Induction of Disseminated *Mycobacterium avium* in Simian AIDS Is Dependent upon Simian Immunodeficiency Virus Strain and Defective Granuloma Formation

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***Mycobacterium avium* complex (MAC) is the most common disseminated bacterial disease in patients infected by the human immunodeficiency virus. Although murine models of disseminated MAC exist, they are primarily based on underlying genetic susceptibilities and cannot adequately address the complex interactions that occur between host, mycobacteria, and immunosuppressive lentivirus. To address this problem we have developed an experimental system to co-inoculate rhesus macaques with the simian immunodeficiency virus (SIV) and a clinical *M. avium* isolate that results in a disease virtually identical to that observed in human cases. Using this experimental system we have found that the development of disseminated MAC is dependent on viral strain. Animals co-infected with SIVmac251 and *M. avium* developed progressive disease, whereas control animals and animals inoculated with closely related viruses (SIVmac239 and SIVmac239MER) developed self-limiting infections. The ability of animals infected with SIVmac239 or SIVmac239MER to eliminate mycobacterial disease was independent of viral load and CD4 T-cell number but was correlated with the size and composition of microgranulomas. This work establishes a novel primate model of disseminated MAC in the context of immunosuppressive lentiviral infection and advances our understanding of why human immunodeficiency virus-infected patients are remarkably sensitive to the development of mycobacterial disease. (Am J Pathol 2001, 159:693–702)**

Mycobacterium avium complex (MAC) is the most common disseminated bacterial disease in patients infected by the human immunodeficiency virus (HIV) and is diagnosed in 30 to 40% of untreated patients dying with the acquired immune deficiency syndrome (AIDS).^{1,2} MAC organisms are found ubiquitously in the environment and

yet rarely cause disease in non-HIV-infected individuals. Furthermore, although the occurrence of MAC is associated with severe CD4 T-lymphocyte depletion in HIV-infected patients, it is uncommon in humans with other immunosuppressive disorders.¹ The reason AIDS patients are uniquely sensitive to infection by these organisms is unknown but evidence suggests that in addition to depletion of CD4 T lymphocytes, viral proteins may have an adverse effect on the host's ability to eliminate MAC organisms.^{3–10}

Current animal models of disseminated MAC have used different strains of laboratory mice to investigate factors associated with disease progression and the host's immune response to mycobacterial infection. Extensive work has been conducted with the beige mouse, a strain that contains a homozygous mutation in the natural resistance associated macrophage protein-1 (*Nramp-1*) gene.^{11–13} The *Nramp-1* gene encodes a transmembrane cationic transport protein that seems to play a fundamental role in the elimination of intracellular pathogens. More recently, knockout mice have been used to examine the role of specific cytokine pathways and cellular immune responses in mycobacterial disease pathogenesis.^{14–22} Although these models have been useful in elucidating many factors associated with disseminated disease, they are primarily dependent on underlying genetic susceptibilities and cannot reproduce the complex interactions between host, mycobacteria, and lentiviral proteins that are likely responsible for the full manifestation of disease in human AIDS patients. For these reasons alternative animal models may prove useful for investigating the pathogenesis of disseminated mycobacterial disease.

Disseminated MAC also occurs in simian immunodeficiency virus (SIV)-inoculated rhesus macaques (*Macaca mulatta*) as a spontaneous disease in the terminal stages of AIDS and this process shares extensive similarities

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with the condition in human patients.²³⁻²⁶ SIV-infected animals acquire *M. avium* from environmental sources and develop spontaneous disease at severe CD4 T-lymphocyte depletion.²⁵ As in man, these animals develop debilitating diarrhea and wasting with progressive disease. In disseminated disease, tissue distribution and histomorphology closely approximates that seen in HIV-infected humans. Retrospective analysis of spontaneous disseminated MAC in rhesus macaques has revealed that the risk of mycobacterial infection is greater after inoculation with the wild-type SIV isolate SIVmac251 than with a closely related molecular clone, SIVmac239.²³ No significant difference in CD4 count near death, survival, or the occurrence of other opportunistic infections have been found between SIVmac251- and SIVmac239-inoculated macaques, eliminating these as confounding variables.

Such findings suggest that independent of CD4 T-lymphocyte depletion viral determinants play a critical role in promoting mycobacterial disease. This hypothesis is supported by *in vitro* work using HIV and MAC, which argues that viral proteins such as gp 120 and *tat* may adversely impact macrophage function.³⁻¹⁰ Here we describe an experimental system to co-inoculate rhesus macaques with SIV and *M. avium* and reproduce disseminated disease. This model has proven useful to study the effect of viral inoculum on progressive disease and the immunopathogenesis of early mycobacterial infection.

