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Authors

Holder, Gerard E
McGary, Christopher M
Johnson, Edward M
et al.

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ORIGINAL ARTICLE

Expression of the Mannose Receptor CD206 in HIV and SIV Encephalitis: A Phenotypic Switch of Brain Perivascular Macrophages with Virus Infection

Gerard E. Holder · Christopher M. McGary · Edward M. Johnson · Rubo Zheng · Vijay T. John · Chie Sugimoto · Marcelo J. Kuroda · Woong-Ki Kim

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Abstract We examined the expression of the mannose receptor CD206 by perivascular macrophages (PVM) in normal human and monkey brains and in brains of HIV-infected humans and of monkeys infected with simian immunodeficiency virus (SIV). Depletion of brain PVM in SIV-infected monkeys by intrathecal injection of liposome-encapsulated bisphosphonates eliminated CD206-expressing cells in the brain, confirming their perivascular location and phagocytic capacity. In vivo labeling with bromodeoxyuridine in normal uninfected and SIV-infected macaques in combination with CD206 immunostaining revealed a CD206+-to-CD206– shift within pre-existing PVM during SIV brain infection and neuroinflammation. These findings identify CD206 as a unique marker of human and macaque PVM, and underscore the utility of this marker in studying the origin, turnover and functions of these cells in AIDS.

Gerard E. Holder and Christopher M. McGary contributed equally to the work.

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G. E. Holder · C. M. McGary · E. M. Johnson · W.-K. Kim (✉)
Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, 700 W Olney Road, Lewis Hall 3174, Norfolk, VA 23507, USA
e-mail: kimw@evms.edu

R. Zheng · V. T. John
Department of Chemical and Biomedical Engineering, Tulane University, New Orleans, LA 70118, USA

C. Sugimoto · M. J. Kuroda
Division of Immunology, Tulane National Primate Research Center, Covington, LA 70433, USA

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Introduction

The normal healthy central nervous system (CNS) contains different populations of macrophages. These myeloid cells are distinguished mainly based on their anatomic locations in the brain, including the choroid plexus, the meninges, and the perivascular spaces as well as the brain parenchyma. Perivascular macrophages (PVM), as the term suggests, are located in the perivascular (Virchow-Robin) spaces surrounding CNS blood vessels (Kim et al. 2005). The brain perivascular compartment is an extension of the subpial space, is in indirect communication with the cerebrospinal fluid (CSF) in the subarachnoid space, and is increasingly recognized for its role for leukocyte trafficking into the brain (Ransohoff and Engelhardt 2012). Due to their location within the perivascular spaces, PVM are likely the first cells encountered in the brain by infiltrating leukocytes and invading pathogens such as HIV.

HIV encephalitis (HIVE), the pathological correlate of HIV-associated dementia, is characterized by a perivascular accumulation of macrophages and multinucleated giant cells (MNGC) in the brain. Difficulties in investigating pathogenesis and lesion formation in HIVE and its animal model, simian immunodeficiency virus encephalitis (SIVE), have been associated with lack of a selective marker to differentiate PVM from infiltrating monocytes and parenchymal microglia. Previously, we and others showed that CD163, a macrophage scavenger receptor, can be used to identify human and monkey PVM (Roberts et al. 2004; Kim et al. 2006; Borda et al. 2008; Fischer-Smith et al. 2008; Soulas et al. 2009). However,

virtually all recruited monocytes/macrophages within encephalitic lesions and, occasionally, microglia directly next to the lesions express CD163, limiting its utility.

CD206, also known as the macrophage mannose receptor (MMR) or as the mannose receptor C, type 1 (MRC1), is a member of the C-type lectin receptor family. Recognition by macrophages of mannosylated carbohydrates of various pathogens including HIV is mediated via CD206 and results in their binding and endocytosis (Nguyen and Hildreth 2003; Trujillo et al. 2007). This endocytic pattern-recognition receptor is expressed by selected macrophages and dendritic cells (Linehan et al. 1999). Unlike other macrophage markers such as CD11b, CD14, CD68, and CD163, it is not expressed by monocytes. The early immunohistochemistry studies on CD206 expression in murine tissues demonstrated that CD206 identifies PVM in the brain and is not present on other brain cell types (Linehan et al. 1999; Galea et al. 2005). Dijkstra and colleagues showed that CD206 expression in frozen brain tissues of multiple sclerosis patients is restricted to PVM (Fabriek et al. 2005; Vogel et al. 2013). The human tissue distribution of CD206 has not been investigated in detail, in part, due to unavailability of CD206 antibodies that are effective on formalin-fixed, paraffin-embedded human tissues. Recently, we immunohistochemically tested new anti-human CD206 antibodies and found some of them to be effective on paraffin-embedded human and nonhuman primate tissues.

In the present study, we examined CD206/MMR expression, using these newly available antibodies, in normal primate brains and in HIV- and SIV-infected primate brains with or without encephalitis. We show that CD206 is a selective marker for PVM in the normal CNS, and that when found, SIV-infected $CD206^+$ macrophages are found always around the vessel located at the center of small SIVE lesions. Targeting the brain PVM in monkeys through intrathecal injection of liposome-encapsulated bisphosphonates resulted in a complete depletion of the cells expressing CD206 from the perivascular space in the CNS, further confirming the perivascular location and phagocytic capacity of the $CD206^+$ cells. Using in vivo thymidine analog labeling to examine the "fate" of $CD206^+$ PVM, we demonstrate that $CD206^+$ PVM shift to a $CD206^-$ subtype in the CNS of infected rhesus monkeys. The MMR CD206 is a unique marker to *phenotypically* differentiate PVM from parenchymal microglia, which will be particularly critical to study the origin, turnover, and longevity of PVM.

Materials and Methods

Tissue Samples

Human Brain Formalin-fixed, paraffin-embedded sections of frontal, temporal and occipital cortices and cerebellum were obtained from the Manhattan HIV Brain Bank (1R24MH59724; New York, NY). A total of 4 HIVE cases, 3 HIV-1-positive cases without encephalitis, and 3 seronegative controls were examined (supplementary Table S1).

