Basic Science Summary

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The 16th Conference on Retroviruses and Opportunistic Infections featured a strong and balanced program that showcased exciting research into cellular restrictions that defend the cell against viral infection as well as cellular cofactors that regulate central steps in viral replication. Immunopathogenesis presentations continued to reveal some surprises such as evidence for pathogenicity in natural simian immunodeficiency virus infection. The identification of novel cellular restrictions and cellular cofactors of viral replication indicate the possibility of numerous opportunities for the development of novel therapeutic agents for the treatment of HIV and AIDS.

Viral Attack and Cellular Defense, Update on Cellular Restrictions

In addition to *gag, pol,* and *env* genes that are common to all retroviruses, primate lentiviruses contain additional small open reading frames. Four of these small open reading frames, namely *vif, vpu, nef,* and *vpr/vpx,* are referred to as accessory genes. For many years, the function of these accessory genes in viral replication has remained elusive, but it is now becoming apparent that they may share a common purpose, that is, to protect the virus from the antiviral effects of cellular restrictions.

Pioneering studies in the laboratories of Malim and Kabat were the first to reveal that the Vif protein of primate lentiviruses counteracted a dominant cellular restriction. This was eloquently demonstrated when researchers generated heterokaryons between cells in which Vif was dispensable for infectious virus production and cells in which Vif was necessary for production of infectious virus. The fact that the resultant heterokaryons produced noninfectious virus indicated that cells in which Vif was required for viral replication harbored a dominant antiviral restriction and that Vif neutralized this restriction.[1,2] Research by Malim and colleagues subsequently revealed the identity of the dominant restriction, a cellular cytidine deaminase known as APOBEC 3G.[3]

In the past year, it has been revealed that the accessory protein Vpu, which is expressed by HIV-1 and certain simian immunodeficiency virus (SIV) lineages such as chimpanzee SIV (SIV_{cpz}), counteracts a cellular restriction that has been independently identified by research groups led by Bieniasz and Guatelli as tetherin (also known as BST-2 or CD317).[1-5] Presentations at the conference this year described evidence that the Vpx protein of HIV-2 and sooty mangabey SIV (SIV_{sm}) and the Nef protein also play roles in neutralizing cellular restrictions of retrovirus infection. Previous studies suggested that Vpx and Vpr may play specific roles in viral replication of myeloid-lineage cells. Heterokaryons formed between macrophages, in which Vpx is necessary for viral infection, and COS cells, in which Vpx is dispensable, were resistant to infection by Vpx-deleted viruses.[6] This evidence suggests that myeloid-lineage cells harbor a restriction that is neutralized by the Vpx protein. Two presentations (Abstracts 25, 238) suggested that this restriction is also active against simple retroviruses including murine leukemia virus (MLV) and may restrict infection of quiescent monocytes by HIV-1.

A central characteristic that distinguishes lentiviruses from simple retroviruses is the ability to infect terminally differentiated, nondividing cells. Whereas retroviruses transduce cells during mitosis, lentiviruses appear to have the capacity to transduce cells at all stages of the cell cycle (with the exception of quiescent cells, see below). For this reason, lentivirus vectors have been exploited for the transduction of nondividing cells including neurons, macrophages, muscle cells, and dendritic cells.

What governs the ability of lentiviruses to transduce nondividing cells is a matter of some debate. A popular model is that the relative abilities of retroviruses and lentiviruses to transduce nondividing cells is dictated by the ability of these viruses to circumvent the nuclear envelope. Upon infection of a cell, viral cDNA is reverse transcribed within the context of a large nucleoprotein reverse transcription complex. In a nondividing cell, these complexes must traverse the nuclear envelope to integrate within chromatin. Therefore, it has been proposed that reverse transcription complexes of lentiviruses harbor nucleophilic determinants that allow the lentiviral reverse transcription complex to translocate across the intact nuclear envelope.

