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Determination of spatial and temporal distribution of microglia by 230nm-high-resolution, high-throughput automated analysis reveals different amyloid plaque populations in an APP/PS1 mouse model of Alzheimer's disease

Katja Scheffler^{1,2,*}, Jan Stenzel^{1,3,*}, Markus Krohn^{1,3}, Cathleen Lange^{1,3}, Jacqueline Hofrichter¹, Toni Schumacher¹, Thomas Brüning¹, Anne-Sophie Plath^{1,2}, Lary Walker⁴, and Jens Pahnke^{1,2,3,‡}

¹University of Rostock, Department of Neurology, Neurodegeneration Research Laboratory (NRL), Gehlsheimer Str. 20, 18147 Rostock, Germany

²German National Center for Neurodegenerative Diseases (DZNE)

³University of Rostock, Interdisciplinary Faculty, Department of Aging Science and Humanities

⁴Yerkes National Primate Research Center and Department of Neurology, Emory University, Atlanta, Georgia, USA

Abstract

One early and prominent pathologic feature of Alzheimer's disease (AD) is the appearance of activated microglia in the vicinity of developing β -amyloid deposits. However, the precise role of microglia during the course of AD is still under discussion. Microglia have been reported to degrade and clear β -amyloid, but they also can exert deleterious effects due to overwhelming inflammatory reactions. Here, we demonstrate the occurrence of developing plaque populations with distinct amounts of associated microglia using time-dependent analyses of plaque morphology and the spatial distribution of microglia in an APP/PS1 mouse model. In addition to a population of larger plaques ($>700\mu\text{m}^2$) that are occupied by a moderate contingent of microglial cells across the course of aging, a second type of small β -amyloid deposits develops ($\leq 400\mu\text{m}^2$) in which the plaque core is enveloped by a relatively large number of microglia. Our analyses indicate that microglia are strongly activated early in the emergence of senile plaques, but that activation is diminished in the later stages of plaque evolution (>150 days). These findings support the view that microglia serve to restrict the growth of senile plaques, and do so in a way that minimizes local inflammatory damage to other components of the brain.

Keywords

Alzheimer's disease; AD; beta-amyloid; neurodegeneration; dementia; microglia; inflammation; immune therapy; blood-brain barrier

[‡]Address correspondence to this author at the University of Rostock, Department of Neurology, Neurodegeneration Research Laboratory (NRL), Gehlsheimer Str. 20, 18147 Rostock, Germany, Tel: +49 (381) 494 4700, Fax: +49 (381) 494 4702; jens.pahnke@med.uni-rostock.de; www.nrl.uni-rostock.de .

*These authors contributed equally to the work.

INTRODUCTION

Alzheimer's disease (AD) is the most frequent cause of dementia and the fourth most common cause of death in aging humans [1]. In addition to the most prevalent pathologic characteristics such as β -amyloid ($A\beta$) plaques, neurofibrillary tangles, and cerebrovascular amyloidosis, many studies provide evidence of inflammatory changes that occur during the pathogenesis of AD, particularly involving microglia [2,3]. As the resident immune cells of the brain, microglia are the first line of defense against pathogens and other agents of brain injury (reviewed in [4-7]). Activated microglia are intimately associated with senile plaques, but the precise role of these inflammatory cells during the development of AD remains controversial. One hypothesis holds that activated microglia exert neurotoxic effects due to the chronic secretion of high levels of pro-inflammatory cytokines or the overproduction of reactive oxygen species (ROS) in response to aggregated $A\beta$ [8,9]. Another hypothesis contends that activated microglia play a neuroprotective role by eliminating $A\beta$ deposits through phagocytosis and the secretion of $A\beta$ -degrading enzymes [2,10,11].

More recently, an effort with microglia-ablated APP/PS1 expressing mice could neither support the beneficial nor the neurotoxic role of microglia during AD pathogenesis as amyloid load and neural dystrophy stayed both unaffected [12]. In a transgenic mouse model of AD, the majority of macrophage-type cells associated with mature plaques were found to originate from the bone marrow, whereas resident microglia were recruited mainly at the onset of plaque formation [13]. Although the blood-derived cells differentiated into microglia-like cells that were morphologically similar to their resident counterparts, the blood-derived cells seemed to be more efficient at eliminating $A\beta$ deposits by a cell-specific phagocytic mechanism [13]. In support of the beneficial role of microglia, recent *in vivo* studies showed that activated microglia migrate toward newly developing $A\beta$ plaques within 1-2 days of the appearance of the aggregated $A\beta$, and also that the microglia are attracted to more mature $A\beta$ deposits. After their recruitment to the plaque, the cells appeared to restrict plaque growth and were capable of reducing plaque size; however, the existing $A\beta$ deposits were not removed [3,14]. Moreover, in an investigation of the dynamic interaction of microglia and plaques, Bolmont and colleagues [14] recognized plaques that were more prone to increase in volume over time, possibly owing to a reduced presence of microglia. Furthermore, an upper limit of the number of microglia associated with plaques, independent of plaque size, may indicate diminished migratory behavior or restricted infiltration of monocytes from the periphery. These findings indicate an important contribution of microglia to the evolution of $A\beta$ deposits, and thus could provide insights into the mechanisms by which $A\beta$ immunotherapy [15] affects these lesions in the brains of AD patients. Both active and passive anti- $A\beta$ immunization approaches have entered human clinical trials [16]. In preclinical studies of transgenic mice, systemic administration of the mouse monoclonal antibody m266 not only decreased plaque formation [17] but also rapidly improved behavioral performance [18,19]. In contrast, the initial phase 2a trial of the active immunization approach was prematurely terminated due to an apparent subacute meningoencephalitis [20]. The first post-mortem analyses revealed increased microglial activation and expression of the phagocytosis marker CD68 [21] on the microglia, apparent clearance of $A\beta$ plaques, and the presence of immunoreactive $A\beta$ within activated microglia [22,23]. Despite these effects on plaques, none of the treated patients showed significant improvement of cognitive abilities [24]. At least with regard to the clearance of senile plaques, these data are largely consistent with $A\beta$ -immunization experiments in animal models [25], and further support the hypothesis that antibody-mediated microglial activation results in the phagocytosis of $A\beta$ and clearance of parenchymal plaques [26,27]. Indeed, recent analyses suggest that CAA, which increases in the early stages of plaque removal, also may be eliminated by immunotherapy after an extended period of time [24]. Hence, if the power and specificity of microglia can be safely harnessed, selective microglial

activation holds promise as a means of reducing A β burden in the brains of patients with AD.

To clarify the temporal and spatial relationships between β -amyloid plaques and microglia, we employed an APP/PS1 transgenic mouse model of cerebral β -amyloidosis [28]. With the help of a newly developed, 230nm-high-resolution, semi-automated, quantitative evaluation method including microscopy and automated computer analysis, we were able to highlight the time-course of microglial activation and β -amyloid deposition in narrow time increments starting with the onset of plaque deposition at day 50. We found that microglia react strongly to early plaque formation, and that they may contribute to plaque size restriction. These results support the potential utility of antibody-mediated microglial activation for therapeutic interventions.

METHODS

Animals

We investigated in an 25 day rhythm, starting at day 50 until day 200 of each three to five male and female transgenic mice harboring mutant human presenilin 1 (L166P variant) and a mutant human β -amyloid precursor protein (APP_{swe}) on a C57Bl/6J background (APP/PS1) [28]. The mice were maintained at 23-25°C on a 12h:12h light:dark cycle, with free access to rodent food and water. All procedures were conducted in accordance with animal protocols approved by the University of Rostock and according to the state law of the government of Mecklenburg-Vorpommern (LALLF M-V/TSD/7221.3-2.3-004/06; LALLF M-V/TSD/7221.3-2.3-003/08).

Immunohistochemistry

Immunohistochemistry was undertaken as previously described [29-32]. In brief, the mice were perfused transcardially with PBS followed by buffered 4% paraformaldehyde. Tissue was postfixed for at least 24 hours in 4% paraformaldehyde. Paraffin-embedded, 4- μ m-thick coronal sections at position bregma -1.5mm to -2.5mm of brain were double-stained using a Bond-Max™ (Leica, Wetzlar, Germany) automated immunostaining system. Sections were first pretreated with 98% formic acid for 5min and immunostained for A β using the anti-human A β clone 6F3D (1:200, 30min, Dako) and the Bond™ Polymer AP-Red Detection kit (red color, Leica, Wetzlar, Germany). Microglia were then immunostained on the same sections using anti-Iba1 (1:1000, 30min, Wako) and the Bond™ Polymer Refine Detection kit (brownish color, Leica, Wetzlar, Germany) (Figure 1).

Digitizing and automated analysis of brain slices

Whole tissue sections were digitized at a resolution of 230nm using a MIRAX Midi slide scanner (Zeiss Microsystems GmbH, Jena, Germany). Scanned brain slices were analyzed using a semi-automated procedure programmed for the AxioVision software package (Zeiss Microsystems GmbH, Jena, Germany). Initially, cortical regions of interest (ROIs) were defined and plaques and microglia were segregated according to their RGB color profile using the appropriate color channels: green for DAB-stained microglia and red for AP-Red-stained A β plaques. The resulting binary pictures were digitally processed to gain the shape corresponding to the initial scan. Then the plaque number and sizes were quantified across all cortical layers. According to the geometrical balance focus of each individual plaque, a rectangle was placed around each plaque, automatically. Then the area occupied by microglia was determined inside the defined rectangle and evaluated according to the plaque size. The obtained data, i.e. ROI size, plaque number, plaque area, microglial area and the percentage of plaque area covered by microglia, were recorded, and the plaque number

finally was normalized to 10mm^2 for each hemisphere to control for ROI differences (Figure 2).

Statistical analyses

Results are presented as means $\pm$ standard error of the mean (SEM). A statistical analysis for value distribution revealed logarithmic-normal (base e) distributed values. Statistical significance was determined using Student's *t*-test with the significance level set at $p < 0.05$ after the values were transformed to a normal distribution.

RESULTS

To analyze the microglial response to β -amyloid plaque development and deposition, double staining of plaques and microglia was necessary for a high-throughput automated analysis of whole brain slices or specific regions. We established immunohistochemical double-staining protocols using anti-Iba1 antibodies and diaminobenzidine (DAB) as substrate for the detection of microglia since in comparison to double immunofluorescence these stainings are more sensitive and enable higher resolutions even after harsh pretreatments, e.g. formic acid. Thus using this method, we were able to visualize the fine morphology of highly ramified resting as well as activated microglia around β -amyloid deposits (Figure 1A). For the presentation of amyloid plaques, the anti-A β 6F3D antibody with two different substrates was tested. The Fast-Green substrate (dark green color) revealed a limited resolution of plaques and an adverse differentiation between plaques and surrounding microglia (Figure 1B). In contrast, AP-Red (light red color) as a substrate showed high-resolution discrimination of plaques and associated microglia (Figure 1C). The latter method was used for the following analyses.

In our APP/PS1 mice, the first β -amyloid deposits were detectable at an age of 50 days (Figure 3), similar to the age of onset reported by Radde et al. [28]. With increasing age, plaque density in the cortical areas increased dramatically. Microglia in the vicinity of senile plaques displayed an activated phenotype, including enlargement of the soma and an amoeboid appearance that distinguished them from the smaller, more delicate-appearing and well-ramified resting microglia. The area occupied by activated microglia that were spatially associated with senile plaques increased with advancing age. Interestingly, the relative plaque area occupied by microglia was greater in a subgroup of small deposits of A β than in the large plaques. In addition, the astrocytic reaction followed a different spatial and temporally delayed pattern. There was a slow increase in astrocytic reactivity at early time points, followed by intense astrogliosis in the cortex starting at the age of 125 days (Figure 3, panels G-I). Spatially, reactive astrocytes accumulated farther from the plaque core than did the reactive microglia, which were more likely to occur within or very near the A β deposits (Figure 3, panels J-L).

To analyze the spatial features of microglia at different stages of AD pathology, appropriate quantitative evaluation methods for stained slides were required. Tissue sections were digitized at 230nm resolution using a MIRAX Midi slide scanner and analyzed using a semi-automated procedure programmed for the AxioVision software package (Zeiss Microsystems GmbH, Jena, Germany) (methods section, Figure 2).

To optimize the temporal resolution of the age-associated changes in plaques and surrounding microglia, we analyzed mice in time-steps of 25 days between the ages of 50 and 200 days. In our analysis, the quantification of a defined cortical area revealed a consistent rise in plaque number through day 200 (Figure 4A). By 200 days, the areal density of plaques reached a mean of 547 lesions per 10mm^2 . Total microglial area also showed a continuous increase over this time period (Figure 4C). Amount of microglia

normalized to plaque number raises constantly till 150 days and remains constant afterwards (Figure 4D). No statistically significant gender differences in either parameter were found.

Using the criteria of Bolmont *et al.* [14], we categorized the plaques according to their size: 1) small, $\leq 400\mu\text{m}^2$; 2) medium, $401\mu\text{m}^2 - 700\mu\text{m}^2$; 3) large, $> 700\mu\text{m}^2$. As expected, the first deposits of A β appeared as small, dense-cored plaques (Figure 3D); as the animals age, the average size of the plaques increases from $336\mu\text{m}^2$ at day 50 to $1176\mu\text{m}^2$ at day 200 (Figure 4B). However, at age 125 days, the relative number of small plaques increases, resulting in a decrease in the fraction of large plaques. Later, the relative proportions of large and small plaques reach a plateau around the age of 150 days (Figure 5). In contrast to the changing proportions of small and large A β plaques, the relative incidence of medium-sized plaques remains fairly stable, particularly after the age of 75 days (Figure 5).

To establish the quantitative spatial relationship between A β plaques and plaque-associated microglia, we calculated the ratio of microglial area to total plaque area (Figure 6). In most plaques, microglia occupied less than 50% of the overall plaque area. However, in a subgroup of mainly small plaques, the relative area occupied by microglia was greater than 50% (Figure 6). This second, distinct population increased in direct relationship to the rising number of small plaques with advancing age (Figure 5). To clarify these data, we compared only the plaques with at least 50% area occupied by microglia at each time point (Figure 6 inset). The measurements overall revealed a burgeoning population of such microglia-rich plaques with age, with a slight dip at day 175.

DISCUSSION

Although microglial activation in AD has been discussed extensively in the literature, no consensus about the contribution of these cells to AD pathogenesis has been reached. The time frame within microglia are linked to senile plaques is an important issue that can be addressed in mouse models of cerebral β -amyloidosis. Amyloid plaques often are surrounded by activated microglia [2,14], and the lesions promote inflammatory responses in the brain [33]. Several lines of evidence indicate that microglia may play a neuroprotective role in AD by mediating A β phagocytosis [13,14], but they also can produce neurotoxins and cytokines, hence promoting neurodegeneration [34].

The 230nm-resolution paradigm has enabled us to generate a detailed time-course of microglial activation in APP/PS1 mice as they increase in age. In agreement with previous studies, our data demonstrate that the first β -amyloid deposits in this mouse model are accompanied by rapid microgliosis, which continuously increases at successively later time points. By following the characteristics of senile plaques over several months, we identified two populations of senile plaques that can be distinguished on the basis of their microglial components, although immunolabeling reflects only one distinct moment of dynamic microglia [35]. Specifically, the smaller plaques are accompanied by a relatively high density of reactive microglia, whereas larger plaques, which are more common in older mice, have a proportionally smaller microglial component. Following the evolution of plaque populations over several months, we found a progressive, age-associated increase in microglial number per plaque, similar to that described by Bolmont and colleagues [14]. In addition, however, we determined that small plaque size was linked to a relatively high microglial load, suggesting that microglia react strongly to newly generated senile plaques, and that they may act to restrict the size of the proteinaceous deposits. However, the microglial response to larger plaques is relatively less, inasmuch as the percent investment of the deposits by microglia is diminished. By around day 175, the mean plaque size and microglial area normalized to plaque number reach a plateau, even though cortical plaque number continues to increase. At the later stages of plaque development, reactive

astrocytosis is substantial, and probably helps to isolate the inflammatory foci from the brain parenchyma as well as may participate in the degradation progress of A β [36,37].

Our results point toward a bifunctional role of microglia in their interactions with A β deposits: an early, reactive phase during which microglia act to restrict plaque size, and a later maintenance phase during which the activated microglial load per plaque is diminished. In support of this hypothesis, Hickman and colleagues [38] found that, in the PS1-APP [B6C3-Tg(APPswe, PSEN1dE9)85Dbo/J] mouse model, the phenotype of accumulating microglia changes as pathology progresses. Early microglial activation seems to contribute to A β clearance, but as the transgenic mice age, the microglia lose their ability to degrade pre-existing amyloid deposits, although they retain their ability to secrete pro-inflammatory cytokines. In conjunction with our findings of diminished microgliosis in larger deposits, these findings suggest a potential protective mechanism by which microglia restrict plaque size while minimizing inflammatory damage to the surrounding tissue that could result from chronic activation in mature deposits. The prominent appearance of reactive astrocytes around the mature lesions suggests that astrocytes and microglia may cooperate in this mechanism.

