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CHAPTER

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Phagocytosis and degradation of neurons by classically versus alternatively activated macrophages

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Manuscript in preparation



ABSTRACT:

Axonal damage is an important feature of MS lesions and contributes to disease progression in MS. Macrophages have been implicated in the phagocytosis of apoptotic neurons and clearance of neuronal debris. Distinct macrophage subsets exist: classically activated (CA/M1), pro-inflammatory macrophages and alternatively activated, anti-inflammatory (AA/M2) macrophages, with distinct functions during inflammation and repair. The aim of the present study was to determine whether phagocytosis and degradation of neurons and neuronal particles differed between CA and AA macrophages.

Neuronal particles, damaged primary neurons, myelin and latex beads were phagocytosed significantly more by CA macrophages compared to control and AA macrophages. The time frame of degradation of neuronal antigens did not differ between the macrophage subtypes. The degradation of β -tubulin and neurofilament (NF) occurred within the first 24 h after neuronal particle ingestion in all subtypes of macrophages. Microtubule associated protein-2 (MAP-2) was degraded at a slower pace, since a significant decrease in immunoreactivity was visible only after 48 h.

Our data suggest that CA macrophages are the primary cells to clear both myelin and axonal debris during MS. In MS lesions cellular debris hinders axonal regeneration, making clearance by macrophages very important for repair. However, due to phagocytosis of intact neurons they could induce damage.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory neurological disease. It is the most common cause of neurological disability in young adults, affecting approximately 2 million people world wide. MS is characterized by multiple lesions in the central nervous system (CNS) separated in space and time, which are associated with perivascular infiltrates, demyelination, astrogliosis, axonal damage and axonal loss^{1,2}. It has been widely accepted that axonal loss is a major correlate for clinical disability³⁻⁵. Axonal damage and loss occur early during disease as well as in later stages, are present in both acute and chronic lesions⁶⁻⁸, and correlate with the degree of inflammation^{9,10}. Macrophages, a major constituent of inflammatory infiltrates, play an important role in axonal damage^{11,12}. They can induce axonal damage due to the release of reactive oxygen species¹³, pro-inflammatory cytokines¹⁴, glutamate¹⁵ and matrix metalloproteinases¹⁶.

Only a few reports are available on the phagocytosis of neuronal debris, whereas phagocytosis of myelin debris has been well documented^{2,17}. Several studies have found that macrophages are in close contact with degenerating axons and can even phagocytose them. In dysmyelinating mice, deficient for the myelin protein P0, macrophages were observed in close contact with degenerating axons¹⁸. In several animal models of neurodegeneration phagocytosis of degenerating neurons has been observed^{19,20}. Activation of p38MAPK in microglia was required for the phagocytosis of axonal debris²¹. During MS pathology there are indications that phagocytosis of degenerating neurons takes place. In the cervical lymph nodes of MS patients phagocytes, including macrophages, contained neuronal antigens, suggesting that after phagocytosis of neurons or neuronal debris macrophages transport the debris outside the CNS and present neuronal antigens to T cells²². During ontogenic development macrophage engulfment of neurons undergoing the first stages of apoptosis is a common occurrence²³. Macrophages are able to phagocytose complete apoptotic neurons²⁴.

Macrophages are a heterogeneous population of cells, with great plasticity in response to environmental cues²⁵⁻²⁷. Already in 1992 a marked upregulation of mannose receptor (MR) on macrophages was observed following stimulation with interleukin-4 (IL-4)²⁸. This newly identified phenotype of macrophages was referred to as alternatively activated (AA). Since then, the two most studied activation subtypes of macrophages are classically activated (CA/M1) macrophages and alternatively activated (AA/M2) macrophages^{25-27,29}. The phenotype and effector functions of macrophages are determined by the environment in which the macrophages are activated³⁰. The CA macrophages are involved in host defense against pathogens and tumor killing, by the production of pro-inflammatory cytokines³¹ and oxidative metabolites³². They also cause harm in the form of bystander damage. In contrast, AA macrophages are growth promoting, due to their role in angiogenesis³³, extracellular matrix remodeling³³, the secretion of growth factors³⁴ and the production of anti-inflammatory cytokines²⁹.

