Cell Tropism of Simian Immunodeficiency Virus in Culture Is Not Predictive of *in Vivo* Tropism or Pathogenesis

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SIVmac239/316 is a molecular clone derived from SIVmac239 that differs from the parental virus by nine amino acids in env. This virus, unlike the parental SIVmac239, is able to replicate well in alveolar macrophages in culture. We have not however, observed macrophage-associated inflammatory disease in any animal infected with SIVmac239/316. Therefore, we sought to examine the cell tropism of this virus *in vivo* in multiple tissues using *in situ* hybridization combined with immunohistochemistry and multilabel confocal microscopy for viral nucleic acid and multiple cell-type-specific markers for macrophages and T lymphocytes. Tissues examined included brain, heart, lung, lymph nodes, spleen, thymus, and small and large intestine. Matched tissues from macaques infected with the parental SIVmac239 and uninfected macaques were also examined. Many infected cells were detected in the tissues of animals infected with SIVmac239 and SIVmac239/316 although there appeared to be fewer positive cells in animals infected with SIVmac239/316. Surprisingly, in light of the cell culture observations, nearly every simian immunodeficiency virus-infected cell in animals inoculated with SIVmac239/316 was a T lymphocyte rather than a macrophage. This was true both during early infection (first 2 months) and in terminal disease. In contrast, as previously described, SIVmac239 was found in both T cells and macrophages in tissues as early as 21 days after infection. These studies indicate that during both acute and chronic SIVmac239/316 infection T lymphocytes rather than macrophages are the principal targets *in vivo*. These

data combined with the absence of macrophage-associated lesions in SIVmac239/316-infected animals indicate that *in vitro* cell tropism is not predictive of *in vivo* tropism or disease pathogenesis. (Am J Pathol 2004, 165:2111–2122)

Simian immunodeficiency virus (SIV) was first isolated in 1984 from captive rhesus macaques (*Macaca mulatta*) at the New England Primate Research Center.^{1,2} SIV-infected macaques develop immunodeficiency characterized by a range of histological changes in lymphoid tissues culminating with lymphoid depletion, opportunistic infections, and in up to 50% of the animals, primary lentivirus-induced inflammatory diseases such as giant cell pneumonia and SIV encephalitis (SIVE).^{3–11} SIVE is characterized by perivascular infiltrates of macrophages and multinucleated giant cells and lesser numbers of lymphocytes, with abundant SIV nucleic acid and antigen within the lesions.^{7,11}

Molecularly cloned SIVmac239 causes acquired immune deficiency syndrome (AIDS) and death in ~25% of inoculated rhesus monkeys within 6 months of inoculation with 50% mortality in a little more than 1 year.¹² The remaining animals develop a more protracted course. Although SIVmac239, the prototypic pathogenic molecular clone, does not replicate efficiently within macrophages in culture, it does infect macrophages *in vivo* as early as 21 days after infection.^{13–15} Furthermore, ~40% of rhesus macaques that die with AIDS after infection with SIVmac239 display lesions associated with infection of macrophages such as encephalitis (SIVE) and giant cell pneumonia.^{1,12,16}

The acquired ability of SIVmac239 to replicate efficiently in monocytes/macrophages in culture was originally mapped to nine amino acid changes in env-

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Table 1. Animals Infected with SIV: Time of Tissue Collection and Major Pathological Findings

Virus	Animal	Days after infection	Major pathologic diagnoses	AIDS
SIVmac239/316	181-94	7	End of experiment, NSL	No
	193-94	7	End of experiment, NSL	No
	106-94	14	End of experiment, follicular hyperplasia and dysplasia	No
	120-94	14	End of experiment, NSL	No
	130-94	21	End of experiment, NSL	No
	229-94	21	End of experiment, follicular hyperplasia and dysplasia	No
	136-94	50	End of experiment, follicular hyperplasia and dysplasia	No
	171-94	50	End of experiment, NSL	No
	387-90	909	Myocardial fibrosis, pulmonary arteriopathy and thrombosis, MAC, PCP, SV40, adenovirus	Yes
	371-90	1031	Myocardial fibrosis, pulmonary arteriopathy and thrombosis, LCV	Yes
	378-90	1049	Sepsis, endocarditis	Yes
	376-90	1556	Thrombus, arteriopathy	Yes
	383-90	1623	End of experiment, LN hyperplasia	No
	369-90	1805	End of experiment, NSL	No
	363-90	2506	MAC, lymphoid depletion	Yes
SIVmac239	247-95	7	End of experiment, NSL	No
	317-96	7	End of experiment, NSL	No
	248-95	14	End of experiment, NSL	No
	318-96	14	End of experiment, NSL	No
	249-95	21	End of experiment, follicular hyperplasia	No
	441-93	21	End of experiment	No
	434-93	50	End of experiment, follicular hyperplasia and dysplasia	No
	364-93	50	End of experiment, follicular hyperplasia and dysplasia	No
	T196	175	Giant cell pneumonia and gastritis, PCP	Yes

