Immunopathology and Infectious Disease

CD163, a Marker of Perivascular Macrophages, Is Up-Regulated by Microglia in Simian Immunodeficiency Virus Encephalitis after Haptoglobin-Hemoglobin Complex Stimulation and Is Suggestive of Breakdown of the Blood-Brain Barrier

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Macrophages and microglia are the major cell types infected by human immunodeficiency virus and simian immunodeficiency virus (SIV) in the central nervous system. Microglia are likely infected *in vivo*, but evidence of widespread productive infection (ie, presence of viral RNA and protein) is lacking. This conclusion is controversial because, unlike lymphocytes, macrophages and microglia cannot be discreetly immunophenotyped. Of particular interest in the search for additional monocyte/macrophage-lineage cell markers is CD163; this receptor for haptoglobin-hemoglobin (Hp-Hb) complex, which forms in plasma following erythrolysis, is expressed exclusively on cells of monocyte/macrophage lineage. We examined CD163 expression *in vitro* and *in vivo* by multiple techniques and at varying times after SIV infection in macaques with or without encephalitis. In normal and acutely SIV-infected animals, and in SIV-infected animals without encephalitis, CD163 expression was detected in cells of monocyte/macrophage lineage, including perivascular macrophages, but not in parenchymal microglia. However, in chronically infected animals with encephalitis, CD163 expression was detected in activated microglia surrounding SIV encephalitis lesions in the presence of Hp-Hb complex, suggesting leakage of the blood-brain barrier. CD163 expression was also induced on microglia *in vitro* after stimulation with Hp-Hb complex. We con-

clude that CD163 is a selective marker of perivascular macrophages in normal macaques and during the early phases of SIV infection; however, later in infection in animals with encephalitis, CD163 is also expressed by microglia, which are probably activated as a result of vascular compromise. (Am J Pathol 2008; 172:725–737; DOI: 10.2353/ajpath.2008.070848)

Multiple studies have reported that macrophages and microglia are the major cell types infected in the central nervous system (CNS) of humans infected with human immunodeficiency virus (HIV) and macaques infected with simian immunodeficiency virus (SIV). Infection of these cells is thought to be critical in the pathogenesis of neuroAIDS and the development of HIV and SIV encephalitis (HIVE/SIVE).^{1–10} Although there is consensus that macrophages in the CNS are infected, these cells are heterogeneous with respect to their morphology, location, and function. At least four distinct populations of CNS macrophages exist. This includes choroid plexus, meningeal, and perivascular macrophages, as well as resident microglia.^{11–13} Although all these populations of brain macrophages have been shown to be infected, perivascular macrophages and microglia are thought to be the most important.^{14,15} Perivascular macrophages and microglia may be distinguished based on the differential expression of myeloid markers.^{16–20} Perivascular macrophages (CD11b⁺CD14⁺CD45⁺) are immunophenotypi-

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cally distinct from microglia (CD11b⁺ CD14⁻CD45⁻) and are considered the primary cell type productively infected in the CNS of macaques with SIVE.²¹ Microglia are widely distributed within brain parenchyma constituting up to 12% of CNS cells.^{22–24} It is likely that microglia are infected *in vivo* but evidence of widespread productive infection (viral RNA and protein) is lacking. This conclusion is somewhat controversial because macrophages cannot be as discreetly categorized by immunophenotypic criteria as is commonly done with lymphocytes. In addition, microglia have been shown to be sensitive to even minor disturbances of CNS homeostasis, and are readily activated.²⁵ Activation of microglia induces changes in cellular morphology (from a ramified resting state to an amoeboid activated state) and in the expression of cell surface receptors that results in an appearance very similar to a tissue macrophage.^{26–28} The difficulty in trying to differentiate perivascular macrophages from inflammatory macrophages (recent immigrants from the blood) and activated microglia complicates our understanding of the neuropathogenesis of AIDS. This has led to a search for new or additional markers of monocyte/macrophage lineage cells, which could enhance our ability to differentiate macrophage populations in the brain, similar to ED2 in the rat, which is specific for perivascular macrophages.^{29–31} Of particular interest has been CD163.^{10,13}

CD163 is a member of the scavenger receptor family with cysteine-rich domains (SCRC) identified as a receptor of haptoglobin-hemoglobin (Hp-Hb) and exclusively expressed in cells of monocyte-macrophage lineage.³² This 130-kDa transmembrane glycoprotein binds with high affinity to the Hp-Hb complex that forms in plasma when Hb is released from ruptured erythrocytes and is exposed to plasma Hp.^{33,34} *In vitro*, CD163 can be suppressed by proinflammatory mediators such as lipopolysaccharide, interferon- γ , and tumor necrosis factor- α , whereas interleukin-6 and the anti-inflammatory cytokine interleukin-10 strongly up-regulate CD163.^{35–38} *In vivo*, CD163-positive macrophages and soluble CD163 are found during late acute and chronic phases of inflammation.^{39–42} Recently, in SIVE and HIVE, it has been reported that CD163 labels perivascular macrophages and amoeboid cells as well as a ramified gray matter microglia population that was not positive for other macrophage markers.¹⁰ In contrast, another report indicated that CD163 expression in encephalitic brains of HIV-infected patients and SIV-infected macaques was confined to perivascular macrophages and that viral antigen-positive cells were all positive for CD163.¹³

Considering that the cellular expression of CD163 is not fully characterized in normal or SIV-infected macaques we examined the expression of CD163 *in vitro* and *in vivo* by multiple techniques and at varying times after SIV infection and in animals with or without SIVE. Our data show that CD163 is expressed by cells of monocyte/macrophage lineage including perivascular macrophages but not microglia in normal and acutely SIV-infected animals. CD163 expression was detected in ramified but activated microglia surrounding SIVE lesions in chronically infected macaques with severe encephalitis in the presence of Hp-Hb complex in the tissue. CD163 expression could also be induced on microglia *in vitro* by stimulation with Hp-Hb. The presence of Hp-Hb complex in tissues is suggestive of leakage of the

blood-brain barrier that is known to occur in SIVE and HIVE.^{43,44} We conclude that CD163 is a selective marker of perivascular macrophages in normal macaques and during the early phases of SIV infection. However, later in infection CD163 also labels microglia that have been activated probably as a result of vascular compromise.