In retroviruses, however, it is generally believed that the reverse transcriptase complexes lack the nucleophilic determinants that permit translocation across the nuclear envelope and consequently, access the nuclear compartment only after the nuclear envelope dissipates during mitosis. Abstract 25 provided the alternative explanation that the relative abilities of retroviruses and lentiviruses to transduce nondividing macrophages are dictated by the ability of these viruses to neutralize a restriction operative in these cells. When HeLa cells permissive to MLV infection were fused with macrophages that are restricted for MLV infection, the resultant heterokaryons were resistant to MLV infection, indicating that nondividing macrophages harbor a restriction that antagonizes MLV. Macrophages harbor a dominant restriction that antagonizes lentiviral

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reverse transcription, and this restriction is counteracted by Vpx of HIV-2 and SIV_{sm}. Kaushik and colleagues suggested that this same restriction was operative against MLV, given that the block to macrophage infection by MLV was at the level of reverse transcription. Furthermore, infection of macrophages with an SIV variant that expressed Vpx rendered macrophages permissive to MLV infection, and an MLV variant that was engineered to package Vpx protein efficiently transduced nondividing macrophage.

Collectively, these results suggest the presence of a dominant restriction that is active against retroviruses and lentiviruses and that this restriction is the obstacle to transduction of nondividing, terminally differentiated cells by retroviruses. Therefore, to establish myeloid cell reservoirs, primate lentiviruses have evolved Vpx (and perhaps Vpr) proteins to neutralize a dominant restriction that otherwise prevents infection of these cells.

In Abstract 238, the same research group demonstrated that the macrophage restriction neutralized by Vpx might also be an obstacle to infection of quiescent monocytes by primate lentiviruses. Although macrophages and dendritic cells are permissive to lentivirus transduction, a large body of experimental evidence demonstrates that quiescent monocytes, which are the circulating precursors to tissue macrophages, are refractory to lentivirus infection. Infection of monocytes is blocked primarily at the level of reverse transcription, and the blocks have been attributed to rate-limiting levels of deoxynucleotide triphosphates that are required for optimal reverse transcription or to the presence of inhibitory complexes of APOBEC 3G. Using a heterokaryon approach, researchers uncovered evidence that monocytes harbor a dominant restriction and that expression of Vpx in these heterokaryons conferred the ability to support HIV-1 infection. Furthermore, packaging of Vpx within HIV-1 variants conferred the ability to infect quiescent monocytes. Susceptibility to infection did not reflect a change in the distribution of APOBEC 3G between low- and high-molecular-mass complexes; it appears that the restriction was unrelated to APOBEC 3G.

A prediction stemming from these observations is that lentiviruses that express a Vpx protein have a greater capacity to infect quiescent monocytes than, for example, HIV-1 does. Whether the ability to establish a reservoir of infected monocytes has an impact on the immunopathogenicity of infection remains to be determined.

Tetherin, or BST-2, is an interferon-inducible, cellular protein that inhibits the dissociation of progeny HIV-1 virions from the surface of the infected cell. To overcome this restriction, HIV-1 has evolved Vpu, which neutralizes tetherin by a poorly understood process. A long-standing question has been how members of the HIV-2 and SIV_{sm} lineages that do not have a *vpu* gene counteract the antiviral effects of tetherin. One study (Abstract 28LB) suggests that the Nef protein of SIV may have assumed this responsibility. Nef is an enigmatic, viral accessory protein required for efficient viral replication and pathogenicity in vivo, and many activities have been ascribed to it. Nef has been shown to down-regulate immunoregulatory molecules from the cell surface, inducing CD4 and major histocompatibility complex class I (MHC-I) receptors, and as a consequence, Nef is believed to play a general role in immune evasion.

Deletion mutants of SIV were tested for virion production in 293T cells that expressed either human or rhesus tetherin, and results revealed that SIV Nef could promote particle release by inhibiting rhesus tetherin. However, SIV Nef was not able to neutralize human tetherin. Conversely, HIV-1 Vpu specifically neutralized human tetherin but not rhesus tetherin. Jia and colleagues identified a 4-amino-acid motif in the cytoplasmic domain of tetherin that was required for its ability to be neutralized by SIV Nef. However, this domain was absent from human tetherin.

