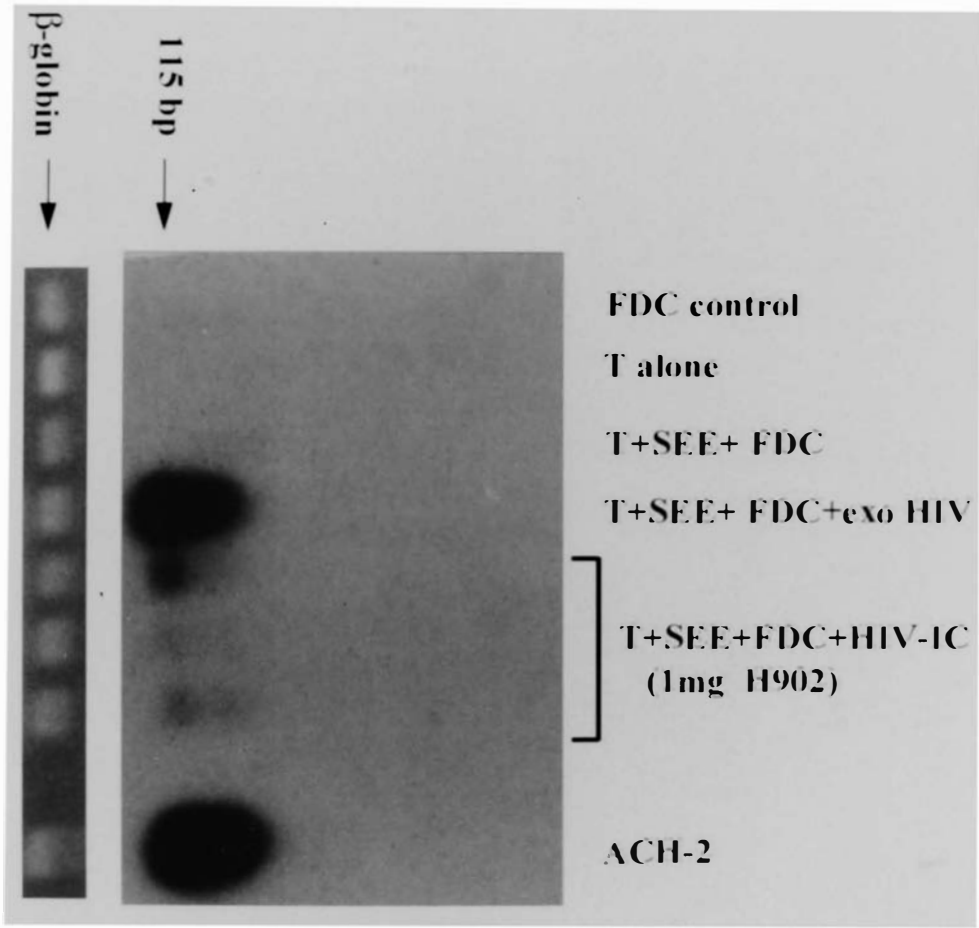
β-globin
115 bp
FDC control
T alone
T+SEE+ FDC
T+SEE+ FDC+exo HIV
T+SEE+FDC+HIV-IC
(1mg H902)
ACH-2

Figure 4b

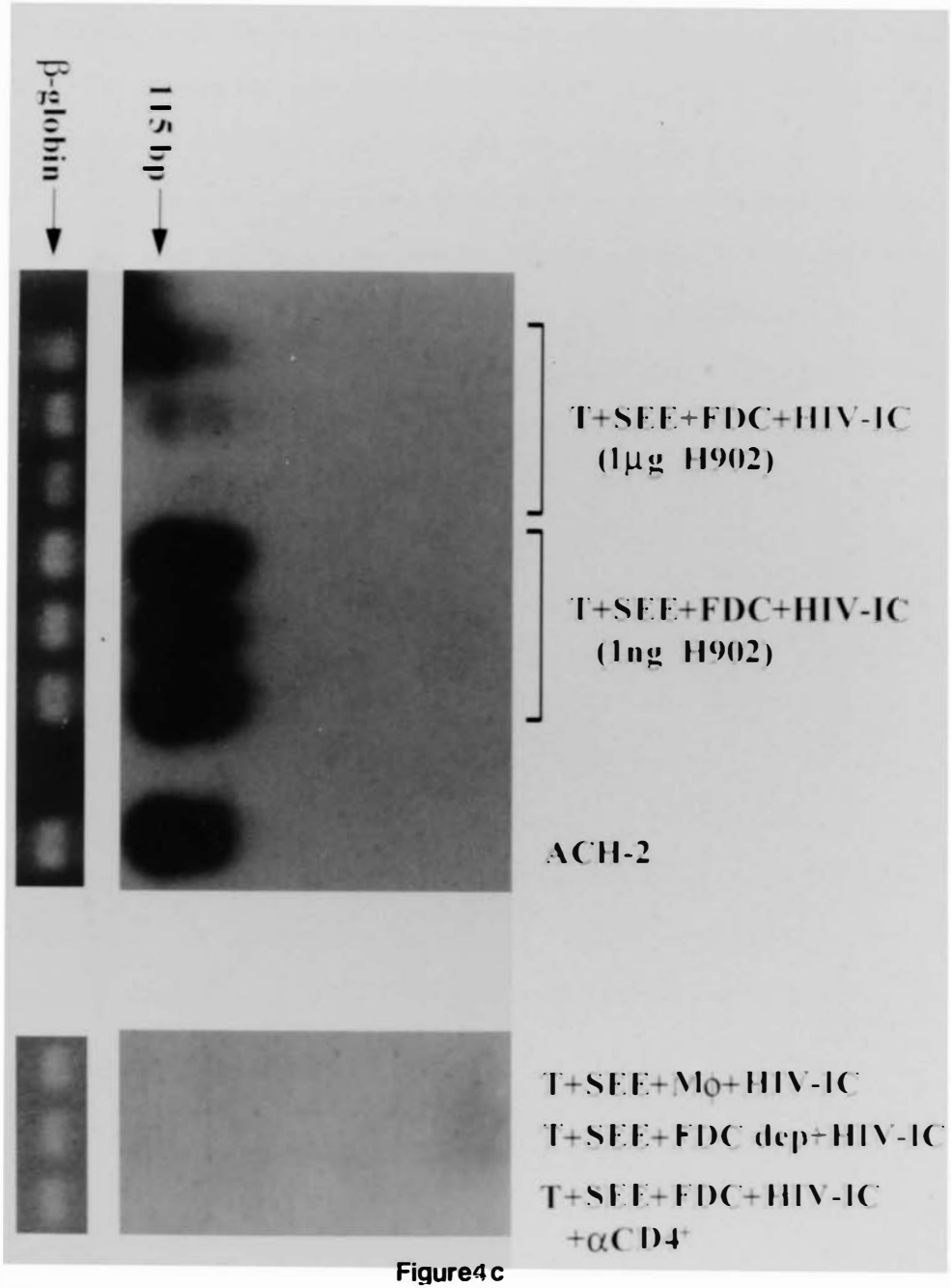
β-globin
115 bp
T+SEE+FDC+HIV-1C
(1μg H902)
T+SEE+FDC+HIV-1C
(1ng H902)
ACH-2
T+SEE+Mφ+HIV-1C
T+SEE+FDC dep+HIV-1C
T+SEE+FDC+HIV-1C
+αCD4+

Figure4 c

it did appear to decrease infection (Fig 4b&c). Cultures were also analyzed by electron microscopy and virus budding from the plasma membrane was observed (figure 5a-c).

Immune complexes were formed with lab isolates HIV-1_{IIIB}, HIV-1_{MN}, and HIV-1_{SF2} and the primary isolate 301714 and human neutralizing monoclonal antibodies (F105 or IgG1b12) in order to determine the general nature of infection by neutralized HIV on FDC (Fig 6). In the absence of FDC, HIV-1 immune complexes were unable to infect T cells regardless of the strain of HIV-1 used. However, when FDC were present, neutralized virus was able to infect the T cells indicating that this FDC mediated process was not restricted to a single antibody or HIV-1 strain. It is noteworthy that the lab isolate SF2, which is easily neutralized by antibody, is rendered infectious when FDC are present. Thus under conditions when antibody is particularly efficient at neutralizing virus, FDC can still negate its effect suggesting that this FDC activity is very potent.

Figure 5: Cultures of HIV-IIIB with neutralizing antibody, human T cells, and Human FDC were examined by electron microscopy. Large amounts of virus were observed budding from the plasma membrane.(a)magnification 5,500x; (b) 16,800x; (c)52,000x.