Rhesus Macaque Brain Necropsy brain tissues including frontal, parietal and temporal cortices and brainstem from 17 adult, male rhesus macaques (*Macaca mulatta*) were used in the present study. These archived specimens were from previous studies involving in vivo labeling with thymidine analogs: four uninfected control monkeys and 13 animals intravenously infected with SIVmac251 virus (20 ng of SIV p27). Of the 13 infected animals, six animals that developed SIVE were included. Evidence of SIVE was defined by the presence of SIV proteins in the brain and the accumulation of macrophages and MNGC. Brain regions were collected in zinc formalin, embedded in paraffin, and sectioned at 5 μm.

Immunohistochemistry

Immunohistochemistry was performed using the antibodies listed in Table 1, as previously described (Kim et al. 2006). Deparaffinized and rehydrated sections were pretreated for antigen retrieval with a citrate-based Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA) in a microwave (800 W) for 20 min. After cooling, the sections were washed with Tris-buffered saline (TBS) containing 0.05 % Tween-20 for 5 min, and incubated with Peroxidase Blocking Reagent (Dako, Carpinteria, CA) for 5 min and then with 5 % normal horse or goat serum in TBS for 15 and 30 min, respectively. Primary antibody incubation was done for 1 h at room temperature or overnight at 4 °C. After washing, the sections were incubated for 30 min with a biotinylated secondary antibody (Vector Laboratories), washed, incubated for 30 min with an avidin-biotin peroxidase complex (Vectastain ABC Elite kit: Vector Laboratories), and developed with diaminobenzidine (DAB; Dako). All primary and secondary antibodies for immunohistochemistry were diluted in Dako Antibody Diluent. Negative controls consisted of omission of the primary antibody or its replacement with an isotype-matched immunoglobulin. To confirm the perivascular location of CD206-positive cells, double-label immunohistochemistry was performed using both Vectastain Elite ABC and ABC-alkaline phosphatase kits, according to the manufacturer's instructions. For this, CD206-stained sections were further stained for glucose transporter 1 (Glut1), a marker of endothelial cells. The color reaction product was developed using Permanent Red (Dako) for Glut1. The sections were visualized using the Nikon Coolscope digital microscope.

Flow Cytometry

Flow cytometry was performed using the antibodies listed in Table 1, as previously described (Kim et al. 2010). Whole

Table 1 Antibodies used in the present study

Antigen	Clone	Isotype	Reactivity	Manufacturer	Application
BrdU	MoBU-1	Mouse IgG1	n/a	Molecular Probes	IHC, IF, FC
CD11b	ICRF44	Mouse IgG1	Hu, Mk	BioLegend	FC
CD14	M5E2	Mouse IgG2a	Hu, Mk	BioLegend	FC
CD16	2H7	Mouse IgG2a	Hu, Mk	Novocastra	IHC, IF
CD16	3G8	Mouse IgG1	Hu, Mk	BioLegend	FC
CD68	KP1	Mouse IgG1	Hu, Mk	NeoMarkers	IHC, IF
CD163	EDHu-1	Mouse IgG1	Hu, Mk	Serotec	IHC, IF, FC
CD163	EPR4521	Rabbit IgG1	Hu, Mk	Epitomics	IHC, IF
CD163	GHI/61	Mouse IgG1	Hu, Mk	BioLegend	FC
CD206	5C11	Mouse IgG1	Hu, Mk	Abnova	IHC, IF
CD206	C-10	Mouse IgG2a	Hu, Mk	Santa Cruz	IHC, IF
CD206	Polyclonal	Rabbit IgG	Hu, Mk	Sigma-Aldrich	IHC, IF
CD206	Polyclonal	Rabbit IgG	Hu, Mk, Rt	Abcam	IHC, IF
CD206	15–2	Mouse IgG1	Hu, Mk	BioLegend	FC
CD206	19.2	Mouse IgG1	Hu, Mk	BD Pharmingen	FC
Glut1	Polyclonal	Rabbit IgG	Hu, Mk, Rt	NeoMarkers	IHC, IF
HLA-DR	L243	Mouse IgG2a	Hu, Mk	BioLegend	FC
IBA1	Polyclonal	Goat IgG	Hu, Mk, Rt,	Abcam	IHC, IF
SIV p28	3F7	Mouse IgG1	n/a	Trinity Biotech	IHC, IF

n/a, not applicable; Hu, human; Mk, monkey; Rt, rat; IHC, immunohistochemistry; IF, immunofluorescence; FC, flow cytometry

blood and single-cell suspensions from spleens of infected animals were stained, and analyzed for HLA-$DR^+CD11b^+CD14^+CD163^+$ monocytes/macrophages co-expressing CD206 using FlowJo software (Tree Star, Ashland, OR).

Immunofluorescence Microscopy

Double- or triple-label immunofluorescence microscopy was used to further phenotype $CD206^+$ cells in brain, to examine whether $CD206^+$ cells were positive for SIV p28, to determine whether nuclei of $CD206^+$ cells were labeled by thymidine analogs injected at different time points, and to determine whether PVM depletion by liposomal bisphosphonates eliminated $CD206^+$ cells in the brain. Sections were deparaffinized, rehydrated and pretreated as described above. The sections were washed with phosphate-buffered saline (PBS) containing 0.2 % fish skin gelatin (FSG) twice for 5 min and permeabilized with 0.1 % Triton X-100 in PBS/FSG for 1 h. The sections were incubated with 5 % normal goat serum in PBS for 30 min at room temperature before incubation for 1 h at room temperature or overnight at 4 °C with primary antibodies diluted in PBS/FSG. After primary antibody incubation, the sections were washed in PBS/FSG and incubated with an Alexa Fluor 488-, 594-, or 350-conjugated secondary antibody (Molecular Probes, Eugene, OR; diluted at 1:1000 in PBS/FSG) for 1 h at room temperature. The sections were washed with PBS/FSG before the addition of the next primary antibody. After immunofluorescence staining, the sections were treated with 10 mM $CuSO_4$ in 50 mM ammonium acetate buffer for 45 min to quench autofluorescence. The sections were rinsed in distilled water, and cover slipped with Aqua-Mount aqueous mounting medium (Thermo Scientific). A Zeiss observer.Z1 fluorescence microscope was used to analyze fluorescent labeled sections. Zeiss AxioVision Release 4.8.2 was used to capture and merge fluorescence images. Adobe Photoshop CS12.1 was also used to merge layers into a single image. The size of the lesions was determined using a computer-assisted image processing and analysis software (ImageJ, NIH). For every lesion, the area was calculated and the number of $CD206^+$ cells in each lesion was counted. A total of 56 lesions were included in the analysis.