NSL, no significant lesions; MAC, *Mycobacterium avium* complex; LCV, lymphocryptovirus; PCP, *Pneumocystis carinii* pneumonia; SV40, Simian virus 40.

lope.^{16–19} The variant of SIVmac239 with these amino acid changes in env was designated SIVmac239/316 env,¹⁷ hereafter referred to as SIVmac239/316. Further work showed that of these nine amino acid changes four (V to M at residue 67, K to E at residue 176, G to R at residue 382, and K to T at residue 573) were strongly associated with the ability of the parental virus, SIVmac239 to replicate well in cultured macrophages.^{17,19} In addition to conferring macrophage tropism these changes were found to result in a significant decrease in the requirement for CD4 before CCR5 binding and to render the virus susceptible to antibody neutralization.^{20–25}

These observations led to speculation that SIVmac239/316 would have a broader tropism than the parental SIVmac239 with a particular preference for CCR5^{high} CD4^{low} populations such as alveolar macrophages, parenchymal microglia in brain and other cell types, particularly those in immunologically privileged sites such as the brain. Contrary to this prediction is the observation that although animals infected with SIVmac239/316 do develop AIDS, macrophage-associated lesions such as SIVE have not been observed (Table 1). To try and resolve these observations we sought to determine the cell types infected *in vivo* in multiple tissues during both acute and chronic SIVmac239/316 infection. To our surprise, not only did we not find evidence of a broadened cell tropism for SIVmac239/316 compared to

SIVmac239 but the only cell type we found infected *in vivo* were T lymphocytes. This raises interesting questions as to the applicability of *in vitro* studies that have defined the cell tropism of SIV and HIV to what is occurring *in vivo*.

Materials and Methods

Viral Inoculum

The viruses used for animal infection in this study were molecularly cloned SIVmac239 and SIVmac239/316.^{17,18,26,27} SIVmac239 is the prototypic pathogenic molecular clone of SIV. SIVmac239/316env (hereafter SIVmac239/316) was derived from SIVmac239 by exchanging the envelope gene of SIVmac316 into SIVmac239.¹⁷ SIVmac316 was obtained from an animal inoculated with SIVmac239 that died of AIDS and disseminated giant cell disease including SIVE and giant cell pneumonia.¹⁶ The differences between the clones of SIVmac239 and SIVmac239/316 used in this study are limited to nine amino acids in env.^{17,18} Therefore differences in tropism and disease pathogenesis can be attributed to the env protein. The stop codons in nef and transmembrane were removed in both the parental SIVmac239 and SIVmac239/316.

Animals and Tissues

Tissues from 24 SIV-infected and 2 uninfected rhesus macaques (*Macaca mulatta*) were obtained from the pathology archives of the New England and Tulane National Primate Research Centers. This included 15 animals infected with SIVmac239/316 and 9 infected with the parental virus SIVmac239. All animals were infected with the same dose of SIV (50 ng of p27) and the same route (intravenous). Of the 15 animals infected with SIVmac239/316, 8 were euthanized within 50 days of infection as part of a serial sacrifice study and 7 were followed long term. Details on the animals including time after inoculation and major pathological findings are provided in Table 1. Matched tissues from nine macaques infected with the parental SIVmac239 and two uninfected macaques, were examined in parallel. Data from numerous additional historical controls infected with SIVmac239 were also available.^{13–15,28–31} All animals received a complete necropsy and histopathological examination. Tissues selected for examination included brain, heart, lung, lymph nodes, spleen, thymus, and small and large intestine. All tissues had been collected immediately after euthanasia with an intravenous overdose of pentobarbital. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 μ m, and stained with hematoxylin and eosin. Adjacent tissues were also embedded in optimal cutting temperature compound (O.C.T.; Miles Inc., Elkhart, IN) and snap-frozen by immersion in 2-methylbutane in dry-ice and cut into sections 6 to 8 μ m thick.