Materials and Methods

Animals, Tissues, and Virus

Tissues from 23 SIV-infected and 2 uninfected Indian-origin rhesus macaques (*Macaca mulatta*) obtained from the pathology archives of the Tulane National Primate Research Center were used for these studies. Twelve of the animals were from a time course study. Three animals were euthanized at 7, 14, 21, and 50 days after inoculation. An additional 11 animals with terminal AIDS were selected based on the presence ($n = 6$) or absence ($n = 5$) of SIVE. All of the animals except one with SIVE were infected with either SIVmac239 or SIVmac251. The one remaining animal was infected with both SHIV162 and SHIV33A. All animals were infected intravenously with 50 ng p27 of virus. Details on the animals, including time after inoculation and major pathological findings, are provided in Table 1. Additional information on some of these animals has been published.⁴⁵

All animals were humanely euthanized with an intravenous overdose of pentobarbital and were immediately necropsied. A complete set of tissues including multiple levels of brain were collected. 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.

Localization of SIV-Infected Cells

In situ hybridization for SIV was performed using both riboprobes and random primed DNA probes as described previously.⁴⁵ 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% formamide with denatured herring sperm DNA and yeast tRNA at 10 mg/ml each). SIV-digoxigenin-labeled antisense riboprobes (Lofstrand Laboratories, Gaithersburg, 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 of an anti-digoxigenin antibody from sheep, conjugated with alkaline phosphatase (Roche, Penzberg, Germany) were used to detect digoxigenin-

Table 1. Animals Infected with Different Strains of SIV, Time of Tissue Collection, and Major Pathological Findings

Animal no.	Days PI	Major pathologic diagnoses	Inoculum	AIDS
250-93	7	End of experiment, NSL	SIVmac239	No
277-93	7	End of experiment, NSL	SIVmac239	No
317-96	7	End of experiment, NSL	SIVmac239	No
285-93	14	End of experiment, NSL	SIVmac239	No
388-93	14	End of experiment, NSL	SIVmac239	No
318-96	14	End of experiment, NSL	SIVmac239	No
406-93	21	End of experiment, NSL	SIVmac239	No
441-93	21	End of experiment, NSL	SIVmac239	No
319-96	21	End of experiment, NSL	SIVmac239	No
364-93	50	End of experiment, NSL	SIVmac239	No
434-93	50	End of experiment, NSL	SIVmac239	No
320-96	50	End of experiment, NSL	SIVmac239	No
G402	86	SIVE, giant cell pneumonia	SIVmac239	Yes
AH01	93	SIVE, giant cell pneumonia, gastric thrichomoniasis, esophageal candidiasis	SIVmac239	Yes
AT71	121	SIVE, giant cell pneumonia, CMV	SIVmac251	Yes
AM93	136	SIVE, giant cell pneumonia, and adenitis	SHIV162 + 33A	Yes
BG98	149	SIVE, giant cell pneumonia, glomerulosclerosis, PCP	SIVmac251	Yes
V791	199	SIVE, optic neuritis, giant cell pneumonia	SIVmac 251	Yes
CE48	300	Meningoencephalitis, demyelination, SV40	SIVmac239	Yes
BV01	303	Giant cell pneumonia, PCP, cryptosporidiosis	SIVmac239	Yes
CI65	377	MAC, lymphoid depletion, colitis granulomatous	SIVmac239	Yes
DD88	383	MAC, lymphoid depletion, colitis granulomatous	SIVmac239	Yes
DD95	455	Giant cell pneumonia, meningitis, glomerulosclerosis	SIVmac239	Yes
DI95			Uninfected	
BV36			Uninfected	

Days PI, days after inoculation; NSL, no significant lesions; SIVE, SIV-encephalitis; MAC, *Mycobacterium avium* complex; PCP, *Pneumocystis carinii* pneumonia; SV40, Simian virus 40; CMV, cytomegalovirus.

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.^{46,47} 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 washed, placed in a humidified chamber, and prehybridized at 37°C with hybridization buffer (containing 50% of formamide with denatured herring sperm DNA and yeast tRNA at 10 mg/ml each) and washed with 2× standard saline citrate. SIV-digoxigenin-labeled DNA probes were used at 0.5 ng/μl 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 nitro blue tetrazolium

chloride/5-bromo-4-chloro-3-indolyl phosphate stock solution (Roche, Mannheim, Germany) or Vector blue (Vector Laboratories, Inc.) as chromogen. If immunofluorescence followed the *in situ* hybridization, 2-hydroxy-3-naphtic acid 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 μl of HNPP/Fast Red TR (10 μg of HNPP in 1 ml of detection buffer plus 10 μl of Fast Red TR solution) was applied. This solution was filtered through a 0.2-μm nylon filter immediately before use, and then the slides were coverslipped and incubated for 30 minutes at room temperature in the dark as described previously.⁴⁵

Immunophenotype of Brain Macrophages

To define the immunophenotype of SIV-infected and non-infected macrophages, we performed combined *in situ* hybridization/immunohistochemistry as described previously.^{21,45,48–50} After *in situ* hybridization for viral nucleic acid as described above, single- or double-label immunohistochemistry or immunofluorescence were performed using a variety of monocyte/macrophage and microglia-specific markers (Table 2).