Materials and Methods

Study Design

Experimental co-infection of rhesus macaques was undertaken to investigate the pathogenesis of mycobacterial disease during simian AIDS. Immunologically normal animals ($n = 4$) were compared to animals infected with SIVmac251 ($n = 3$), SIVmac239 ($n = 2$), and SIVmac239MER ($n = 2$). *M. avium* was administered 2 weeks after SIV infection to coincide with the period of peak viremia. All animals were bled and had biopsies of peripheral lymph node, colon, and duodenum before mycobacterial inoculation and at 2, 4, 6, 8, and 10 weeks after inoculation. Tissues were processed for routine histopathology, acid-fast stains, immunohistochemistry, and lymphocyte isolation and subset analysis. Blood samples were processed for complete blood counts, serum chemistries, mycobacterial culture, viral isolation, and lymphocyte subset analysis. All three SIVmac251 co-infected animals were euthanized between 45 and 70 days after *M. avium* challenge because of progressive wasting and diarrhea. To allow comparisons, one animal in each of the normal, SIVmac239, and SIVmac239MER groups was euthanized and necropsied on day 45. Complete post-mortem examinations were performed on all animals, and representative samples of tissue were taken for formalin fixation, and freezing. All sections were stained with hematoxylin and eosin (H&E) for routine evaluation and with Ziehl-Neelson acid-fast stain to determine the distribution of mycobacterial organisms.

Animals and Housing

Rhesus macaques (*M. mulatta*) were housed at the New England Regional Primate Research Center in a centralized animal biolevel 3 containment facility in accordance with standards of the Association for Assessment and Accreditation of Laboratory Animal Care and Harvard Medical School's Animal Care and Use Committee. Animals were tested and found free of simian retrovirus type-D, SIV, simian T lymphotropic virus-1, and herpes B virus before assignment to experimental protocols. Lymph node, gastrointestinal, and hepatic biopsies were performed as previously described.²⁷ Animals received commercial monkey chow and to reduce exposure to environmental mycobacteria autoclaved water *ad libitum*. The monkeys were monitored closely and euthanized when moribund or deemed necessary by the veterinary staff.

Animal Inoculations

Animals were inoculated intravenously with equivalent doses of SIVmac251, SIVmac239, or SIVmac239MER. The *in vitro* growth properties and *in vivo* disease characteristics of these viruses have been described extensively.²⁸⁻³³ Briefly, SIVmac251 is a pathogenic wild-type viral isolate that replicates to high titer in cells of lymphocyte and monocyte lineage. SIVmac239 is a pathogenic molecular clone derived from a SIVmac251-infected animal and replicates well in lymphocytes.³⁴ T-cell tropism in SIVmac239 results from the decreased efficiency at which this virus utilizes the CD4 receptor present on tissue macrophages at low density.³⁵ SIVmac239MER is derived from the SIVmac239 parental clone and has three amino acid changes in the envelope protein that confers the ability of the virus to replicate in tissue macrophages and monocytes.^{36,37}

To develop a pathogenic *M. avium* isolate, a SIVmac251-infected rhesus macaque (155-84) was injected intravenously with a splenic tissue homogenate from a second SIV-infected animal (31-83) that had died with massive splenomegaly and mesenteric lymphadenopathy. *M. avium* was confirmed in the donor animal by histopathology, mycobacterial culture, and DNA probe (Gene-Probe, San Diego, CA).³⁸ Rhesus macaque 155-84 subsequently developed severe diarrhea and wasting and died from disseminated *M. avium* 52 days later. Clinical signs, tissue distribution, and histopathology were identical to spontaneous cases recognized in SIV-infected macaques. A smooth transparent *M. avium* clone (clinical isolate no. 88415) was recovered from frozen mesenteric lymph node and used to inoculate 50 ml of 7H9 broth (Difco, Detroit, MI). The broth was harvested during log-phase growth and aliquots of 10^8 colony-forming units (cfu) were stored at -70°C . For inoculation, aliquots were rapidly thawed at 37°C and sonicated for 10 seconds before administration intravenously.

Cell Isolation and Flow Cytometry

Peripheral blood, lymph node, and intestinal lymphocytes were stained and analyzed by flow cytometry as previously described.^{27,39} Briefly, jejunal and colonic endoscopic biopsies were incubated with 1 mmol/L ethylenediaminetetraacetic acid in Hanks' balanced salt solution for 30 minutes, followed by 1 hour in RPMI containing 20 U of collagenase per ml while rapidly shaking at 37°C. Biopsies were further disrupted, and single-cell suspensions were prepared by pipetting 5 to 10 times with a 16-gauge feeding needle. Lymphocytes were enriched by Percoll density centrifugation. Lymph node biopsies were processed similarly without ethylenediaminetetraacetic acid. Cells were stained by incubating 10⁶ cells from each of the above-described samples with excess amounts of monoclonal antibodies at 4°C for 30 minutes, followed by a wash (400 × g, 7 minutes) and fixation in 2% paraformaldehyde. Blood was stained by incubating 100 μl of whole blood with monoclonal antibodies for 30 minutes at 4°C, followed by a 7-minute lyse with FACS lysing solution (Becton Dickinson, San Jose, CA). Cells were then washed (400 × g, 7 minutes) and resuspended in 2% paraformaldehyde. All antibodies were directly conjugated to either fluorescein isothiocyanate, phycoerythrin, or peridinin chlorophyll protein. Monoclonal antibodies directed at CD2 (Leu-5b, Becton Dickinson), CD3 (6G12; provided by J. Wong, Massachusetts General Hospital, Boston, MA), CD4 (Leu3a, Becton Dickinson), CD8 (Leu-2a, Becton Dickinson), CD20 (Leu-16, Becton Dickinson), CD45RA (Leu-18, Becton Dickinson), CD56 (Leu19, Becton Dickinson), and HLA-DR (Becton Dickinson) were used. All antibodies have been used extensively in rhesus macaques.^{27,40} Samples were acquired on a FACS Calibur flow cytometer and analyzed with Cell Quest software (Becton Dickinson).

