Recently Infiltrating MAC387⁺ Monocytes/Macrophages

A Third Macrophage Population Involved in SIV and HIV Encephalitic Lesion Formation

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Monocytes/macrophages are critical components of HIV and SIV encephalitic lesions. We used *in vivo* BrdU labeling and markers specific to stages of macrophage differentiation or inflammation to define macrophage heterogeneity and to better define the role of macrophage populations in lesion formation and productive infection. Lesions were heterogeneously composed of resident macrophages (CD68⁺HAM56⁺), perivascular macrophages (CD163⁺ CD68⁺MAC387⁻), and recently infiltrated MAC387⁺ CD68⁻CD163⁻ monocytes/macrophages. At 24 and 48 hours after BrdU inoculation, 30% of MAC387⁺ monocytes/macrophages were BrdU⁺, consistent with their being recently infiltrated. In perivascular cuffs with low-level SIV replication, MAC387⁺ monocytes/macrophages outnumbered CD68⁺ macrophages. Conversely, lesions with numerous SIV-p28⁺ macrophages and multinucleated giant cells had fewer MAC387⁺ monocytes/macrophages. The MAC387⁺ cells were not productively infected nor did they express detectable CCR2, unlike perivascular macrophages. Overall, we found that the proportion of MAC387⁺ cells tends to be higher than the proportion of CD68⁺ macrophages in the brain of animals with mild encephalitis; the ratio was reversed with more severe encephalitis. These results suggest that development of SIV and HIV encephalitis is an active and ongoing process that involves the recruitment and accumulation of: i) non-

productively infected MAC387⁺ monocytes/macrophages that are present with inflammation (potentially M1-like macrophages), ii) CD163⁺ perivascular macrophages (consistent with M2-like macrophages), and iii) CD68⁺ or HAM56⁺ resident macrophages. The latter two populations are cellular reservoirs for productive infection. (*Am J Pathol* 2011, 178:2121–2135; DOI: 10.1016/j.ajpath.2011.01.023)

Encephalitis associated with infection by HIV and SIV (HIVE and SIVE, respectively) is characterized by perivascular accumulation of mononuclear cells (predominantly macrophages) and multinucleated giant cells (MNGCs).^{1,2} We and others^{3–5} have shown that perivascular macrophages (CD14⁺CD163⁺ cells) are preferentially productively infected and are significant components of encephalitic lesions. In addition to these cells, recruitment of parenchymal macrophages to HIV and SIV lesions and traffic of monocytes/macrophages from outside the central nervous system (CNS) likely occur; however, this is not clearly studied because efforts to differentiate between these cell populations are understudied. Overall, brain macrophages are heterogeneous regarding their phenotype, origin, turnover rate, and stage of differentiation/activation.^{6–8} Thus, understanding macrophage heterogeneity in HIVE and SIVE is likely important for defining viral reservoirs and the “age” and inflammatory activity of encephalitic lesions.

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Pan-macrophage markers CD68 and HAM56 have been generally used to identify brain macrophages in classic histopathological studies,^{9–11} primarily using paraffin-embedded sections. Others^{3,12,13} have used CD14, CD16, and, more recently, CD163 to differentiate perivascular macrophages, activated macrophages, and microglia in viral and inflammatory encephalitis. Macrophages accumulated within the perivascular/Virchow-Robins space express CD163, CD68, CD14, and CD16,^{5,10} whereas the parenchymal cells are consistently CD68⁺, HAM56⁺, and CD16⁺. In addition to these markers, intracellular myeloid-related proteins (MRPs) 8 and 14 or MRP8/MRP14 (a heterocomplex also called calprotectin) has been used to define macrophage differentiation and/or different stages of inflammatory lesions in the CNS (early acute, late acute, or chronic).^{14–16} The antibody known as MAC387 is described as recognizing MRP14 and, to a lesser extent, the MRP8/MRP14 heterocomplex.^{17,18} MRP14 is expressed on recently infiltrating monocytes/macrophages during early acute inflammation.¹⁹ In contrast, MRP8⁺ macrophages are found during chronic inflammation.¹⁶ In addition, the 25F9 antibody recognizes antigens on fully differentiated resident macrophages.²⁰ Combinations of these antibodies have characterized CNS lesions in multiple sclerosis (MS) and other pathological conditions.^{16,21} Their expression on monocytes/macrophages in CNS pathogenesis of AIDS has not been used to characterize encephalitic lesions.

We hypothesized that active recruitment of populations of monocytes/macrophages is involved in lesion formation and expansion, whereas other macrophage populations function to inhibit lesion expansion. To test this hypothesis and to better define macrophage populations in SIV and HIV lesions, we used markers differentially expressed on monocytes/macrophages previously described and *in vivo* 2-bromo-2'-deoxyuridine (BrdU) labeling to characterize patterns of macrophage differentiation/activation in encephalitis. In addition to resident and perivascular macrophages, we distinguished a third population of MAC387⁺ macrophages that is not productively infected and is actively recruited to the CNS with inflammation. Our data underscore the role of these MAC387⁺ monocytes/macrophages as markers of active inflammation in SIV- and HIV-infected brains. These inflammatory macrophages can be distinguished from perivascular and resident brain macrophages that likely play immune regulatory roles in the brain.

Materials and Methods

Tissue Samples

Human Brain

The FFPE sections of frontal white and gray matter from 14 different individuals were used. Tissues used were from patients with the following pathological diagnosis: HIVE (*n* = 7), HIV-1⁺ individuals without encephalitis (*n* = 5), and HIV-1-seronegative controls (*n* = 2). All CNS tissues were obtained from the Manhattan HIV Brain Bank

Table 1. SIVE Lesions in SIVmac251-Infected Rhesus Macaques

Animals	DPI	Severity of CNS disease*
Conventional progressors (>6 months)		
265-95	407	Severe
530-98	523	Severe
456-98	176	Mild
Rapid progressors (≤6 months)		
156-99	118	Mild
147-99	157	Severe
249-97	111	Severe
142-99	124	Mild
153-99	67	Severe
CD8 ⁺ T-lymphocyte-depleted rapid progressors		
226-86	78	Severe
169-90	85	Severe
224-93	63	Mild
169-94	70	Severe
217-93	57	Mild
272-82	76	Severe

DPI, days post infection.

*Severe indicates large, numerous, perivascular lesions and MNGCs; mild, smaller lesions with fewer MNGCs.

(1R24MH59724; New York, NY) or the AIDS and Cancer Specimen Resource (U01CA066531, U01CA066535, U01CA066529, and U01CA096230; San Francisco, CA).