With these new findings on the role of Nef in neutralization of tetherin, it now appears that all 4 viral accessory proteins play a role in counteracting cellular antagonists of HIV and SIV replication. The fact that lentiviruses have evolved these counter-defense mechanisms supports the notion that these viral accessory proteins represent highly attractive targets for therapeutic intervention.

A number of presentations featured research aimed at improving understanding of how viral accessory proteins counteract cellular restrictions. Two (Abstracts 129LB, 130LB) presented nuclear magnetic resonance structures of the deaminase domain of APOBEC 3G. These structures have begun to reveal insight into loops within the active site that are directly involved in binding to substrate. Although the 2 structures presented very different orientations of the substrate groove, they are important first steps toward identifying elements in APOBEC 3G that can aid in the rational design of small molecules that enhance substrate binding and antiviral activity of APOBEC 3G.

Additional APOBEC Studies

Although considerable insight into the mechanism by which Vif counteracts the antiviral activity of APOBEC 3 proteins has been obtained, gaps remain in our understanding of the mechanisms by which APOBEC 3 proteins inhibit viral replication. APOBEC 3G is a cytidine deaminase that is packaged into viral particles, and the consensus is that it restricts viral replication by catalyzing deamination of cytosines in minus-strand viral cDNA. Whereas APOBEC 3G has 2 cytidine deaminase domains, only the C-terminal domain has catalytic activity.

Several studies provide evidence that the antiviral activity of APOBEC 3G is dependent upon cytidine deaminase activity. However, studies by Malim, Sheehy, and colleagues have provided evidence that APOBEC 3G may exhibit antiviral activity independent of APOBEC 3G enzymatic activity. For example, packaging of APOBEC 3G protein into Vif-deleted HIV-1 virions correlates with an inhibition of endogenous reverse transcription within the virion.[7] This would suggest that APOBEC 3G has the capacity to inhibit viral reverse transcription. Abstract 240 continued in this vein by describing a

large panel of APOBEC 3G proteins, some of which retain cytidine deaminase activity yet do not have antiviral activity. Studies presented in Abstract 233 reinforced the model that the extent of deamination of viral cDNA correlated inversely with viral infectivity and that packaging of a single molecule of APOBEC 3G was sufficient to affect HIV-1 infectivity, which further implicated that restriction is dependent upon enzymatic activity.

The level to which APOBEC 3G is expressed in cells also shapes the outcome of infection. At higher levels of APOBEC 3G expression, neutralization by Vif may be incomplete, which would create the scenario in which even wild-type viruses are suppressed by APOBEC 3G. Abstract 241 presented evidence that the differential expression of APOBEC 3G in T helper lymphocyte subsets (T_H1 or T_H2) correlated with their susceptibility to infection. APOBEC 3G was expressed at higher levels in T_H1 than T_H2 cells, and virus produced from T_H1 cells was less infectious than virus obtained from T_H2 cells. This suggests that strategies that augment APOBEC 3G expression in vivo may be effective in inhibiting the replication of HIV-1.

Abstract 26 employed biochemical procedures to evaluate the interaction between the cellular restriction TRIM5 and lentiviral capsid proteins. TRIM5α from rhesus monkeys potently inhibits HIV-1 infectivity at an early step in the viral life cycle. It has been hypothesized that TRIM5α interferes with retroviral uncoating. However, study of the uncoating process in the context of an infected cell has been a difficult challenge. Abstract 26 presented evidence that a recombinant form of TRIM5α, containing the RING domain of human TRIM21, formed monomers and dimers and bound directly to synthetic capsids of HIV-1 CA-NC proteins as well as to core particles of equine infectious anemia virus. Furthermore, TRIM5-TRIM21 chimeric proteins were able to autoubiquitylate in vitro. The ability to recapitulate ubiquitin ligase activity and Gag binding in vitro with recombinant TRIM proteins should allow a more detailed investigation into the mechanism by which TRIM5 proteins affect capsid function and viral uncoating.