Immunohistochemistry and in Situ Hybridization

To examine the immunophenotype of cells within tissues, immunohistochemistry was performed on formalin-fixed paraffin-embedded tissues. Tissue sections were cut at 5 μm and immunostained using an avidin-biotin-horseradish peroxidase complex technique with diaminobenzidine chromogen as previously described.⁴¹ Sections were stained for CD68 (EBM-11; DAKO, Carpinteria, CA), CD3 (A0452, DAKO), CD20 (L26, DAKO), HLA-DR (CR3/43, DAKO), CD4 (Nu/Th1; Nicheri Research Institute, Tokyo, Japan) and CD8 (DK25, DAKO) and examined with an Olympus Vanox-S microscope interfaced with a Quantimet image analyzer (Leica, Cambridge, UK) via a DEI 750 charge-coupled device camera (Optronics, Goleta, CA). For quantitative measurements, images were captured and analyzed with Quantimet software as previously described.^{27,42} Briefly 5-μm sections of liver were stained for CD3, CD4, CD8, and HLA-DR. Twenty random hepatic microgranulomas from two animals in each group were identified in tissue sections and their images captured. Based on diaminobenzidine chromogen staining the total number of positive and negative cells for CD3, CD4, and CD8 were detected within individual micro-

granulomas and used to calculate relative (%) cell number. To obtain density, the area of each granuloma in a section was concurrently measured with data expressed as positive cells/10³ μm². Sections stained with irrelevant isotype-matched antibodies (DAKO) were uniformly below background. For HLA-DR, diaminobenzidine-staining intensity was measured by microdensitometry for individual microgranulomas and expressed as mean gray scale intensity/granuloma. *In situ* hybridization was performed on formalin-fixed, paraffin-embedded tissues to demonstrate SIVmac-infected cells as previously described.³⁹

Virus Isolation and Quantitation

Peripheral blood was collected for viral isolation before inoculation and at 3, 7, 14, 28, 42, 56, and 70 days after SIV infection. Quantitative viral cultures were performed on each blood sample as previously described.²⁹ Briefly serial threefold dilutions beginning with 10⁶ peripheral blood mononuclear cells were co-cultured with 10⁵ CEMx174 cells in a volume of 1 ml and viral production assayed by enzyme-linked immunosorbent assay for SIV p27 (Coulter, Hialeah, FL). Results are expressed as the number of SIV-positive cells/10⁶ peripheral blood mononuclear cells.

Statistical Analysis

Groups were compared statistically using commercially available software (Sigma Stat, Jandel Scientific) by *t*-test, one-way analysis of variance with multiple comparisons (Dunn's method or Tukey test) where appropriate.

Results

Co-Inoculation of Rhesus Macaques Produces Progressive Mycobacterial Disease

Three rhesus macaques were inoculated with SIVmac251 and challenged intravenously with 10⁸ cfu of *M. avium* 2 weeks after primary SIV infection (Table 1). Peak viral load (729 to 2188/10⁶ peripheral blood mononuclear cells) occurred at 14 days after inoculation and coincided with *M. avium* administration. As has been previously described during acute SIV infection, there was a temporary reduction in the relative and absolute number of CD3+CD4⁺ lymphocytes detected in blood and peripheral lymph node that reached its nadir at 2 to 4 weeks after inoculation and then rebounded to near normal values (Figure 1, A and B). Despite nearly normal peripheral CD4 T-lymphocyte values, all SIVmac251-co-infected animals developed severe clinical signs requiring euthanasia 45 to 70 days after mycobacterial challenge. The syndrome paralleled the course observed in spontaneous *M. avium* disease seen in simian AIDS and was characterized by anorexia, diarrhea, and progressive wasting. These clinical signs were accompanied by peripheral lymphadenopathy, a febrile response, and marked hepatosplenomegaly. Complete blood counts revealed a modest leukopenia (mean, 3100 cells/μl) 7 days after mycobacterial infection but values returned to nor-

Table 1. Viral Inoculum, Survival, and Clinical Findings in Rhesus Macaques Inoculated with *Mycobacterium avium*