Viral Load

Viral RNA loads in plasma of SIV-infected rhesus macaques were measured by quantitative real-time polymerase chain reaction as previously described.^{32–34}

Localization of SIV-Infected Cells

In situ hybridization for SIV was performed using both riboprobes and random primed DNA probes as described previously.³⁵ Briefly, for RNA *in situ* hybridization formalin-fixed, paraffin-embedded tissue sections were pretreated in a microwave with citrate buffer (antigen unmasking solution; Vector Laboratories, Burlingame, CA) for 20 minutes at high power according to the manufacturer's instructions. Thereafter, sections were thoroughly washed, placed in a humidified chamber, and prehybridized at 45°C with hybridization buffer (containing 50% of formamide with denatured herring sperm DNA and yeast tRNA at 10 mg/ml each). SIV-digoxigenin-labeled anti-sense riboprobes (provided by Drs. V. Hirsch and C. Brown, National Institutes of Health, Rockville, MD) were used at a concentration of 10 ng/slide in hybridization buffer and hybridized overnight at 45°C. After hybridization slides were washed with 2 $\times$ standard saline citrate, 1 $\times$ standard saline citrate, 0.1 $\times$ standard saline citrate, and blocking solution was applied. Fab fragments from an anti-digoxigenin antibody from sheep, conjugated with alkaline phosphatase (Roche, Penzberg,

Germany), were used to detect digoxigenin-labeled probes. Controls included matched tissues from known positives and negatives and hybridization with digoxigenin-labeled sense RNA labeled with digoxigenin.

For DNA *in situ* hybridization the DNA probe used was a combination of two plasmids: a subclone of p239SpE3' in phosphate-buffered saline (PBS), which contains *tat*, *rev*, *env*, *nef*, and a small part of the 3'LTR, and p239SpSp5', which contains *gag*, *pol*, *vif*, *vpx*, *vpr*, and the 5'LTR in PBS. This combination provides essentially the entire SIVmac239 genome. The probes were labeled with digoxigenin-11-dUTP by random priming (Boehringer-Mannheim, Indianapolis, IN) as described previously.^{36,37} Hybridization was performed under denaturing conditions to detect both viral DNA and RNA. Formalin-fixed, paraffin-embedded tissue sections were pretreated in a microwave with antigen unmasking solution (Vector Laboratories) for 20 minutes at high power and according to the manufacturer's instructions. Thereafter, sections were thoroughly sequentially washed, placed in a humidified chamber, and prehybridized at 37°C with hybridization buffer (containing 50% formamide with denatured herring sperm DNA and yeast tRNA at 10 mg/ml each) and washed with 2 $\times$ standard saline citrate. SIV digoxigenin-labeled DNA probes were used at 0.5 ng/ μ l in hybridization buffer and hybridized overnight at 37°C. After hybridization, sections were treated as previously described above for RNA *in situ* hybridization. Selected positive tissues and serial sections of the test tissues hybridized with plasmid pUC19 labeled with digoxigenin were used as controls.

When immunohistochemistry followed *in situ* hybridization the slides were developed using NBT/BCIP stock solution (Roche, Mannheim, Germany) or Vector blue (Vector Laboratories) as chromogen. If immunofluorescence followed the *in situ* hybridization, 2-hydroxy-3-naphthoic acid-2'-phenylaniide phosphate (HNPP) fluorescent detection system (Boehringer Mannheim) was used. Briefly, slides were rinsed in detection buffer (0.1 mol/L Tris base, 0.1 mol/L NaCl, 0.01 mol/L MgCl₂) and then 200 μ l of HNPP/Fast Red TR (10 μ g of HNPP in 1 ml of detection buffer plus 10 μ l of Fast Red TR solution) was applied. This solution was filtered through a 0.2- μ m nylon filter immediately before use, and then the slides were coverslipped and incubated for 30 minutes at room temperature in the dark.

Immunophenotype of Infected Cells

To define the immunophenotype of infected cells we performed combined *in situ* hybridization/immunohistochemistry as described previously.^{13–15,31} After *in situ* hybridization for viral nucleic acid as described above, single- or double-label immunohistochemistry or immunofluorescence was performed using a variety macrophage- and T lymphocyte-specific markers (Table 2).

In situ hybridization followed by single-label immunohistochemistry for macrophages or T cells was performed as previously described.^{13,15} To identify macrophages we initially used CD68 (KP-1, IgG1, M0814; DAKO Corp.,

Table 2. Antibodies Used in Immunohistochemistry and Immunofluorescence

Antigen	Cell type	Source	Antibody type*	Dilution	Technique
CD68 (Kp1)	Macrophage	DAKO	IgG1	1:125, 1:25	IHC, IFA
HAM56	Macrophage	DAKO	IgM	1:25, 1:5	IHC, IFA
MAC387	Macrophage	DAKO	IgG1	1:20	IFA
MRP8	Macrophage	Accurate Chemical & Scientific Corp.	IgG1	1:640	IFA
CD3	T lymphocyte	DAKO	Polyclonal	1:350, 1:100	IHC, IFA
CD5	T lymphocyte	DAKO	IgG1	1:10	IFA
CD7	T lymphocyte	DAKO	IgG2b	1:10	IFA

*For monoclonal antibodies the isotype is indicated. All polyclonal antibodies were made in rabbit.