Abstract 24 presented studies aimed at monitoring the uncoating process in vivo. The investigators incorporated a fluorescent label into the membranes of HIV-1 virions that had also packaged green fluorescent protein (GFP) through its association with Vpr. Cells were then infected and virions visualized with antibody to capsid. The investigators followed the timing of uncoating from the percentage of virions that had lost the membrane fluorescent signal. They observed that capsid remained associated with the viral core after fusion and gradually associated over the first 4 hours after infection. Curiously, when cells were treated with reverse transcriptase inhibitors, the percentage of capsid-associated virions increased with time. Collectively, these findings suggest that capsid can remain associated with the viral core for hours after infection and that reverse transcription may be important for HIV-1 uncoating because the process was delayed in the presence of reverse transcriptase inhibitors.

Cellular Cofactors and Viral Replication

Over the past year, considerable attention has focused on cellular cofactors that regulate early events in the retroviral life cycle and in particular, cellular proteins that aid in translocation of viral complementary DNA (cDNA) into the nucleus. Last year, a short interfering RNA (siRNA) screen by Brass and colleagues identified more than 200 potential HIV-1 cofactors required for viral replication.[8] Among these were nuclear pore proteins and nuclear shuttling proteins that may play a role in trafficking of viral reverse transcription complexes from the cytoplasm to the nucleus.

This aspect of retroviral replication has remained highly elusive. After infection of a cell and synthesis of viral cDNA, viral integrase remains associated with viral cDNA as it translocates to the host cell nucleus. Because these nucleoprotein reverse transcription complexes approach the size of a ribosome, some specialized mechanism must permit them to translocate across the nuclear envelope during infection of a nondividing cell. Abstract 23 presented evidence that transportin-SR2 binds to HIV-1 integrase but not MLV integrase. Upon siRNA-mediated knockdown of transportin-SR2, nuclear translocation of HIV-1 reverse transcription complexes was blocked. Interestingly, transportin-SR2 knockdown inhibited nuclear translocation of HIV-1 in cells that had not been growth arrested. This suggests that infection of the cell by HIV-1 may depend upon nuclear transport factors irrespective of the cycling state of the target cell.

In a plenary presentation, Debyser (Abstract 74) discussed ongoing studies aimed at developing small molecule inhibitors of the interaction between HIV-1 integrase and its cellular cofactor, lens epithelium-derived growth factor/p75 (LEDGF/p75). This cellular protein was originally identified in association with integrase complexes expressed in cells.[9] Subsequent studies using RNA interference (RNAi)-mediated knockdown of LEDGF indicated that it played an important role in viral integration. Crystallographic information on LEDGF/p75-integrase interaction has aided in the design of inhibitors of the interaction by computer modeling. Debyser discussed a lead compound that fits in the LEDGF binding pocket. This compound was antiviral and was active against viruses resistant to the integrase inhibitor raltegravir. Importantly, this small molecule appeared to act at the interface between LEDGF and integrase rather than the interface of LEDGF with cellular binding partners such as JPO2. Therefore, inhibition of LEDGF-integrase interaction may be possible without affecting the normal cellular function of LEDGF.

Studies were presented of HIV replication using analyses of single molecules aimed at visualizing the pathway that viruses use to navigate in and out of the cell (Session 44). Abstract 167 presented studies using virions in which integrase was fused to a fluorescent protein. This permitted analysis of the spatial and dynamic distribution of viral reverse transcription com-

plexes in the nucleus. This strategy indicated that viral reverse transcription complexes localized primarily to decondensed regions of chromatin but not to heterochromatin, suggesting the existence of a mechanism by which viral reverse transcription complexes are oriented toward particular integration sites. In a parallel presentation (Abstract 166), fluorescently tagged derivatives of Gag were used to follow the assembly of HIV-1 virions at the surface of the infected cell. Using a variety of fluorescent imaging approaches, virions were found to appear at the cell surface sequentially, and assembly of a single virion typically required 5 minutes to 6 minutes.