Animal	Viral inoculum	Survival days	Clinical findings
51-97	SIVmac251	45	Diarrhea, hepatosplenomegaly, peripheral lymphadenopathy, dermatitis
271-96	SIVmac251	70	Diarrhea, hepatosplenomegaly, peripheral lymphadenopathy
85-96	SIVmac251	45	Diarrhea, hepatosplenomegaly, peripheral lymphadenopathy; dyspnea
372-90	None	+365	None
318-90	None	+365	None
109-96	None	45	None; euthanized as control
192-96	None	+365	None
65-97	SIVmac239	45	None; euthanized as control
355-96	SIVmac239	304	Euthanized when moribund with AIDS; no evidence of <i>M. avium</i>
154-96	SIVmac239MER	45	None; euthanized as control
221-96	SIVmac239MER	410	Euthanized when moribund with AIDS; no evidence of <i>M. avium</i>

mal by 14 days. Serum chemistries indicated a gradual rise in serum globulin and marked elevations in liver enzymes (alkaline phosphatase and alanine aminotransferase) by 4 weeks after inoculation.

Figure 1. Temporal alterations in CD3CD4-positive lymphocytes. Changes observed after *M. avium* challenge in SIVmac251 (51-97 and 271-96) and SIVmac239-infected (65-97 and 355-96) rhesus macaques. **A:** Blood; **B:** peripheral lymph node; and **C:** duodenum. Each bar represents the mean values from two infected animals after mycobacterial challenge (day 0).

Sequential biopsies of peripheral lymph node, liver, duodenum, and colon were performed to determine the temporal changes in mycobacterial distribution. Acid-fast bacilli were first detected in scattered macrophages within lymph nodes and the lamina propria of the small intestine at 28 days after mycobacterial challenge. Mycobacterial blood cultures became positive at this time and subsequent biopsies indicated an increase in the number of infected cells and mycobacterial tissue load (Table 2). By 6 weeks after *M. avium* inoculation morphological features typical of disseminated MAC were apparent.

At necropsy acid-fast bacilli were found in numerous tissues including peripheral and mesenteric lymph nodes, small and large intestine, liver, spleen, lung, kidneys, and bone marrow. Tissue distribution and morphological findings were identical to that seen in spontaneous cases of disseminated MAC found in human and simian AIDS. In lymph nodes, sheets of pale eosinophilic histiocytes effaced normal architecture and contained large numbers of acid-fast bacilli (Figure 2, A and B). Similar histiocytic infiltrates were found throughout the spleen, mesenteric lymph nodes, and lamina propria of the small intestine (Figure 2C). Typical microgranulomas, all containing bacilli, were found in liver, spleen, lung, and bone marrow and consisted of small aggregates of CD68-positive histiocytes and variable numbers of CD4- and CD8-positive lymphocytes. Microgranulomas rarely contained CD20-positive B lymphocytes and expressed low levels of MHC II antigen. As previously described,²⁴ co-infection of macrophages by SIVmac and *M. avium* as detected by *in situ* hybridization and acid-fast stains was rarely identified. Other opportunistic infections were not identified at necropsy.

Immunologically Normal Animals Develop Disseminated Disease but Resolve Mycobacterial Infection

To investigate host responses in immunologically normal animals, 10^8 cfu of *M. avium* was administered to four non-SIV-infected rhesus macaques. These non-SIV-in-

Table 2. Distribution of *M. avium* in Biopsy Sections of Peripheral Lymph Node and Small Intestine after Intravenous Mycobacterial Challenge in Normal, SIVmac251-Infected, and SIVmac239-Infected Animals

	51-97 SIVmac251 LN/SI*	271-96 SIVmac251 LN/SI	109-96 Normal LN/SI	192-96 Normal LN/SI	65-97 SIVmac239 LN/SI	355-96 SIVmac239 LN/SI
-2 wk PI†	-/-‡	-/-	-/-	-/-	-/-	-/-
2 wk PI	-/-	-/-	-/-	-/-	-/-	-/-
4 wk PI	+/+	+/-	+/-	+/-	+/-	+/-
6 wk PI	+++ /++	++ /+	-/-	-/-	-/-	-/-
8 wk PI		+++ /++		-/-		-/-
10 wk PI		++++ /++		-/-		-/-
12 wk PI				-/-		-/-

*LN/SI, lymph node/small intestine.

†PI, Weeks after mycobacterial challenge.

‡-, No acid fast bacteria evident; +, <3 infected cells per high-power field; ++, >3 infected cells per high-power field; +++, 10-50% of cells contain acid-fast bacilli; +++++, >50% of cells contain acid-fast bacilli.

infected animals remained clinically normal throughout the study period (Table 1). Appetite, activity, and stool consistency remained unchanged during acute infection and long-term follow-up conducted in three animals for >365 days has been unremarkable. As in SIVmac251-infected animals, complete blood counts performed biweekly revealed minimal changes. Likewise lymphocyte subset analysis performed on peripheral blood was unremarkable. Lymph node biopsies were obtained at 2-week intervals from these animals and scattered acid-fast bacilli were recognized in histiocytes within medullary sinuses at 4 weeks after mycobacterial challenge but not at later time points (Figure 2D and Table 2). Bacilli were not detected in gastrointestinal biopsies and mycobacterial blood cultures remained negative.