Carpinteria, CA) or HAM56 (IgM, M0632; DAKO) whereas for T cells we used a polyclonal primary antibody specific for CD3 (DAKO). Briefly, after *in situ* hybridization for SIV, sections were incubated sequentially with the primary, cell-type-specific antibody for 60 minutes (monoclonal) or 30 minutes (polyclonal) at room temperature followed by biotinylated horse anti-mouse or goat anti-rabbit (Vector Laboratories) secondary antibodies, respectively. Finally, sections were incubated with avidin-biotin-complex (ABC alkaline phosphatase, Vector Laboratories), and the reaction was visualized with Vector Red (Vector Laboratories). As negative control, serial sections were processed identically using equivalent concentrations of irrelevant primary antibodies of the same isotype.

To more rigorously examine the cell types infected we performed additional multilabel techniques combining *in situ* hybridization with immunohistochemistry or immunofluorescence for two cell-type-specific markers as described previously^{14,31} using combinations of antibodies as shown in Table 3. For these multilabel techniques, *in situ* hybridization was performed as described above except that the results of *in situ* hybridization were visualized with HNPP/Fast Red, which fluoresces intensely red. In addition to CD68, HAM56, and CD3 we used several additional T cell- and macrophage-specific antibodies (Tables 2 and 3). The cell type-specific antibodies of differing isotypes or species origin were applied sequentially followed by isotype-specific anti-mouse or species-specific secondary antibodies applied simultaneously. The secondary antibodies were coupled to either Alexa 488 (green) or Alexa 633 (far red) (Molecular Probes, Eugene, OR) as shown in Table 3. After antibody treatment, sections were washed twice for 15 minutes in PBS with 0.2% fish skin gelatin. Finally, the sections

were rinsed in doubly distilled H₂O and mounted with aqueous mounting medium.

Confocal Microscopy

Confocal microscopy was performed using a Leica TCS SP2 confocal microscope equipped with three lasers (Leica Microsystems, Exton, PA). Individual optical slices represent 0.2 μ m and 32 to 62 optical slices were collected at 512 $\times$ 512 pixel resolution. NIH Image (version 1.62) and Adobe Photoshop (version 7.0) were used to assign colors to the four channels collected: HNPP/Fast Red substrate that fluoresces red when exposed to a 568-nm wavelength laser appears red, Alexa 488 (Molecular Probes) is green, Alexa 633 (Molecular Probes) appears blue, and the differential interference contrast (DIC) image is gray scale. The four channels were collected simultaneously. In some tissues and to differentiate between individual cells, To-pro3 (nuclear marker, Molecular Probes) was used at 1 μ g/ml, incubated for 5 minutes, and tissues were washed in PBS. Co-localization of antigens is demonstrated by the addition of colors as indicated in the figure legends.

Quantitation of SIV-Infected Cells in Situ

Both semiquantitative and quantitative means were used to quantify the total number of infected cells and the number of infected macrophages. For subjective quantitation (Table 4) sections were scored from negative (–) to ++++ as follows: absence of positive cells per section, –; 1 to 10 positive cells per $\times$ 10 field, +; 10 to 30

Table 3. Antibody and Fluorochrome Combinations for Double and Triple Label Confocal Microscopy

Channel 1 Red with HNPP/Fast Red		Channel 2 Green with Alexa 488		Channel 3 Far Red with Alexa 633	
Riboprobes or DNA probes	Anti-digoxigenin/sheep	CD68 (IgG1) mouse	Anti-mouse (IgG1)	CD3 rabbit	Anti-rabbit
Riboprobes or DNA probes	Anti-digoxigenin/sheep	HAM56 (IgM) mouse	Anti-mouse (IgM)	CD3 rabbit	Anti-rabbit
Riboprobes	Anti-digoxigenin/sheep	MAC387 (IgG1) mouse	Anti-mouse (IgG1)	CD3 rabbit	Anti-rabbit
Riboprobes	Anti-digoxigenin/sheep	MRP8 (IgG1) mouse	Anti-mouse (IgG1)	CD3 rabbit	Anti-rabbit
Riboprobes	Anti-digoxigenin/sheep	CD3 rabbit	Anti-rabbit		
Riboprobes	Anti-digoxigenin/sheep	HAM56 (IgM) mouse	Anti-mouse (IgM)	CD5 (IgG1) mouse	Anti-mouse (IgG1)
Riboprobes	Anti-digoxigenin/sheep	HAM56 (IgM) mouse	Anti-mouse (IgM)	CD7 (IgG2b) mouse	Anti-mouse (IgG2b)

In situ hybridization was visualized with HNPP/Fast Red, which fluoresces intensely red (channel 1) followed by additional labels detected using secondary antibodies conjugated to Alexa 488 (channel 2) or Alexa 633 (Far Red, channel 3). The color of the Far Red channel is changed to blue at the time of data acquisition on the confocal microscope.

positive cells per $\times 10$ field, ++; 30 to 100 positive cells per $\times 10$ field, +++; greater than 100 positive cells per $\times 10$ field, ++++.