Studies of Nef

Abstract 258 presented research aimed at identifying small molecule inhibitors of HIV-1 Nef. As discussed above, of the myriad of activities that have been described for lentiviral Nef proteins, its ability to down-regulate MHC-I receptor expression on the surface of the infected cells is generally considered an important function of Nef because it would protect the infected cell from immune surveillance by cytotoxic T cells. A cell-based assay was described that screens for small molecules that stabilize surface expression of MHC-I in the presence of HIV-1 Nef. From a library of 70,000 compounds, 2 were identified that could prevent down-regulation of MHC-I on the cell surface in the presence of HIV-1 Nef. Studies are underway to determine whether these small molecules restore the sensitivity of HIV-1-infected cells to killing by cytotoxic T lymphocytes.

Although Nef plays a central role in viral replication, it is also implicated as a determinant of pathogenicity in HIV and SIV infection. Nef may be a determinant that contributes to an increased risk of cardiovascular disease in HIV infection (Abstract 147). There is an increased incidence of dyslipidemia and cardiovascular disease in HIV-1-infected individuals. Examining high-density lipoprotein (HDL) metabolism in SIV-infected macaques fed an atherogenic diet, Bukrinsky and colleagues observed an SIV-specific block in HDL remodeling in the liver and down-regulation of ABCA1 (adenosine triphosphate-binding cassette, subfamily A, member 1) activity in the liver. Soluble extracellular Nef was found in liver tissue and in plasma. In vitro, Nef inhibited ABCA1-dependent cholesterol efflux from macrophages and hepatocytes, suggesting a model in which Nef released from infected cells inhibits reverse cholesterol transport. This effect may lead to changes in HDL metabolism that may contribute to increased risk of cardiovascular disease.

Viral Replication and Pathogenicity

Pathogenic lentivirus infection (HIV-1 infection of humans and SIV infection of rhesus macaques) is associated with high-level viral replication and viremia, accelerated turnover of CD4+ lymphocytes and CD4+ cell depletion, and generalized immune activation. In contrast, primate lentiviruses are not considered pathogenic in their natural hosts (SIV_{cpz} infection, SIV_{smm} infection). Nonpathogenic infection exhibits most of the characteristics of pathogenic infection including efficient viral replication, high-level viremia, and accelerated CD4+ lymphocyte turnover. However, the CD4+ lymphocyte depletion and generalized immune activation characteristic of pathogenic infection are not apparent in nonpathogenic infection.

Abstract 80 presented the surprising finding that SIV_{cpz} may have a substantial impact on the health of infected chimpanzees as well as their survival and reproduction, indicating that SIV_{cpz} is pathogenic in its natural host. Because chimpanzees are a protected species, it has been difficult to study the natural history of SIV_{cpz} infection in these animals. However, the investigators exploited 50 years of observational data, combined with antibody and DNA polymerase chain reaction status, to study SIV_{cpz} infection in chimpanzees in Gombe National Park, Tanzania. SIV_{cpz} prevalence rates increased from 8% in 2001 to 17% in 2007. There was a higher mortality risk in infants born to SIV_{cpz}-infected mothers than in infants born to uninfected mothers, and infected chimpanzees had a 6- to 17-fold increased death hazard. CD4+ lymphocyte depletion was also evident in 2 of 3 SIV_{cpz}-infected animals but not in 4 uninfected controls. Thus, in terms of the extent of pathogenicity, mortality data in SIV_{cpz} infection in chimpanzees is lower than that of HIV-1-infected humans but higher than in SIV_{smm} infection. Whether the extent of pathogenicity in SIV_{cpz}-infected monkeys correlates with levels of generalized immune activation remains to be determined.

In a plenary presentation, Douek discussed the role of immune activation in pathogenic lentivirus infection as well as factors that may drive immune activation (Abstract 20). The mucosal barrier is damaged as a consequence of depletion of gut-associated lymphoid tissue (GALT), with the preferential loss of T_H17 cells in the gastrointestinal tract as a result of HIV infection. Translocation of microbial products from the gut lumen into the systemic circulation, as a consequence of this damaged mucosa, drives immune stimulation. In contrast, levels of T_H17 cells in the gastrointestinal tract of SIV-infected sooty mangabeys remain normal, and there is no evidence for increased translocation of immunostimulatory microbial products.