In situ hybridization followed by single-label immunohistochemistry for macrophages or microglia was performed as previously described.^{45,48,49} To identify macrophages we initially used CD163 (clone 10D6: Novocastra Laboratories Ltd., Newcastle, UK; and clone EDHu-1: Serotec Ltd., Oxford, UK), whereas for microglia we used a rabbit polyclonal primary antibody specific for Iba1 (ionized calcium-

Table 2. Antibodies Used in Immunohistochemistry (IHC) and Immunofluorescence (IFA)

Antigen	Cell type	Source	Antibody type*	Dilution	
				IFA	IHC
CD163	Macrophage	Novocastra	IgG1	1:50	1:100
CD163	Macrophage	Serotec	IgG1		1:100
Iba1	Microglia	BD PharMingen	IgG1, κ	1:100	1:250
Iba1	Microglia	Aves Labs Inc.	Polyclonal	1:100	
GLUT-5	Microglia	Chemicon	Polyclonal		1:500
CD11b	Microglia/macrophage	Novocastra	IgG1	1:10	
RCA-1	Microglia	DAKO	Lectin		1:500
HLA-DR	Activation	Zymed	IgG2b	1:20	1:50
NEU-N	Neuron	Chemicon	IgG1	1:10	1:200
MAP2	Neuron	Novocastra	IgG2a		
GFAP	Astrocyte	Sigma	IgG1 conjugated with CY3	1:700	1:3000
Peripherin	Astrocyte	Novocastra	IgG2b	1:50	
MAB 328	Oligodendrocyte	Chemicon	IgM	1:50	
CNPase	Oligodendrocyte	Sigma	IgG1		1:200
Haptoglobin	Haptoglobin	Sigma	Polyclonal	1:3000	

*For monoclonal antibodies the isotype is indicated. Most of the polyclonal antibodies were made in rabbit with the exception of Iba1, which was made in chicken.

binding adapter molecule-1, Wako Pure Chemical Industries Ltd., Richmond, VA) or a chicken polyclonal antibody specific for Iba1 (Aves Labs Inc., Tigard, OR). This antibody was produced by immunization of chickens with a synthetic peptide (CZ YEE KAR EKE KPT GPP AKK) which represents residues 124 to 141 of the human Iba1 gene (accession no. D86438). The specificity of the affinity-purified chicken IgY antibody was compared to the commercially available Iba1 made in rabbits. Both antibodies labeled the same cells in brain tissue and *in vitro*-cultured microglia. The only discernable difference was that the cellular staining pattern with the chicken antibody was more punctate than the rabbit antibody.

After *in situ* hybridization for SIV, sections were incubated sequentially with the primary, cell-type-specific antibody for 60 minutes 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 Elite or alkaline phosphatase, Vector Laboratories), and the reaction was visualized with AEC (DAKO, Carpinteria, CA) or Vector Red (Vector Laboratories) as the chromogen. As negative control, serial sections were processed identically using equivalent concentrations of irrelevant primary antibodies of the same isotype.

To more carefully examine the cell types infected we performed additional multilabel techniques combining *in situ* hybridization with immunofluorescence for two cell-type-specific markers as described previously^{21,45,50} using combinations of antibodies as shown in Table 3. For these multilabel techniques, *in situ* hybridization was performed as described above except that

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

Channel 1 Red with HNPP/Fast Red or Red with Alexa 568		Channel 2 Green with Alexa 488		Channel 3 Far-Red with Alexa 633	
Iba1 (rabbit)	Anti-rabbit	CD163 (IgG1) mouse	Anti-mouse (IgG1)	Nuclear staining Topro3	
GFAP (IgG1) mouse/CY3		CD163 (IgG1) mouse	Anti-mouse (IgG1)		
Peripherin (IgG2b) mouse	Anti-mouse (IgG2b)	CD163 (IgG1) mouse	Anti-mouse (IgG1)		
GFAP (IgG1) mouse/CY3		CD163 (IgG1) mouse	Anti-mouse (IgG1)	Peripherin (IgG2b) mouse	Anti-mouse (IgG2b)
Riboprobes or DNA probes	Anti-digoxigenin/sheep	CD163 (IgG1) mouse	Anti-mouse (IgG1)		
HLA-DR (IgG2b) mouse	Anti-mouse (IgG2b)	CD163 (IgG1) mouse	Anti-mouse (IgG1)		
HLA-DR (IgG2b) mouse	Anti-mouse (IgG2b)	Iba1 (rabbit)	Anti-rabbit	CD163 (IgG1) mouse	Anti-mouse (IgG1)
CD163 (IgG1) mouse	Anti-mouse (IgG1)	Hp (rabbit)	Anti-rabbit	Nuclear staining Topro3	
Iba1 (chicken)	Anti-chicken	Hp (rabbit)	Anti-rabbit		
CD163 (IgG1) mouse	Anti-mouse (IgG1)	Hp (rabbit)	Anti-rabbit		
CD163 (IgG1) mouse	Anti-mouse (IgG1)	Hp (rabbit)	Anti-rabbit	Iba1 (chicken)	Anti-chicken

Each column shows the primary and secondary antibody (if relevant) used. Channel 1 was used for: 1) visualization of *in situ* hybridization using HNPP/ Fast Red, which fluoresces intensely red; 2) primary antibodies conjugated to CY3; or 3) secondary antibodies conjugated with Alexa 568. Channel 2 was used to detect additional labels using secondary antibody conjugated to Alexa 488 and Channel 3 was used to detect secondary antibodies conjugated to Alexa 633. The color of the Far Red channel is changed to blue at the time of data acquisition on the confocal microscope.