For objective quantitation of the number of infected cells/mm² of tissue and the number of infected macrophages/mm² we used computer-assisted image analysis as described previously.^{13,38} Briefly, combined *in situ* hybridization/immunohistochemistry for SIV and macro-

phages (HAM56) was performed on sections of lymph node and spleen and examined by light microscopy as described above. The use of two different chromogens allowed for identification of SIV-infected cells in blue (Vector blue) and HAM56⁺ macrophages in red (Vector red). Macrophages infected with SIV would appear purple because of the mixing of the red and blue chromogens. The number of infected cells/mm² and infected macrophages/mm² was

Figure 1. Principal lesions in SIVmac239/316-infected macaques. **A:** Follicular hyperplasia and dysplasia in lymph node of an animal during acute infection. **C** and **E:** Enlarged hypocellular germinal centers containing eosinophilic material without discernible mantle zones in chronically infected animals in lymph node (**C**) and spleen (**E**). Several chronically infected animals also had extensive myocardial fibrosis (**B**), arterial thrombi (**D**), and arteriopathy (**F**) involving small- to medium-sized vessels in heart, lung, brain, and kidney. Examples from brain (**D**) and heart (**F**) are shown.

Table 4. SIV *in Situ* Hybridization Results

Virus	Animal	Survival (days)	Brain	Lungs	Heart	GI	LN	Spleen	Thymus
SIVmac239/316	181-94	7	—	—	—	+	+++	++	NA
	193-94	7	—	—	—	—	++	+	NA
	106-94	14	—	—	—	+	—	+	—
	120-94	14	—	+	—	—	+	+	—
	130-94	21	—	—	—	—	+	+	—
	229-94	21	+	—	—	—	+++	++	—
	136-94	50	—	+	—	+	++++	+++	+++
	171-94	50	—	—	—	—	+++	NA	—
	387-90	909	—	+	—	+	+	+	NA
	371-90	1031	—	—	—	—	—	—	—
	378-90	1049	—	—	—	+	+	+	+
	376-90	1556	—	NA	—	—	—	—	—
	383-90	1623*	—	—	—	—	—	—	—
	369-90	1805*	—	—	—	—	+	+	NA
	363-90	2506	—	—	—	+	—	—	—
SIVmac239	247-95	7	—	—	—	++	+++	+++	NP
	317-96	7	—	—	—	+	+++	+++	NP
	248-95	14	—	—	—	++	+++	++	NP
	318-96	14	—	—	—	++	++	++	NP
	249-95	21	—	—	—	+	+++	++	NP
	441-93	21	NP	NP	NP	+	++	+++	NP
	434-93	50	NP	NP	NP	+	++++	+++	NP
	364-93	50	NP	NP	NP	++	+++	++	NP
	T196	175	—	+++	—	+++	+++	++	—

Results of RNA *in situ* hybridization for SIV were quantified as follows: no positive cells, negative; 1 to 10 positive cells per $\times 10$ field, +; 10 to 30 positive cells per $\times 10$ field, ++; 30 to 100 positive cells per $\times 10$ field, +++; greater than 100 positive cells per $\times 10$ field, +++++. *, End of experiment. NA, Tissue not available; NP, not performed.

determined by using a Leica DMLB microscope with a SPOT Insight digital camera (Digital Instruments Inc., Sterling Heights, MI) interfaced to Image-Pro Plus (Media Cybernetics, Inc., Silver Spring, MD) image analysis software. The difference in staining characteristics (blue, red, or purple) allowed for the software to automatically identify positive cells. The absolute number of SIV-infected cells versus infected macrophages was determined from an average of five different 1-mm² areas per slide from each tissue (lymph node and spleen) in two animals per time point.

Results

Histopathology

In acute infection the principal lesions in macaques infected with SIVmac239/316 were in the lymphoid tissues with follicular hyperplasia and dysplasia the most common finding (Figure 1). In long-term SIVmac239/316-infected macaques, five of seven animals developed AIDS at different times after SIV inoculation (Table 1). These animals had generalized lymphoid depletion and follicular involution characterized by enlarged hypocellular germinal centers containing amorphous eosinophilic material without discernible mantle zones (Figure 1, C and E). These changes are consistent with pathogenic SIV infection and AIDS.^{3–5,11,28} In addition, several chronically infected animals had extensive myocardial fibrosis, arterial thrombi, and arteriopathy involving small- to medium-sized vessels in heart, lung, brain, and kidney (Figure 1; B, D, and F). These vascular lesions were previously

described as unique to macaques infected with SIV.^{11,39} Three of the five animals that developed AIDS also had a variety of opportunistic infections including SV40, adenovirus, lymphocryptovirus, *Pneumocystis carinii*, and *Mycobacterium avium* complex (Table 1). With the exception of inflammation associated with these opportunistic infections, SIVmac239/316-infected animals did not have typical primary SIV-associated inflammatory lesions such as giant cell pneumonia and SIVE.