Rhesus Macaque Brain

Tissues from 14 adult rhesus macaques (*Macaca mulatta*) infected with SIVmac251 (20 ng of SIV p27) that developed AIDS and SIVE were used (Table 1). These include “rapid” progressors (SIVE <6 months after infection, *n* = 5), “conventional” progressors (SIVE ≥6 months after infection, *n* = 3), and animals that were CD8⁺ lymphocyte depleted and developed rapid AIDS (SIVE <3 months after infection, *n* = 6).²² In addition, we examined tissues from normal uninfected animals (*n* = 3), SIV-infected CD8⁺ lymphocyte-depleted animals with AIDS but without SIVE (*n* = 4), and animals sacrificed 21 days after infection without AIDS or SIVE (*n* = 4). Brain necropsy specimens were collected in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 6 μm. Alternate tissues were blocked in optimal cutting temperature compound (Miles Scientific, Elkhart, IN), snap frozen, and sectioned at 10 μm.

Ethics Statement

All animals were handled in strict accordance with good animal practice, as defined by Harvard University's Institutional Care and Use Committee; and all animal work was approved by this committee.

Immunohistochemistry

IHC was performed using the antibodies listed in Table 2, as previously described.³ Deparaffinized rehydrated sections

Table 2. Markers for Monocytes/Macrophages Used in the Study

Antigen	Ab clone/type	Expression/function	Manufacturer
CD14	AML-2-23/IgG2a	Mo, MΦ/LPS-R ¹⁰	Medarex (Princeton, NJ)
CD16	2H7/IgG2a	Mo, MΦ/FcR ¹⁰	Novocastra (Newcastle, UK)
CD68	KP1/IgG1	Pan-MΦ and Mo/scavenger R ¹⁰	Dako (Carpinteria, CA)
CD163	EDHu-1/IgG1	CNS perivascular MΦ/scavenger R ³	Serotec (Raleigh, NC)
CD163	10D6/IgG1		Novocastra
Not defined	HAM56/IgM	Pan-MΦ/not defined ⁹	Dako
86-kDa protein	25F9/IgG1	Mature tissue MΦ/not defined ²⁰	AbCam (Cambridge, MA)
MRP8	8-5C2/IgG1	Late stages or chronic inflammation/proinflammatory mediator ¹⁴	BMA Biomedicals Inc. (Augst, Switzerland)
MRP14 or MRP8/MRP14	MAC387/IgG1	Mo, recently infiltrated Mo/MΦ/proinflammatory mediator ¹⁷	Dako
Glut-1	Rabbit polyclonal	CNS endothelial cells/glucose transporter	Chemicon (Temecula, CA)
SIV-p28	MX-0322/IgG1 FITC conjugated	SIVmac251	Microbix Biosystems (Toronto, Canada)

The references cited indicate studies in which these markers were used in the CNS.

Mo, monocytes; MΦ, macrophages; R, receptor; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; Ab, antibody.

were incubated with peroxidase block (Dako, Carpinteria, CA) and then protein block (Dako) for 30 minutes before incubation with primary antibody. After incubation with a peroxidase-conjugated polymer, slides were developed using a diaminobenzidine chromogen (Dako). Controls consisted of isotype-specific immunoglobulins corresponding to the test antibody. For double staining, commercially available kits (Vectastain Elite ABC and ABC-AP kits) were used according to the manufacturer's recommendations (Vector Laboratories, Burlingame, CA). Primary antibodies were detected using a biotinylated anti-mouse IgG, followed by ABC reagent, and developed with the corresponding chromogen (diaminobenzidine or Vector Blue). An avidin-biotin blocking kit (Vector Laboratories) was used to ensure that all endogenous biotin or biotin receptors were blocked before the addition of biotin-labeled antibodies. Sections were visualized using a microscope (Zeiss Axio Imager.M1; Carl Zeiss MicroImaging, Inc., Thornwood, NY). Cells immunoreactive with the MAC387 antibody recognizing MRP14 and, to a lesser extent, MRP8/MRP14 heterocomplex are designated MAC387⁺ cells to simplify the nomenclature.¹⁸

In Vivo BrdU Labeling and Detection in Brain Tissues

After i.v. injection, BrdU (Sigma-Aldrich, St Louis, MO) labels divided premonocytes and promonocytes in bone marrow that can subsequently be detected in blood and tissue as Ki-67⁺ BrdU⁺ nonproliferating cells.²³ Briefly, BrdU was administered by i.v. injection to two SIV-infected CD8⁺ T-lymphocyte-depleted animals 24 hours or 48 hours before necropsy. BrdU expression in SIVE brain tissues was assessed by IHC on paraffin sections, as previously described.²³

In Situ Hybridization for SIV RNA

In situ hybridization for SIV RNA was performed using digoxigenin-labeled antisense riboprobes (Lofstrand

Labs, Gaithersburg, MD), as previously described.²⁴ *In situ* hybridization for SIV RNA was followed by immunofluorescence for MAC387, CD68, and glucose transporter type 1 (Glut-1), as described later for the detection of SIV RNA in monocytes/macrophages and endothelial cells.

Immunofluorescence and Confocal Microscopy

Multilabel immunofluorescence using antibodies and fluorochromes described in Table 3 was performed on frozen and paraffin sections. Frozen sections were thawed for 20 minutes at room temperature, fixed in cold acetone, and washed with PBS. They were permeabilized with 0.1% Triton X-100/PBS/fish skin gelatin (FSG) and washed with PBS/FSG. Then, they were incubated with PBS/FSG/10% normal goat serum before incubation overnight at 4°C or for 1 hour at room temperature with primary antibodies diluted in PBS/FSG/normal goat serum. Sections were washed with PBS/FSG before the addition of the primary or secondary antibody. Sections were rinsed in PBS/FSG and washed in distilled water before treatment with 50 mmol/L Cu₃SO₄ in ammonium acetate buffer for 45 minutes to quench endogenous autofluorescence.⁵ Slides were coverslipped with or without DAPI (Vectashield; Vector Laboratories) and visualized under a microscope (Zeiss Axio Imager.M1). Individual channels were collected simultaneously using computer software (Axio-Vision, version 4.6.3; Carl Zeiss MicroImaging, Inc.). Confocal microscopy was performed as previously described.⁸

Quantitative Immunofluorescence Densitometry

We used quantitative immunofluorescence densitometry on a microscope (Zeiss Axio Imager.M1) to distinguish MAC387⁺ from CD68⁺ cells in SIVE lesions in a nonbiased automated fashion. All densitometry readings were performed at ×200 with exposure times of 60 millise-

Table 3. Antibody and Fluorochrome Combinations for Immunofluorescence and Confocal Microscopy