Figure 2. Morphological features of disseminated *Mycobacterium avium* after experimental inoculation in simian AIDS. **A:** Mesenteric lymph node from an SIVmac251-infected animal 6 weeks after *M. avium* challenge reveals characteristic histiocytic infiltrates (H&E stain; original magnification, $\times 140$). **B:** Ziehl-Neelson stain reveals acid-fast bacilli within an infected mesenteric lymph node (Ziehl-Neelson acid-fast stain; original magnification, $\times 424$). **C:** *M. avium* found within lamina propria of ileum (Ziehl-Neelson acid-fast stain; original magnification, $\times 140$). Animals were challenged intravenously with *M. avium* and disseminated infection resulted including extensive involvement of the gastrointestinal tract. Localization here likely resulted in severe diarrhea noted clinically. **D:** Scattered acid-fast bacilli were noted in the peripheral lymph node of immunologically normal animals 4 weeks after *M. avium* challenge (axillary lymph node, Ziehl-Neelson acid-fast stain; original magnification, $\times 424$). As in SIVmac239 and SIVmac239MER co-infected animals, disseminated infection resolved by 6 to 8 weeks after *M. avium* challenge.

Serum chemistry profiles on these normal animals revealed a transient increase in serum globulin and hepatic enzymes. Serum alkaline phosphatase values peaked 4 weeks after *M. avium* inoculation and were significantly higher than base-line values (5667 IU/L versus 1099 IU/L, respectively). H&E-stained sections of liver obtained at necropsy and by hepatic biopsy revealed large numbers of hepatic microgranulomas at 4, 6, and 8 weeks after challenge. Although morphologically similar to those seen in disseminated *M. avium* arising spontaneously in simian AIDS, Ziehl-Neelson stains uniformly failed to demonstrate acid-fast organisms within histiocytes. In three animals followed long term, elevations in hepatic enzymes returned to pre-inoculation values and hepatic microgranulomas resolved. Hepatic morphology was normal by 6 months after *M. avium* administration. Similarly, although lymph node biopsies obtained at 4 weeks after inoculation contained acid-fast bacilli, subsequent biopsies evaluated at 6, 8, and 10 weeks failed to demonstrate *M. avium* indicating resolution of mycobacterial infection.

As a control and to investigate tissue distribution of *M. avium*, one normal animal was euthanized and a complete necropsy performed on day 45 after challenge. Typical microgranulomas were identified in multiple organs including liver, spleen, lung, and bone marrow, however as with hepatic lesions these they were uniformly devoid of acid-fast bacilli. Necropsy findings confirmed that although normal animals developed disseminated infection, a vigorous host response developed that eliminated mycobacterial organisms and prevented the establishment of progressive disease and death.

SIVmac Strain Differences Exist in Production of Progressive Disease

Retrospective analysis of spontaneous disseminated MAC in simian AIDS has revealed a strong association between SIVmac251 and the occurrence of mycobacterial disease as compared to SIVmac239 and its macrophage-tropic derivative.²³ The mechanism behind this association is unknown but it seems to be independent of viral load and CD4 T-lymphocyte count at death. To investigate whether a similar association could be repro-

Table 3. Immunophenotypic Composition of Hepatic Microgranulomas after Intravenous Mycobacterial Challenge in Normal, SIVmac251-Infected, and SIVmac239-Infected Animals

	Mean granuloma size ($\mu\text{m}^2 \times 10^3$)	%CD3 cells	No. CD3 cells/ $10^3 \mu\text{m}^2$	%CD4 cells	No. CD4 cells/ $10^3 \mu\text{m}^2$	%CD8 cells	No. CD8 cells/ $10^3 \mu\text{m}^2$
Normal	93.0	50.1	0.79	32.4	0.40	33.6	0.47
SIVmac239	80.9	54.5	0.95	39.5	0.56	33.9	0.54
SIVmac239MER	91.2	47.6	0.94	33.1	0.56	22.8	0.39
SIVmac251	31.1*	33.5*	0.50†	3.0*	0.05*	25.2	0.36

* $P < 0.001$ Kruskal-Wallis one-way analysis of variance on ranks with multiple comparisons versus control (normal) group (Dunn's method, $P < 0.05$).

† $P < 0.001$ one-way analysis of variance with multiple comparisons versus control (normal) group (Tukey test, $P < 0.05$).

duced in a more tightly controlled experimental setting, animals were infected with SIVmac239 ($n = 2$) and SIVmac239MER ($n = 2$) and challenged with *M. avium* 2 weeks later. After SIV inoculation, parameters of viral infection including peripheral viral load and alterations in lymphocyte subsets were similar to those seen in SIVmac251-infected animals. As with immunologically normal animals, these macaques failed to develop overt clinical signs after *M. avium* challenge.