the results of *in situ* hybridization were visualized with HNPP/Fast Red, which fluoresces intensely red. In addition to CD163 and Iba1 we used several additional cell-type-specific antibodies to define specific cell types including NeuN (neuronal nuclei) and MAP2 (microtubule-associated protein-2) for neurons; GFAP (glial fibrillary acidic protein) and peripherin for astrocytes⁵¹; MAB328 (myelin oligodendrocyte-specific protein) and CNPase (cyclic nucleotide phosphodiesterase) for oligodendrocytes; and Glut5 (glucose transporter-5) and RCA-1 (Ricin communis agglutinin-1) for microglia. To differentiate resting cell populations from activated cell populations we used HLA-DR as an activation marker. To determine the presence of Hp in the brain parenchyma we used anti-human polyclonal Hp (Table 2). 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 (if the primary was not directly conjugated) applied simultaneously as previously described.⁴⁵ The secondary antibodies were coupled with either Alexa 488 (green), Alexa 568 (red), or Alexa 633 (far red) (Molecular Probes, Eugene, OR). After antibody treatment, sections were washed twice for 15 minutes in phosphate-buffered saline (PBS) with 0.2% fish skin gelatin. Finally, the sections were rinsed in doubly distilled water 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). Thirty-two to sixty-two optical slices were collected at 512×512 pixel resolution. Each individual slice represented $0.2 \mu\text{m}$. NIH Image (version 1.62; National Institutes of Health, Bethesda, MD) and Adobe Photoshop (version 7.0; Adobe, San Jose, CA) were used to assign colors to the four channels collected: HNPP/Fast Red, which fluoresces when exposed to a 568-nm wavelength laser, appears red; Alexa 488 (Molecular Probes) appears 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 $1 \mu\text{g/ml}$, incubated for 5 minutes, and tissues were then washed in PBS. Colocalization of antigens is demonstrated by the addition of colors as indicated in the figure legend.

Microglial Culture to Assess CD163 Expression

Microglia were isolated from normal adult brain as previously described.⁵² After 1 week in culture the microglia were removed from the flask by gentle rotation on an environmental incubator at 100 rpm for 1 hour. The cells were collected and centrifuged at $400 \times g$ and the cell pellet was resuspended and

plated in eight-well chambers (Nalgene Nunc International, Rochester, NY) for microscopy studies and in 75-cm² cell culture flasks for RNA and protein studies (Corning Inc., Corning, NY) as described below. The cells were plated at the same density (same number of cells) in each 75-cm² flask. The resulting cultures were 99% microglia as evidenced by Iba1 labeling. The remaining cells (less than 1%) were GFAP⁺ astrocytes (data not shown).

Hp-Hb complex was prepared as follows: blood from normal rhesus macaques was collected in two vacutainer tubes, one containing sodium heparin and the other containing silica clot activator and polymer gel to obtain serum. The red blood cells were separated from the plasma and rinsed twice with isotonic PBS. The packed red blood cells were lysed mechanically by adding 2-mm glass beads and vigorously mixing the tube for 5 to 10 minutes on a vortex (Fisher Scientific, Inc., Pittsburgh, PA). Disruption of the red blood cells was confirmed by microscopic examination. The blood lysate was centrifuged at 15,000 rpm in a microcentrifuge (International Equipment Company, Needham Heights, MA) and the supernatant (soluble Hb) was separated from the particulate pellet. The soluble Hb was mixed at a 1:10 ratio with serum from the same animal. The mixture was left at room temperature for 10 minutes to allow binding of Hp to the free Hb (Hp-Hb complex formation). The complex was further diluted 1:100 in RPMI media before addition to tissue culture media.

After 24 hours of culture, the microglia were treated with Hp-Hb complex, prepared as described above, for either 2 hours followed by culture for 0, 6, 12, or 18 hours or constantly with Hp-Hb complex for 18 hours. In addition, one eight-well chamber culture dish was cultured for 48 hours after 2 hours of incubation with Hp-Hb complex. In those cultures that were only exposed to Hp-Hb complex for 2 hours, at the end of this time the cultures were rinsed three times with tissue culture media (RPMI-10% fetal calf serum) to remove the Hp-Hb complex. The control microglia tissue culture received the same serum used to make the Hp-Hb complex at the same concentration (final concentration 1:1000 in the tissue culture media). After the culture period the cell cultures in chambers for microscopy were washed with warm PBS two times and fixed in 2% paraformaldehyde for 10 minutes, and immunostained as described above with anti-CD163 and anti-Iba1 antibodies to identify macrophages¹³ and microglia,⁵³ respectively. This experiment was performed using cells from three different animals and each microglia culture was examined in quadruplicate. For RNA and protein extraction one 75-cm² flask was used for each assay at each time point. The extraction of RNA and protein were performed as described below.

Quantitative Real-Time SYBR Green One-Step Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Gene expression for CD163 in cultured microglia from three animals was evaluated by quantitative real-time SYBR green one-step RT-PCR assay (QRT-PCR). Total

Table 4. Primer Sequence, Concentration, and Product Size Used for QRT-PCR

Name of gene	Primer sequence	Product size (bp)	Primer concentration
CD163	Forward: 5'-AGCATGGAAGCGGTCTCTGTGATT-3' Reverse: 5'-AGCTGACTCATTCCCACGACAAGA-3'	138	500 nmol/L
β -Actin	Forward: 5'-AGGCTCTCTTCCAACCTTCCTT-3' Reverse: 5'-CGTACAGGTCTTTACGGATGTCCA-3'	108	300 nmol/L

RNA was extracted from untreated and Hp-Hb-treated microglial cultures for 0, 6, 12, or 18 hours or constantly with Hp-Hb complex for 18 hours and was assayed in triplicate wells. Each QRT-PCR reaction (25 μ l) contained the following: 2 $\times$ Master mix without uracil-*N*-glycosylase (12.5 μ l), reverse transcriptase (0.25 μ l), target forward and reverse primer, and total RNA (25 ng) quantified spectrophotometrically based on $A_{260}:A_{280}$ ratios. Forward and reverse primer sequence, concentration, and product size including β -actin are shown in Table 4. The PCR amplification was performed in the ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster City, CA). Thermal cycling conditions were 50°C for 30 minutes, 95°C for 15 minutes, followed by 40 repetitive cycles of 95°C for 15 seconds, 55°C for 30 seconds, 72°C for 30 seconds. As a normalization control for RNA loading, parallel reactions in the same multiwell plate were performed using β -actin mRNA.