Red channel: Alexa 568/permanent red		Green channel: Alexa 488		Far red channel: Alexa 647/Alexa 633		Blue channel
Primary	Secondary	Primary	Secondary	Primary	Secondary	
CD16(IgG2a)	Anti-Ms IgG2a	CD163(IgG1)	Anti-Ms IgG1	MAC387(IgG1)biotin	SA	Nuclei DAPI
CD14(IgG2a)	Anti-Ms IgG2a	MAC387(IgG1)	Anti-Ms IgG1			
MAC387(IgG1)	Anti-Ms IgG1	Glut-1(pAb)	Anti-Rb	CD68(IgG1)biotin	SA	Nuclei DAPI
Riboprobe	Anti-DIG/sheep	MAC387(IgG1)	Anti-Ms IgG1			
Riboprobe	Anti-DIG/sheep	MAC387(IgG1)	Anti-Ms IgG1	CD68(IgG1)biotin	SA	
MAC387(IgG1)	Anti-Ms IgG1	CD163(IgG1)-FITC		CD68(IgG1)biotin	SA	
MAC387(IgG1)	Anti-Ms IgG1	CCR2(IgG)	Anti-Rb			Nuclei DAPI
MAC387(IgG1)	Anti-Ms IgG1	SIV p28(IgG1)-FITC		Nuclei ToPro3		
HIV-p24(IgG1)	Anti-Ms IgG1	CD68(IgG3)	Anti-Ms IgG3	MRP8(IgG1)biotin	SA	Nuclei DAPI

Each column shows the primary and secondary (italic) antibody used for the channel indicated. Each line shows a combination of two or three antibodies with or without counterstaining for nuclei.

Ms, mouse; SA, streptavidin; pAb, polyclonal antibody; Rb, rabbit; DIG, digoxigenin; FITC, fluorescein isothiocyanate.

onds for CD68 (green: Alexa488) and 100 milliseconds for MAC387 (red: Alexa568). All SIVE lesions detected per section of cortical area ($n = 9$ to 28 lesions per section) were analyzed using software (AxioVision). Red and green fluorescence backgrounds were subtracted from their respective channels. We calculated a relative fluorescence intensity ratio between the MAC387 and CD68 signals to distinguish between two groups of cells: MAC387⁺ cells had a ratio >1 , and CD68⁺ cells had a ratio <1 . Only a few cells ($<2\%$) with equivalent MAC387 and CD68 fluorescence levels (ratio = 1) were detected.

Flow Cytometry Analysis of Blood Monocytes

Whole blood from five SIV-infected animals was incubated with a cocktail of cross-reactive monoclonal antibodies specific for human CD16, HLA-DR, and CD14 [phycoerythrin (PE), PerCP-Cy5.5, and allophycocyanin (APC), respectively; all from BD Biosciences, San Diego, CA] and CD163 [fluorescein isothiocyanate (FITC); Trilium Diagnostics, Brewer, ME]. Intracellular expression of MAC387 and CD68 was analyzed after cell permeabilization with solution (Cytofix/Cytoperm; BD Biosciences), used according to manufacturer's instructions. Cells were then incubated with FITC-conjugated MAC387 (Serotec; Raleigh, NC) or anti-CD68 (clone KP1; Dako, Carpinteria, CA) antibodies. Cells were washed with PBS and resuspended in 2% formaldehyde. Data were acquired on an FACS Aria (BD Biosciences) and further analyzed with software (FlowJo version 7.2.2; Tree Star Inc., Ashland, OR).

Results

MAC387⁺ Cells Accumulate in Encephalitic Lesions of SIV-Infected Rhesus Macaques

We examined MAC387 expression in brain tissues from SIV-infected animals that were sacrificed with AIDS and SIVE. These included conventional progressors (≥ 6 months after infection) and rapid progressors (< 6 months after infection). MAC387⁺ cells were typically round and morphologically consistent with monocytes.

MAC387⁺ cells were in the parenchyma and scattered blood vessels of conventional (Figure 1, A and B) and rapid (Figure 1, D and E) progressors. Similarly, all brain tissues from CD8⁺ lymphocyte-depleted animals with SIVE animals had MAC387⁺ cells (Figure 1, C and F). Few MAC387⁺ cells ($\leq 2\text{mm}^2$) were found in the CNS of SIV-infected macaques without encephalitis, SIV-infected animals sacrificed by 21 days after infection, or uninfected controls (Figure 1G).

To study whether MAC387⁺ cells in the CNS are recently emigrated monocytes/macrophages from blood, as it has been described in lymph nodes,²⁵ we examined BrdU labeling of MAC387⁺ cells in the lesions of two animals with SIVE that were injected i.v. with BrdU before necropsy. At 24 hours or 48 hours after BrdU injection, approximately 30% of the MAC387⁺ cells detected in SIVE lesions were BrdU⁺ (Figure 1H). Moreover, most (80% to 90%) of the BrdU⁺ cells were also MAC387⁺. This strongly supports the notion that MAC387⁺ monocytes/macrophages labeled with BrdU in bone marrow have recently infiltrated the CNS and play a role in lesion formation.

Heterogeneous Populations of Macrophages Compose SIVE Lesions

AIDS-associated encephalitic lesions are composed mainly of monocytes/macrophages and $< 5\%$ scattered CD3⁺CD8⁺ T lymphocytes.²⁶ We performed IHC using defined markers characteristic of different stages of monocyte/macrophage differentiation to characterize subsets that comprise lesions (Table 2). SIVE lesions in CD8⁺ lymphocyte-depleted SIV-infected animals were characterized by numerous SIV-p28⁺ cells (Figure 2G) and MNGCs (Figure 2). Lesions were heterogeneously composed of MAC387⁺ (Figure 2A), CD163⁺ (Figure 2B), CD68⁺ (Figure 2C), and HAM56⁺ (Figure 2D) cells, some of which had overlapping antigen expression and others that did not have this overlapping. CD163⁺ macrophages were homogeneously distributed in lesions. CD68⁺ and HAM56⁺ pan-macrophages had overlapping distributions, in contrast to MAC387⁺ monocytes/macrophages (Figure 2, upper row). MNGCs, previously identified as CD14⁺, CD16⁺, and CD163⁺