Sequential biopsies of peripheral lymph node revealed scattered mycobacterial organisms at 4 weeks after infection (Table 2). Blood samples and gastrointestinal biopsies remained negative. However in contrast to SIVmac251-infected animals and paralleling our observations made in the challenged normal macaques described above, biopsies of peripheral lymph node and gastrointestinal tract revealed resolution of mycobacterial infection. Acid-fast bacilli could not be detected in tissues obtained at 6, 8, and 10 weeks after mycobacterial challenge (Table 2 and Table 3). To serve as controls, SIVmac239-infected animal 65-97 and SIVmac239MER-infected animal 154-96 were euthanized at 45 days after *M. avium* administration and a complete necropsy performed. As in immunologically normal animals challenged with *M. avium*, microgranulomas were present in multiple organs including spleen, lung, liver, kidney, and bone marrow but these were uniformly devoid of acid-fast bacilli. Small numbers of acid-fast bacilli were evident focally within colonic sections from the SIVmac239-infected animal 65-97.

Although animals remained clinically normal, elevations in alkaline phosphatase and alanine aminotransferase were detected as early as 2 weeks after *M. avium* challenge. Histological examination of liver sections obtained during weeks 6, 8, and 10 revealed numerous microgranulomas. In striking contrast to SIVmac251-infected macaques and similar to immunologically normal animals, these microgranulomas contained no acid-fast bacilli (Figure 3). One SIVmac239 (355-96) and one SIVmac239MER-infected (221-96) animal were followed long term and euthanized 304 and 410 days after *M. avium* challenge. Both animals died with opportunistic infections including *Pneumocystis pneumonia* and *Cryptosporidial enteritis*. Evaluation of tissues obtained at necropsy revealed complete resolution of granulomatous hepatitis and absence of acid-fast bacilli in all tissues examined.

Co-inoculation experiments revealed that all SIV-infected and immunologically normal animals developed disseminated disease as evidenced by the presence of widespread microgranulomas. However only animals infected with SIVmac251 developed progressive infection characterized by continued mycobacterial growth, severe clinical signs, and death. The ability of SIVmac239- and SIVmac239MER-infected animals to eliminate mycobacterial disease in a manner similar to non-SIV-infected controls seems to be independent of viral load and CD4 T-lymphocyte number present in blood, peripheral lymph node, and gastrointestinal tract as these did not differ among groups.

Figure 3. Comparison of microgranulomas from animals experimentally co-infected with SIVmac and *M. avium* (original magnification, $\times 140$). Although animals co-infected with SIVmac239 or SIVmac251 and *M. avium* developed hepatic microgranulomas, only granulomas from SIVmac251-infected animals contained acid-fast bacilli. **A:** Rhesus macaque 65-97 infected with SIVmac239 followed by 10^8 cfu of *M. avium* was euthanized on day 45 and hepatic tissue collected. Typical microgranulomas were found scattered throughout the liver (liver, H&E). **B:** Rhesus macaque 51-97 infected with SIVmac251 followed by 10^8 cfu of *M. avium* was euthanized on day 45 and hepatic tissue collected. Typical microgranulomas were found scattered throughout the liver (H&E stain). In comparison to the SIVmac239-co-infected animals, granulomas were smaller in size and involved less of the hepatic parenchyma. **C:** Granulomas from rhesus macaque 65-97 co-infected with SIVmac239 and *M. avium* did not contain acid-fast bacilli (liver, Ziehl-Neelson acid-fast stain). **D:** Granulomas from rhesus macaque 51-97 co-infected with SIVmac251 and *M. avium* contained large numbers of acid-fast bacilli (liver, Ziehl-Neelson acid-fast stain).

Figure 4. CD3-positive lymphocytes within hepatic microgranulomas from rhesus macaques 45 days after *M. avium* challenge (original magnification, $\times 140$). The relative and absolute number of CD3-positive lymphocytes recruited to microgranulomas in SIVmac251-infected animals was significantly less than those recruited in normal and SIVmac239/SIVmac239MER-infected animals. **A:** Hepatic microgranuloma from normal rhesus macaque 109-96. **B:** Hepatic microgranuloma from SIVmac251-infected rhesus macaque 51-97. **C:** Hepatic microgranuloma from SIVmac239-infected rhesus macaque 65-97. **D:** Hepatic microgranuloma from SIVmac239MER-infected rhesus macaque 154-96.

Failure to Recruit CD3-, CD4-, and CD8-Positive Lymphocytes Predicts Disease Progression

Microgranulomas are commonly found in hepatic tissue of macaques with AIDS and spontaneous disseminated MAC. To determine whether there were immunomorphological correlates of protection after experimental inoculation, immunohistochemistry was performed on liver sections obtained at 6 weeks after *M. avium* challenge to identify the immunophenotype of cells within newly formed microgranulomas. Quantitative image analysis revealed fundamental differences between microgranulomas from SIVmac251-infected animals compared to normal and SIVmac239 and SIVmac239MER-infected animals (Table 3). Microgranulomas from SIVmac251-inoculated animals were significantly smaller than microgranulomas from immunologically normal and SIVmac239/SIVmac239MER-infected animals. Furthermore, histiocytes within such granuloma expressed decreased levels of the MHC II antigen compared to normal animals (127.2 versus 159.5 mean gray scale intensity/granuloma, $P < 0.001$ *t*-test) suggesting a lesser degree of macrophage activation.