Quantification of gene amplification after RT-PCR was made by determining the threshold cycle (C_T) number for SYBR Green fluorescence within the geometric region of the semilog plot generated during PCR. Within this region of the amplification curve, each difference of one cycle is equivalent to a doubling of the amplified product of the PCR. The relative quantification of target gene expression across treatments was evaluated using the comparative C_T method. The ΔC_T value was determined by subtracting the β -actin C_T value for each sample from the target C_T value of that sample. Calculation of $\Delta\Delta C_T$ involved using the highest sample ΔC_T value (ie, sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔC_T sample values. Fold changes in the relative gene expression of target was determined by evaluating the expression, $2^{-\Delta\Delta C_T}$.

Immunoprecipitation and Western Blotting

Cultured microglial cells were dislodged, pelleted, and protein extraction was performed in ~250 μ l of lysis buffer (Cell Signaling Technology, Inc., Beverly, MA) containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na_2EDTA , 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β -glycerophosphate, 1 mmol/L Na_3VO_4 , 1 μ g/ml leupeptin, protease inhibitor cocktail, and phosphatase inhibitor cocktail (Sigma Chemical Co., St. Louis, MO). The protein lysates were first precleared with 5 μ l of normal rabbit immunoglobulin for ~1 hour and then immunoprecipitated with ~10 μ l of a rabbit polyclonal antibody against CD163 (Santa Cruz Biotechnology, Santa Cruz, CA), overnight at 4°C followed by incubation with 30 μ l (50% w/v) of protein G

agarose beads (Millipore Corp., Billerica, MA) at 4°C for 4 to 5 hours. The supernatant was removed and transferred to a separate 1.5-ml microcentrifuge tube and immunoprecipitated using a goat polyclonal antibody against β -actin (~5 μ l) (Santa Cruz Biotechnology). The immunoprecipitation for β -actin was performed at 4°C overnight on a shaker. Immunoprecipitated CD163 and β -actin proteins were heat denatured for 5 minutes at 100°C in sample loading buffer containing 62.5 mmol/L Tris-HCl, 5% 2-mercaptoethanol, 10% glycerol, 2% sodium dodecyl sulfate, and bromophenol blue, resolved on 8% and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, respectively, and transferred to 0.2- μ m nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were probed with a rabbit polyclonal primary antibody against CD163 (Santa Cruz Biotechnology), and β -actin (Santa Cruz Biotechnology) followed by a anti-rabbit polyclonal horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Membranes were treated with West Pico chemiluminescent substrate (Pierce Biotechnology Inc., Rockford, IL) for 5 minutes and the signal was developed by exposing the membrane to Kodak X-OMAT film (Eastman-Kodak, Rochester, NY) for 5 and 10 minutes for CD163 and β -actin, respectively.

Flow Cytometry

Flow cytometry on peripheral blood was used to confirm CD163 expression on circulating monocytes. One hundred μ l of EDTA-treated fresh whole blood from eight uninfected and six SIVmac239- or SIVmac251-infected rhesus macaques were stained with phycoerythrin-conjugated anti-human CD163 monoclonal antibody (mAb) (Mac2-158; Trillium Diagnostics, LLC., Scarborough, ME) and PerCP-Cy5.5-conjugated anti-human CD14 mAb (M5E2; BD Pharmingen, San Jose, CA) for 15 minutes at room temperature. Red blood cells were then lysed using a Coulter Immunoprep reagent system and a TQ-prep Workstation (Beckman Coulter, Hialeah, FL). The cells were then washed with 2% fetal calf serum-PBS, fixed with 1% formaldehyde-PBS, and were analyzed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

Results

Histopathology

As indicated in Table 1, of the 11 animals with AIDS 6 had SIVE characterized by nodular perivascular aggregates

of mononuclear cells and multinucleated giant cells along with variable areas of gliosis as described previously (Supplemental Figure S1A at <http://ajp.amjpathol.org>).^{21,54–56} The SIVE lesions were observed in both gray and white matter of brain but predominantly in white matter. The remaining five animals with AIDS had no histopathological abnormalities in the CNS, however, all 11 animals with AIDS had AIDS-defining lesions such as multiple opportunistic infections and generalized lymphoid depletion. The 12 animals from which tissues were collected within 50 days of infection had mild perivascular cuffs in the CNS (Supplemental Figure S1B at <http://ajp.amjpathol.org>) as described previously.^{21,54,55,57}

CD163 and SIV Infection in Vivo

Localization of SIV-infected cells in brain was examined by *in situ* hybridization using both riboprobes and random primed DNA probes to detect viral RNA and/or DNA. To define the immunophenotype of infected brain macrophages, we performed single- and double-labeled immunohistochemistry or multilabel confocal microscopy for CD163 and a variety of cell markers combined with *in situ* hybridization for SIV (Tables 2 and 3). We also confirmed that CD163 was expressed exclusively on circulating CD14⁺ monocytes of both normal and SIV-infected macaques (Supplemental Figure S2 at <http://ajp.amjpathol.org>).