Figure 1. MAC387 expression in brains of SIV-infected macaques. IHC showing MAC387 expression [brown, diaminobenzidine (DAB)] in brains of animals with encephalitis (**A–F** and **H**) or uninfected animals (**G**). MAC387⁺ cells accumulate in lesions (**black arrows**) characterized by microglial nodules, perivascular cuffs, and MNGCs (**black arrowheads**). **A** and **B**: Conventional progressors with AIDS and SIVE longer than 6 months after infection. **D** and **E**: Rapid progressors with AIDS and SIVE <6 months after infection. **C** and **F**: CD8⁺ T-lymphocyte-depleted macaques with AIDS and SIVE <3 months after infection. **Insets**: Higher magnification of MAC387⁺ cells (**D** and **F**). **G**: MAC387⁺ cells were not detected in uninfected animals. Data are representative of IHC staining on four different CNS regions per animal ($n = 14$ animals with SIVE and $n = 3$ uninfected animals). **H**: Recently recruited MAC387⁺BrdU⁺ (**insets**: brown + blue) are present in SIVE lesions of a CD8⁺ T-lymphocyte-depleted macaque that had received a BrdU injection 24 hours before necropsy.

cells,³ were also CD68⁺ and HAM56⁺ but MAC387[−] (Figure 2, A–D, insets). Although present in the spleen of infected monkeys, we did not detect MRP8⁺ or 25F9⁺ macrophages in CNS lesions of the CD8⁺ lymphocyte-depleted animals (Figure 2, E and F, insets) or in the nondepleted animals with or without SIVE (data not shown).

Next, we investigated the expression of CD14, CD16, CD68, CD163, and CCR2 on MAC387⁺ cells to differentiate between perivascular macrophages and resident brain macrophages that have been previously described. Many MAC387⁺ macrophages coexpressed CD16 within SIVE le-

sions (Figure 3A, white arrowheads) and CNS vessels (Figure 3B). A minor scattered population of MAC387⁺ monocytes/macrophages also expressed CD14 (Figure 3C). All MAC387⁺ monocytes/macrophages were CCR2[−] (Figure 3D). Most MAC387⁺ cells were CD163[−] and CD68[−] (Figure 3, E–G), with only few scattered MAC387⁺CD163⁺ cells (<2%; Figure 3G, black arrowheads). In contrast, most CD163⁺ perivascular macrophages coexpressed CD68 (Figure 3, E and F), although a few CD163⁺ cells that were CD68[−] were also detected (Figure 3E). These data demonstrate a heterogeneity of monocytes/macrophages composing SIVE lesions and underscore at least three subsets of macrophages

Figure 2. Heterogeneity of macrophages accumulated in SIVE lesions. IHC showing macrophage heterogeneity in adjacent sections from a representative SIVE lesion from an SIV-infected CD8⁺ T-lymphocyte-depleted animal. Tissue was stained for MAC387 (early-stage acute inflammation, **A**), CD163 (brain perivascular macrophages, **B**), CD68 and HAM56 (pan-macrophage markers, **C** and **D**), MRP8 (late-stage acute inflammation, **E**), 25F9 (resident histiocytes, **F**), and SIV-p28 protein (**G**). Negative control (Neg., **H**) is shown. **Insets:** Higher-magnification of MAC387⁺ cells (**A**) or MNGCs (black arrowheads, **B–D** and **G**) expressing CD163, CD68, HAM56, and SIV-p28. MRP8⁺ and 25F9⁺ cells were detected in spleen (**E** and **F**, upper insets) but not in SIVE lesions in the same animal. Scale bars = 20 μm (insets).

in SIVE lesions: i) MAC387⁺CD68[−]CD163[−]CCR2[−] recently recruited monocytes/macrophages, ii) CD68⁺CD163⁺CCR2⁺ perivascular macrophages, and iii) CD68⁺CD163[−] macrophages.

MAC387, CD163, and CD68 Are Differentially Expressed on Blood Monocytes

“Activated” CD14^{low}CD16^{high} blood monocytes expressing CD163 could be potential precursors of brain perivascular macrophages.^{3,4} We found that MAC387 is expressed by monocytes/macrophages recently infiltrated to the CNS that appear to be distinct from CD163⁺ perivascular macrophages and CD68⁺ resident macrophages (Figures 1–3). To investigate whether such heterogeneity in tissue is also observed on blood monocytes, we analyzed MAC387, CD163, and CD68 expression on monocytes from SIV-infected CD8⁺ lymphocyte-depleted animals before infection and when terminally ill with AIDS. Before infection, all monocytes co-express MAC387, CD163, and CD68 at varying levels. MAC387 expression on total monocytes tended to decrease with the development of AIDS, whereas expression of CD163 and CD68 increased (Figure 4A). Similar observations were made on CD14^{high}CD16[−] and CD14^{low}CD16^{high} monocytes; the latter expressed higher levels of CD163 with AIDS (Figure 4B). Although the mean fluorescence intensity (MFI) of MAC387 decreases with the development of AIDS and the MFI of CD68 increases, significant coexpression of MAC387 and CD68 exists. Similarly, monocytes also express CD163 at varying levels. Coexpression of these molecules is less frequent on monocyte/macrophage populations in the brain.

Accumulation of MAC387⁺ versus CD68⁺ Macrophages in Lesions Reflects Severity of SIVE

We investigated the distribution of recently recruited MAC387⁺ cells versus resident CD68⁺ macrophages in encephalitic lesions. Confocal microscopy confirmed that encephalitic lesions were mainly composed of distinct populations of CD68⁺ macrophages and recently infiltrated MAC387⁺ monocytes/macrophages (Figure 5, A and B). Few cells (<2%) coexpressed both markers. Depending on the lesion and/or the animal studied, we observed different proportions of MAC387⁺ or CD68⁺ cells within individual lesions. There were few CD68⁺ macrophages around blood vessels in brain tissues of SIV-infected macaques without SIVE (Figure 5C). We detected almost no MAC387⁺ cells in the parenchyma of SIV-infected animals without SIVE but did find few scattered cells in CNS blood vessels (Figure 5C).