PVM Depletion by Intracisternal Liposomal Bisphosphonates

Liposome-encapsulated bisphosphonates including clodronate injected into the lateral cerebral ventricle were able to selectively deplete PVM in mice, rat, and rabbits (Trostdorf et al. 1999; Polfliet et al. 2001, 2002; Galea et al. 2005; Hawkes and McLaurin 2009; Steel et al. 2010). We used the same approach to rhesus monkeys to deplete PVM, using a more potent bisphosphonate, alendronate. Alendronate liposomes were prepared using the lipid film hydration method using the method described by Epstein and colleagues (Epstein et al. 2008). 1,2-dipalmitoyl-sn-glycero-3-

phosphocholine (DPPC) (Avanti Polar Lipids), 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DMPG) (Avanti Polar Lipids) and cholesterol (Sigma-Aldrich), at a molar ratio of 3:1:2, were dissolved in a 2:1 (*v/v*) chloroform-methanol mixture. The solution was completely dried on a rotary evaporator, and the dried film was hydrated with 1X PBS containing sodium alendronate at 50 °C for 1 h to obtain a liposome suspension. The liposome suspension was subsequently extruded through a series of 400- and 200-nm pore size polycarbonate membranes (Whatman) at 65 °C to downsize the liposomes (11 times extrusion through each). The obtained liposomes were purified on a Sephadex G-25 column (GE Healthcare Life Sciences) and eluted with 1X PBS buffer pH 7.4 to remove free alendronate. The liposomes were suspended at a concentration of 24 mM total lipids in 1X PBS. The final alendronate concentration in the liposomal solution is approximately 5.1 mg/ml, which was measured by UV-vis spectrophotometry at a wavelength of 300 nm (Kuljanin et al. 2002). The value varies a little for different batches of alendronate liposomes. Drug-free liposomes were prepared by the same procedure, excluding the alendronate. Three SIV-infected macaques received an intracisternal injection of liposomal alendronate, and were euthanized either 2 days or 6 h later. Two uninfected macaques received intracisternal liposomes 24 h before euthanasia.

In vivo BrdU/EdU Labeling and Detection

Thymidine analogs, such as 5-bromo-2′deoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) and 5-ethynyl-2′-deoxyuridine (EdU; Molecular Probes, Eugene, OR), can be used to pulse-label cells undergoing DNA synthesis including dividing monocyte precursors in the bone marrow and proliferating macrophages in inflamed tissues (Jongstra-Bilen et al. 2006; Zhu et al. 2009; Robbins et al. 2013; Amano et al. 2014). We examined a total of ten (4 normal uninfected and 6 infected) rhesus macaques: uninfected animals received a single intravenous (i.v.) infusion of BrdU (60 mg/kg), 4 days (n=2) or 2 days (n=2) prior to euthanasia; two infected animals received BrdU either at 8 days post-infection (dpi) or at 47 dpi, and were euthanized at 49 dpi. Four additional SIV-infected rhesus macaques received a single i.v. infusion of EdU (50 mg/kg) and were euthanized 20 to 51 days after EdU injections.

We used flow cytometry to investigate BrdU/EdU labeling of bone marrow and blood monocyte subsets in monkeys (data not shown). BrdU/EdU labeling in tissues including brain was assessed by immunofluorescence on paraffin sections. After pretreatment, permeabilization, and washing with PBS/FSG, paraffin sections were then incubated with an anti-BrdU monoclonal antibody that did not cross-react with 5-ethynyl-2′-deoxyuridine. To detect EdU, the Click-it EdU Alexa Fluor 488 Imaging kit (Molecular Probes) was used according to the manufacturer's instructions. Briefly, paraffin-embedded sections were pretreated, washed and permeabilized as described above. The reagents in the Click-it EdU Alexa Fluor 488 Imaging kit (175 μl of reaction buffer, 4 μl of $CuSO_4$, 1 μl of azide, and 20 μl of buffer additive required per test) were prepared in the order as listed and mixed, and this reaction cocktail was used within 15 min. The sections were then incubated with EdU cocktail for 1 h at room temperature. Quenching of lipofuscin-like autofluorescence was done with $CuSO_4$ treatment as described above. The sections were then washed in PBS/FSG and mounted with Aqua-Mount aqueous mounting solution (Thermo Scientific). For multi-label immunofluorescence, sections were washed and blocked with 5 % normal goat serum in TBS for 30 min at room temperature after BrdU/EdU staining.

Statistical Analysis

The Spearman correlation coefficient was calculated to determine relationship between lesion size and the number of $CD206^+$ cells in the lesion. A two-tailed P-value was presented.

Results

CD206 Expression is Limited to PVM in HIVE, SIVE, and Control Brains

Previously, Dijkstra and colleagues showed that the mannose receptor CD206 was selectively detected on PVM in both control and multiple sclerosis brains, using a monoclonal antibody that is effective only on fresh frozen tissues (Fabriek et al. 2005; Vogel et al. 2013). Fresh frozen tissues from human pathological specimens are often unavailable, which limits the utility of this antibody. Therefore, we tested by immunohistochemistry six commercially available antibodies to human CD206 and identified 4 of them that work on formalin-fixed, paraffin-embedded human and monkey tissues. Using these newly characterized antibodies, we first sought to determine whether CD206 selectively identifies PVM in normal human and monkey brains, without labeling other brain cell types. In normal brains, CD206 expression was observed in cells reminiscent of PVM as well as in macrophages of the leptomeninges and choroid plexus. No CD206 immunoreactivity was detected in the brain parenchyma (Fig. 1a, b). Both monoclonal and polyclonal antibodies against CD206 presented consistently similar staining patterns and revealed a largely uniform morphology. Consistent with the morphology of PVM, the stained cells were elongated, flattened, and found only directly adjacent to CNS vasculature.