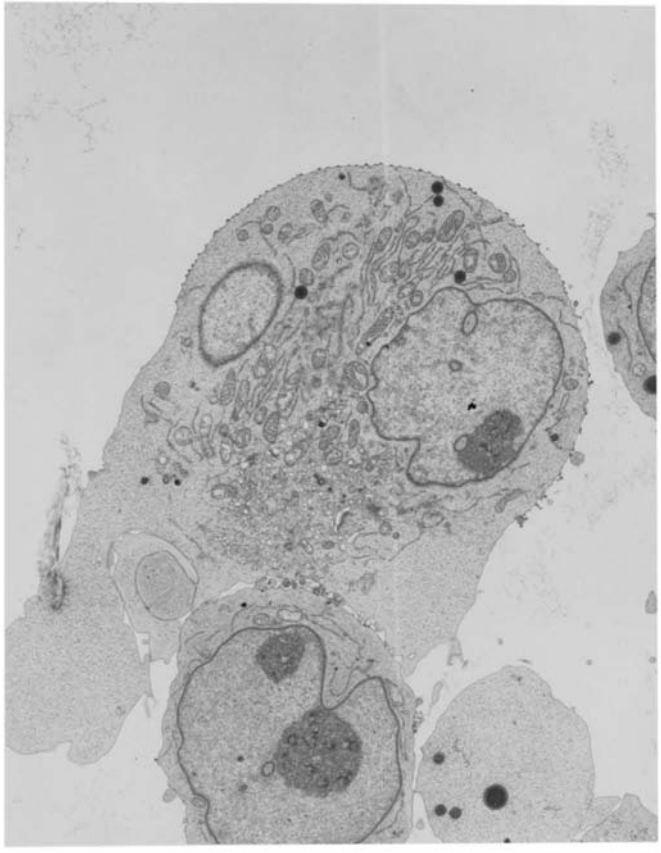

Figure 5a

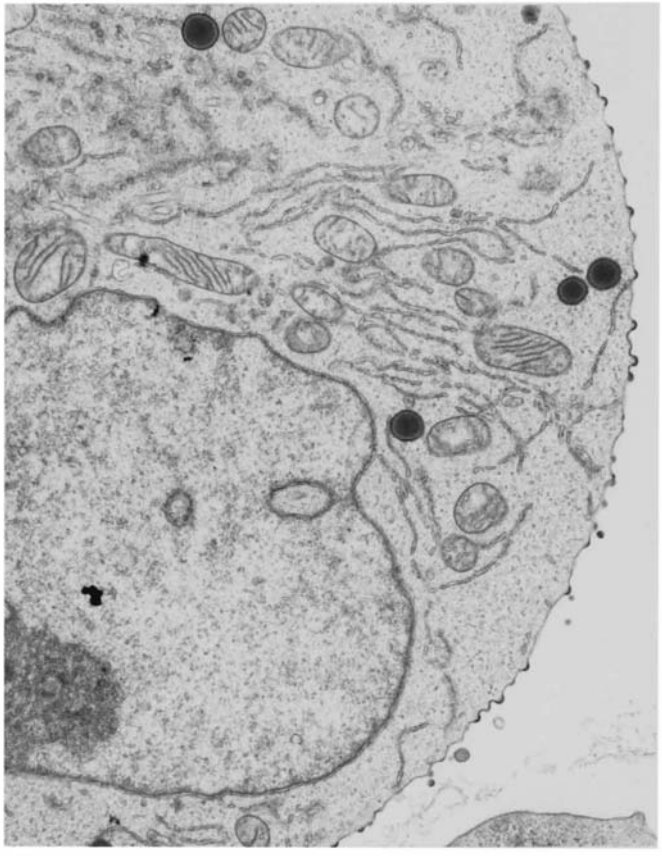

Figure 5 b

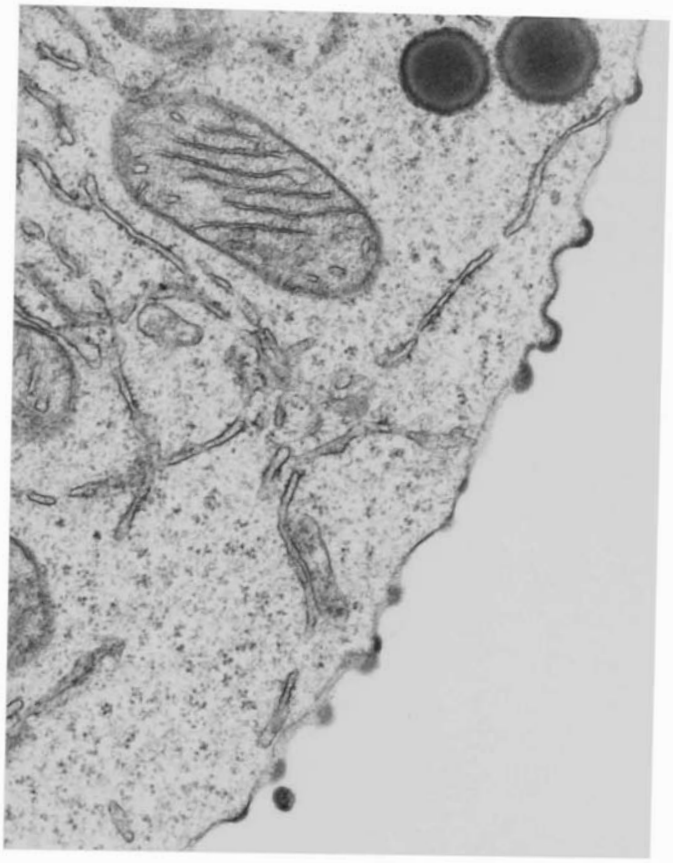

Figure 5c

Figure 6: FDC mediated infection by neutralized HIV-1 immune complexes is not restricted to viral strain or antibody. PCR analysis of HIV-1 *gag* proviral DNA (115bp) and β-globin in cultures containing various strains of neutralized HIV-1 immune complexes [HIV-IC (F105)] and SEE activated T cells (T+SEE)[$2.6x10^4$] or SEE activated T cells and human FDC ($5x10^4$). Infection by HIV-1 immune complexes formed with human, neutralizing anti-gp120 (1µg F105 or 20 mg IgG1b12) was blocked in the absence of FDC with HIV-1 strains IIIB, MN and SF2 as reported (ARRRP, Data Sheet)and with the primary strain 301714. In contrast, infection was apparent in cultures when FDC were present indicating that the FDC's ability to reverse the effect of neutralizing antibody was not restricted to a single strain of virus or monoclonal antibody.

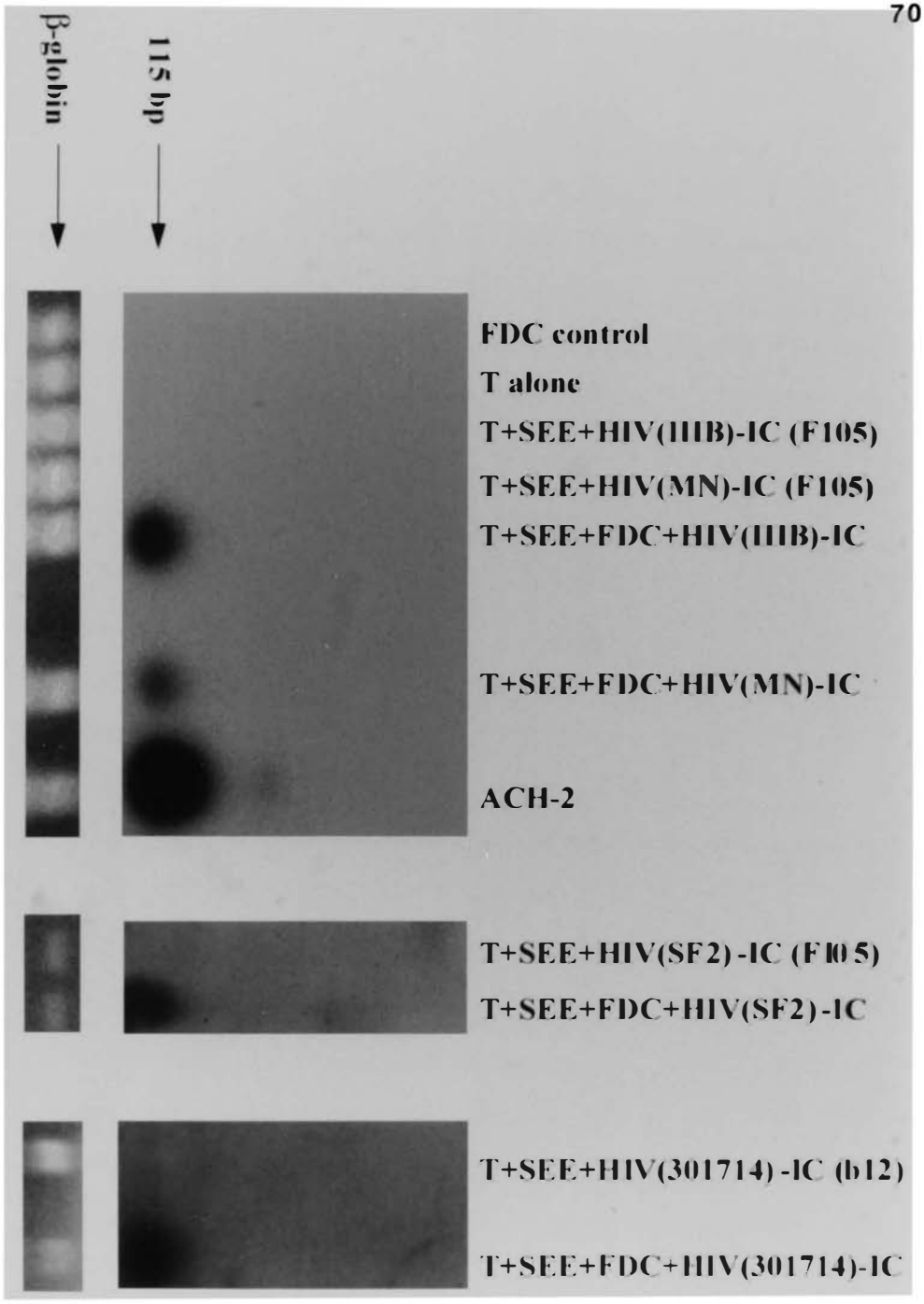
β-globin
115 bp
FDC control
T alone
T+SEE+HIV(IIIB)-IC (F105)
T+SEE+HIV(MN)-IC (F105)
T+SEE+FDC+HIV(IIIB)-IC
T+SEE+FDC+HIV(MN)-IC
ACH-2
T+SEE+HIV(SF2)-IC (F105)
T+SEE+FDC+HIV(SF2)-IC
T+SEE+HIV(301714)-IC (b12)
T+SEE+FDC+HIV(301714)-IC

Figure 6

Discussion

HIV is active and progressive in secondary lymphoid tissue during the clinically latent stage of disease. In particular, HIV is trapped, as are other antigens, on the dendritic processes of FDC in the form of immune complexes. This trapping involves antibody, complement, or both. Since the major site of active HIV infection throughout clinical latency is the germinal center surrounding HIV laden FDC, we hypothesized that the germinal center and FDC provide a unique environment which plays a major role in HIV infection. The objective of this thesis project was to begin to test this hypothesis by determining if HIV immune complexes on the surface of FDC were infectious.