We quantified the number of MAC387⁺ and CD68⁺ cells in SIVE lesions using quantitative double immunofluorescence. We calculated the relative fluorescence ratio between MAC387⁺ and CD68⁺ cells on a single-cell basis within individual lesions and counted all cells immunoreactive for each corresponding antibody within lesions and perivascular cuffs from SIV-infected CD8⁺ lymphocyte-depleted animals. We confirmed that CD68⁺ cells (MAC387/CD68 relative fluorescence ratio, <1) do not overlap with MAC387⁺ cells (MAC387/CD68 relative fluorescence ratio, >1) (Figure 6A). Few cells (<2%) were MAC387⁺CD68⁺ (MAC387/CD68 relative fluorescence ratio = 1) (Figure

Figure 3. Phenotype of MAC387⁺ monocytes/macrophages in SIVE lesions. Immunofluorescence and confocal images (A–F) showing expression of CD16 (red: A and B), CD14 (red: C), CCR2 (red: D), CD68 (red: E and F), and CD163 (blue: E and F) on MAC387⁺ cells (green) within SIVE lesions of SIV-infected CD8⁺ T-lymphocyte-depleted animals. Side panels represent individual channels and differential interference contrast (DIC, gray); large panels, merged images combining all channels plus the DIC image. **A:** SIVE lesions are composed of MAC387⁺ cells, CD16⁺ cells, and some MAC387⁺CD16⁺ cells (yellow; **white arrowheads**). **B:** Confocal image showing double-positive MAC387⁺CD16⁺ monocyte-like cells inside a blood vessel. **C:** Confocal image showing the accumulation of CD14⁺ monocytes (red) and recently infiltrating MAC387⁺CD14⁺ monocytes/macrophages (yellow) within an SIVE lesion. **D:** CCR2 expression is detected in SIVE lesions but not on MAC387⁺ cells. **E and F:** MAC387⁺ cells with CD68⁺ macrophages and CD163⁺ perivascular macrophages in SIVE lesions. MAC387⁺ cells do not coexpress CD68 or CD163. Although most CD163⁺ perivascular macrophages express CD68 (**F:** magenta), distinct CD68⁺CD163⁺ macrophages are found in lesions (**E**). **G:** Double IHC showing no or few (**black arrowheads**) cells positive for MAC387 (blue) and CD163 (brown) in an SIVE lesion. **White arrows** indicate CD163⁺ MNGCs. All images are from paraffin sections, except for **C** (frozen section).

6A, gray dotted line). The MAC387/CD68 relative fluorescence ratio was significantly inversely correlated with the corresponding cell areas (Figure 6B, $P < 0.0001$), confirming that MNGCs (large cells with an area $\geq 400 \mu\text{m}^2$, associated with severe SIVE; animals 226-86 and 169-90) expressed CD68⁺ but not MAC387. We calculated the relative percentage of MAC387⁺ cells compared with CD68⁺ macrophages per lesion and perivascular cuff of cortical areas (Figure 6C). Interestingly, percentages of MAC387⁺ cells were significantly different between mild and severe encephalitis ($P < 0.0001$). Animals with mild encephalitis tend to have numerous MAC387⁺ cells (mean $\pm$ SD of MAC387⁺ cells in 31 lesions analyzed in animals 224-93 and 217-34, $67.1\% \pm 26.5\%$) (Figure 6C, white symbols). In contrast, animals with severe SIVE that had numerous MNGCs (Figure 6B) tend to have fewer MAC387⁺ cells compared with CD68⁺ macrophages (mean $\pm$ SD of MAC387⁺ cells in 28 lesions analyzed in animals 226-86 and 169-90, $25.8\% \pm 32.4\%$) (Figure 6C, black symbols). These results suggest that mild encephalitis is associated with more MAC387⁺ than CD68⁺ macrophages and that this ratio is reversed when encephalitis is more severe.

Recently Infiltrated MAC387⁺ Macrophages in Encephalitic Lesions Are Not Productively Infected

HIVE and SIVE lesions are composed of macrophages with various percentages of productively infected cells. Because we found many MAC387⁺ monocytes/macrophages in SIVE lesions, we investigated whether these cells were productively SIV infected. By using *in situ* hybridization, we found that MAC387⁺ cells did not overlap with SIV RNA⁺ cells (Figure 7, A and B). This was confirmed by MAC387 and SIV RNA fluorescence densitometry analysis of CNS tissues from animals 226-86, 217-93, and 169-90 (Figure 7C). SIV RNA⁺ cells within SIVE lesions are CD68⁺ macrophages (Figure 7B) and CD163⁺CD68⁺ perivascular macrophages, as previously reported.³ Consistent with *in situ* hybridization results, MAC387⁺ cells within SIVE lesions (Figure 7D) and inside blood vessels (Figure 7E) were uniformly SIV-p28⁻. These data suggest that, in contrast to CD68⁺ macrophages, recently infiltrating MAC387⁺ monocytes/macrophages accumulated in encephalitic lesions are not reservoirs of productive viral replication. The results of assays to determine whether the MAC387⁺ cells harbor SIV DNA were inconclusive. Whether the MAC387⁺

Figure 4. Differential expression of MAC387, CD163, and CD68 on blood monocytes. MAC387, CD163, and CD68 expression was analyzed by flow cytometry on total monocytes (**A**) and monocyte subsets (**B**) from the whole blood of animals before infection (Pre) and after SIV infection and terminal AIDS ($\dagger$). **A** (right column) and **B**: Each symbol represents an individual animal. Expression levels are shown as the mean of fluorescence intensity (MFI). **A**: Expression of MAC387, CD163, and CD68 (MFI) on total monocytes before infection and terminally with AIDS. **B**: Mean of fluorescence intensity of MAC387, CD163, and CD68 on monocyte subsets from five animals, distinguished as CD14^{high}CD16⁻ (left column) and CD14^{low}CD16^{high} (right column).

monocytes/macrophages can mature *in situ* to become productively infected was not determined.

MAC387⁺ Monocytes/Macrophages Accumulate in Encephalitic Lesions from Patients with HIV

We investigated whether MAC387⁺ cells also accumulated in encephalitic lesions in patients who died of chronic HIV infection with HIVE. MAC387⁺ monocytes/macrophages accumulated in brain tissues of patients with HIV who also had encephalitis (Figure 8, A–D) but not in patients with HIV who did not have encephalitis or in HIV-negative individuals (data not shown). In contrast to the SIV-infected macaques, MRP8⁺ cells were identified in the HIVE lesions (Figure 8, A and B). All of the MRP8⁺ cells were also MAC387⁺ (Figure 8A), but the proportion of MAC387⁺ cells that coexpress MRP8 varied depending on the patient (Figure 8, A and B). Because MRP8 is associated with late stages of MS and other CNS inflammatory diseases, these data suggest that HIVE lesions with MAC387⁺MRP8⁺ cells are from a more advanced disease stage and likely represent older and more chronic lesions compared with those in SIVE. These data are in agreement with the chronicity of disease in such HIV-infected patients receiving antiretroviral therapy. We found few to no MAC387⁺ cells that were CD163⁺ (Figure 8, C and D). In addition, the MRP8⁺ cells were HIV-p24⁻ and CD68⁻ (Figure 8E). MAC387⁺ monocytes/macrophages in human brain also did not express CCR2, whose expression was restricted to MNGCs and other MAC387⁻ macrophages (Figure 8F). In summary, in HIVE, we detected the same three populations of macrophages found in SIV-infected macaques. In addition, we found marked expression of MRP8 in HIVE brains but

not in SIVE macaques, which is likely characteristic of more chronic disease in humans.