The percentage of CD3-positive cells within granulomas and the number of CD3-positive lymphocytes/ $10^3 \mu\text{m}^2$ within microgranulomas in SIVmac251-infected animals was significantly less than those recruited in normal and SIVmac239/SIVmac239MER-infected animals (Figure 4 and Table 3). Image analysis also confirmed a striking reduction in both the percent and density of CD4-positive cells within hepatic microgranulomas from SIVmac251-infected animals (Table 3). This difference did not represent a global phenomenon as little difference was observed between groups in CD4 relative num-

ber in blood, peripheral lymph node, or gastrointestinal tract (Figure 1). Rather, the decreased number of CD4 cells reflects a decreased recruitment of such cells to microgranulomas in animals with progressive *M. avium* disease.

CD8-positive lymphocytes constitute the principal cytotoxic T-cell population in defense against many intracellular pathogens. Although most experimental work has indicated a dominant role for CD4 T cells in protection against mycobacterial infection, murine studies have suggested a role for cytotoxic T lymphocytes as well.^{21,43} There was no significant difference in the percent or density of CD8-positive cells within hepatic microgranulomas. Thus, microgranulomas differed in the composition of specific T-lymphocyte subsets and these differences correlated with the presence of acid-fast bacilli and the development of progressive disease.

Discussion

In this study, we describe an experimental system to co-inoculate rhesus macaques with *M. avium* and SIV to reproduce disseminated mycobacterial disease. We have used this model system to demonstrate the complex interactions that occur between host, mycobacteria, and immunosuppressive lentiviruses. We demonstrate that the development of progressive disease with continued mycobacterial growth is dependent on the strain of SIV. Specifically, co-infection with uncloned SIVmac251 resulted in progressive infection whereas co-infection with closely related T-cell tropic SIVmac239 or macrophage tropic SIVmac239MER did not. This occurred despite an absence of differences between the groups with respect to viral load or CD4 T-cell number but was correlated with significant differences in the composition of microgranulomas. These data suggest that viral determinants play a critical role in facilitating MAC infection through modulation of monocyte/macrophage effector function.

The clinical and morphological features of this process closely parallels that seen in humans and rhesus macaques with AIDS. SIVmac251-co-infected animals developed persistent diarrhea and progressive wasting. Physical examination revealed a febrile response, peripheral lymphadenopathy, and massive hepatosplenomegaly. At death mycobacterial infection was widespread with acid-fast bacilli found in multiple organs. Despite intravenous challenge tissue distribution was nearly identical to that found in spontaneous disease including significant involvement of the gastrointestinal tract. This suggests that the sites of MAC infection are determined by factors other than route of infection. Furthermore, histomorphology revealed typical histiocytic infiltrates and microgranulomas characteristic of disseminated MAC in AIDS.

In human patients and rhesus macaques, spontaneous disseminated MAC develops at severe CD4 T-lymphocyte depletion. In this model, animals were challenged intravenously with 10^8 cfu of *M. avium* 2 weeks after primary SIV infection and developed disseminated disease despite preservation of CD4 T lymphocytes in blood and peripheral lymph node. An alternative experi-

mental approach would be to wait until CD4 T-lymphocyte count dropped below a critical level before mycobacterial challenge. We have reproduced disease in such severely immunodeficient animals (CD4 T lymphocytes <100 cells/dL) after oral and intravenous inoculation of *M. avium* (data not shown). Although this alternative approach will undoubtedly have potential applications, the long interval between primary SIV infection and the occurrence of a suitable level of immunodeficiency poses a serious drawback. Mean survival to AIDS in rhesus macaques infected with either SIVmac251 or SIVmac239 is 18 months. Furthermore, the occurrence of spontaneous opportunistic infections during the course of progressive immunodeficiency could confound interpretation of experimental mycobacterial inoculation. Our interests are focused on elucidating interactions between the virus, host, and mycobacteria that may promote disseminated disease. A 2-week time frame was chosen to coincide with the period of peak viremia and to decrease the degree of genetic variation that would occur in SIV with time and disease progression.

Granuloma formation likely represents a critical event in the orchestration of an effective host immune response to mycobacterial infection and is dependent on the recruitment of inflammatory cells and macrophage activation. In our studies morphological evaluation of hepatic microgranulomas revealed differences in granuloma composition that predicted progressive mycobacterial disease. In SIVmac251-infected animals, fewer T lymphocytes were recruited to microgranulomas primarily reflecting a decrease in the CD4-positive subtype. CD4 T lymphocytes have been shown to play a critical role in the elimination of intracellular mycobacteria through the elaboration of cytokines that result in macrophage activation and increased mycobacteriocidal activity. Decreased staining of microgranulomas for MHC-II supports the presence of ineffective macrophage activation in SIVmac251-infected animals.