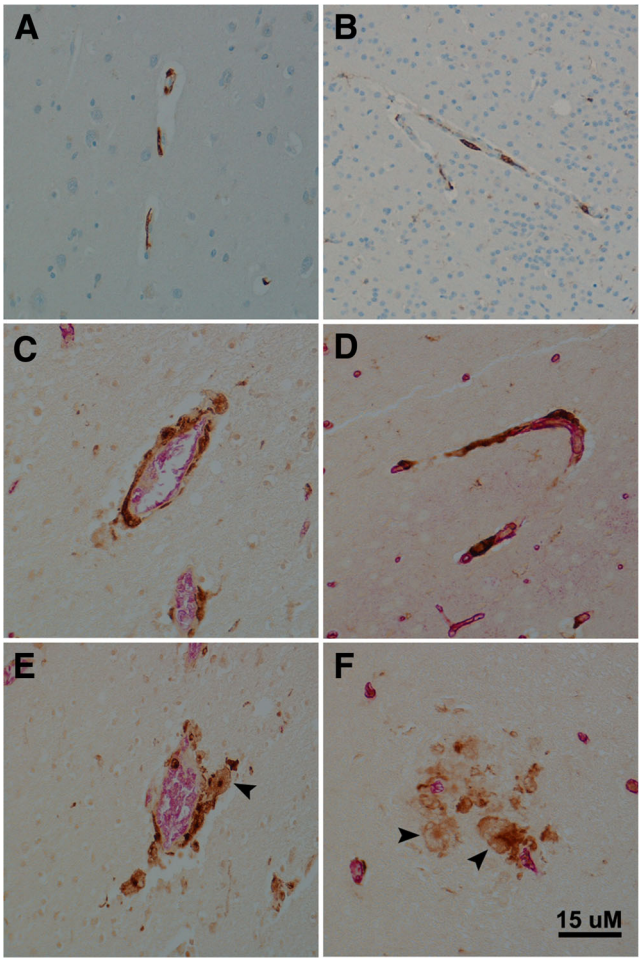

Fig. 1 CD206 expression in encephalitic brains of HIV-infected patients and SIV-infected rhesus macaques. Immunohistochemistry studies of human brains (**a**, **c**, and **e**) and monkey brains (**b**, **d**, and **f**) demonstrate macrophages that are CD206-positive next to CNS vessels. CD206 immunoreactivity (DAB; *brown*) was found in brain PVM in normal uninfected brains (**a** and **b**) and HIVE (**c**) and SIVE (**d**) brains. $CD206^+$ cells were intimately associated with $Glut1^+$ CNS vessels (Permanent Red; *red*; **c** and **d**). Anti-CD206 antibodies also weakly stained very few foamy macrophages and MNGC (*arrowheads*) in HIVE (**e**) and SIVE (**f**) lesions. Data are representative of immunohistochemistry staining on two or three different brain regions each from seven animals (n=4 with SIVE and n=4 uninfected) as well as six human subjects (n=4 HIVE and n=2 uninfected)

We then investigated CD206 expression in the brains of patients and animals with terminal AIDS. In patients and animals without histological evidence of encephalitis, the expression of CD206 was the same as for normal uninfected controls (data not shown). No difference in levels of CD206 expression or number of CD206-reactive cells was found in non-encephalitic subjects compared with uninfected controls. In the brains of HIV-infected humans with HIVE and SIV-infected macaques with SIVE, increased numbers of $CD206^+$ cells were present, corresponding to multiple perivascular cuffs (Fig. 1c, d). CD206 immunoreactivity remained restricted to cells tightly associated with CNS vessels, which include MNGC that exhibited weak cytoplasmic staining (Fig. 1e, f). To confirm localization of CD206 to the PVM in human and monkey brains, we performed double-label immunohistochemistry with anti-CD206 and anti-Glut1 (a marker of endothelial cells). Virtually, all $CD206^+$ cells, while morphologically diverse, are found intimately associated with CNS vessels (Fig. 1c, d). We further investigated if $CD206^+$ cells were found in encephalitic lesions. Round foam cells with weak CD206 immunoreactivity were occasionally found in smaller lesions, and more so in SIVE than HIVE (Fig. 1e, f). Again, there was no indication that CD206 is present on other brain cell types including $Iba1^+$ microglia and $GFAP^+$ astrocytes (Fig. S1).

$CD206^+$ Perivascular Macrophages Co-express CD16, CD68 and CD163

We and others previously showed that PVM exhibit a $CD14^+CD16^+CD163^+$ phenotype similar to the subset of blood monocytes, which expands in response to HIV/SIV infection (Kim et al. 2006). However, when examined by flow cytometry, CD206, a selective marker for PVM, was not expressed on human or rhesus monkey monocytes (Fig. 2a) while it was expressed by a subset of spleen macrophages (Fig. 2b). Therefore, we sought to determine if $CD206^+$ PVM express the same $CD16^+CD163^+$ macrophage phenotype that was identified in inflammatory perivascular cuffs and encephalitic lesions. Multi-label immunofluorescence for CD206 with CD16, CD68 or CD163 and Glut1 in uninfected control brains showed colocalization, which demonstrates co-expression of these macrophage/monocyte markers on $CD206^+$ cells in the perivascular spaces (Fig. 3). In SIV-infected animals with or without encephalitis, CD206 expression remained colocalized with these markers. However, unlike uninfected controls, the perivascular spaces in the brains of infected animals harbored a subset of PVM that did not overlap with CD206 ($CD206^-$). A majority of macrophages in the perivascular cuffs and nodular lesions did not express CD206. Interestingly, down-regulation of CD206 was previously observed in macrophages exposed to HIV (Koziel et al. 1998; Caldwell et al. 2000; Vigerust et al. 2005) and correlated with their increased migration (Swain et al. 2003). Although the biological significance of these $CD206^-$ PVM is unknown, it is tempting to speculate that these cells may be derived from $CD206^+$ PVM and involved in lesion growth.