Discussion

The literature has classically described SIVE and HIVE lesions being composed of monocytes/macrophages expressing CD68, HAM56,²⁷ and CD163.^{3–5,13} Budka²⁸ and Burudi et al²⁹ used MAC387 antibodies in studies of HIV- and SIV-infected brains, respectively; but did not note differential expression of this marker within macrophage populations. Interestingly, in the article by Burudi et al, MAC387 appeared to function primarily in the CNS of animals that were rapid disease progressors, which is in agreement with our results. In addition to productively infected CD68⁺ macrophages, we report herein MAC387⁺ monocytes/macrophages that do not contain SIV RNA or SIV-p28 in SIVE or HIV-p24 in HIVE.

Approximately 30% of MAC387⁺ cells that accumulated in SIVE lesions were BrdU⁺ 24 hours or 48 hours after BrdU injection, establishing that they are recently recruited from the bone marrow. This finding is consistent with the notion that MAC387 is a marker of monocytes/macrophages that have recently infiltrated tissues and is perhaps the earliest macrophage marker expressed on such cells as they enter tissues.¹⁹ The stimulus for such traffic is not clear but is likely dependent on chemokine gradients, the death of macrophages in target organs, translocated lipopolysaccharide (LPS), and inflammatory events in the periphery. The hypothesis that MAC387⁺ cells migrate to the brain to renew macrophage populations does not seem likely. In contrast to CD68⁺ macrophages, MAC387⁺ monocytes/macrophages were not detected in normal human brain without inflammation.³⁰

Figure 5. MAC387⁺ and CD68⁺ cells represent two distinct populations of macrophages that accumulate in brains of SIV-infected animals. Confocal images of brain tissues from SIV-infected CD8⁺ T-lymphocyte-depleted animals with (A and B) or without (C) encephalitis, showing the distribution of recently infiltrating MAC387⁺ monocytes/macrophages (red) and resident CD68⁺ macrophages (green). Side panels represent individual channels and differential interference contrast (DIC) (gray); large panels, a merged image combining all channels plus the DIC image. CNS vessels are stained with Glut-1 (A–C, blue), and nuclei are stained with BoPro1 (A and B, gray). **A:** MAC387⁺ and CD68⁺ cells accumulate in an SIVE lesion in the vicinity of Glut-1⁺ blood vessels. MNGC express CD68 but not MAC387 (A, white arrowhead). MAC387⁺ cells that have not infiltrated the tissue yet are located in the lumen of a Glut-1⁺ blood vessel (B). **C:** Representative confocal image of brain tissue from an SIV-infected animal that developed AIDS but not encephalitis. Few CD68⁺ macrophages are detected in the vicinity of blood vessels compared with SIVE brains. Rare MAC387⁺ cells are detected only inside blood vessels.

We did not detect MAC387⁺ monocytes/macrophages in normal human and monkey brain or in the brains of HIV- and SIV-infected humans and monkeys without encephalitis. In contrast to the MAC387⁺ cells, we showed that CD163⁺ perivascular macrophages are renewed by CD34⁺ hematopoietic stem cell–derived monocytic precursors in normal noninfected monkeys.⁸ We did not find evidence that these CD34⁺ hematopoietic stem cells produced MAC387⁺ cells in normal CNS. This observation, paired with our demonstration that increased BrdU⁺ monocytes in blood correlate with the rate of AIDS and the severity of CNS disease in SIV-infected monkeys,^{23,31} underscores that the presence of MAC387⁺ cells is closely linked with inflammation.^{14,21}

Virus is detected in the CNS early after infection (as early as 3 days to 7 days after infection) in humans and nonhuman primates.^{2,5} In the CNS, the presence and possible levels of viral DNA, once present, remain unchanged throughout disease while viral RNA is down-regulated after the acute phase.^{5,32} Virus replication is thought to be reactivated during the late stage of infection, which correlates with macrophage activation and infection and/or recruitment of perivascular macrophage precursors.³² We found an accumulation of MAC387⁺ cells in SIV-infected monkeys that were rapid or conventional progressors, corresponding to acute or more chronic disease, respectively, and in HIVE lesions in patients with AIDS who had chronic disease. This led us to speculate that recently infiltrated MAC387⁺ cells are recruited to sites of active infection and inflammation, and the presence of these cells might be a marker of active inflammation (Figure 9). Moreover, we found few scattered MAC387⁺ cells in the CNS of animals 21 days after infection, suggesting that the massive recruitment of MAC387⁺ cells to the brain is initiated at a later time of disease, probably when virus replication is reactivated concomitant with macrophage activation and the development of encephalitis.

Monocytes are considered to be a primary cell type carrying virus to CNS in HIVE and SIVE, but they first have to differentiate into macrophages for viral replication.^{33,34} Classic CD14⁺CD16[−] and activated CD14⁺CD16⁺ monocytes in macaques express the highest levels of MAC387 and the lowest levels of CD68 compared with activated CD14[−]CD16⁺ monocytes, as demonstrated by flow cytometry and gene arrays.³⁵ Transcriptional analyses in humans have indicated that MAC387 is also up-regulated in CD14⁺CD16[−] monocytes.³⁶ However, flow cytometric analysis clearly shows that these monocyte populations coexpress varying levels of MAC387, CD68, and CD163. This suggests that there is differential regulation of MAC387, CD68, and CD163 on monocytes in blood that is found in tissues as they become macrophages.

We and others^{3,4} have previously shown that a subset of CD14⁺CD16⁺ blood monocytes could be precursors of brain CD163⁺ perivascular macrophages. Whether CD163⁺ perivascular macrophages or CD68⁺ brain macrophages eventually arise from recently infiltrating MAC387⁺ cells is not known. However, it is clear that

Figure 6. Immunofluorescence densitometry analysis of MAC387 and CD68 in SIVE lesions. **A:** MAC387⁺ and CD68⁺ cells detected by immunofluorescence (left) were counted, and the corresponding fluorescence of each marker was measured by densitometry. Three SIVE lesions from the same cortical section of animal 226-86 show different proportion of MAC387⁺ and CD68⁺ cells in lesions. The ratio between MAC387 and CD68 relative immunofluorescence calculated for each cell (white dots, right graph) allows the distinction between double-positive cells (ratio = 1; gray dotted line), MAC387⁺ cells (ratio >1; right to dotted line), and CD68⁺ cells (ratio <1; left to dotted line) detected in SIVE lesions (left). **B:** Inverse significant correlation (Spearman's rank test) between cell area (μm²) and MAC387/CD68 relative immunofluorescence ratio. MAC387 immunofluorescence is associated with small cells (ie, monocytes/early differentiated macrophages) compared with CD68 immunofluorescence, which is associated with small to large cells (ie, MNGCs with an area >400 μm² shown in the gray box). All cells (black dots) detected in cortical sections of all four animals and characterized by a MAC387/CD68 relative immunofluorescence ratio are shown. **Inset:** MNGCs detected in the cortical sections analyzed are shown, illustrating mild (animals 224-93 and 217-34) and more severe (animals 226-86 and 169-90) encephalitis. **C:** Percentages of MAC387⁺ cells (MAC387/CD68 ratio >1; circles) and CD68⁺ cells (MAC387/CD68 ratio <1; squares) accumulated in mild (animals 224-93 and 217-34; white symbols) and severe (animals 226-86 and 169-90; black symbols) encephalitic lesions. Each symbol represents the percentage of one subset relative to total MAC387⁺ and CD68⁺ cells detected per lesions analyzed in one cortical section per animal. Horizontal bars are medians with interquartile ranges. Cell percentages are significantly different between mild and severe encephalitis (Mann-Whitney nonparametric test, $P < 0.0001$).

CD34⁺CD14⁺CD163⁺ cells traffic to the CNS as perivascular macrophage precursors normally and with HIV and SIV infection and are a significant site of productive replication.^{3,8} It is highly likely that the CD68⁺CD163[−] brain macrophages that we described are not perivascular macrophages but instead are resident brain macrophages that move within brain regions. Others¹² have found CD163 on these microglial cells and suggest that this is evidence of turnover of these cells directly from the blood.

The MAC387 antibody used in this study is thought to primarily recognize MRP14 but also reportedly the MRP8/MRP14 heterocomplex called calprotectin.^{17,18} Significantly elevated levels of serum calprotectin have been detected in asymptomatic HIV-infected individuals and patients with AIDS³⁷ and other pathological

conditions, such as active MS³⁸ and rheumatoid arthritis.³⁹ Detectable plasma calprotectin levels in asymptomatic patients with HIV suggest continuous monocyte/macrophage activation is occurring. However, granulocytes can also secrete calprotectin; thus, calprotectin in serum might not be strictly from monocytes.⁴⁰ Unfortunately, serum samples from the animals used in our study were not available for calprotectin analysis. After tumor necrosis factor-α secretion by infected cells or injured tissues, calprotectin is secreted during transendothelial migration of monocytes⁴¹ and induces increased expression of integrins and adhesion molecules on phagocytes and endothelial cells, respectively, leading to increased extravasation and activation of leukocytes.⁴⁰ Thus, it is likely that calprotectin secretion could be induced by tumor ne-

Figure 7. Recently infiltrated MAC387⁺ monocytes/macrophages are not productively infected. Confocal images (**A**, **B**, **D**, **E**) showing MAC387⁺ (green), CD68⁺ (**B**, blue), and SIV RNA⁺ (**A** and **B**, red) or SIV-p28⁺ (**D** and **E**, red) cells in lesions of SIV-infected CD8⁺ T-lymphocyte-depleted animals. Side panels represent individual channels and differential interference contrast (DIC) (gray); large panels, a merged image combining all channels plus the DIC image. Nuclei are stained with BoPro-1 or ToPro3 (gray). SIVE lesions contain MAC387⁺ cells that are negative for SIV RNA (**A** and **B**) and for SIV-p28 (**D** and **E**). In contrast, CD68⁺ cells were productively infected, showing costaining for SIV RNA (**B**). Data presented herein are representative of immunohistochemical studies performed on four CNS regions of animals with SIVE ($n = 4$). **C:** The proportion of MAC387⁺ cells and SIV RNA⁺ cells, as determined by fluorescence densitometry analysis, confirming that MAC387⁺ cells are not productively infected. The relative immunofluorescence of MAC387 and SIV RNA detected in each cell was measured by densitometry in individual sections from animals 226-86, 217-93, and 169-90. Relative cell percentages are indicated.

crisis factor- α secretion from CD68⁺ brain macrophages at sites of SIV and HIV infection.⁴² Consequently, MAC387⁺ monocytes/macrophages might be continuously recruited to the sites of infection during active inflammation, contributing to the amplification of the inflammatory response *in situ* and leading to the recruitment of additional leukocytes and infected cells (Figure 9). Regarding cell traffic, in contrast to CD68⁺

and CD163⁺ macrophages, recently infiltrated MAC387⁺ monocytes/macrophages are CCR2⁻, suggesting that they might not migrate toward CCL2 gradients, as it has been described for macrophages in the CNS.⁴³ Because the MAC387⁺ monocytes/macrophages express CCR1,⁴⁴ they may migrate instead in response to CCL3 [macrophage inflammatory protein 1- α (MIP 1- α)] that is secreted in HIV brain.⁴⁵

Figure 8. MAC387⁺MRP8⁺ macrophages in HIV lesions do not coexpress CD68, CD163, or CCR2 and are negative for HIV-p24. **A** through **D**: Double IHC showing expression of MAC387 (**A** and **B**, brown; **C** and **D**, blue), MRP8 (**A** and **B**, blue), and CD163 (**C** and **D**, brown) in brains of two HIV-infected individuals who developed encephalitis. Depending on the patient, MAC387⁺ cells accumulating in perivascular cuffs and encephalitic lesions either coexpress (**A**) or do not coexpress (**B**) MRP8. In contrast to MNGCs (**white arrows**, **D**), most human MAC387⁺ recently recruited macrophages do not express CD163 (**C** and **D**). **E** and **F**: Multi-color immunofluorescence showing MRP8 (green: **E**), CD68 (blue: **E**), HIV-p24 (red: **E**), MAC387 (green: **F**), and CCR2 (red: **F**) in HIV lesions. Nuclei labeled with DAPI are shown (gray). Side panels represent individual channels and differential interference contrast (DIC) (gray); large panel, a merged image combining all channels plus the DIC image. **White arrows** indicate MNGCs expressing CD68 and HIV-p24 (**E**) and CCR2 (**F**). HIV lesions characterized by HIV⁺ cells and MNGCs contain MRP8⁺ cells that are negative for CD68 and HIV-p24. Images in **A** and **C** through **F** are from the same individual.