The results indicate that HIV immune complexes trapped on the surface of FDC are highly infectious. HIV immune complex formation and subsequent virus trapping on FDC was performed *in vitro*(figure 2) and *in vivo* (figure 3). FDC were washed to remove unbound virus and then cultured (as the only source of virus) with susceptible CD4+ human T cells. In both systems infection was detected. Furthermore, infection was detected even when HIV immune complexes were formed with potent

neutralizing antibody indicating that FDC were able to negate the effect of the antibody, even when it was present in vast excess(figure 4 and 5). We believe that these results will have a significant impact on our understanding of HIV pathogenesis, and intervention strategies.

Infectivity of FDC trapped HIV

Our first experiment which tested the infectivity of HIV trapped on the dendritic processes of FDC (Figure 2) mimicked *in vivo* events in an *in vitro* culture system. We formed HIV immune complexes, using serum from an HIV infected individual (as a source of antibody), and then allowed these to be trapped by FDC. FDC bearing HIV were then cultured with human T cells over a four day period and proviral DNA was detected indicating that trapped virus was still infectious.

To more closely mimic *in vivo* conditions, we repeated this experiment using a xenogeneic model(murine) where immune complex formation and FDC localization would occur *in vivo*. This model of trapping virus on murine FDC was prompted by previous work using conventional antigen in which immunization of mice with specific antibody followed by antigen led to rapid immune complexes formation followed by FDC localization. In our system, we passively immunized mice with anti-gp120

followed by injection of HIV. After injection of HIV, we waited for five days prior to isolating FDC. Previous experiments with conventional antigen had shown that by this time, the only antigen remaining in the lymph node was that trapped by FDC. Therefore, after five days, murine FDC bearing HIV were removed and cultured with human T cells as previously done in figure 2. Again infection was clearly seen indicating that the virus remained infectious (Figure 3).

The results from the murine studies suggested that the environment that FDC provide is protective for the virus. We reason that within the mouse, immune complex formation and FDC localization would occur in a similar manner to that occurring in humans. However, since the mouse lacks cells bearing the human CD4 receptor, the virus cannot replicate. Therefore, in our murine model, the virus was able to remain infectious for four days before it could come in contact with susceptible cells to initiate viral replication. This is particularly significant when one considers the half life of the HIV in the absence of replication. HIV is estimated to have a half-life of six hours *in vivo*(Verbal communication- D. Ho; International Congress of Immunology Meeting 7/95). In our experiment, the mice were injected with 25,000 $TCID_{50}$,(5000 $TCID_{50}$ in each of five different sites). Calculations based on

the six hour half-life would suggest that by the end of the first six hours, 12,500 $TCID_{50}$ would remain in the mouse. In one day (i.e. four half-lives), the amount of virus would fall to 3,125 $TCID_{50}$ and by the end of the four day experiment, HIV would have undergone a decrease of sixteen half lives. If all the injected virus were trapped on FDC and if each FDC bearing virus could be isolated this would indicate a maximum of 6 $TCID_{50}$ that could be present in the entire mouse. We further reason that not all of the injected virus would have been trapped by FDC and that not all FDC would have been isolated. Thus, even though little if any virus would be expected to remain in the mouse for four days if it were not protected from immune clearance and/or inactivation, we saw substantial infection in our cultures! These data support the hypothesis that the FDC trapped virus is infectious and that FDC can protect that infectivity beyond what would be expected to be the normal lifespan of the virus. Thus, we reason that FDC may provide a substantial reservoir of infectious virus that is maintained for long periods of time.

The environment of the germinal center has a unique cellular composition which influences the events that occur there. The presence of FDC and their ability to trap antigen is important for HIV because FDC serve as a reservoir for

virus. As a reservoir, FDC may protect the virus from immune events which attempt to destroy the virus. Clearance of HIV by the immune system is mediated by a variety of immune responses. For example, cell mediated immunity including cytotoxic HIV specific T cells appear to play a significant role in reducing viremia during the acute phase of the disease. In addition, an antibody response is mounted against the virus which would facilitate immune clearance as well as follicular localization. Antibody may also result in destruction of infected cells by antibody dependent cellular cytotoxicity (ADCC) mediated by natural killer cells (NK).

Finally, the complement pathways may also contribute to decreasing viral load *in vivo*. These immune events and perhaps others help rid the body of circulating virus.

Furthermore, FDC may also protect FDC trapped immune complexes which could potentially activate complement from destruction via as yet unknown mechanisms. We reason that complement activation by FDC trapped HIV immune complexes might be predicted to result in destruction of the retained antigen along with the FDC network. However, since antigen (in the form of immune complex) is maintained for long periods of time, this supports the concept that FDC have a mechanism which prevents complement lysis. Thus, FDC and the germinal

center appear to provide a safe site for the virus to reside without being affected by the immune system's attempts at clearance.

In addition to providing a reservoir safe from the harsh environment, FDC may actively increase infectivity of the virus by further activating healthy T cells and maintaining their viability. Szakal, et. al., found that antigen trapped on FDC within a germinal center was not randomly retained on FDC but that a given antigen was trapped on a single FDC network in a highly ordered periodic fashion. Furthermore, it was observed that one germinal center could have more than one antigen retaining FDC network. In this fashion, germinal centers would have different antigenic specificities and several antigens could be found in one follicle (unpublished) [121,122]. Therefore, we reasoned that within a lymph node there are some germinal centers with FDC trapped HIV and some without depending on the stage of disease and the amount of virus being produced. We further envision that both types of germinal centers would play a role in increasing infection and maintaining cell viability. HIV infected individuals have CD4+ T helper cells which are both noninfected and latently infected (i.e., harbor proviral DNA). Cells from either group may be specific for HIV or another antigen. As the cells

enter various germinal centers, they may become activated as they interact with antigen presenting cells at that site. Once activated, they may receive a costimulatory signal from FDC. FDC have been shown to provide costimulatory signaling to both T and B cells which results in marked increase in proliferation[131,147].

We envision three unique scenario's in the lymph nodes of HIV infected individuals. First, a non-infected T cell can enter a germinal center with virus laden FDC. This cell, specific for another antigen, may become activated by B cells presenting that antigen. It can then receive costimulatory signals from FDC, become fully activated, and proliferate. Once in the activated state, the cell is highly susceptible to infection by the HIV trapped on FDC. The second scenario involves latently infected cells entering germinal centers with FDC lacking HIV on their processes. In this environment, the cell can again be activated by B cells presenting the required specific antigen followed by costimulation by FDC. Once fully activated, the infected cell will begin active replication of HIV and will proliferate thereby increasing viral load. Finally, in the last scenario, HIV specific latently infected cells enter a germinal center with HIV laden FDC. These cells will interact with processed antigen

expressed on B cells and will also receive FDC costimulation. As before, they will proliferate and replicate virus. In addition, these cells will be susceptible to superinfection by the HIV trapped on FDC. In all of these situations, FDC and their costimulatory signals are activating T cells, causing increased proliferation, increasing susceptibility to HIV, and ultimately contributing to the overall infection.

In addition to activating cells and causing proliferation, FDC are also able to maintain cell viability[147]. In previous work, cultures of B cells stimulated with anti-μ and IL-4 $\pm$ FDC both showed increased proliferation. Those cultures with FDC had a three-fold higher proliferative response. Along with this, FDC were able to maintain proliferation for several days. While cultures of B cells alone had little or no detectable proliferation after 4 days, those containing FDC maintained proliferation. Furthermore, examination by trypan blue exclusion indicated that after 3 days only 24% of the cells were viable in B cell cultures alone whereas nearly 100% were viable in cultures containing FDC. The ability of FDC to not only increase cell proliferation but also maintain viability may contribute to the maintenance of HIV infection within germinal centers during clinical latency.

The maintenance of cell viability may in part be attributable to the ability of FDC to decrease apoptosis (Xiong & Tew unpublished). In similar experiments, mitogen stimulated B cells were cultured with FDC or reducing agents such as thiols to further characterize the costimulatory effect. Both showed augmentation of proliferation above mitogen stimulation alone. Furthermore, thiols induced significantly higher levels of proliferation above that seen with FDC. After seven days of culture, cell viability was examined by microscopic analysis. While cultures containing FDC remained healthy and viable, those with thiols had few if any healthy cells left. Thiols were able to induce proliferation but cultures were highly apoptotic. FDC containing cultures, on the other hand, had lower levels of apoptotic cells.

To further elucidate this phenomenon, B cells and anti-fas antibody were cultured with or without FDC (Xiong & Tew unpublished). In cultures without FDC, fas mediated apoptosis occurred at a high frequency as expected. However, in cultures with FDC, apoptotic levels were greatly decreased suggesting that FDC, and microenvironment they provide, spare cells from death. We believe that FDC may prevent apoptosis in germinal center T cells in a similar manner.