$CD206^+$ PVM in SIV Lesions Inversely Correlate with Lesion Size

Since PVM are major targets of HIV/SIV infection in the brain, we sought to determine whether the $CD206^+$ subtype of PVM are productively infected by the virus. For this, triple-label immunofluorescence was performed on brain tissues from SIVE macaques to detect the SIV Gag protein p28 in

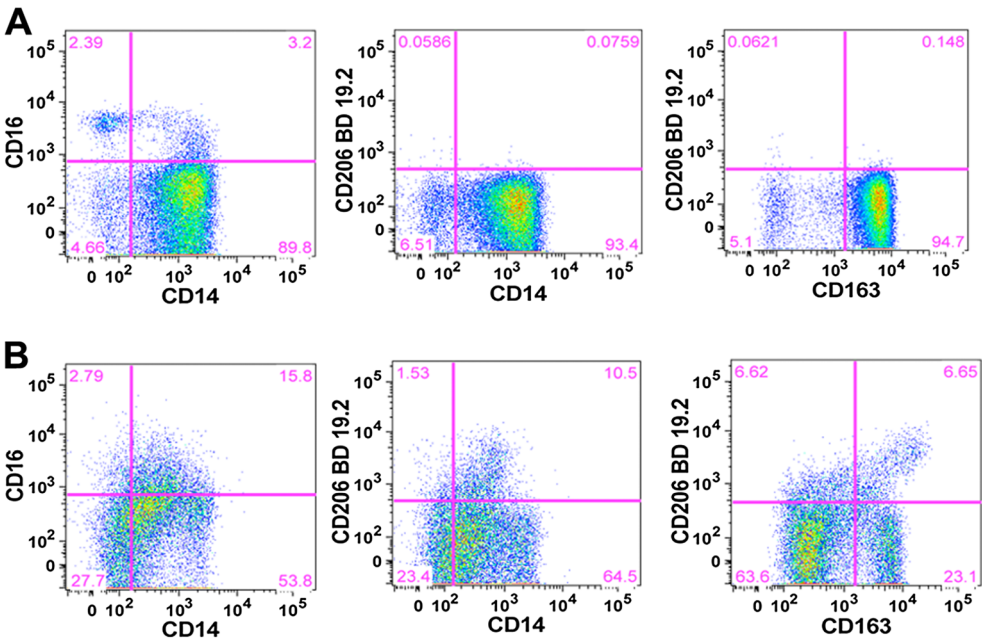

Fig. 2 CD206 expression on blood monocytes and splenic macrophages in SIV-infected rhesus macaques. Flow cytometric analysis of whole blood (**a**) and splenocytes (**b**) was performed to examine CD206 expression in the blood and spleen. Monocyte populations are gated by co-expression of HLA-DR and CD11b. One representative of four experiments is shown. **a**: In whole blood, there is no CD206 staining on both $CD14^+$ and $CD163^+$ monocytes. **b**: Spleen was used to show that $CD14^+$ and $CD163^+$ splenic macrophages express CD206

$CD206^+$ cells located around $Glut1^+$ CNS vessels (Fig. 4a). SIV p28 could only be demonstrated at low frequency in these $CD206^+$ cells which were located in perivascular cuffs and in small lesions (Fig. 4a, b and c). In contrast, the SIV p28 protein was expressed more abundantly and more frequently in CD206-negative lesional foamy macrophages and a significant majority of $CD206^-$ PVM. These results suggest *either* that $CD206^+$ PVM are less susceptible to virus infection than $CD163^+CD206^-$ PVM *or* that $CD163^+CD206^+$ PVM differentiate in the perivascular cuff of a growing lesion into $CD163^+CD206^-$ cells that are more productively infected by the virus. Interestingly, a highly significant negative

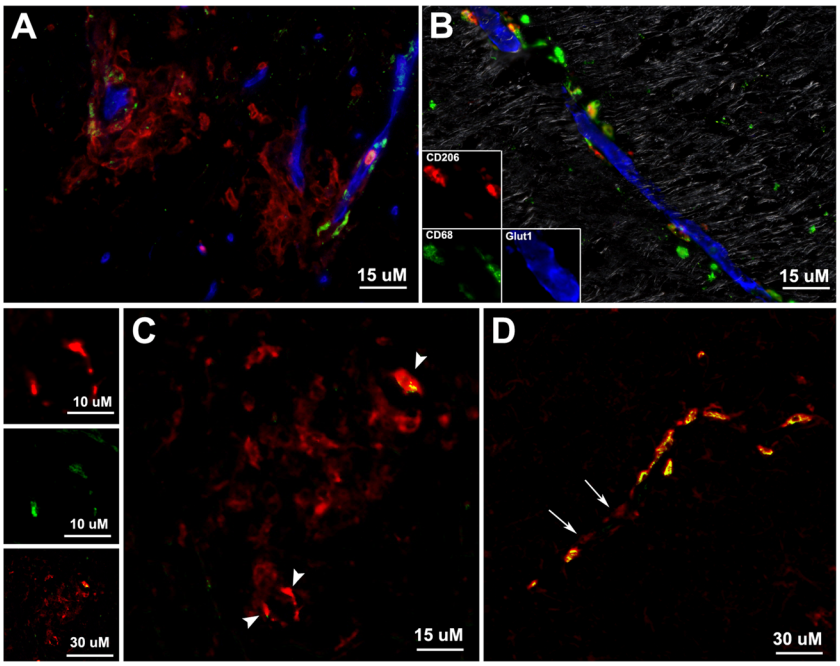

Fig. 3 Co-expression of CD16, CD68 and CD163 in $CD206^+$ PVM in the CNS of macaques infected by SIV. Multi-label immunofluorescence studies of SIVE brains demonstrate $CD206^+$ PVM that are positive for CD16 (**a**), CD68 (**b**), and CD163 (**c** and **d**). **a**: Colocalization of CD206 (Alexa Fluor 488; *green*) with CD16 (Alexa Fluor 594; *red*) restricted to $Glut1^+$ (Alexa Fluor 350; *blue*) vessels at the center of small SIVE lesions. **b**: Colocalization of CD206 (*red*) with CD68 (*green*) along CNS vessels (*blue*). Note that not all $CD68^+$ PVM expressed CD206. **c**,**d**: Double staining with anti-CD206 (*green*) and anti-CD163 (*red*) revealed double-labeled (*yellow*; *arrowheads*) cells within an SIVE lesion (**c**) and along a CNS vessel (**d**). Note that not all $CD163^+$ cells are positive for CD206 (*arrows*). Data shown are representative of frontal and parietal cortices from three animals with SIVE

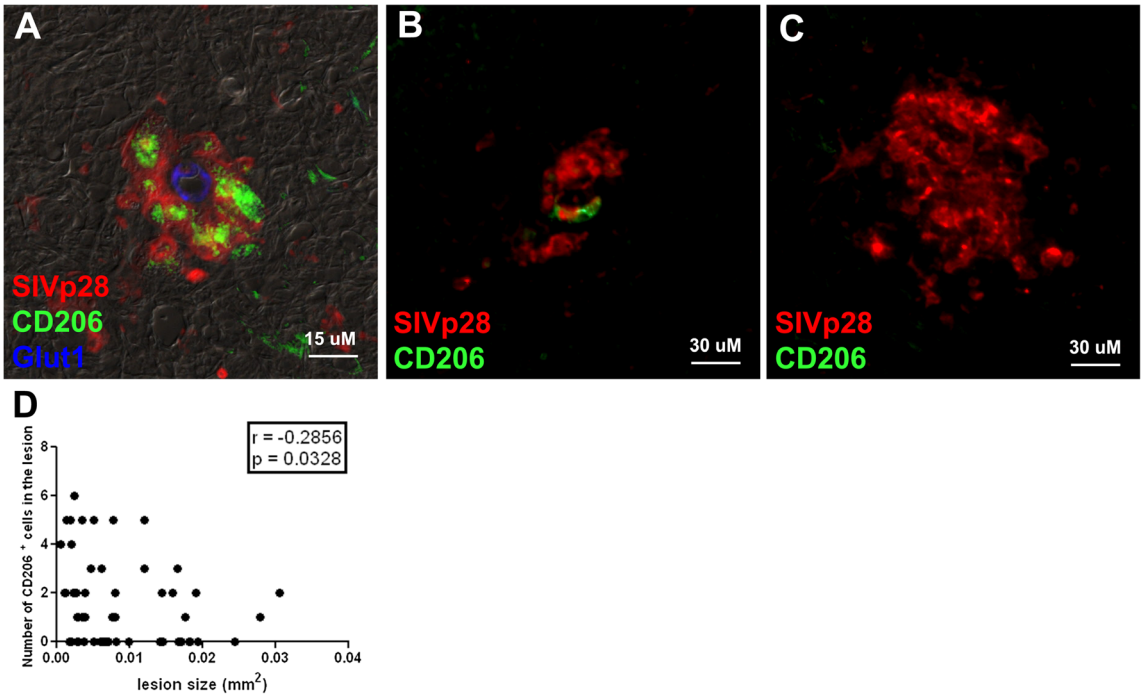

Fig. 4 SIV infection of CD206$^+$ PVM and correlation with encephalitis lesion size. Multi-label immunofluorescence with CD206 (*green*), SIV Gag p28 (*red*), and Glut1 (*blue*) show a few of CD206$^+$ PVM are productively infected, exclusively located in the perivascular cuff of small SIVE lesions. **a**–**c** show increasing lesion areas with fewer CD206$^+$ PVM. **d** illustrates a significant negative correlation between lesion area and the number of CD206$^+$ cells in the lesion ($P<0.05$). A total of 56 lesions in frontal or parietal cortices from 4 animals with SIVE were included in the analysis

correlation was observed between SIVE lesion size and the number of CD206$^+$ cells in the lesion (Fig. 4d). We found fewer CD206$^+$ cells as lesion area increased; yet CD68$^+$, CD163$^+$ or Iba1$^+$ cells continued to increase with encephalitic lesion development (Fig. S2). CD206$^+$ cells were consistently located in close proximity to CNS vessels, lending the idea that this CD206$^+$ subtype is lost in mature lesions.

CD206$^+$ PVM are Selectively Depleted by Intrathecal Liposomal Bisphosphonates

We and others have successfully targeted rodent PVM to demonstrate the roles of PVM in brain inflammation and infection using liposome-encapsulated clodronate (Polfliet et al. 2001, 2002; Audoy-Remus et al. 2008; Hawkes and McLaurin 2009; Serrats et al. 2010; Steel et al. 2010). Bisphosphonates including clodronate and alendronate have been extensively used in small animal models to effectively deplete monocytes and macrophages in vivo when administered in liposomal form (Huitinga et al. 1992; Danenberg et al. 2003; Sunderkotter et al. 2004). Our preliminary study with clodronate and alendronate indicates that in a liposome encapsulated form these two bisphosphonate compounds are effective in rhesus macaques to deplete CD14$^+$ monocytes when injected intravenously. We, therefore, injected bisphosphonate-encapsulated liposomes intracisternally in two uninfected and three chronically SIV-infected macaques to explore the potential utility of liposomal alendronate in depleting PVM and to confirm the identity of CD206$^+$ cells as PVM. The animals were euthanized 6 h, 24 h or 2 days later. PVM depletion was partial (~50 %) in the animals that were euthanized 24 h later, but a near-complete depletion of PVM was achieved 2 days post liposome injection. In the animal euthanized 2 days post liposome injection, virtually no positive staining for CD206 or CD163 was detected in the perivascular space (Fig. 5b, g). This directly demonstrated that the cells expressing CD206 and CD163 in the brain were capable of internalizing liposomal alendronate and located in the perivascular space. The meninges were not completely devoid of CD206 immunoreactivity. Choroid plexus macrophages were least affected by liposomal alendronate treatment. This is likely due to subarachnoid CSF flow, impeding the access of intracisternally injected liposomes to choroid plexus macrophages. Unlike CD206$^+$ or CD163$^+$ cells, CD206$^-$Iba1$^+$ parenchymal microglia were not depleted by intracisternal liposomal treatment (Fig. 5d).

Phenotypic Switching of CD206$^+$ PVM in the CNS of SIV-infected Macaques

To estimate the "birth date" of CD206$^+$ PVM, we used in vivo pulse-labeling with BrdU, which was injected at different time points. Normal uninfected animals received a single i.v. injection of BrdU either 4 days or 2 days prior to euthanasia. In

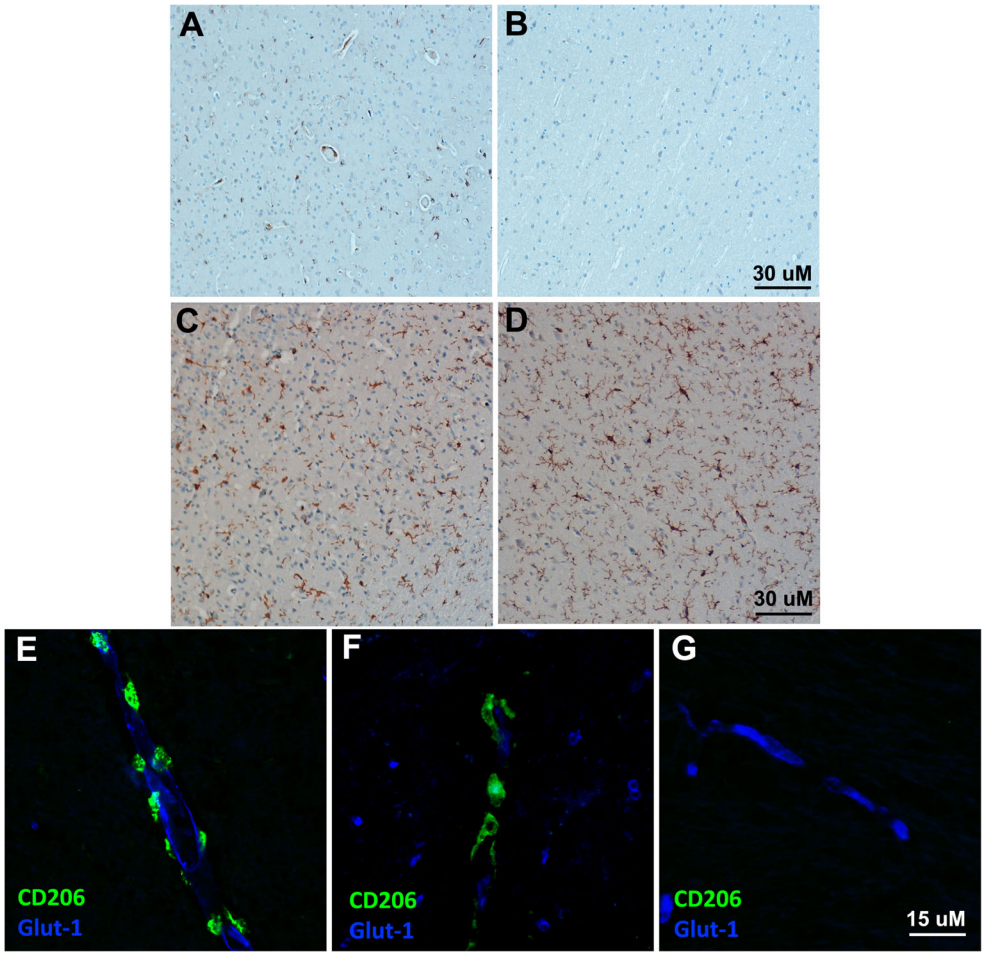

Fig. 5 Selective depletion of CD206$^+$ PVM by liposome-encapsulated alendronate injected into the CSF of SIV-infected monkeys. Chronically SIV-infected macaques received liposomal alendronate intracisternally and were euthanized 6 h or 2 days later. A near-complete depletion of PVM was achieved as early as 2 days post-treatment. **a**,**b**: More than 10 brain areas were stained for CD163 (DAB; *brown*), a marker for PVM. The control brain tissues from untreated SIV-infected macaques showed CD163 immunoreactivity exclusively around CNS vessels (**a**). There was a lack of CD163 immunoreactivity in the parietal cortex of an alendronate-treated animal (**b**). Perivascular spaces were completely devoid of PVM. **c**,**d**: The same areas used for CD163 staining were stained for Iba1 (DAB; *brown*), a marker for activated microglia. Brain tissues from PVM-depleted macaques (**d**) and controls (**c**) demonstrate that Iba1+ microglia were not depleted. **e**,**f**,**g**: Double-label immunofluorescence was used to confirm successful depletion of CD206$^+$ PVM. **e** and **f** show CD206$^+$ PVM (*green*) in untreated normal uninfected and chronically SIV-infected animals, respectively. **g** shows the loss of CD206$^+$ PVM after depletion

these animals, a few (less than 20 labeled nuclei per section) cells which stained positive for BrdU were exclusively found in close association with blood vessels in the brain. Virtually all of the BrdU-labeled cells in the perivascular space were positive for CD206 (Fig. 6a). One SIV-infected animal received a single i.v. injection of BrdU at 47 dpi, 2 days prior to euthanasia (49 dpi). Once again, the majority of BrdU-labeled cells in this animal were found perivascularly and were positive for CD206 (Fig. 6b). However, in an SIV-infected animals injected with BrdU at 8 dpi, 41 days prior to euthanasia (49 dpi), a majority of BrdU-labeled cells at 41 days after BrdU injection were negative for CD206 (Fig. 6c) while they were positive for CD163 (Fig. 6d). We further examined the phenotype of thymidine analog-labeled PVM in the brains of four additional SIV-infected animals that were euthanized between 20 and 51 days after thymidine analog injection. Thymidine analog-labeled PVM were also largely CD163$^+$CD206$^-$ (data not shown). This observation suggest that thymidine analog-labeled, pre-existing CD163$^+$CD206$^+$ cells differentiated into CD163$^+$CD206$^-$ cells in the brain 20–51 days after thymidine analog injection during SIV infection. Taken together, these results show that by using animals at various time points after BrdU injection, one can establish a detailed time course of the phenotypic and functional changes of PVM induced by SIV infection in the brain.

Discussion

In this study, we validated the mannose receptor CD206 as a selective marker for PVM in the CNS of normal uninfected

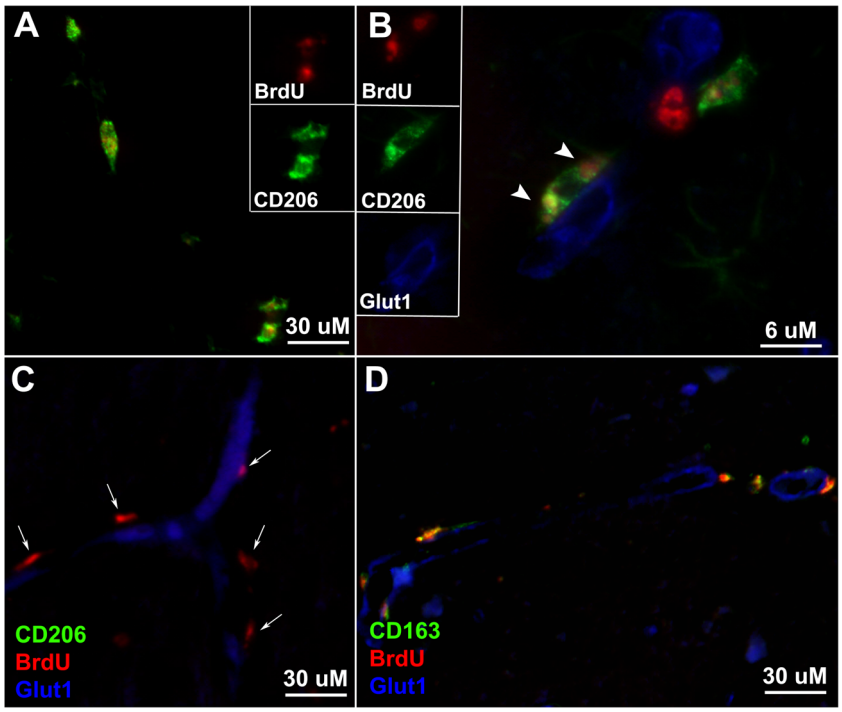

Fig. 6 Phenotypic switching of pre-existing resident CD206$^+$ PVM in SIV infection. **a** and **B** show BrdU-labeled CD206$^+$ PVM in animals euthanized 2 days post BrdU injection. In normal uninfected (**a**) and chronically SIV-infected (**b**) animals, virtually all BrdU-labeled cells in the perivascular space were CD206$^+$ PVM. **c** and **d** show an animal receiving a single BrdU injection 41 days prior to euthanasia. The vast majority of BrdU-labeled cells were negative for CD206 (**c**; *arrows*), while most were CD163-positive (**d**). Note that BrdU-labeled "doublets" (mitotic figures) were occasionally observed in the perivascular cuffs of the CNS of SIV-infected animals. In **b**, a single CD206$^+$ cell appears to have two BrdU-labeled, dividing (dumbbell-shaped) nuclei (*arrowheads*)

and HIV- or SIV-infected subjects. CD206 exclusively identified macrophages localized in close association with CNS vessels without labeling surrounding cells. Such utility was not yet been demonstrated by any existing markers. We utilized the SIV macaque model to demonstrate the utility of this marker during disease progression and contemplate the response of this population of macrophages to HIV infection. We further verified the perivascular location and phagocytic activity of CD206-expressing cells in the brain by injecting liposomal bisphosphonates intracisternally to deplete PVM. To our knowledge, this is the first demonstration to date of macrophage depletion by liposomal bisphosphonates in primates. This is a significant first step in eliminating SIV-infected PVM in the brain and in the future developing intrathecal liposomal anti-HIV drugs for the treatment of HIV CNS infection.

Previously, we showed that CD163 can be used to identify human and monkey PVM in normal and encephalitic CNS tissues (Kim et al. 2006). However, monocytes within the lumen of CNS vessels, inflammatory macrophages within encephalitic lesions and microglia in the surrounding parenchyma also express CD163. Unlike CD163, CD206 remained restricted to the ablumenal side of CNS vessels, indicating CD206 is specific for a large subset of PVM. Thus far, CD206 is a more stringent PVM-specific marker that is significant for assessing their contribution to disease.

In this study, we also describe two phenotypically distinguishable PVM subpopulations, CD206$^+$CD163$^+$ and CD206$^-$CD163$^+$ in the brains of SIV-infected macaques. We and others have demonstrated either protective or pathogenic functions of PVM in brain inflammation by depleting rodent PVM using clodronate liposomes (Polfliet et al. 2001, 2002; Audoy-Remus et al. 2008; Hawkes and McLaurin 2009; Serrats et al. 2010; Steel et al. 2010). This may reflect functional plasticity or heterogeneity of PVM. In fact, several recent studies, including ours, have shown heterogeneity within this population in rhesus macaques and humans (Soulas et al. 2009, 2011). In future studies, it would be valuable to add more markers that reveal changes in the phenotypes of the PVM, e.g. dendritic cell markers and macrophage activation markers. The current study strongly points toward a shift within pre-existing resident CD206$^+$ PVM to the CD206$^-$ phenotype during HIV brain infection and neuroinflammation, although due to the presence of Iba1 microglial marker on lesional macrophages we cannot rule out the possibility of CD206$^-$Iba1$^+$ microglia accumulating at lesions. The functional relevance of this phenotypic switch in PVM to HIV brain infection and inflammation remains to be established. Interestingly, infection with HIV-1 or exposure to HIV-1 proteins, Tat and Nef, are known to down-regulate CD206 expression on macrophages (Koziel et al. 1998; Caldwell et al. 2000; Vigerust et al. 2005). Increased activation and migration of macrophages have also been shown to correlate with down-regulation of CD206 (Swain et al. 2003). Down-regulation of CD206 expression in HIV-infected macrophages may represent mechanisms by which HIV alters host innate immunity against HIV and opportunistic pathogens, thereby affecting HIV disease progression. It remains to be explored whether the virus-induced phenotypic switch in PVM supports or restricts HIV/SIV replication by PVM.

The field of macrophage biology has increasingly focused on identification, characterization and pathobiological significance of classically (M1) and alternatively activated (M2) macrophages, and now has also shifted into investigating an intermediate activation status of macrophages in the inflamed brain (Vogel et al. 2013). Given the reported association between CD206 expression and M2 activation, extending the current study to further address macrophage activation in the context of HIV brain infection would be interesting and potentially important.

In summary we have identified and validated the MMR CD206, as a marker that can be used to identify perivascular macrophages in normal and HIV/SIV-infected brains. This marker will be critically important for studying biological functions of PVM during development of HIV-1-associated with neurological disorders.

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Conflict of Interest The authors declare that they have no conflict of interest.

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