Contrasting results have been observed when addressing phagocytosis in CA and AA macrophages. Some studies describe a higher phagocytosis rate in AA macrophages³⁵, while others report decreased phagocytosis after stimulation

with IL-4³⁶. The aim of this study was to determine whether the phagocytosis of damaged neurons and neuronal particles differs between the different subtypes of macrophages. We also established the rate of degradation of neuronal antigens. The phagocytosis of neuronal particles, neurons, myelin and latex beads was higher by CA macrophages compared to both AA and control macrophages. The degradation of β -tubulin and neurofilament (NF) occurred within the first 24 h after neuronal particle ingestion in all subtypes of macrophages. Microtubule associated protein-2 (MAP-2) was degraded at a slower pace.

MATERIALS AND METHODS

Animals

Timed pregnant C57BL/6 mice were bred in the animal facility for neuronal cultures of neonatal cortex. In order to obtain bone marrow macrophages adult C57BL/6 mice were used.

All experiments were performed according to the guidelines of the local University Committee on Animal Welfare, which follow the European Communities Council Directive (86/609/EEC).

Neuronal cultures

Neuronal monolayers were generated as described earlier^{37,38}. In brief the brains of gestational day 19 mice were isolated and meninges were removed. The cortex was isolated and divided into fragments. A single cell suspension was generated by both chemical and mechanical methods: 1% trypsin (Sigma-Aldrich, Zwijndrecht, the Netherlands) with 0.1 mM DNase (Sigma-Aldrich) in HBSS (Invitrogen, Breda, the Netherlands), and five times trituration in Neurobasal medium (Invitrogen) containing 0.1 mM DNase. The cell concentration was adjusted to 0.25×10^6 cells/ml in complete neurobasal medium (containing Neurobasal medium, B27 supplement (Invitrogen), glutamax (Invitrogen) and gentamycin (Invitrogen)). The cells were plated on 96-wells plates coated overnight with poly-L-lysine (Sigma-Aldrich).

After 2 days in culture the primary neurons were prepared for the phagocytosis assay. The neurons were labeled by incubation with $1 \mu\text{M}$ calcein (Sigma-Aldrich) during 15 min at 37°C , in order to label viable cells. The cells were washed and harvested using trypsin. Subsequently the neurons were carefully centrifuged, by using a slow start and low brake, and were immediately used to study phagocytosis.

To generate neuronal particles, neuronal monolayers were harvested by roughly pipetting the medium containing the neurons after 2 days in culture. The neuronal particles were washed with PBS. The protein content was measured using a bicinchoninic acid (BCA) assay (Pierce, Ettenleur, the Netherlands), according to the manufacturer's description. The concentration of neuronal particles was adjusted to $200 \mu\text{g/ml}$ protein. Finally, they were labeled with the lipophilic fluorescent dye 1,1',di-octadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (DiI) (Sigma-Aldrich), by incubating with $100 \mu\text{M}$ of this solution for 30 min at 37°C .

We used immunofluorescence to analyze the neuronal proteins present on the

neuronal particles. The neuronal particles were washed with PBS and exposed to the first antibody (for list see Table 1) for 1 h. After washing, the Alexa-488 labeled secondary antibody was added for 1 h. The particles were analyzed using flow cytometry (FACS Calibur, Becton Dickinson) combined with Cellquest Pro software (Becton Dickinson, Erembodegem, Belgium). Omission of the primary antibody served as a negative control.

Bone marrow derived macrophages

Bone marrow derived macrophages were obtained as described ³⁹. Briefly, the femurs and tibias of C57BL/6 mice were flushed to obtain bone marrow and this was cultured in complete macrophage medium (DMEM (Invitrogen) containing 10% fetal calf serum (FCS) (VWR, Amsterdam, the Netherlands), 15% conditioned medium from macrophage-colony stimulating factor-secreting L929 fibroblasts (ECAAC, Sigma-Aldrich) and 2% penicillin/streptomycin-glutamine (Lonza, Breda, the Netherlands) for 1 week. After 7-10 days the adherent cells formed an approximately 95% pure population of macrophages. The macrophages were washed, to remove non-adherent cells, and used in experiments.

The CA phenotype was induced by exposure to 5×10^3 U/ml IFN- γ (U-Cytech, