CD163 Identifies a Unique Population of Ramified Microglia in HIV Encephalitis (HIVE)

ELEANOR S. ROBERTS, PhD, ELEIZER MASLIAH, MD, AND HOWARD S. FOX, MD, PhD

Abstract. The idea that CNS ramified microglia are quiescent has been challenged by studies that show that microglia without the classic signs of activation can be phagocytic and appear with shorter, thicker ramifications. These semi-activated cells may constitute a form of microglia that has not been previously recognized in neuropathological conditions and may contribute to the pathology and dysfunction in these disorders. This study investigated the expression of CD163, a cell surface marker whose normal expression is restricted to monocytes/macrophages, in cases of HIV or SIV encephalitis (HIVE/SIVE), Alzheimer disease, and variant Creutzfeldt-Jakob disease. In HIVE/SIVE, in addition to reacting with CNS macrophages, CD163 antibody staining was shown to highlight ramified microglia. Such reactivity was especially notable in grey matter ramified microglia and was greater than that of another typically used marker, HLA-DR. CD163 expression was only observed in infected/affected tissue, in contrast to that shown with another microglia marker, GLUT5, which has recently been shown to identify all microglia regardless of disease state. Although activated microglia were present in the other disorders, as evidenced by strong HLA-DR expression, there was very little CD163 immunoreactivity. The activation state identified by CD163 has not been previously recognized and may have a positive or negative impact on neuronal damage shown in HIV-associated dementia.

Key Words: Alzheimer disease; CD163; HIVE; Microglia; SIVE; Variant Creutzfeldt-Jakob disease (vCJD).

INTRODUCTION

Resident central nervous system (CNS) microglia are very long lived, show little turnover, and constitute up to 20% of CNS cells (1). In their ramified resting state, where they show many processes, they have no phagocytic ability and few surface markers to distinguish them in paraffin-embedded tissue (2). By contrast, in their amoeboid activated state, where they have a more ovoid appearance with short or no processes, there is upregulation of a number of factors including MHC antigens. Activated microglia can produce immunomodulatory molecules such as cytokines and chemokines, as well as potential neurotoxins, including nitric oxide, proteases, arachidonic acid derivatives, and quinolinic acid (3, 4).

While many consider ramified microglia as quiescent, there are reports that these cells can be phagocytic, such as shown in a study where anterograde axonal degeneration was carried out with the active participation of ramified microglia (5). This form of microglia may be in an intermediate, so-called, hyper-ramified state between ramified (resting) and amoeboid (activated) states, as has been observed in normal aging and slow degenerative disorders (6, 7).

There are many markers of activated microglia, such as the antibodies HAM56, anti-HLA-DR, anti-CD68, and the lectin RCA1, all of which will also highlight vascular cells and infiltrating macrophages. However, markers of ramified microglia are harder to find, especially when dealing with formalin-fixed, paraffin-embedded tissue. Silver staining can be used to envisage the whole macrophage population in the brain, but this involves a difficult and capricious methodology (8). Recently, anti-GLUT5, a fructose transporter of undefined CNS function, has been shown by our group and others to highlight microglia in both control and disease states (9, 10). However, both silver staining and GLUT5 immunostaining can highlight all ramified microglia, regardless of their activation state; therefore, they are not of use when investigating differences between control and pathogenic situations. While HAM56 can be used to highlight ramified microglia in inflammatory conditions, it is also shown to react with endothelial cells, and thus is not useful when examining perivascular microglia/macrophages that may be important in the pathology of conditions such as human immunodeficiency virus-associated encephalitis (HIVE). In our previous study of the frontal lobes of rhesus macaques with simian immunodeficiency virus-associated encephalitis (SIVE), we found that immunostaining for the product of one of the upregulated genes, CD163, may be useful in defining neuropathology. There we demonstrated that an antibody toward CD163 highlighted ramified grey matter microglia in SIVE that were not positive for HLA-DR or HAM56. These were not found in control, non-SIV-infected tissue (9), thus CD163 may provide a useful marker for ramified microglia in a unique state of activation.

CD163 (a.k.a. M130, RM3/1, or p155) is a membrane-bound scavenger receptor detected on monocytes and most tissue macrophages (11–14). Expression of CD163 can be induced by interleukins (IL)-6 and –10, macrophage colony stimulating factor (M-CSF), and glucocorticoids (15, 16), and it is downregulated by lipopolysaccharide (LPS), interferon (IFN)-γ, and tumor necrosis...
factor (TNF-α) (15). Increased occurrence of CD163-positive (CD163+) macrophages appear in the days following acute inflammatory reactions in chronic inflammatory diseases and during wound healing (13, 14, 17). Many authors argue that CD163 expression is important in the anti-inflammatory process because cross-linking of CD163 can lead to the secretion of histamine (which inhibits TNF-α and IL1 expression (18, 19)) and an unknown anti-inflammatory agent (20, 21). In blood monocytes, activation of CD163 by hemoglobin:haptoglobin complexes leads to the secretion of the anti-inflammatory cytokine IL-10 (22). However, cross-linking of CD163 also increases the secretion of the pro-inflammatory mediators IL-1β, IL-6, and M-CSF (23), indicating a more complicated relationship to the inflammatory process. The CD163 receptor also has a role in monocyte adhesion to activated endothelium, independent of integrins, E-selectin, ICAM-1, or VCAM-1 (16, 24).

To further investigate the relationship of CD163 expression to neuropathological disorders we used tissue from individuals infected with HIV or with variant Creutzfeldt-Jakob Disease (vCJD) or Alzheimer disease (AD), along with rhesus macaques infected with simian-immunodeficiency virus (SIV). The virus-infected human and monkey cases displayed encephalitis (HIVE/SIVE) on autopsy, which is diagnosed by the appearance of infected cells of the monocyte/macrophage lineage, microglia/macrophage nodules, and multinucleated giant cells. vCJD is a non-inflammatory neurodegenerative disease characterized by astrocystosis, neuronal loss, and neuropil vacuolation with widespread upregulated expression of MHC class II on microglia, especially in cortical grey areas (25, 26). AD is not generally characterized as an inflammatory disorder because there is little to no infiltration of leukocytes (27), however, microglia activation is found from early on in the disease, which has caused many researchers to characterize it as potentially inflammatory in nature (28, 29). We also used a case of systemic sepsis with pneumonia to investigate whether a peripheral infection can lead to increased expression of CD163 on microglia in the CNS. CD163 staining was compared to HAM56, a commonly used immunostain to activated endothelium, independent of integrins, E-selectin, ICAM-1, or VCAM-1 (16, 24).

In this study we demonstrate that in the SIVE and HIVE cases, expression of CD163 was far more widespread in grey matter ramified microglia than was expression of HLA-DR. However, there was little expression of CD163 in vCJD or in most of the AD cases. Thus, CD163 differentiates a unique population of semi-activated, yet ramified, microglia, and its expression pattern is distinguishable from that of other markers of microglia activation. Further investigation will ascertain the role of these microglia identified by CD163 in HIV/SIV encephalitis.

**MATERIALS AND METHODS**

**Tissue Samples**

Frontal lobe tissue was obtained at autopsy from cases with AIDS, either with HIVE (n = 3) or without (n = 3); AD cases (n = 4) with age-matched controls (n = 2); a case with systemic sepsis and pneumonia (n = 1) (all obtained from Dr. Eliezer Masliah, UCSD, La Jolla, CA); and vCJD cases (n = 3) with an age-matched control (n = 1) (obtained from the London Neurodegenerative Diseases Brain Bank, Institute of Psychiatry, Camberwell, UK). For the AD cases, the Blessed score, which reflects level of cognition from 0 (normal) to 33 (totally demented), ranged from 18 to 33. In addition, brain tissue was obtained from rhesus macaques that developed SIVE following inoculation with a cell-free stock of SIVmac182 and a CD8-depleting regime and compared to uninfected control monkeys. SIVE monkeys were killed at an average 95.2 (standard deviation 16.6) days following inoculation. More details of these SIVE cases can be found in Roberts et al (9). SIVE and HIVe were defined on autopsy as including areas (in one or more parts of the brain) of microglia activation, macrophage infiltration, perivascular cuffing, macrophage/microglia nodules, and multinucleated giant cells. For SIVE/HIVE cases, tissue sections were chosen that represented areas both with and without immediate pathology to enable examination of microglia activation in concert with, and remote from, inflammatory centers, for example nodules and perivascular cuffing.

**Immunohistochemistry and Image Capture**

Formalin-fixed, paraffin-embedded sections were deparaffinized with xylene and hydrated in graded alcohols. Immunohistochemical staining followed a basic indirect protocol, using an antigen retrieval method (heated to 95°C in 0.01 M citrate buffer for 45 min, then left for 20 min to steep). The primary antibody was detected with PicturePlus universal secondary antibody-horseradish peroxidase polymer reagent (Zymed, San Francisco, CA) and developed with NovaRed chromogen (Vector Laboratories, Burlingame, CA), followed by a hematoxylin counterstain (Sigma-Aldrich, St. Louis, MO). All cases were stained with antibodies toward CD163 (Novocastra, Newcastle-upon-Tyne, UK, 1:100), GLUT5 (Chemicon, Temecula, CA, 1:200), HAM56 (Dako, Carpinteria, CA, 1:200), and HLA-DR (Zymed, 1:50). To examine whether CD163 was limited to microglia, SIVE tissue was double stained with GFAP (Zymed, 1:500) to highlight astrocytes, and CNPase (Sigma, 1:200) to highlight oligodendrocytes. Examination was also made of morphologically identified neurons and endothelial cells to assess CD163 reactivity. Control slides included omission of the primary antibody and use of irrelevant primary antibodies. vCJD cases were handled according to the Biosafety in Microbiological and Biochemical Laboratories Manual (4th Edition) (30). Image capture was performed with a Spot RT Color CCD camera and Spot RT software, version 3.4.2 for MacOS (Spot Diagnostic Instruments, Sterling Heights, MI) using a Leica Diaplan microscope (Leica Inc., Deerfield, IL). Figures were
assembled with Adobe Photoshop, version 7.0 for MacOS (Adobe Systems Inc., San Jose, CA).

RESULTS

We show here that anti-CD163 highlights cells with the morphological appearance of microglia and macrophages in the brains of rhesus monkeys with SIVE and human cases with HIVE. High magnification figures (×1,000) reveal that these included ramified cells (Fig. 1A) identical to those immunostained with anti-GLUT5 (Fig. 1B), HAM56 (Fig. 1C), or anti-HLA-DR (Fig. 1D); perivascular cells, and amoeboid cells (Fig. 1E), which may be either macrophages of systemic origin or activated microglia (31), and are also shown with anti-GLUT5 (Fig. 1F), HAM56 (Fig. 1G), or anti-HLA-DR (Fig. 1H). In double labeled SIVE sections, cells stained with anti-GFAP (astrocytes) or CNPase (oligodendrocytes) did not stain with anti-CD163 (data not shown), and there was no CD163 staining of neurons or endothelial cells.

Confirmation was made of the diagnosis of encephalitis in SIVE and HIVE cases with the appearance of reactive microglia, perivascular cuffing, and microglia/macrophage nodules, which were positive for CD163, GLUT5, HAM56, and HLA-DR. In serial sections of SIVE, low magnification figures (×100) reveal that CD163 reactivity occurred both in areas with encephalitic pathology and in areas with little to no proximal pathology (Fig. 2A). This occurred in cells in both grey and white matter. Similar staining was found with anti-GLUT5 (Fig. 2B), which was also seen in a small number of neurons. HAM56 (Fig. 2C) and HLA-DR (Fig. 2D) expression was mainly concentrated in parenchymal lesions and perivascular cuffs, and only the cells immediately surrounding these regions (Fig. 2D), but not those more remote from them. HAM56 reactivity was also present on endothelial cells, rendering identification of perivascular microglia/macrophages problematic. In uninfected monkey control cases, higher magnification figures (×250) reveal that there was very little CD163 expression, with reactivity limited to perivascular cells (asterisk) (Fig. 2E). There was still expression of GLUT5 on microglia but staining was lighter than in the SIVE cases (Fig. 2F), with no expression of HAM56 (Fig. 2G) or HLA-DR (Fig. 2H).

A very similar pattern to the SIVE cases was seen in the HIVE cases, although there was not as widespread staining with CD163 (Fig. 3A) (×100). This is possibly due to the accelerated disease regime used in the monkeys in the SIVE cases. GLUT5 highlighted all microglia/macrophages (Fig. 3B), with a large number of these cells also being highlighted with HAM56 (Fig. 3C). HLA-DR staining in the HIVE cases was also seen but to a far lesser extent that with the other immunostains (Fig. 3D). In cases of individuals who died of an AIDS-related illness but without encephalitis, anti-CD163 (×250) highlighted a few rare patches of positive ramified microglia/macrophages in the white matter. Along with perivascular cells (Fig. 3E), GLUT5-positive (GLUT5+) cells were also shown, although it was to varying degrees with the case in this figure (Fig. 3F) showing a greater GLUT5+ population than the other 3 cases. There was no HAM56 (Fig. 3G) or HLA-DR expression (Fig. 3H).