In Normal Brain

In normal brain the expression of CD163 was observed in meningeal, choroid plexus, and perivascular macrophages (Figure 1, A–C). No labeling of cells in the brain parenchyma where microglia reside was detected in normal controls (Figure 1C). In contrast, using a variety of microglial markers (Iba1, Glut-5, and RCA-1, which label resting and activated microglia) we were able to clearly label microglia with elongated nuclei, scanty perikaryon, and multiple fine cytoplasmic processes characteristic of resting microglia (Figure 1D). Double-label immunohistochemistry using CD163 plus Iba1 clearly demonstrate that the Iba1⁺ cells in the parenchyma were not CD163⁺ (Figure 1E).

In Acute Infection

Similar to normal brain, in acutely SIV-infected macaques, the expression of CD163 was observed in meningeal, choroid plexus, and perivascular macrophages and no positive cells resembling microglia were observed in the brain parenchyma. In addition, consistent with previous observations using other macrophage markers⁵⁸ increased numbers of CD163⁺ cells consistent with perivascular macrophages were present corresponding to the mild perivascular cuffs observed (Supplemental Figure S1B at <http://ajp.amjpathol.org>).

In Terminal AIDS without SIVE

In animals with terminal AIDS but no histological evidence of SIVE the expression of CD163 was the same as for acutely SIV-infected animals.

In Terminal AIDS with SIVE

In contrast to the other groups of animals, in animals with SIVE the expression of CD163 in the CNS was not limited to the three populations of brain macrophages, but was also observed in cells morphologically compatible with activated microglia (Figure 1F). Generally, the staining pattern of putative activated microglia was focal or focally extensive but always near a SIVE lesion. This was true in both the gray and white matter. Most of the CD163⁺ cells had short crenellated cytoplasmic projections and there appeared to be progressive diminution of cytoplasmic projections the closer the proximity to an SIVE lesion. No such labeling was seen in brains of normal animals nor acutely or chronically SIV-infected animals without encephalitis. In brain of acutely infected macaques and in macaques with terminal AIDS without SIVE, the expression of CD163 was restricted to meningeal, choroid plexus, and perivascular macrophages (similar to normal brain). To confirm the impression that CD163 was labeling microglia in the vicinity of SIVE lesions we used double-label immunofluorescence plus confocal microscopy combining CD163 and Iba1; this demonstrated co-localization of both markers indicating that CD163 was labeling microglia in encephalitic brain (Figure 2A).

To evaluate the possibility that cells with ramified morphology may represent cytoplasmic projections of other cells from the brain parenchyma such as neurons, astrocytes, or oligodendrocytes, we used MAP2, GFAP, peripherin, CNPase, and myelin/oligodendrocyte-specific protein. MAP2 selectively labels the dendritic tree of neurons, GFAP and peripherin label astrocytes, and CNPase and myelin/oligodendrocyte-specific protein label oligodendrocytes. Double- and triple-label immunofluorescence plus confocal microscopy did not show co-localization between CD163 and any of the other cell-type-specific markers (Supplemental Figure S3 at <http://ajp.amjpathol.org> illustrates this for astrocytes) providing additional support to the notion that the CD163⁺ cells were microglia.

In acutely infected macaques and in macaques with terminal AIDS without SIVE, SIV-infected cells were infrequent whereas in animals with SIVE large numbers of infected mononuclear cells and multinucleated giant cells were present within lesions as described previously.^{21,54,55,57,58} In the lesions most of the individual macrophages and multinucleated giant cells expressed CD163 and these cells were often infected with SIV (Figure 2B).

CD163 Is Expressed on Activated Microglia

We hypothesized that the CD163⁺ microglia were activated based on their morphology. To further evaluate

Figure 1. Immunohistochemistry of brain macrophage populations. In normal brain immunohistochemistry reveals CD163 expression by meningeal macrophages (**A**), choroid plexus macrophages (**B**), and perivascular macrophages (**C**), but not cells morphologically compatible with microglia. **D:** Immunohistochemistry for Iba1 labels resting microglia throughout the parenchyma characterized by elongated nuclei with scanty perikaryon and multiple cytoplasmic extensions. **E:** Double-label immunohistochemistry in normal brain clearly shows that CD163⁺ perivascular macrophages (blue, vector blue chromogen) and Iba1⁺ microglia (red, AEC chromogen) are mutually exclusive. **F:** In contrast to normal brain, immunohistochemistry for CD163 in animals with SIVE reveals focally extensive aggregates of cells morphologically consistent with microglia in the areas surrounding SIVE lesions. Original magnifications: $\times 200$ (**A**, **B**, **F**); $\times 1000$ (**C**); $\times 400$ (**D**).

this we examined the expression of HLA-DR (human leukocyte antigen) on multiple cell types. In normal brain, HLA-DR expression was restricted to perivascular cells whereas in SIVE a large number of HLA-DR⁺ cells were observed, which were associated with SIVE

lesions (Figure 2C). HLA-DR⁺ cells had a round to oval morphology within the lesions and a more ramified morphology compatible with microglia in the area around the lesions. We then performed triple-label studies with Iba1, HLA-DR, and CD163 (Figure 3). This

Figure 2. Multilabel confocal microscopy of CD163 in SIVE brain. Individual channels are on the **left** with a larger merged image on the **right**. **A:** Triple-label confocal microscopy showing co-localization of CD163 (green) and Iba1 (red), indicating that CD163 is expressed on microglia during SIVE (CD163 with Alexa 488, green; Iba1 with Alexa 568, red; and cell nuclei with ToPro3, co-localization of both markers results in a yellow-orange). **B:** Double-label confocal microscopy showing that CD163 is expressed by individual macrophages and multinucleated giant cells, and these cells are often infected with SIV (CD163 with Alexa 488, green; SIV *in situ* hybridization with Fast Red, red; and differential interference contrast, DIC). **C:** Double-label confocal microscopy for CD163 and HLA-DR to assess immune activation in SIVE. Extensive co-localization of CD163 and HLA-DR can be observed. In addition to CD163⁺HLA-DR⁺ cells with a round to oval morphology within the lesion, cells with a more ramified morphology are present at the margins of the lesion. These cells are consistent with parenchymal microglia (CD163 with Alexa 488, green; and HLA-DR with Alexa 568, red).