Viral Loads

Viral loads in the animals infected with SIVmac239/316 have previously been reported³² as have viral loads in numerous animals infected with SIVmac239.^{32,33,40,41} Consistent with *in vitro* data^{17,42} early replication of SIVmac239/316 is similar to that of the parental SIVmac239 from which it was derived (Table 4). Viral loads at

Table 5. Viral RNA Load Comparisons

Time of load detection	Viral RNA load $\times 10^6$ (no. of animals)	
	SIVmac239	SIVmac239/316
Peak height	47 $\pm$ 28 (10)	84 $\pm$ 58 (6)
Set point	4.2 $\pm$ 2.3 (5)	<0.01 (6)

Viral loads are expressed as SIV RNA copy equivalents per ml of plasma. The numbers indicate means $\pm$ SD for the indicated number of animals. The detection limit for SIVmac239/316 was 10,000 copies/ml because of the historic nature of the study and the way the samples were stored.

peak height (week 2) and at set point (weeks 20 to 50) were averaged and compared between 6 of the animals infected with SIVmac239/316 for which such data were available and 10 animals infected with SIVmac239 (Table 5). Although peak viral loads were similar, SIVmac239/316 infection is rapidly controlled with much lower viral loads at set point. Consistent with this observation, animals infected with SIVmac239/316 tend to have a prolonged survival³² but ultimately progress to AIDS as indicated in Table 1.

Localization of SIV-Infected Cells

Localization of SIV-infected cells in tissues was examined by *in situ* hybridization using both riboprobes and random-primed DNA probes to detect viral RNA and/or DNA. Both techniques gave similar results, which are summarized in Table 4. In animals infected with either SIVmac239 or SIVmac239/316, lymphoid tissues (lymph nodes, spleen, and gut-associated lymphoid tissue) harbored the majority of infected cells. In SIVmac239/316-infected animals, chronically infected animals generally had fewer SIV-positive cells compared to animals in the acute phase of infection consistent with viral load data (Tables 4 and 5). In acutely infected animals the pattern of viral infection was similar to what has been described in animals infected with the parental molecular clone SIVmac239.^{15,28,43} In addition, by 21 days after infection, a diffuse staining pattern was noted over germinal centers consistent with trapping of viral particles (antigen/antibody complexes) on follicular dendritic cells (Figure 2A). Although there were marked similarities between the tissue distribution of SIVmac239/316 and SIVmac239, animals infected with SIVmac239/316 had relatively less virus in tissues by *in situ* hybridization at later time points consistent with lower viral loads in these animals (Tables 4 and 5 and reference 32). In addition, little if any infection of the central nervous system was detected (Table 4) and the morphology of infected cells was more consistent with lymphocytes than macrophages (Figure 2).

Immunophenotype of Cells Infected with SIVmac239/316

To examine the immunophenotype of infected cells we performed combined *in situ* hybridization for SIV followed by immunohistochemistry for macrophage or T-lymphocyte markers individually and in combination (Tables 2 and 3). Initially we performed routine *in situ* hybridization for SIV followed by immunohistochemistry for CD68 (a macrophage marker) in acute and long-term SIVmac239/316-infected animals using different chromogenic substrates. This technique revealed few if any SIV-infected macrophages (Figure 2C). To more rigorously examine the immunophenotype of the infected cells and avoid potential problems with color mixing when using chromogenic substrates we performed fluorescent *in situ* hybridization for SIV followed by immunofluorescence for macrophage- and T cell-specific markers individually and in combination (Table 3). Tissues examined included

Figure 2. Localization of virus in macaques infected with SIVmac239/316. **A:** SIV *in situ* hybridization reveals a dendritic staining pattern at 21 days after infection in lymph node consistent with antigen/antibody trapping on follicular dendritic cells. **B:** Numerous *in situ* hybridization-positive cells at 50 days after infection in lymph node. The higher magnification **inset** illustrates the cell morphology. **C:** *In situ* hybridization for SIV (blue) followed by immunohistochemistry for the macrophage marker CD68 (red) in LN at 50 days after infection. No co-localization of the two labels (would appear purple) is evident.

spleen, lymph node, and small and large intestine from animals infected with SIVmac239/316 and SIVmac239.

In animals infected with SIVmac239/316 the majority of the infected cells were T cells (positive for CD3, CD5, CD7) (Figure 3). In similar experiments with macrophage markers (CD68, HAM56, MAC387, and MRP8) we were able to find only a single SIV-infected macrophage in one tissue from one of the animals infected with SIVmac239/316. This lone infected macrophage was present in the subcapsular sinus of the axillary lymph node (Figure 3C).