T_H17 cells may be selectively depleted in HIV-1 infection because the majority of T_H17 cells are CD4+ and are thus susceptible to infection (Abstract 78). Although the majority of T_H17 cells expressed both CC chemokine receptor 5 (CCR5) and CXC chemokine receptor 4 (CXCR4) coreceptors, CCR5 expression was higher in the T_H17 subset that secretes interleukin-17 and interferon-gamma (IFN-γ). These cells were highly susceptible to infection by X4-tropic and R5-tropic HIV-1 and to the cytopathic effects of infection in vitro. There was a substantial decrease in this IFN-γ-positive T_H17 population in HIV-1-infected individuals. This T_H17 subset was also decreased in HIV-1-infected individuals receiving suppressive antiretroviral therapy who have a CD4+ count greater than 200 cells/μL. Therefore, targeting of these cells by HIV-1 may occur even with effective

antiviral therapy, or this subpopulation of T_H17 cells may not recover if viral replication is subsequently controlled by antiretroviral therapy.

Rowland-Jones discussed features of HIV-2 infection and pathogenicity (Abstract 56). The majority of HIV-2-infected individuals do not show signs of disease, and as such, HIV-2-infected individuals share similar characteristics with HIV-1-infected long-term nonprogressors. About 20% of HIV-2-infected individuals develop a pathogenic infection similar to AIDS in HIV-1 infection. Rowland-Jones presented evidence that proviral DNA load in HIV-2 progressors and HIV-1-infected individuals at the same stage of disease are similar. However, HIV-2-infected nonprogresssors have lower plasma viremia. HIV-2-infected individuals with nonprogressive infection are more able to mount an effective immune response and preserve HIV-specific T-helper-cell responses and strong CD8+ cell responses. Low viral load correlated with the presence of T-cell responses to HIV-2 Gag.

Surprisingly, there are variations in HIV-2 Nef that affected its ability to down-regulate T-cell-receptor complex, and although this correlated with levels of systemic immune activation, there was no correlation with the extent of disease progression. It was previously hypothesized that down-regulation of the T-cell-receptor–CD3 complex from infected T cells by Nef may inhibit their response to activation and that this ability to down-regulate the T-cell receptor was lost in HIV-1 Nef.[10] The interpretation was that an inability to down-regulate the T-cell receptor and prevent infected cell responsiveness to activation may contribute to the extent of pathogenicity. Although the ability to down-regulate the T-cell receptor may not directly correlate with clinical outcome in HIV-2 infection, accumulating evidence suggests that Nef plays a role either in contributing to the state of immune activation or in regulating the ability of the infected cell to respond to immune stimuli.

As in some HIV-2-infected individuals, rare SIV_{sm}-infected sooty mangabeys show profound CD4+ lymphocyte depletion. This lymphocyte depletion is accompanied by emergence of SIV that is able to use several coreceptors for entry (R5/X4/R8/R2). To determine whether viral or host determinants defined the extent of lymphocyte depletion, plasma from sooty mangabeys with depleted CD4+ cells was used to infect additional animals. This led to a rapid depletion of CD4+ cells within 4 weeks of plasma transfer. In addition, virus recovered from 3 additionally infected monkeys retained its ability to infect cells by several coreceptors. Despite the profound depletion of CD4+ lymphocytes, levels of immune activation remained at levels observed in uninfected monkeys. Furthermore, plasma lipopolysaccharide levels remained low, which suggested that profound depletion of CD4+ lymphocytes was not accompanied by an impairment of gut epithelial integrity.

Collectively, these results indicate that SIV variants capable of using numerous coreceptors can induce severe systemic CD4+ cell loss in the natural host without inducing simian AIDS. This indicates that the natural host of SIV infection is able to maintain mucosal integrity and adaptive immune responses without the presence of high levels of CD4+ cells.

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A list of all cited abstracts appears on pages 89-95.

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