Recent studies by Kupfer *et. al.* examined apoptosis in HIV infection by double staining immunohistochemistry[157]. Using one stain for HIV RNA and another for apoptosis, they found that in the lymph node those cells undergoing apoptosis were "in no case" productively infected (i.e. expressing viral message). They hypothesized that HIV infected T cells express a virally encoded protein which prevents apoptosis. We entertain another hypothesis envisioning that T cells, as they interact with FDC, not only become infected with FDC trapped HIV but also receive signals from FDC sparing them from apoptosis. If FDC are able to spare cells, some of which are infected, they may be inadvertently maintaining infection. By maintaining an environment which saves cells and induces proliferation, FDC may be increasing viral load of infected T cells within the germinal center.

Negation of neutralizing antibody by FDC

The second major finding of our work is that FDC can negate the effect of neutralizing antibodies. In our studies, the virus was incubated with several doses of neutralizing antibody ranging from one nanogram (ng) to one milligram (mg). At all doses of neutralizing antibody, the virus was unable to cause infection when cultured with activated T cells verifying

that the virus was fully neutralized. However, when FDC were added to the cultures, infection was detected indicating that FDC were able to render the neutralized immune complex into an infectious form. This phenomenon was not restricted by viral strain or antibody (figure 6).

These experiments utilized a wide range of neutralizing antibody doses (Figure 4). At this lowest neutralizing dose, 1 nanogram of neutralizing antibody, all of the virus was neutralized. In the presence of FDC the signal was very intense indicating high levels of infection. This impressive response was seen in all three cultures of this sample. Furthermore, the signal was much more intense than that of the positive control ACH-2 containing 50,000 cells each with one copy of proviral DNA. This indicates that FDC were able to take fully neutralized viral immune complexes and render them highly infectious such that infection/integration occurred in greater than 50,000 cells.

Although the intensity of the signal was decreased considerably when 1µg or 1 mg of antibody was present, the signal was still apparent indicating that neutralizing antibody when present in vast excess could not prevent infection. Milligram levels of neutralizing antibody are not found naturally in infected individuals but we wanted to

determine if there was a threshold level at which the effects of FDC would be lost. Milligram levels of antibody did not achieve this. FDC were still able to negate the effect of the antibody.

Although FDC in our system are able to negate the effects of the neutralizing antibody in all cases shown, our data also suggests that increasing titers of neutralizing antibody are beneficial. Figure 4 shows decreasing signal representative of HIV infection in the presence of increasing doses of neutralizing antibody. This suggests that high levels of antibody may dampen the viral load in individuals infected with the virus.

The ability of FDC to facilitate HIV infection in the presence of neutralizing antibody may help to explain the reason some individuals with high titer neutralizing antibody have ongoing infection. It may also help explain the reason that much of the viral replication occurring during the clinically latent stage of HIV infection is confined to lymphoid follicles where virus laden FDC reside in intimate contact with germinal center T and B cells. Although the mechanism converting neutralized virus into an infectious form by FDC is unclear, there are similarities to events which occur in anamnestic responses where antigens are trapped by

FDC in the form of immune complexes. Even though antibody should "mask" epitopes and prevent recognition by the immunoglobulin receptors of B cells, experiments have shown that antigen-specific B cells are able to recognize the immune complexed antigen in vast antibody excess[158,159]. We envision a similar process in the case of FDC trapped HIV. The V3 loop of HIV-1 gp160 would be potentially "masked" by neutralizing antibody but the FDC can display the virus in a manner that gp120 can bind CD4 on adjacent T cells and thus cause infection. The finding that FDC can convert neutralized HIV immune complexes into an infectious form may have important implications for the design of therapeutic and vaccine strategies, regardless of the mechanism(s) involved.

Neutralization Assays

Currently there are three main protocols for researching neutralization of HIV. Common *in vitro* methods culture virus, neutralizing antibody, and susceptible T cells prior to assaying for viral integration and/or p24 levels. Infectivity can be measured by assaying for integrated proviral DNA using PCR analysis as used in these studies. Primers specific for *gag* are chosen since *gag* is among the last part of the genome transcribed prior to integration. Detection of *gag* usually

indicates that a full provirus has been integrated. The other method assays for the presence of p24 in culture supernatants by ELISA. P24 can be directly correlated to infectivity but this value may misrepresent infectivity since p24 can also be generated from defective virus particles.

The most recent *in vivo* model generated to study HIV pathogenesis is the hu-HIV/PBL-SCID mouse[157]. The SCID mouse lacks murine T and B cells and mature FDC. In the hu-HIV/SCID model, mice are engrafted with human PBL and therefore can maintain HIV infection and replication. The advantage of this system is the presence of the characteristic CD4+ T cell depletion during active infection. This model has been utilized to study some therapies including anti-viral agents such as nucleoside analogs. Studies using this model and treatment demonstrated increases in circulating T cells *in vivo.* This model is expected to be a useful technique for the study of neutralizing antibody and other potentially beneficial therapies.

The above described protocols neglect to address the role of FDC and the germinal center. Our assay demonstrated effective neutralization in the absence of FDC yet in their presence effective neutralization was not observed. Since FDC appear to have an important role in the maintenance of HIV

infection, protocols must include an accurate representation of *in vivo* conditions, including germinal centers with FDC, as our *in vivo* and *in vitro* models do. Culturing *in vitro* systems with and without FDC will allow researchers to address all aspects of disease stages. *In vivo* methodologies must also not exclude the potential role of FDC. Models like the hu-HIV/PBL-SCID do not accurately represent *in vivo* conditions. Furthermore, reconstitution of human lymph nodes with germinal centers and functional FDC is near impossible (personal communication, M. Connners). Without addressing the role of FDC the research neglects many of the events surrounding the late acute stage, all of the clinically latent stage, and much of AIDS. A full understanding of pathogenesis requires an understanding of these events.

Vaccination Strategies

We believe the data presented here may also cause us to reshape our thinking of current vaccination strategies. When developing a vaccine against any microbial agent one of the first questions asked is: Which immune response mechanisms are protective? This knowledge is fundamental in the engineering of vaccines. Vaccines attempt to prime the immune response so that subsequent exposure to the organism will result in a more

rapid vigorous response. Not only do we not know which response are protective, but we also do not know which responses prevent sequestration of the virus which prefaces clinical latency. More research on the pathogenesis of HIV will bring us closer to answering these questions.

Current vaccination attempts focus primarily on blocking viral entry into susceptible cells by eliciting an anti-gp120 antibody response. In fact, the first tested vaccine was recombinant gp120 which attempted antibody mediated prevention. None of these trials were successful. Since then, gp120 has been mapped and humoral epitopes have been determined. Many researchers are now focusing on one area, the V-3 loop, which contains a neutralizing domain. Many patients produce high levels of neutralizing antibody yet infection persists. Furthermore, there is no solid correlation between neutralizing antibody titers and disease progression. Antibody responses normally prevent viral infection. However, data presented here demonstrates that viral-antibody complexes are still infectious, even in the presence of high levels of neutralizing antibody. Vaccines which attempt to elicit a strong antibody response therefore may provide little or no protection.

Some vaccines not only fail to protect but also enhance

disease. This has been the case in several diseases including respiratory syncytial virus and measles in humans. In addition, other lentiviruses have shown this feature including arthritis-encephalitis virus in goats and visna-maedi virus in sheep[160]. A vaccine for HIV which elicits an antibody response may also result in enhancing infection. Some people who are exposed to HIV fail to become infected. Cytotoxic T lymphocytes and complement mechanisms are the first responses to HIV known to clear the initial viremia. It is conceivable that some people are able to clear the virus quickly before it becomes trapped on the FDC. If vaccination which elicited circulating antibodies against HIV were available, it is possible that more people would become infected after exposure. The circulating antibodies would become complexed with the virus and would quickly be carried to the most proximal lymph node. Here the viral complex would be trapped on FDC and be retained for several years. The virus would be in a environment which makes it more immunogenic and brings it close to highly activated CD4+ T cells. The long term slow progressive disease would begin.

Rather than engineer vaccines to elicit an antibody response, we believe that a CMI response would be more protective in light of the data presented here. As previously

stated, the design of vaccines should attempt to mimic the natural immune response which results in protection. The first responses which occur in HIV infection are CD8 lymphocyte and complement mediated events. Although these responses are not wholly protective, they are successful at clearing the initial viremia.

Additional data also supports the hypothesis that the cellular immune response is protective. HIV-specific T cell responses have been detected in human and animals who have been multiply exposed to HIV or SIV, respectively. Several high risk or HIV exposed populations have been studied including gay men, intravenous drug users, accidentally exposed health workers, and newborns of HIV infected mothers[161-163](Pinto; manuscript submitted for publication). PBMC from these people were challenged with HIV envelope synthetic peptides. In the groups, 35% to 75% of the individuals responded to at least two of the five peptides as compared to controls which responded 0-2%. More recently, CD8+ CTL's specific for NEF were detected in women partners of HIV+ men[164]. Studies using the animal model SIV in macaques demonstrates that low dose exposure induces a cellular response but not a humeral response. Later challenge with SIV resulted in strong T proliferative responses, no

seroconversion, no detectable provirus by PCR analysis, and no disease whereas controls, not receiving the subinfectious dose previously, did seroconvert and develop disease[165].

In addition, some cell mediated activities have been correlated with a positive prognosis. Long term survival has recently been associated with a strong cellular immune response characterized by anti-HIV suppressor activities[166]. This suppressor activity is believed to prevent viral replication and viral expression. Furthermore, it may prevent some of the harmful CTL responses. These functions may be important in maintaining virological latency and thereby allowing normal T cell function. These data and our results support the hypothesis that a cellular response is protective and that vaccinations should be engineered to elicit a cellular response as opposed to a humoral response.