Figure 3. Immunophenotype of SIV-infected cells in SIVmac239/316 infection. **A:** Double-label confocal microscopy (three channel) in LN at 50 days after infection. Images for individual channels (CD3 with Alexa 488, green; SIV *in situ* hybridization with Fast Red, red, and differential interference contrast, DIC) are shown on the **left** and a large merged image containing two channels plus DIC showing many double-positive cells on the **right**. At higher magnification (**inset** with scale bar) the infected cells can be seen to be morphologically consistent with lymphocytes. **B:** In lymph node in long-term infected animals, images for individual channels (SIV-ISH, red; Ham56-macrophages, green; and DIC) are shown on the **left**. No evidence of macrophage infection (co-localization of the two markers) is present in the larger merged image. **C:** Infection of macrophages *in vivo* is extremely rare in SIVmac239/316 infection. **C** shows the only instance in which macrophage infection could be demonstrated (CD68 with Alexa 488, green; SIV-ISH, red; and DIC). The double-positive cell is present in the subcapsular sinus of the axillary lymph node. **D:** Co-localization of SIV (red), macrophages (HAM56, green), and T cells (CD5, blue) in the lymph node of a chronically infected animal. In the larger merged image several SIV-infected T cells are present and appear pink/purple because of the mixing of red and blue labels.

Thus macrophage infection *in vivo* by SIV mac239/316 is very rare. In contrast, in acute and long-term SIVmac239 infection many SIV-infected macrophages were found in lymph node, spleen, lamina propria of small and large intestine, and Peyer's patches as early as 21 days after infection (Figure 4; A to C) consistent with previous published reports.^{13–15} Giant cell pneumonia, enteritis, and SIVE with SIV infection of macrophages and multinucleated giant cells was a common finding in terminal SIVmac239 infection (Figures 4 and 5).

Quantitation of SIV-Infected Macrophages in Situ

Although macrophage infection by SIVmac239/316 was rare, the total number of infected cells/mm² in these animals was less than in SIVmac239-infected animals (Figure 6). Therefore we also quantitated the number of infected macrophages in animals infected with each virus. As can be seen in Figure 6 the number of infected macrophages in lymph node and spleen of animals infected with SIV-

mac239/316 is much lower than in animals infected with SIVmac239 (ninefold less at the closest point, 50 days after infection) and cannot be explained simply on the basis of differences in the frequency of infected cells.

Discussion

The ability of SIV to infect macrophages *in vivo* is clearly required for the development of macrophage-associated inflammatory lesions such as SIVE and giant cell pneumonia. Data supporting this conclusion is diverse and robust, originating from multiple laboratories, and *in vitro* and *in vivo* studies.^{16–19,44–46} This data has consistently implicated a series of amino acid changes in envelope that often overlap with the nine amino acid differences in env of SIVmac239/316 compared to SIVmac239. Thus it is somewhat surprising that in this study, in which animals were inoculated with SIVmac239/316, a macrophage tropic virus that originated from an animal with AIDS,

Figure 4. Immunophenotype of SIV-infected cells in SIVmac239 infection. **A:** Double-label confocal microscopy in lymph node at 50 days after infection showing SIV infection of macrophages. Images for individual channels (CD68 with Alexa 488, green; SIV-ISH with Fast Red, red) are shown on the **left** with the larger merged image on the **right**. Several infected macrophages are present (**arrow**). **B** and **C:** Infection of macrophages is also observed in the spleen in a long-term infected animal (**B**) using the same markers and in the intestine (**C**) using HAM56 in place of CD68 for detection of macrophages. **D:** Co-localization of SIV (red), macrophages (HAM56, green), and T cells (CD3, blue) in the lymph node of an animal chronically infected with SIVmac239. Infection of both T cells (pink/purple) and macrophages (yellow) can be seen.

SIVE, and giant cell pneumonia, that not only did the animals not develop similar lesions but infection of monocyte/macrophage lineage cells *in vivo* was limited or non-existent.