Although variability is noted in the number of acid-fast bacilli and granuloma composition in spontaneous cases of disseminated MAC, this likely represents differences in disease stage. In contrast to experimental inoculation, granulomas in spontaneous cases do not resolve and are therefore fundamentally different. Although staining for acid-fast bacilli lacks the sensitivity of mycobacterial culture, complete resolution of granulomas in normal, SIVmac239-inoculated, and SIVmac239MER-inoculated animals indicates that these granulomas were effective in eliminating mycobacterial organisms.

Recent evidence has also suggested a role for CD8-positive cytotoxic T lymphocytes in host defenses against disseminated mycobacterial infection.^{21,43} The preservation of CD4- and CD8-positive T lymphocytes in blood and peripheral lymph node and the absence of significant differences between SIVmac251- and SIVmac239-infected animals at these sites suggests that the decreased number of T lymphocytes within hepatic microgranulomas did not simply represent a global phenomenon. Rather, mycobacterial-infected macrophages in SIVmac251-inoculated animals failed to recruit an effective T-cell response.

The reason SIVmac251-infected animals are more susceptible to spontaneous *M. avium* disease compared to SIVmac239-infected animals is unknown.²³ This conclusion was based on a retrospective analysis of animals with AIDS and was independent of CD4 count at death. In this report we reproduce this phenomenon through inoculation of animals with *M. avium* during the acute phase of SIV infection. During this period severe depletion of CD4 T cells does not occur and opportunistic infections are not seen. We suspect that viral determinants present within the SIVmac251 wild-type inoculum are absent from the SIVmac239 molecular clone and that these differences promote pathogen survival through adverse effects on the host's ability to eliminate mycobacteria. The *in vitro* and *in vivo* characteristics of SIVmac251 and SIVmac239 have been described extensively.³³ SIVmac251 represents the first SIV wild-type isolate and replicates and causes cytopathology in lymphocytes and macrophages. SIVmac239 was the first pathogenic molecular clone and was derived from a SIVmac251-inoculated animal. This virus grows to high titer in lymphocytes but decreased utilization of the CD4 receptor present at low density in tissue macrophages restricts its ability to replicate in cells of monocyte/macrophage lineage.^{35,44} Genetic differences between the consensus sequence of SIVmac251 and the SIVmac239 molecular clone are primarily concentrated within the envelope region; overall they share 98% nucleotide identity.³⁴ Although the *in vitro* tropism of SIVmac239 and SIVmac251 differ significantly, the clinical disease course with these two viruses is nearly identical with no significant difference in mean survival and CD4 T-lymphocyte count at death.^{23,45} With the exception of disseminated MAC the incidence of other spontaneously occurring opportunistic infections including *Pneumocystis carinii*, cytomegalovirus, *Cryptosporidium parvum*, *Enterocytozoon bieneusi*, Adenovirus, simian virus 40, and lymphocryptovirus is similar.⁴⁶

It is tempting to speculate that in comparison to SIVmac239 the ability of SIVmac251 to replicate in macrophages may have been responsible for its association with mycobacterial disease. However, macrophage tropic variants arise frequently in SIVmac239-infected animals²⁹ and SIVmac239 infects macrophages *in vivo* within 2 to 3 weeks of primary inoculation.^{39,42} Furthermore, we could find no evidence that the macrophage tropic clone, SIVmac239MER, altered disease course from that seen in the parental strain. SIVmac239MER is a variant of SIVmac239 containing three amino acid differences in the envelope protein that confer the ability to enter and replicate within macrophages presumably through changes in CD4-receptor affinity. Therefore it is unlikely that macrophage tropism in-and-of-itself can account for the differences in mycobacterial disease pathogenesis observed between SIVmac251- and SIVmac239-infected animals.

In vitro evidence suggests direct and indirect growth-promoting effects of viral proteins on MAC organisms.^{3-5,8-10} At physiological concentrations recombinant gp120 has been shown to enhance growth of *M. avium* within human monocytes and macrophages.^{5,10} Recombinant gp120 induces phenotypic changes in human monocytes in-

cluding decreased expression of HLA-DR, CD64, and interferon- γ -receptor 1 and causes alterations in tumor necrosis factor production that may adversely impact the ability of macrophages to eliminate *M. avium*.⁴⁷ Impaired phagolysosomal fusion has been identified in peripheral blood monocytes from HIV-1-infected patients, a finding that could be reproduced by the addition of recombinant gp 120 to cells from normal individuals.⁴⁸ The *in vivo* relevance of these findings have been difficult to ascertain. We suspect that in addition to the destructive effect of HIV on CD4 T lymphocytes, that viral determinants may play a critical role in facilitating infection by MAC through modulating monocyte/macrophage effector function. Such interactions are likely to be most relevant during the late stages of HIV infection when CD4 T lymphocytes are depleted and can no longer orchestrate an effective immune response. The rhesus macaque model of disseminated *M. avium* provides the opportunity to investigate these *in vitro* observations and evaluate the *in vivo* consequences of differences in viral determinants on mycobacterial disease.

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