Treatment Strategies

We believe the data presented here will also benefit those already infected with HIV as it may restructure treatment strategies. Although the immune response ultimately fails at defeating the virus, it is effective at maintaining low viremia levels for several years. The antibody response is believed to contribute both to the initial clearing of the

virus and to the maintenance of low plasma virus levels throughout clinical latency. The repertoire of antibodies which exist often includes neutralizing antibodies. Neutralizing antibodies are usually detectable within one year of the acute phase[167,168]. Although high titers of neutralizing antibody do not correlate with prevention of AIDS and death[169], some researchers have correlated them with good short term prognosis[170]. Furthermore, infected mothers who did not transmit the virus to their babies were reported to have better neutralizing capabilities[171-174]. This suggests that the antibody response does play a beneficial role. In addition to this, our data also suggest that increasing titers of neutralizing antibody are beneficial(Figure 4).

Treatment for HIV should continue to include the use of antibodies especially those which may be neutralizing since they may result in some protection. However, we believe that these antibodies should be altered to prevent interaction with FDC via F_c or complement receptors. Viral immune complexes which do not become trapped on FDC do not contribute to the existing reservoir of virus. Instead these complexes may be cleared from the system via phagocytosis. This type of therapy may slow progression of the disease and delay onset of

AIDS. The viral burden within the germinal center would take longer to develop therefore follicular hyperplasia, destruction of FDC, and immunosuppression leading to AIDS would be delayed.

To be effective, treatment antibodies should not bind to F_c receptors. For example, Fab molecules could be produced from neutralizing antibodies. These would bind to and neutralize the virus in the same manner as whole antibody. They could not, however, bind to FDC without their F_c region. One potential drawback of Fab molecules is their short half life. Alternatively, "designer antibodies" could be developed which have a mutated F_c region. These molecules again would lack the ability to bind F_c receptors but they have the added benefit that they will remain in circulation longer than Fab fragments and will therefore be of greater clinical value. As patients progress from HIV asymptomatic to AIDS they experience a variety of impairments in the immune systems including a decline in neutralizing antibody titers. Immunizations with the described designer antibodies in conjunction with other treatments which decrease viral load such as AZT, ddI, and protease inhibitors may prolong the clinically latent stage of disease.

The results reported here will bring us closer to

understanding how the virus is able to use the host to its advantage. The finding that FDC are able to convert neutralized virus into an infectious form increases our understanding of HIV pathogenesis and the clinically latent stage. The implications of this research may help explain failure in previous treatment and vaccine attempts and enlighten us to improved designs for the future.

Bibliography

Bibliography

1. Abbas AK, Lichtman AH, Pober JS. Congenital and Acquired Immunodeficiences. In: Wonsiewicz MJ, editor. Cellular and Molecular Immunology. Philadelphia: W. B. Saunders Company, 1991:386-394.

2. Dalgleish AG, Beverly PCL, Clapham PR, Crawford DH, Greaves MF, Weiss RA. The CD4(T4) antigen is an essential component of the receptor for the AIDS virus. Nature 1984; 312:763-676.

3. Klatzmann D, Champagne E, Chamaret S, Gruest J, Guetard D, Hercend T, et al. T lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature 1984; 312:767-768.

4. McDougal JS, Kennedy MS, Sligh JM, Cort SP, Mawle A, Nicholson JKA. Binding of HTLV-III/LAV to T4 positve T cells by a complex of the 110K viral protein and the T4 molecule. Science 1986; 231:382-365.

5. Schnittman SM, Psallidopoulos MC, Lane HC. The reservior for HIV-1 in human peripheral blood is a T cell that maintians expression of CD4. Science 1989; 245:305-308.

6. Embretson J, Zupanic M, Beneke J. Analysis of human immunodeficiency virus infected tissues by amplification and in situ hybridization reveals latent and permissive infections at single cell resolution. Proc.Natl.Acad.Sci.USA 1993; 90:357-361.

7. Ahmad N, Venkatasan S. Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. Science 1988; 241:1481.

8. Luciw PA, Cheng-Mayer C, Levy JA. Mutational analysis of the human immunodeficiency virus: The orf-B region down regulates virus repilcation. Proc.Natl.Acad.Sci.USA 1987; 84:1434.

9. Niederman TMJ, Thielan BJ, Ratner L. Human immunodeficiency virus type-1 negative factor is a transcriptional silencer. Proc.Natl.Acad.Sci.USA 1989; 86:1128.

10. Kestler HW, Ringler DJ, Mori K. Importance of the nef gene for the maintenance of high virus loads and for the development of AIDS. Cell 1991; 65:651.

11. Anderson S, Srugers DC, Swanstrom R. Nef from primary isolates of human immunodeficiency virus type 1 suppresses surface CD4 expression in human and mouse T cells. J Virol 1993; 67:4923.

12. Rhee SS, Marsh JW. Expression of HIV nef protein in human T cells induces rapid internalization of surface CD4. J Cell Biochem 1994; S18.

13. Benson RE, Sanfridson A, Ottinger JS. Downregulation of surface CD4 expression by simian immunodeficiency virus nef prevents viral superinfection. J Exp Med 1993; 177:1561.

14. Garcia JV, Miller AD. Serine phosphorylation-independent downregulation of cell surface CD4 by nef. Nature(Lond.) 1991; 350:508-11.

15. Kao SY, Calman AF, Luciw PA, Peterlin BM. Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. Nature(Lond.) 1987; 330:489-493.

16. Feinberg MB, Baltimore D, Frankel AD. The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation Proc.Natl.Acad.Sci.USA 1991; 77:4045.

17. Laspia MF, Rice AP, Mathews MB. HIV-1 tat protein increases transcriptional initiation and stabilizes elongation. Cell 1989; 59:283.

18. Sodroski J, Goh WC, Rosen C, Dayton A, Terwillinger E, Haseltine W. a second post-transcriptional transactivator gene required for HTLV-III replication. Nature(Lond.) 1986; 321:412-417.

19. Feinberg MB, Jarrett RF, Aldovini A, Gallo RC, Wong-Staal F. Cell 1986; 46:807-817.

20. Pomerantz RJ, Trono D, Feinberg MB, Baltimore D. Cells nonproductively infected with HIV-1 exhibit an aberrant pattern of viral RNA expression: a molecular model for latency. Cell 1990; 61:1271-1276.

21. Constantoulakis PM, Campbell BK, Felber BK, Nasioulas G, Afonia E, Pavlakis GN. Inhibition of Rev-mediated HIV-1

expression by an RNA binding protein encoded by the interferon-inducible 9-27 gene. Science 1993; 259:1314-1318.

22. Mervis RJ, Ahmad N, Lillehoj EP, Raum HW, Salazer FHR, Chan HW, et al. The gag gene products of human immunodeficieancy virus type 1: alignment within the gag open reading frame, identification of posttranslational modfications, and evidence for alternative gag precursors. J Virol 1988; 62:3993-4002.

23. Yu X, Yu QC, Lee TH, Essex M. The C terminus of human immunodeficiency virus type 1 matrix protein is invovled in early steps of the virus life cycle. J Virol 1992; 66:5667-5670.

24. Dupraz P, Spahr PF. Specificity of Rous sarcoma virus nucleocapsid protein in genomic RNA packaging. J Virol 1992; 66:4661-4670.

25. Lillehoj EP, Salazer FHR, Mervis RJ, Raum HW, Chan HW, Ahmad N, et al. Purification and structural characterization of the putative gag-pol protease of human immunodeficiency virus. J Virol 1988; 63:111-121.

26. Mous J, Heimer EP, Le Grice SFJ. Processing protease and reverse transcriptase from human immunodeficiency virus type 1 polyprotein in Escherichia coli. J Virol 1988; 62:1433-1436.

27. Park J, Morrow CD. Overexpression of the gag-pol precursor from human immunodeficiency virus type 1 proviral genomes results in efficient protelytic processing in the abscence of virion production. J Virol 1991; 65:5111-5117.

28. Peng C, Ho BK, Chang TW, Chang NT. Role of human immunodeficiency virus type-1 specific protease in core protein maturation and viral infectivity. J Virol 1989; 63:2550-2556.

29. Fujiwara T, Craigie R. Integration of mini retroviral DNA: a cell free reaction for biochemistry analysis of retroviral integration. Proc.Natl.Acad.Sci.USA 1989; 86:3065-3069.

30. Von Schwedler U, Song J, Aiken C, Trono D. Vif is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. J Virol 1993; 67:4945-4955.

31. Hansen J, Schulze W, Moelling K. Rnase H activity associated with bacterially expressed reverse transcriptase of

human T-cell lymphotropic virus III/lumphadenopathy-associated virus. J Biol Chem 1987; 262:12393-12396.

32. Hansen J, Schulze W, Mellert W, Moelling K. Identification and characterization of HIV-specific RNase H by means of monoclonal antibodies. EMBO J 1988; 7:239-243.

33. Brown PO. Integration of retroviral DNA. In: Swanstrom R, Vogt PK, editors. Retroviruses, Strategies of Replication. New York: Springer, 1990:19-48.

34. Grandgenett DP, Mumm SR. Unraveling retrovirus integration. Cell 1990; 60:3-4.

35. Van Gent D, Elgersma Y, Vink C, Plasterek RHA. DNA binding properties of the integrase proteins of human immunodeficiency virus types 1 and 2. Nucleic Acids Res 1991; 19:3821-3827.