The vCJD cases, which had very notable spongiform pathology, showed extensive HLA-DR expression (as shown on low magnification figures, ×100), especially in the grey matter (Fig. 4A). HAM56 immunoreactivity was far less extensive (Fig. 4B) and, in contrast, there was extremely limited expression of CD163 (Fig. 4C) or GLUT5 (Fig. 4D), except for perivascular cells and limited areas with gross pathology. High magnification figures (×1,000) reveal that cells immunopositive for HLA-DR were mostly ameboid with some bushy, ramified microglia cells with short processes (Fig. 4E). Both of these cell types were also shown with HAM56 (Fig. 4F), whereas the small numbers of CD163+ (Fig. 4G) or GLUT5+ (Fig. 4H) cells were all ameboid. An age-matched control case (shown at ×250) showed almost no staining with HLA-DR (Fig. 4I) or HAM56 (Fig. 4J) reactivity (note the neurons highlighted in this picture are counterstained with hematoxylin). There were only a few CD163+ perivascular cells (Fig. 4K, asterisk), however, anti-GLUT5 highlighted perivascular cells and a few ramified microglia in the grey matter (Fig. 4L).

Three of the 4 AD case showed little CD163 expression (Fig. 5A, ×100). While there was some CD163 reactivity in microglial clusters in 1 case, because this case had a moderate Blessed score (cognition of 18) and one of the cases without CD163 reactivity had a high score (cognition of 33), this expression did not correspond with level of dementia. In contrast, HLA-DR staining showed diffuse microgliosis (Fig. 5B). There was very little staining with HAM56 (Fig. 5C), and anti-GLUT5 only faintly highlighted a limited number of microglia (Fig. 5D). High magnification images (×1,000) of microglia in AD cases revealed that CD163 expression was limited to perivascular cells (Fig. 5E), whereas HLA-DR was shown in many instances of microglial clusters typical of AD (Fig. 5F). Immunostaining with both HAM56 (Fig. 5G) and anti-GLUT5 (Fig. 5H) also highlighted some of these microglia clusters. Finally, in the case of systemic sepsis (×100), which displayed scattered CNS microabscesses, there was very little staining with anti-CD163 (Fig. 5I), anti-HLA-DR (Fig. 5J), or HAM56 (Fig. 5K), whereas GLUT5 highlighted microglia only in places of microabscess pathology (Fig. 5L).

DISCUSSION

Macrophages and microglia are the primary cells infected with HIV/SIV in the CNS and, whether infected...
or not, are postulated to be the primary effectors of neuronal damage. For example, the severity of HIV-associated dementia (HAD) resulting from damage to neuronal synapses and dendrites in the grey matter (32, 33) correlates more with the degree of macrophage infiltration and microglial activation than with the extent of infection (34). In this study we have shown that in the SIVE and HIV cases, anti-CD163 revealed a far greater extent of grey matter microglial staining than did anti-HLA-DR and did not highlight endothelial cells as was seen with
HAM56. The CD163+ cells were shown not only in and around lesions, but also in regions more remote from these in both grey and white matter. This was in contrast to the control, uninfected monkey cases that did not show positivity of any of these antibodies. Staining with an antibody against GLUT5, a fructose transporter recently shown to be a useful microglia stain (10), was similar to that shown with anti-CD163 in that it highlighted grey matter microglia in the SIVE/HIVE cases. However, there was also GLUT5 expression in control tissue and in some
neurons and thus is not as useful for examining different activation states in neuropathological disorders.

While neuronal damage in HIVE is generally attributed to fully activated microglia/macrophages, such as the white matter population in these cases, CD163 staining here points to a much more widespread cortical grey matter upregulation of ramified microglia in SIVE and HIVE cases. These cells may be akin to the hyper-ramified microglia described in the aging CNS that are activated to some extent but still retain the morphological appearance
Fig. 4. Low magnification images (×100) of serial sections of vCJD cases immunostained with anti-HLA-DR (A), HAM56 (B), anti-CD163 (C), and anti-GLUT5 (D). High magnification images (×1,000) of these cases: anti-HLA-DR (E), HAM56 (F), anti-CD163 (G), and anti-GLUT5 (H). Medium magnification images (×250) of sections from control cases immunostained with anti-HLA-DR (I), HAM56 (J), anti-CD163 (K, asterisk) and anti-GLUT5 (L).