Figure 3. Multilabel confocal microscopy demonstrating co-localization of CD163, Iba1, and HLA-DR indicative of CD163 expression on activated microglia. Images for individual channels from the same field (Iba1 with Alexa 488, green; HLA-DR with Alexa 568, red; and CD163 with Alexa 633, blue) are shown on the **left** and a larger merged image containing all three channels showing co-localization of the three markers is on the **right**. Microglia (Iba1⁺ cells) expressing HLA-DR appear yellow, CD163⁺ cells expressing HLA-DR appear purple, and the co-localization of CD163 and Iba1 appear light blue. Note that the co-localization of all three colors indicates that CD163 is expressed by activated microglia.

revealed that the CD163⁺ microglia (defined by morphology and Iba1⁺) were also positive for HLA-DR indicating that CD163 was expressed by activated microglia.

Co-Localization of CD163 Expression and Its Ligand Hp-Hb Complex

We hypothesized that expression of CD163 on microglia was a result of damage to the blood-brain barrier and formation of Hp-Hb complexes that up-regulated CD163 expression. To assess this possibility we examined the presence of Hp in relation to the presence of activated microglia by single-, double- and triple-label immunofluorescence. In brain of normal animals, acutely SIV-infected animals and SIV-infected animals without SIVE, labeling for Hp was limited to the lumen of vessels (Figure 4A). In contrast, in encephalitic brain, Hp labeling also occurred in the brain parenchyma in areas where CD163 was expressed by microglia (Iba1⁺ CD163⁺) (Figure 4, B–D). Clear co-localization of Hp and Iba1 (Figure 4B) and Hp and CD163 (Figure 4C) were observed. Moreover, clear co-localization of CD163, Iba1, and Hp (Figure 4D) was also observed. These data strongly suggested that breakdown of the blood-brain barrier was associated with the activation of microglia and their expression of CD163.

CD163 Expression in Microglia in Vitro

To further evaluate the connection between Hp-Hb and CD163 expression on microglia we performed *in vitro* experiments on the effect of Hp-Hb on microglia. Microglia were prepared and cultured in eight-well chambers with or without Hp-Hb complex as described in the Materials and Methods. At all times the cultures were 99 to 100% positive for Iba1 (Figure 5A). In contrast, expression of CD163 was observed only in cells treated with the Hp-Hb complex for at least 2 hours (Figure 5C). No

Figure 4. Multilabel confocal microscopy for Hp in animals without (A) or with (B–D) encephalitis. **A:** In normal animals, acutely SIV-infected animals and SIV-infected animals without SIVE, labeling for Hp was limited to the lumen of vessels. Images for individual channels (Hp with Alexa 488, green; CD163 with Alexa 568, red; and cell nuclei with Topro3, blue and DIC) are shown on the **left** and a larger merged image is on the **right**. **B:** Hp is present in the brain parenchyma and co-localizes with microglia (Iba1⁺) resulting in an orange-yellow color. Images for individual channels [Hp with Alexa 488, green; Iba1 (chicken polyclonal) with Alexa 568, red; and DIC] are shown on the **left** and a larger merged image on the **right**. **C:** Similar to B, this image shows the presence of Hp that co-localizes with CD163 in cells morphologically compatible with microglia giving an orange-yellow color. Images for individual channels (Hp with Alexa 488, green; CD163 with Alexa 568, red) are shown on the **left** and a larger merged image is on the **right**. **D:** Co-localization of Hp (green), CD163 (red), and Iba1 (blue) can be seen in this image. Images for individual channels from the same field [Hp with Alexa 488, green; CD163 with Alexa 568, red; and Iba1 (chicken polyclonal) with Alexa 633, blue] are shown on the **left** with a larger merged image on the **right**.

CD163 expression was observed in untreated wells or in wells treated with serum only. This result suggests that the interaction of the Hp-Hb complex is required to trigger the up-regulation of CD163, the receptor for Hp-Hb complex in cultured microglia *in vitro*.

CD163 Protein Up-Regulation in Cultured Microglia in Response to Treatment with Hb/Hp Complexes Is Not Preceded by a Concomitant Increase in CD163 mRNA

To confirm that the activation of microglia was associated with the presence and up-regulation of CD163 mRNA and protein we treated *in vitro*-cultured microglia isolated from rhesus macaque brain (frontal cortex and mid brain) with Hp-Hb complexes (100 μ l) for either 2 hours followed by culture for 0, 6, 12, 18 hours or constantly for 18 hours and performed quantitative real-time SYBR Green one-

step RT-PCR and immunoprecipitation/Western blotting, respectively, for CD163 mRNA and protein analysis. Treatment for either 2 hours followed by culture for 0, 6, 12, or 18 hours or 18 hours constant treatment with Hp-Hb complexes did not have any effect on CD163 mRNA transcription (data not shown). The data indicates that CD163 mRNA is constitutively expressed and is not influenced by the presence of Hp-Hb complexes. However, these findings along with the fact that the CD163 protein was detectable by immunofluorescence/confocal microscopy only after treatment with Hp-Hb complexes (Figure 5C) led us to hypothesize the presence of post-transcriptional regulation. To confirm the latter hypothesis, total protein was extracted at each time point and changes in CD163 protein expression was evaluated using immunoprecipitation/Western blotting. Figure 5B shows the band density for CD163 and β -actin at each time point and the bars represent the ratio of CD163 to β -actin levels. Interestingly, we observed no significant