36. Bushman FD, Craigie R. Activities of human immunodeficiency virus (HIV) integration protein in vitro: specific cleavage and integration of HIV DNA. Proc.Natl.Acad.Sci.USA 1991; 88:1339-1343.

37. Craigie R, Mizuuchi D, Bushman FD, Engelman A. A rapid in vitro assay for HIV DNA integration. Nucleic Acids Res 1991; 19:2729-2734.

38. Vink C, Van Gent D, Elgersma Y, Plasterek RHA. Human immunodeficiency Virus integrase protien requires a sunterminal position of its viral DNA recognition sequence for cleavage. J Virol 1991; 65:4636-4644.

39. Planelles V, Jowett J, Bachelerie F, Haislip A, Xie Y, Chen ISY. The fate of the HIV-1 provirus in infected cells: a novel role for vpr. 1995; J Cell Biochem - Keystone Symposia on Molecular & Cellualar Biochemistry S21B:19540. Willey RL, Maldarelli F, Martin MA. Human immunodeficiency virus Type-1 Vpu protein induces rapid degradation of CD4. J Virol 1992; 66:7193.

41. Willey RL, Maldarelli F, Martin MA. Human immunodeficiency virus type-1 Vpu protein regulates the formation of intracellular gp160-CD4 complexes. J Virol 1992; 66:226.

42. Klimkait T, Strebel K, Hoggan MD. The human immunodeficiency virus type 1-specific protein vpu is required for efficient virus maturation and release. J Virol 1990; 64:3708.

43. Jones KA, Luciw PA, Duchange N. Structural arrangements of transcription control domains within the 5'-untranslated leader regions of the HIV-1 and HIV-2 promoters. Genes Dev 1988; 2:1101-1114.

44. Kato H, Horikoshi M, Roeder RG. Repression of HIV-1 transcription by a cellular protein. Science 1991; 251:1476-1479.

45. Garcia JA, Wu FK, Mitsuyasu R, Gaynor RB. Interactions of cellular proteins involved in the transcriptional regulation of the human immunodeficiency virus. EMBO J 1987; 6:3761-3770.

46. Lu YC, Touzjian N, Stenzel M, Dorfman T, Sodroski J, Haseltine WA. Identification of cis-acting repressive sequences within the negative regulatory element of human immunodeficiency virus type 1. J Virol 1990; 64:5226-5229.

47. Siekevitz M, Josephs SF, Dukovich M, Peffer N, Wong-Staal F, Greene WC. Activation of the HIV-1 LTR by T cell mitogens and the tranactivator protein of HTLV-1. Science 1987; 238:1575-1578.

48. Sodroski J, Goh WC, Rosaen CA, Salahuddin SZ, Aldovini A, Franchini G, et al. Transactivation of the human T-cell leukemia virus long terminal repeat correlates with expression of the x-lor protein. J Virol 1985; 55:831-835.

49. Gartner S, Markovits P, Kaplan MH, Gallo RC, Popovic M. The role of mononuclear phagocytes in HTLV-iii/LAV infection. Science 1986; 233:215-219.

50. Lyerly HK, Cohen OJ, Weinhold KJ. Transmission of HIV by antigen presenting cells during T-cell activation: prevention by 3'-azido,3'-deoxythymidine. AIDS Res Hum Retroviruses 1987; 3:87-94.

51. Lifson JD, Reyes GR, McGrath MS, Stein BS, Engleman EG. AIDS retrovirus induced cytopathology: giant cell formation and involvement of CD4 antigen. Science 1986; 232:1123-1127.

52. Lifson JD, Feinberg MB, Reyes GR, Rabin L, Banapour B, Chakrabarti S, et al. Induction of CD4-dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. Nature 1986; 323:725-728.

53. Tersmette M, Lange JM, de Goerde RE. Association between biological properties of human immunodeficiency virus variants and risk for AIDS and AIDS mortality. Lancet 1989; 1:983-985.

54. Tersmette M, Gruters RA, de Wolf F. Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: studies of sequential HIV isolates. J Virol 1989; 63:2118-2125.

55. elAhmer W, Poumbourios P, McPhee DA, Kemp BE. N-terminal residues 105-117 of HIV-1 gp120 are not involved in CD4 binding. AIDS Res Hum Retroviruses 1991; 7:855-858.

56. Pollard SR, Meier W, Chow P, Rosa JJ, Wiley DC. Cd4 binding regions of human immunodeficiency virus envelope glycoprotein gp120 defined by proteolytic digestion. Proc.Natl.Acad.Sci.USA 1991; 88:11320-11324.

57. Pollard SR, Rosa JJ, Rosa MD, Wiley DC. Truncated variatns of gp120 bind CD4 with high affinity and suggest a minimum CD4 binding region. EMBO J 1992; 11:585-591.

58. La Rosa GJ, Davide JP, Weinhold KJ. Conserved sequence and structural elements in the HIV-1 principle neutralizing determinant. Science 1990; 249:932-935.

59. Fauci AS, Lane HC. The acquired immunodeficiency syndrome (AIDS). In: Wilson JD, Braunwald E, Isselbacher KJ, editors. Harrison's principles of internal medicine. 12th ed. New York: McGraw-Hill, 1991:1402-1410.

60. Lemp GF, Payne SF, Neal D, Temelso T, Rutherford GW. Survival trends for patients with AIDS. JAMA 1990; 263:402-406.

61. Tindall B, Cooper DA. Primary HIV infection: Host responses and intervention strategies. AIDS 1991; 5:1-14.

62. Clark SJ, Saag MS, Decker WD, Campbell-Hill S, Roberson JL, Veldkamp PJ, et al. High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection. New Engl J Med 1991; 324:954-960.

63. Daar ES, Moudgil T, Meyer RD, Ho DD. Transient high levels of viremia in patients with primary human immunodeficiency virus type I infection. New Engl J Med 1991; 324:961-964.

64. Pantaleo G, Graziosi C, Demarest JF, Butini L, Montroni M, Fox CH, et al. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature 1993; 362:355-358.

65. Kroese FGM, Timens W, Nieuwenhuis P. In: Grundman E, Vollmer E, editors. Reaction Patterns of the Lymph Node. Springer-Verlag, 1990:103-148.

66. Fox CH, Tenner-Racz K, Racz P, Firpo A, Rizzo PA, Fauci AS. Lymphoid germinal centers are reserviors of human immunodeficiency virus type 1 RNA. J Infect Dis 1991; 164:1051-1057.

67. Pantaleo G, Butini L, Graziosi C. Human immunodeficiency virus (HIV) infection in CD4+ T lymphocytes genetically deficient in LFA-1: LFA-1 is required for HIV-mediated cell fusion but not for viral transmission. J Exp Med 1991; 173:511-514.

68. Hildreth JEK, Orentas RJ. Involvement of leukocyte adhesion receptor (LFA-1) in HIV-induced syncytium formation. Science 1989; 244:1075-1078.

69. Garry RF. Potential mechanisms for the cytopathic properties of HIV. AIDS 1989; 3:683-694.

70. Rosenburg ZF, Fauci AS. The immunopathogenesis of HIV infection. Adv Immunol 1989; 47:377-431.

71. Rosenburg ZF, Fauci AS. Immunopathogenesis of HIV infection. FASEB J 1991; 5:2382-2390.

72. Rosenburg ZF, Fauci AS. Immunopathogenic mechanisms of HIV infection: cytokine induction of HIV expression. Immunol Today 1990; 11:176-180.

73. Biberfeld P, Ost A, Porwit A, Sandstedt B, Pallsen G, Bottinger B, et al. Histopathology and immunohistology of HTLV-III/LAV related lymphadenopathy and AIDS. ActaPatholMicrobiolImmunolScand 1987; 95:47-65.

74. Armstrong JA. Ultrastructure and significance of the lymphoid tissue lesions in HIV infection. In: Racz P, Dijkstra CD, Gluckman JC, editors. Accessory Cells in HIV and Other Retroviral Infections. Basel: Karger, 1991:69-82.

75. Tenner-Racz K, Racz P, Bofill M. HTLV-III/LAV viral antigens in lymph nodes of homosexual men with persistentgeneralized lymphadenopathy and AIDS. Am J Pathol 1986; 123:9-15.

76. Tenner-Racz K, Racz P, Dietrich M, Kern P. Altered follicular dendritic cells and virus-like particles in AIDS and AIDS related lymphadenopathy. Lancet 1985; 1:105-106.

77. Pantaleo G, Graziosi C, Butini L, Pizzo PA, Schnittman SM, Kotler DP, et al. Lymphoid organs function as major reservoirs for human immunodeficiency virus. Proc.Natl.Acad.Sci.USA 1991; 88:9838-9842.

78. Pantaleo G, Fauci AS. New concepts in the immunopathopgenesis of HIV infection. Ann Rev Immunol 1995; 13:487-512.

79. Terai C, Kornbluth RS, Pauza CD, Richman DD, Carson DA. Apoptosis as a mechanism of cell death in cultured T lymphoblasts acutely infected with HIV-1. J Clin Invest 1991; 87:1710-1715.

80. Laurent-Crawforg AG, Krust B, Muller S. The cytopathic effect of HIV is associated with apoptosis. Virology 1991; 185:829-839.

81. Amiesen JC, Capron A. Cell dysfunction and depletion in AIDS: The programmed cell death hypothesis. Immunol Today 1991; 12:102-105.

82. Groux H, Torpier G, Monte D, Mouton Y, Capron A, Amiesen JC. Activation induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals. J Exp Med 1992; 175:331-340.