of resting microglia (6, 7). It may be that despite being HLA-DR-negative, and thus not fully activated cells in the classic sense, the ramified CD163+ microglia in the grey matter shown here may contribute to neuronal damage, as has been shown in HAD cases (32, 33). Studies have shown that ramified microglia can participate in mechanisms that may cause harm to neurons. For instance, these cells have been shown to be involved in the displacement of synaptic boutons following facial nerve transection (35) and can be phagocytic while still ramified and without upregulation of CR3, MHCI, CD4, and CD45 (7, 36). In addition an in vitro study of primary ramified microglia showed that upon stimulation with LPS or TNF-α these cells can produce nitric oxide (37), which has been linked to neuronal damage in HAD cases (38, 39). Increased expression of CD163 itself has been linked to harmful mechanisms. For instance, CD163+ macrophages have been shown to exude the proinflammatory cytokines M-CSF, IL-6, and IL-1β (23), and levels of the latter two increase in the CNS over the course of SIV disease in infected...
monkeys correlating with gliosis (40). Thus the CD163+ grey matter microglia we see here may be involved in the synaptic and dendritic damage shown in HAD through synaptic displacement, phagocytosis of dendrites, and/or upregulation of proinflammatory cytokines and neurotoxins.

However, CD163 expression on microglia may alternatively be linked to protective mechanisms. Many studies have shown CD163 to be present on macrophages involved in the healing phase of inflammatory conditions, with the postulate that CD163+ cells are partly responsible for the downregulation of inflammation (41), as has been shown following infiltration/upregulation of CD163+ macrophages in cases of psoriasis (17, 42). An anti-inflammatory and angiogenic factor has been shown to be secreted from CD163+ macrophages (21, 43, 44), which may be the soluble form of CD163 (45). CD163 is also highly upregulated on placental and alveolar macrophages, as shown in human and mouse studies, with a postulated function of protection from inflammatory and...
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immune reactions (46, 47). Future studies may clarify the relationship between microglial CD163 in the HIV-infected brain, neuronal damage, and level of dementia to help elucidate whether CD163+ microglia are involved detrimentally or protectively in this pathology.

The grey matter microglia in the vCJD cases displayed distinct ameboid morphology and, as has been shown previously (26), pronounced expression of HLA-DR. They did not, however, show much CD163 expression, unlike the HLA-DR-positive cells in the SIVE/HIVE cases in the current study. CD163 expression has been shown to be induced by the cytokine IL-6 and because this cytokine has been shown to be upregulated in both SIV (40) and vCJD cases (48, 49), we were surprised not to see immunoreactivity in the vCJD cases. Therefore, there must be distinct mechanisms that are causing the expression of CD163 in the HIV/SIVE cases (e.g. the presence of the viral pathogen) that are not present in the vCJD cases. In the AD cases there was also increased amounts of HLA-DR staining in ramified microglia, which corroborated the findings of a previous study (50), but no consistent expression of CD163, with staining being shown in some cases and not others, unrelated to the Blessed score of cognition. Neither AD nor vCJD are normally classed as inflammatory disorders because there is no notable influx of systemic macrophages (29) and they have not been shown to be caused by a viral or bacterial pathogen unlike in the SIVE/HIVE cases. We may thus be able to use CD163 expression in the categorization of neuropathological disorders.

It has been postulated that as SIV/HIV was not just a disorder of the CNS, unlike vCJD or AD, and because microglia activation can occur from signals originating from systemic HIV-infected cells (51), the expression of CD163 in the SIVE/HIVE cases could be occurring via a systemic process. To this end, we investigated AIDS cases without encephalitis and whether, in a case of systemic sepsis, CD163 would be upregulated in the CNS. There was little CD163 expression in the AIDS cases and no expression in the sepsis cases, suggesting that the mechanism of CD163 expression is more probably due to local changes in the CNS in concurrence with factors released by the infiltrating macrophages shown in encephalitis, combined with the action of HIV and HIV proteins.

In conclusion, CD163 was shown to highlight ramified grey matter microglia in cases with SIVE or HIV, in contrast to controls, and to a far greater extent than HLA-DR. Although GLUT5 also highlighted this microglia population, because it also stained cells in control tissue it was not a useful pathological indicator. In contrast, the HLA-DR reactivity found in vCJD and AD was not, in general, accompanied by CD163 reactivity. This may point to a role of CD163+ microglia in the pathogenesis of HIV/SIVE and neuronal disruption shown in cases of HAD, but the function of these microglia, and whether they play a detrimental or protective role, awaits further investigation. Thus, we conclude that CD163 is not a marker of ramified microglia upregulation in general, rather that it represents a particular form of microglial activation requiring certain factors, perhaps present only in certain pathogen-induced inflammatory disorders of the CNS.

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