In summary, our findings indicate that cerebral microglia may exhibit beneficial effects during early disease states due to their plaque size restriction/degradation function. However, during the evolution of senile plaques, the number of microglia only rises linearly, in parallel with the number of plaques. This restraint of microglial reactivity serves to impede a fulminant inflammatory reaction to the growing population of persistent A β deposits in brain. It is possible that current A β -immunization therapy trials for AD might disrupt this self-limiting process, thus, leading to inflammatory side effects in patients with substantial amounts of intracerebral β -amyloid [39]. Mitigating the disinhibition of inflammatory cells by immunization therapy could improve the side-effect profile of this promising therapeutic approach.

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Fig. 1. Establishment of a double-labeling method for A β plaques and microglia for high-throughput automatic scanning. A: Detection of well-ramified resting as well as activated microglia around A β deposits. B: Double labeling of microglia using anti-Iba1 antibody (brown) and A β plaques using anti-A β antibody 6F3D (developed with Fast-Green substrate). C: Double labeling of microglia using anti-Iba1 antibody (brown) and A β plaques using anti-A β antibody 6F3D (developed with AP-Red substrate). Using AP-Red, microglia and A β deposits are better delineated and distinguishable than with Fast-Green.

Fig. 2.

230nm-high-resolution, high-throughput determination of plaque size and corresponding microglial area in whole-section digital microscopic scans. A: Segregated brain tissue in blue. B: Segregated β -amyloid plaques enclosed by a rectangle calculated according to the geometric balance focus of the individual plaque in red. C: Microglial area in green, corresponding to the region of interest (ROI). D: Merged picture of tissue (blue), amyloid plaques (red) and microglia (green). The overlay of microglia and plaques is presented in yellow. E: Table of calculated data for the corresponding relative microglial area as a function of total plaque area, the values that were used for the analyses.

Fig. 3.

Age-dependent changes in A β plaque-associated microglia and astrogliosis in APP/PS1 mice. Early A β deposits appear at day 50 (A) and increase in number with increasing age (B - 125 days, C - 200 days). Insets (D-F) highlight the microglial infiltration of A β plaques. Microglial coverage increases over time until day 125 (E), after which the relative microglial load is reduced in large plaques, whereas small plaques are accompanied by a relatively large microglial component (F). GFAP immunostaining reveals increasing astrogliosis with advancing age (G-I). Insets (J-L) show that reactive astrocytes around A β plaques are farther from the plaque core than are reactive microglia (Insets D-F).

Fig. 4.

Analysis of plaque number, plaque size and microglial area in 25-day time steps, beginning at age 50 days, in APP/PS1 mice. Quantification of (A) plaque number, (B) plaque size (μm^2), (C) microglial area, and (D) the microglial area normalized to plaque number in the neocortex of APP/PS1 mice (all measurements were normalized to 10mm^2 cortical area). Plaque number rises continuously through this period (A), as does plaque size, which passes through a possible plateau around day 125 (B). The total microglial area rises constantly over time (C), whereas normalized to plaque number a plateau is reached after 150 days (D). These data show that the relative microglia number is reduced in older mice suggesting a reduction in microglia function. Data are presented as means $\pm$ SEM.

Figure 5.

Analysis of plaque size distribution over time in APP/PS1 mice. Relative plaque numbers fluctuated between different plaque size categories. A sharp decline of smaller plaques (dashed line) at the early age is followed by a slight increase at day 125. The number of large plaques (continuous line) developed in the opposite direction of small plaques, whereas the number of medium-sized plaques (dotted line) remained nearly constant over time. Data are presented as means $\pm$ SEM.

Fig. 6.

Formation of two distinct plaque populations over time in APP/PS1 mice. Plotting microglial coverage to corresponding plaque size resulted in two distinct groups of plaques. Larger plaques are enveloped by a constant number of microglia (area occupied by microglia, blue dots), whereas small plaques are accompanied by a proportionally higher number of microglia (increased area occupied by microglia, red dots). *Diagram inset:* Quantification of plaques covered by at least 50% of their area by microglia revealed a consistently growing percentage versus total plaque number interrupted by a decrease at the age of 175 days finally leading to 50% of all plaques covered by at least half of their size by microglia. Data are presented as means $\pm$ SEM.