The explanation for this observation is not immediately obvious but is likely related to the fact that these animals, as opposed to animals from which macrophage tropic viruses have been derived, were not also infected with virulent T-cell tropic viruses such as SIVmac239 from which they evolved. It is important to remember that the changes in SIVmac239/316 envelope occurred naturally and that uncloned SIVmac316 (from which the env in SIVmac239/316 was obtained) was isolated from lung macrophages in an animal infected with SIVmac239. This animal had AIDS and wide spread infection of macrophages manifest as SIVE and giant cell pneumonia.^{16,17} The development of macrophage tropism is also associated with a decreased dependence on CD4. This likely reflects an evolutionary process to adapt to infection of macrophages, some of which express little CD4.¹⁹ An additional consequence of the amino acid changes that confer macrophage tropism is that the virus is rendered much more susceptible to antibody neutralization.^{23,25,32,47,48}

This is reflected in the longer survival and lower viral set points in animals infected with SIVmac239/316³² as compared to SIVmac239 but similar peak viral loads. This suggests that macrophage tropic viruses such as SIVmac239/316 are relatively easy to control in the absence of a more virulent virus such as SIVmac239 and that viral replication may be insufficient to cause severe giant cell disease. In this scenario, macrophage tropic viruses such as SIVmac239/316 are conceptually opportunists that require destruction of the immune system to have a significant impact.

Although SIVmac239/316 may indeed be easier to control than SIVmac239 this does not explain why infection of monocyte/macrophages was essentially nonexistent. Based on *in vitro* data, robust infection of monocyte/macrophage lineage cells would have been expected at least during the peak of viremia, 7 to 14 days after infection. Although it is possible that SIVmac239/316 may evolve away from macrophage tropism to escape neutralizing antibodies²⁵ this would not be an issue during the early time points examined. We initially considered that perhaps the infection was altering expression of selected macrophage or T cell-specific molecules used

Figure 5. Multilabel confocal microscopy in lung of an animal infected with SIVmac239 that died with giant cell pneumonia. The individual channels (SIV *in situ* hybridization, red; Ham56-macrophages with Alexa 488, green; all cellular nuclei with Topro3, blue and DIC) are shown on the **left** with the larger merged image on the **right**. Several infected macrophages and multinucleated giant cells are present.

in immunohistochemistry but after examining a series of macrophage and T-cell-specific markers (Table 2), two different *in situ* hybridization probes and performing multilabel confocal microscopy we are confident that the *in vivo* lack of macrophage infection is real. This leads us to believe that the conditions under which macrophage infection has been examined *in vitro* are not reflective of macrophages *in vivo*. From work by Becher and Antel⁵⁰ and Williams and colleagues⁴⁹ it has been shown that tissue macrophages such as brain microglia rapidly change their phenotype within hours of isolation from tissue becoming more activated with up regulation of a variety of immunologically important molecules such as MHCII, CD4, and so forth. This leads us to speculate that the immune dysfunction caused by a virulent virus such as SIVmac239 manifests as increased cytokine production,^{29,51,52} up-regulation of adhesion molecules, and may cause activation of tissue macrophages making them more like macrophages *in vitro* and susceptible to

infection by SIVmac239/316. Although SIV rarely uses co-receptors other than CCR5 it is possible that altered co-receptor usage could contribute to macrophage tropism and differences in pathogenesis. In studies by Puffer and colleagues²³ using envs from SIVmac239, SIVmac239/316 and other macrophage tropic viruses no evidence that co-receptor choice was responsible for altered tropisms was found: all used CCR5.

In contrast to the unexpected *in vivo* cell tropism of SIVmac239/316, the tropism of SIVmac239 for T cells and macrophages was in agreement with previously published work.^{11,14,15,53,54} It is worth noting however that infection of macrophages by SIVmac239 has been consistently observed *in vivo* by 21 days after infection, before any evidence of SIVE or other pathology associated with macrophage infection. This has been presumed to reflect the evolution of variant strains of SIVmac239 that had acquired SIVmac239/316-like changes in envelope and the ability to replicate efficiently in macrophages.

Figure 6. Total numbers of SIV-infected cells/mm² (left axis) versus numbers of SIV-infected macrophages/mm² (right axis) detected by combined RNA *in situ* hybridization/immunohistochemistry for SIV and macrophages using HAM56 in LN and spleen. Bars represent averages of five 1-mm² areas per slide from each tissue in two animals per time point. In SIVmac239/316-infected animals both the total numbers of infected cells and infected macrophages were lower than animals inoculated with SIVmac239. All animals infected with SIVmac239 for 21 days or more had evidence of SIV-infected macrophages. In terminal SIVmac239-infected animals, the number of macrophages was primarily represented by multinucleated giant cells. These cells were not observed in terminal SIVmac239/316-infected animals.

However, it is possible that as was proposed above for SIVmac239/316, the *in vitro* culture conditions used to define the inability of SIVmac239 to replicate in macrophages do not adequately reflect *in vivo* conditions. In support of this, using microdissection techniques it has been recently shown that SIVmac239 env RNA is present in individual SIV giant cells although this was interpreted as reactivation of latent infection of microglia by fusion into multinucleated giant cells.³⁰ Nevertheless, the *in vivo* data examining cell tropism of SIV suggests that further study examining the timing of acquisition of macrophage-tropism *in vivo* and how, or if, those changes affect disease pathogenesis is warranted.

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