Many molecules are involved in proinflammatory responses, including Toll-like receptors (TLRs) and their agonists. Elevated levels of plasma LPS, a TLR4 ligand, similar to high levels of serum calprotectin, are detected in patients with chronic illnesses and in SIV-infected rhesus macaques.⁴⁶ Moreover, high circulating LPS levels correlate with increased monocyte activation and dementia in patients with AIDS⁴⁷ and might increase the ability of monocytes to enter the brain by compromising blood-brain barrier integrity.⁴⁸ MRP8 and MRP14 (recognized by the MAC387 antibody) also can function as endogenous ligands for TLR4 to exacerbate LPS-induced inflammatory responses.⁴⁹ The potential contribution of MRP8 and MRP14 as TLR4 ligands in persistent immune activation in HIV pathogenesis needs to be further investigated, but it is likely that they are key players in the control of proinflammatory responses to HIV and SIV and are potentially linked to elevated levels of plasma LPS.

The MAC387, MRP8, and 25F9 antibody immunoreactivity on cells of monocyte/macrophage lineage has been used to distinguish different types of macrophage activation in MS lesions¹⁶ and autoimmune neuropathies.¹⁴ MAC387⁺ monocytes/macrophages accumulate in lymph nodes in SIV-infected animals during primary SIV antigenemia and indicate acute inflammatory activity.²⁵ MAC387⁺ (or MRP14) cells are associated with early

active lesions in MS. However, the presence of MRP8⁺ macrophages has been used to define late active lesions.⁵⁰ The 25F9 is expressed by resident histiocytes and, like MRP8⁺ cells, is associated with the long-term nature of inflammation.²⁰ MRP8⁺ and 25F9⁺ macrophages were not detected in the CNS of macaques with SIVE, although they were found in the corresponding control splenic tissues. This led us to speculate that SIVE lesions in these animals are active lesions associated with acute inflammatory activity. We detected MAC387⁺MRP8⁺ macrophages in HIV lesions, corresponding to chronic inflammation. Because the natural development of HIV in humans is a long-term process (>10 years), HIV lesions might be expected to be more “chronic” than SIVE lesions, which occur for a shorter period (from 3 months to 1 to 3 years) and more likely reflect acute disease.

We found that the proportion of MAC387⁺ cells tends to be higher than CD68⁺ macrophages in the CNS of animals with mild encephalitis, and the ratio is reversed if encephalitis is more severe. The numbers of CD68⁺ or HAM56⁺ macrophages positively correlated with the severity of encephalitis in monkeys and humans.^{27,51} We propose that stages of inflammation and severity of SIVE lesions can be distinguished based on the proportion of MAC387⁺ monocytes/macrophages versus CD68⁺ mac-

A Proportion of heterogeneous macrophage populations in encephalitic lesions

Human	Acute	Chronic HIVE	> 10 yrs
NHP	Early stage	Late stage SIVE	< 3 mo (CD8+ T depl.) 1-2 yrs
MAC387+ Mo/MΦ			
CD163+ CD68+ PVM			
CD68+ resident MΦ			
HIV or SIV+ MΦ			

B Proposed mechanism for the formation of encephalitic lesions

Figure 9. Proposed mechanism for the formation of encephalitic lesions. **A:** Heterogeneous macrophage populations in encephalitic lesions. **B:** Early after infection, infected cells cross the blood-brain barrier (1). Brain-infected macrophages release proinflammatory signals (2) that, in turn, activate endothelial cells (3). With a chemokine gradient (4), phagocytes (MAC387⁺ cells and macrophage precursors, some of which are infected) are recruited to tissues (5). The accumulation of MAC387⁺ cells and other macrophages in perivascular cuffs (6) amplifies proinflammatory signals (7), leading to the recruitment of more phagocytes (8). The recruitment of infected cells (9) and the excessive accumulation of productively infected CD163⁺ perivascular macrophages established an anti-inflammatory environment in favor of the infection, leading to severe encephalitis (10).

rophages in lesions (Figure 9). In this study, the accumulation of MAC387⁺ cells corresponds to acute inflammatory activity occurring during early formation of perivascular cuffs, composing few CD68⁺ cells and infected cells. A transitional stage preceding the SIVE lesions with active viral replication and numerous MNGCs would contain similar percentages of MAC387⁺ cells and CD68⁺ macrophages. Then, with excessive perivascular recruitment and accumulation of monocytes/macrophages, encephalitic lesions become more severe with formation of MNGCs and more productively infected cells, CD163⁺ perivascular macrophages, and CD68⁺ HAM56⁺ macrophages (Figure 9). Therefore, it is likely that three important cell populations are involved in inflammatory and SIVE lesions: i) nonproductively infected MAC387⁺ inflammatory monocytes/macrophages, ii) productively infected CD163⁺CD68⁺ perivascular mac-

rophages, and iii) CD163⁺CD68⁺ resident macrophages, the parenchymal microglia.

Regarding the proinflammatory activities of MRP14 and the absence of CD163 and CCR2 expression, the MAC387⁺ cells could be considered as M1-polarized macrophages. In contrast, the CD163⁺CD68⁺ perivascular macrophages that are also CCR2⁺ could be associated with an anti-inflammatory phenotype and are likely M2-polarized alternatively activated macrophages.^{4,12,52} The M1-polarized macrophages thought to amplify immune response and inflammation are associated with higher suppression of HIV-1 replication *in vitro* compared with M2-polarized macrophages.⁵³ In SIV-associated cardiac diseases, CD163⁺ macrophages correlate with numerous SIV-infected cells and decreased infiltration of inflammatory cells, such as dendritic cells.⁵⁴ This scenario suggests that the MAC387⁺ inflammatory macro-

phages are likely involved in active lesion formation and perhaps expansion, whereas the CD163⁺ perivascular cells might potentially play a role in down-regulating immune responses. This is particularly interesting in light of the fact that productively infected CD163⁺ perivascular macrophages may eventually contribute to lesions by attempting to establish an immune-suppressive milieu in the CNS. Conversely, the MAC387⁺ cells that we find in active lesions perhaps function to expand antiviral immune responses. Thus, the MAC387⁺ cells have a minor presence in established, chronic, nonactive lesions.

We conclude that SIVE lesions are heterogeneously composed of nonproductively infected MAC387⁺ monocytes/macrophages, CD163⁺CD68⁺ perivascular macrophages that can be productively infected, and CD163⁺CD68⁺ resident parenchymal microglia. We further propose that the relative age or immune activity of lesions can be estimated on the basis of their proportion of recently infiltrating MAC387⁺ monocytes/macrophages and CD68⁺ macrophages. We also suggest that recently infiltrating MAC387⁺ cells in humans and monkeys could represent inflammatory cells recruited via classic proinflammatory mechanisms, initiated with virus infection, rather than be reservoirs for the virus. These findings support the notion that an active and continuous recruitment of monocytes/macrophages is likely required for the formation of SIVE and HIVE, providing new insights about the kinetics of development of AIDS-associated neuropathogenesis.

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