83. Abbas AK, Lichtman AH, Pober JS. Cells and Tissues of the Immune System. In: Wonsiewicz MJ, editor. Cellular and Molecular Immunology. Philadelphia: W.B. Saunders Company, 1991:26.

84. Kroese GM, Wubenna A, Seijen HG, Nieuwenhuis P. Germinal Centers develop oligoclonally. Eur J Immunol 1995.

85. Jacob J, Kelsoe G, Rajewsky K, Weiss U. Intraclonal generation of antibody mutants in germinal centres. Nature 1991; 354:389-392.

86. Jacob J, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. a common clonal origin for PALS-associated foci and germinal centers. J Exp Med 1992; 176:679-687.

87. Berek C, Berger A, Apel M. Maturation of the immune response in germinal centers. Cell 1991; 67:1121-1129.

88. Kallberg E, Gray D, Leanderson T. Analysis of somatic mutation activity in multiple Vk genes invovled in the response to 2-phenyl-5-oxazalone. Int Immunol 1993; 5:573-82.

89. Liu YJ, Joshua D, Williams GT, Smith C, Gordon J, Maclennan I. Mechanism of antigen-driven selection in germinal centres. Nature 1989; 342:929-931.

90. Coico RF, Bhogal BS, Thorbecke GJ. Relationship of germinal centers in lymphoid tissue to immunologic memory VI. Transfer of B cell memory with lymph node cells fractionated according to their receptors for peanut agglutinin. J Immunol 1983; 131:2254-2257.

91. Klaus GGB, Humphrey JH, Kunkl A, Dongworth DW. The follicular dendritic cell: its role in antigen presentation in the generation of immunological memory. Immunol Rev 1980; 53:3-28.

92. Benner R, Hijmans W, Haaijman JJ. The bone marrow: the major source of serum immunoglobulins, but still a neglected site of antibody formation. Clin Exp Immunol 1981; 46:1-8.

93. Kosco MH, Burton GF, Kapasi ZF, Szakal AK, Tew JG. Antibody-forming cell induction during an early phase of germinal centre development and its delay with ageing. Immunology 1989; 68:312-318.

94. Tew JG, DiLosa RM, Burton GF, Kosco MH, Kupp LI, Masuda A, et al. Germinal centers and antibody production in bone marrow. Immunol Rev 1992; 126:1-14.

95. Humphrey JH. The fate of antigen and its relationship to the immune response; the complexity of antigens. Antibiot Chemother 1969; 15:7-23.

96. Miller JFAP, Mitchell GF. Thymus and antigen-reactive cells. Transplant Rev 1969; 1:3-42.

97. Tew JG, Thorbecke GJ, Steinman RM. Dendritic cells in the immune response: characteristics and recommended nomenclature. JReticuloendothelialSociety 1982; 31:371-380.

98. Schnizlein CT, Kosco MH, Szakal AK, Tew JG. Follicular dendritic cells in suspension: identification, enrichment, and initial characterization indicating immune complex trapping

and lack of adherence and phagocytic activity. J Immunol 1985; 134:1360-1368.

99. Tew JG, Kosco MH, Burton GF, Szakal AK. Follicular dendritic cells as accessory cells. Immunol Rev 1990; 117:185-211.

100. Szakal AK, Gieringer RL, Kosco MH, Tew JG. Isolated follicular dendritic cells: cytochemical antigen localization, Nomarski, SEM, and TEM morphology. J Immunol 1985; 134:1349-1359.

101. Humphrey JH, Grennan D. Isolation and properties of spleen follicular dendritic cells. In: Nieuwenhuis P, van der Broek AA, Hanna MG, Jr. editors. In vivo immunology. New York: Plenum Press, 1982:823-827.

102. van der Valk P, van der Loo EM, Daha MR, Meijer CJ. Analysis of lymphoid and dendritic cells in human lymph node, tonsil and spleen a study using monoclonal and heterologous antibodies. Virchows Arch 1984; 45:169.

103. Schriever F, Freedman AS, Freeman G, Messner E, Lee G, Daley J, et al. Isolated human follicular dendritic cells display a unique antigenic phenotype. J Exp Med 1989; 169:2043.

104. Sellheyer K, Schwarting R, Stein H. Isolation and antigenic profile of follicular dendritic cells. Clin Exp Immunol 1989; 78:431.

105. Petrasch S, Perez AC, Schmitz J, Kosco MH, Brittinger G. Antigenic phenotyping of human follicular dendritic cells isolated from nonmalignant and malignant lymphatic tissue. Eur J Immunol 1990; 20:1013-1018.

106. Gerdes J, Stein H, Mason DY, Ziegler A. Human dendritic reticulum cells of lymphoid follicles: their antigenic profile and their identification as multinucleated giant cells. Virchows Arch (cell Pathol) 1983; 42:161.

107. Johnson GD, Hardie DL, Ling NR, Maclennan I. Human follicular dendritic cells (FDC): a study with monoclonal antibodies (MoAb). Clin Exp Immunol 1986; 64:205.

108. Mitani S, Takagi K, Oka T, Mori S. Increased immunoglobulin E Fc receptor bearing cells in germinal centers of hyperimmunoglobulinemia E patients. Int Arch Allergy Appl Immunol 1988; 87:63-69.

109. Masuda A, Kasajima T, Mori N, Oka K. Immunohistochemical study of low affinity Fc receptor for IgE in reactive and neoplastic follicles. Clin Immunol Immunopathol 1989; 53:309-320.

110. Kosco MH, Tew JG, Szakal AK. Antigenic phenotyping of isolated and in situ rodent follicular dendritic cells (FDC) with emphasis on the ultrastructural demonstration of Ia antigens. Anat Rec 1986; 215:201-213.

111. Heinen E, Radoux D, Kinet-Denoel C, Moeremans M, DeMey J, Simar LJ. Isolation of follicular dendritic cells from human tonsils and adenoids III. analysis of their Fc receptors. Immunology 1985; 54:777.

112. Carbone A, Manconi R, Poletti A, Volpe R. Heterogeneous immunostaining patterns of follicular dendritic cells in human lymphoid tissue with selected antibodies reactive with different cell lineages. Hum Pathol 1985; 19:51.

113. Takei F. Inhibition of mixed lymphocyte response by a rat monoclonal antibody to a novel murine lymphocyte activation antigen (MALA-2). J Immunol 1985; 134:1403.

114. Prieto J, Takei F, Gendelman R, Biberfeld B, Patarroyo M. MALA-2, mouse homologue of human adhesion molecule ICAM-1 (CD54). Eur J Immunol 1989; 19:1551-1557.

115. Maeda K, Kosco MH, Burton GF, Szakal AK, Tew JG. Expression of the intercellular adhesion molecule-1 (ICAM-1) on HEV and on non-lymphoid antigen handling cells: interdigitating cells, antigen transport cells, and follicular dendritic cells. Cell Tissue Res 1994; (In Press).

116. Kosco MH, Pflugfelder E, Gray D. Follicular dendritic cell-dependent adhesion and proliferation of B cells *in vitro*. J Immunol 1992; 148:2331-2339.

117. Gordon J, Flores-Romo L, Cairns JA, Millisum MJ, Lane PJ, Johnson GD, et al. CD23: a multi-functional receptor/lymphokine?. Immunol Today 1989; 10:153-157.

118. Tew JG, Mandel TE. The maintenance and regulation of serum antibody levels: evidence indicating a role for antigen retained in lymphoid follicles. J Immunol 1978; 120:1063-1069.

119. Kasajima T, Yamakawa M, Imai Y. Immunohistochemical study of intrathyroidal lymph follicle. Clin Immunol Immunopathol 1987; 43:117-128.

120. Szakal AK, Holmes KL, Tew JG. Transport of immune complexes from the subcapsular sinus to lymph node follicles on the surface of nonphagocytic cells, including cells with dendritic morphology. J Immunol 1983; 131:1714-1727.

121. Szakal AK, Kosco MH, Tew JG. A novel *in vivo* follicular dendritic cell-dependent iccosome-mediated mechanism for delivery of antigen to antigen-processing cells. J Immunol 1988; 140:341-353.

122. Szakal AK, Kosco MH, Tew JG. Microanatomy of lymphoid tissue during the induction and maintenance of humoral immune responses: structure function relationships. Ann Rev Immunol 1989; 7:91-109.

123. Tew JG, Kosco MH, Szakal AK. The alternative antigen pathway. Immunol Today 1989; 10:229-231.

124. Sordat B, Sordat M, Hess MW, Stoner RD, Cottier H. Specific antibody within germinal center cells of mice after primary immunization with horseradish peroxidase: a light and electron microscopic study. J Exp Med 1970; 131:77-91.

125. Terashima K, Imai Y, Kasajima T, Tsunoda R, Takahashi K, Kojima M. An ultrastructural study on antibody production of the lymph nodes of rats with special reference to the role of germinal centers. Acta Pathol Jpn 1977; 27:1.

126. Tew JG, Phipps RP, Mandel TE. The maintenance and regulation of the humoral immune response: persisting antigen and the role of follicular antigen-binding dendritic cells as accesory cells. Immunol Rev 1980; 53:175-201.

127. Mandel TE, Phipps RP, Abbot A, Tew JG. The follicular dendritic cell: long term antigen retention during immunity. Immunol Rev 1980; 53:29-59.

128. Gray D, Skarvall H. B-cell memory is short lived in the absence of antigen. Nature 1988; 336:70-73.

129. Jacobson EB, Caporale LH, Thorbecke GJ. Effect of thymus cell injections on germinal center formation in lymphoid tissues of nude (thymusless) mice. Cell Immunol 1974; 128:416.