Figure 5. CD163 protein induction *in vitro* in microglia. **A:** Untreated microglia in an eight-well chamber labeled with Iba1 (Iba1 with Alexa 568 red). **B:** Microglial cells were either treated for 2 hours with Hp-Hb complexes and cultured *in vitro* for 6, 12, or 18 hours or constantly treated for 18 hours. After incubation, cell homogenates were immunoprecipitated and immunoblotted with a rabbit anti-human CD163 polyclonal antibody. The figure **inset** on the **top** of the histogram shows protein bands of CD163 and β -actin used as a loading control for Western blotting. The graph depicts the density value for each treatment as a ratio of CD163 to β -actin levels. 0 = no addition of Hp-Hb; 2 + 6 hours = treated 2 hours with Hp-Hb, wash, and 6 hours of incubation; 2 + 12 = treated 2 hours with Hp-Hb, wash, and 12 hours of incubation; 2 + 18 = treated 2 hours with Hp-Hb, wash, and 18 hours of incubation; 18 hours constant = 18 hours of incubation with Hp-Hb. **C:** Microglia in an eight-well chamber labeled for Iba1 (red) and CD163 (green) after treatment with the Hp-Hb complex showing co-labeling for both Iba1 and CD163. CD163 expression is predominantly on the cell surface in green. This demonstrates up-regulation of CD163 in cultured microglia in response to stimulation with Hp-Hb complex. (Iba1 with Alexa 568, red; CD163 with Alexa 488, green; and cell nuclei with Topro3, blue).

increase in CD163 protein expression in microglia cultured *in vitro* for 0, 6, 12, or 18 hours after a 2-hour treatment with Hp-Hb complexes. In contrast, constant treatment of *in vitro*-cultured microglia with Hp-Hb complexes for 18 hours resulted in a significant increase (~ 3.4 -fold) in CD163 protein expression compared to the 2-hour treatment/incubation time points. The data clearly indicates that continued stimulation of *in vitro*-cultured microglia with Hp-Hb complexes ($100 \mu\text{l}$) results in increased translation of the CD163 protein from a message (mRNA) that is basally expressed, implying posttranscriptional regulation.

Discussion

Understanding the neuropathogenesis of AIDS is hampered by difficulty in identifying the specific subset of monocytes/macrophages involved. Several recent studies have suggested that CD163, the macrophage receptor for endocytosis of Hp-Hb, which is restricted to monocyte/macrophage lineage cells,^{32,59,60} may be a marker for subsets of macrophages important in the neuropathogenesis of AIDS.

Previous studies in macaques with SIV encephalitis demonstrated that CD163 was expressed by perivascular macrophages and/or a population of hyperramified microglia in the gray matter.^{10,13} In the present study, we examined the expression of CD163 in brain of normal and SIV-infected macaques with or without encephalitis. We determined that the expression of CD163 in normal animals and SIV-infected animals without encephalitis is limited to perivascular macrophages. In contrast, in animals with SIVE, CD163 was expressed by perivascular macrophages as well as activated microglia. Using multilabel confocal microscopy we demonstrated co-localization of CD163 with Iba1 and HLA-DR, indicating that CD163 was up-regulated on activated microglia surrounding SIVE lesions.

Based on this we hypothesized that the localized expression of CD163 on microglia was a result of immune activation possibly associated with damage to the blood-brain barrier, which would facilitate formation of Hp-Hb complexes and could increase CD163 expression via the high affinity between Hp-Hb complexes and CD163.³⁴ To assess this possibility we examined the presence of Hp in the CNS of normal macaques and SIV-infected animals with and without SIVE. In brain of normal macaques, acutely SIV-infected animals and SIV-infected animals without SIVE, the presence of Hp was limited to the lumen of vessels. In contrast, in animals with SIVE, Hp was also present in the brain parenchyma in areas where CD163 was up-regulated by microglia (Iba1⁺ CD163⁺).

To examine the relationship between extravascular Hp-Hb complexes and up-regulation of CD163 we performed *in vitro* studies. This work demonstrated that CD163 protein was undetectable on microglia by immunofluorescence under normal culture conditions but was detectable by immunoprecipitation/Western blotting. After treatment of microglia with Hp-Hb complexes up-regulation of CD163 was shown by immunoprecipitation/Western blotting and CD163 became detectable by immunofluorescence. Together these data strongly suggest that Hp-Hb complexes bind to existing, but rare, CD163 molecules on microglia that results in up-regulation of CD163 to a level detectable by immunofluorescence and immunohistochemistry. This finding prompted us to further investigate the mechanisms by which Hp-Hb complexes regulate CD163 protein expression in microglia. Interestingly, despite evidence from two different techniques that up-regulation of CD163 occurs in microglia as a result of exposure to Hp-Hb complexes, no changes in CD163 mRNA expression were observed suggesting the presence of posttranscriptional or translational regulation or alterations in intracellular trafficking or degradation of CD163. Similar increases in protein synthesis in the absence of a concomitant increase in

mRNA synthesis have been previously described for ferritin and insulin-like growth factor-1.^{61,62} Hb released from ruptured erythrocytes is a molecule with toxic and proinflammatory properties and consequently requires immediate clearance from the circulation and areas of tissue damage.⁶³ In the present study, the strong expression of CD163 on macrophages and microglia in encephalitic brain may enable these phagocytic cells to clear Hb/Hp complexes and in doing so perform an anti-inflammatory role. Finally, the up-regulation of CD163 protein expression in response to Hp-Hb complexes without any increase in its cognate mRNA represents a highly efficient mechanism in the brain and can be attributed to a need to hasten the clearance of Hb and protect the brain from its untoward toxic effects.

We conclude that CD163 is a selective immunohistochemical marker of perivascular macrophages in normal macaques and during the early phases of SIV infection. However, later in infection in animals with encephalitis, CD163 also labels microglia, probably activated as a result of vascular compromise and the generation of Hp-Hb complexes.

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