130. Fuller KA, Kanagawa O, Nahm M. T cells within germinal centers are specific for the immunizing antigen. J Immunol 1993; 151:4505-4512.

131. Tew JG, Burton GF, Szakal AK. Follicular dendritic cells in antibody responses. In: Nossal GJV, editor. Handbook of Experimental Immunology, The Lymphoid System Antibody Responses and Affinity Maturation. Oxford: Rockwell Scientific Publications, 1994:In Press.

132. Schnizlein CT, Szakal AK, Tew JG. Follicular dendritic cells in the regulation and maintenance of immune responses. Immunobiol 1984; 168:391-402.

133. Heinen E, Cormann N, Lesage F, Kinet-Denoel C, Tsunoda R, Simar LJ. Follicular dendritic cells act as accessory cells. In: Schook LB, Tew JG, editors. Antigen presenting cells diversity, differentiation, and regulation. N.Y. Alan R. Liss,Inc, 1988:69.

134. Zack JA, Arrigo SJ, Weitsman SR, Go AS, Haislip A, Chen ISY. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell 1990; 213-222.

135. Popovic M, Sarngadharan MG, Read E, Gallo R. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 1984; 224:497-500.

136. Mann DL, O'Brien SJ, Gilbert DA, Reid Y, Popovic M, Read-Connole E, et al. Origin of the HIV-susceptible human CD4+ cell line H9. AIDS Res Hum Retroviruses 1989; 5:253-255.

137. Ratner L, Haseltine W, Patarca R, Livak KJ, Starcich B, Josephs SF, et al. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature 1985; 313:277-283.

138. Popovic M, Read-Connole E, Gallo R. T4 positive human neoplastic cell lines susceptible to and permissive for HTLV-III. Lancet 1984; ii:1472-1473.

139. Levy JA, Hoffman AD, Kramer SM, Landis JA, Shimabukuro JM, Oshiro LS. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. Science 1984; 225:840-842.

140. Clouse KA, Powell D, Washington I, Poli G, Strebel K, Farrar W, et al. Monokine regulation of human immunodeficiency

virus-1 expression in a chronically infected human T cell clone. J Immunol 1989; 142:431-438.

141. Folks TM, Clouse KA, Justement J, Rabson A, Duh E, Kehrl JH, et al. Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. Proc.Natl.Acad.Sci.USA 1989; 86:2365-2368.

142. Chesebro B, Wehrly K. Development of a sensitive quantitative focal assay for human immunodeficiency virus infectivity. J Virol 1988; 62:3779-3788.

143. Pincus S, Wehrly K, Chesebro B. Treatment of HIV tissue culture infection with monoclonal antibody-ricin A conjugates. J Immunol 1989; 142:3070-3075.

144. Posner MR, Cavacini LA, Emes CL, Power J, Byrn R. Neutralization of HIV-1 by F105, a human monoclonal antigbody to the CD4 binding site of gp120. J Acquired Immune Defic Syndr 1995; 6:7-14.

145. Posner MR, Hideshima T, Cannon T, Mukherjee M, Mayer KH, Byrn RA. An IgG human monoclonal antibody that reacts with HIV-1/gp120 inhibits virus binding to cells, and neutralizes infection. J Immunol 1991; 146:4325-4332.

146. Burton DR, Pyati J, Koduri R, Sharp SJ, Thornton GB, Parren PW, et al. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science 1994; 266:1024-1027.

147. Burton GF, Conrad DH, Szakal AK, Tew JG. Follicular dendritic cells (FDC) and B cell co-stimulation. J Immunol 1993; 150:31-38.

148. Butch A, Chung G, Hoffmann J, Nahm M. Cytokine expression by germinal center cells. J Immunol 1993; 150:39.

149. Butch AW, Hug BA, Nahm MH. Properties of human follicular dendritic cells purified with HJ2, a new monoclonal antibody. Cell Immunol 1994; 155:27-41.

150. Cameron PU, Freudenthal PS, Barker JM, Gezelter S, Inaba K, Steinman RM. Dendritic cells exposed to human immunodeficiency virus type-I transmit a vigorous cytopathic infection to CD4+ T cells. Science 1992; 257:383-387.

151. Ou C, Kwok S, Mitchell SW, Mack DH, Sninsky JJ, Krebs JW, et al. DNA amplification for direct detection of HIV-1 in DNA

of peripheral blood mononuclear cells. Science 1988; 239:295-297.

152. Bauer HM, Ting Y, Greer CE, Chambers JC, Tashiro CJ, Chimera J, et al. Genital human Papillomavirus infection in female university students as determined by a PCR-based method. JAMA 1991; 265:472-477.

153. Gelderblom HR, Hausmann ESH, Ozel M, Pauli G, Koch MA. Fine Structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. Virology 1987; 156:171-176.

154. Tenner-Racz K, Racz P, Schmidt H, Taveres LM, de Noronha F, Stahl-Henning C, et al. Virus trapping by follicular dendritic cells in retrovirus infections inducing follicular hyperplasia of lymph nodes. In: Racz P, Dijkstra CD, Gluckman JC, editors. Accessory Cells in HIV and Other Retroviral Infections. Basel: Karger, 1991:83-97.

155. Embretson J, Zupancic M, Ribas JL, Burke A, Racz P, Tenner-Racz K, et al. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature 1993; 362:359-362.

156. Tew JG, Mandel TE, Miller GA. Immune retention: immunological requirements for maintaining an easily degradable antigen *in vivo*. Aust J exp Biol med Sci 1979; 57:401-414.

157. Boyle MJ, Conners M, Flanigan ME, Geiger SP, Ford Jr. H, Baseler M, et al. The human HIV/peripheral blood lymphocyte (PBL)-SCID mouse. J Immunol 1995; 154:6612-6623.

158. Gray D, Kosco MH, Stockinger B. Novel pathways of antigen presentation for the maintenance of memory. Int Immunol 1991; 3:141-148.

159. Tew JG, Greene EJ, Makoski MH. *In vitro* evidence indicating a role for the Fc region of IgG in the mechanism for the long-term maintenance and regulation of antibody levels *in vivo*. Cell Immunol 1976; 26:141-152.

160. Matsuda T. AIDS; Current Status & Prospect. Masui 1988; 37:1321-1327.

161. Clerici M, Sison AV, Berzofsky JA, Rakusan TA, Brandt CD, Ellaurie M, et al. Cellular immune factors associated with mother-to-infant transmission of HIV. AIDS 1993; 7:1427-1432.

162. Clerici M, Levin JM, Kessler HA, Harris A, Berzofsky JA, Landay JA, et al. Human immunodeficiency virus (HIV)-specific T helper activity in HIV seronegative health workers accidentally exposed to HIV contaminated blood. J Amer Med Assoc 1994; 271:42-46.

163. Clerici M, Shearer GM. Cellular Immunity and a type 1 cytokine profile in protection against HIV infection and progression to AIDS. Res Immunol 1994; 145:635-643.

164. Langlade-Demoyen P, Ngo-Giang-Huong N, Ferchal F, Oksenhender E. Human immunodeficiency virus (HIV) nef-specific cytotoxic T lymphocytes in noninfected heterosexual contact of HIV-infected patients. J Clin Invest 1994; 93:1293-1297.

165. Clerici M, Clark EA, Polacino P, Axeberg I, Kuller L, Casey NI, et al. Induction of cellular immune response by subinfectious dose of SIV: protection from virus challenge. AIDS 1995; in press.

166. Levy M.D. JA. Feature of HIV pathogenesis that warrant close attention in approaches at therapy. 1995; Spirat Conferance-Novel HIV Therapies: From Discovery to Clinical Proof-of-Concept 68167. Groopman JE, Benz PM, Ferriani R. Characterization of serum neutralization response tothe human immunodeficiency virus (HIV). AIDS Res Hum Retroviruses 1987; 3:71-85.

168. Goudsmit J, Thiriart C, Smit L. Temporal development of cross-neutralization between HTLV-IIIB and HTLV-III RF in experimantally infected chimpanzees. Vaccine 1988; 6:229-232.

169. Prince AM, Pascual D, Kosolapov LB. Prevalence, clinical significance, and strain specificity of the neutralizing antibody of the human immunodeficiency virus. J Infect Dis 1987; 156:268-272.

170. Weber JN, Weiss RA, Roberts C. Human immunodeficiency virus type-1 (HIV-1) infection in two cohorts of homosexula men: Neutralizing sera and association of anti-gag antibody with prognosis. Lancet 1987; 1:110-121.

171. Scarlatti G, Albert J, Rossi A. Mother to child transmission of human immunodeficiency virus type-1:

Correlation with neutralizing antibodyies against primary isolates. J Infect Dis 1993; 168:207-210.

172. Goedert JJ, Drummond JE, Minkoff HL, Stevens R, Blattner WA, Mendez H, et al. Mother to infant transmission of numan immunodeficiency virus type 1: Association with prematurity or low anti-gp120. Lancet 1989; 2:1351-1354.

173. Rossi P, Moschese V, Broliden PA, Fundaro C, Quinti I, Plebani A, et al. Presence of maternal antibodies to human immunodeficiency virus 1 envelope glycoprotein 120 epitopes correlates with the uninfected status of children born to seropositive mothers. Proc.Natl.Acad.Sci.USA 1989; 87:8055-8058.

174. Devash Y, Calvelli TA, Wood DG, Reagan KJ, Rubenstein A. Vertical transmission of human immunodeficiency virus is correlated with the absence of high-affinity/avidity maternal antibodies to the gp120 principle neutralizing domain. Proc.Natl.Acad.Sci.USA 1990; 87:3445-3449.

Vita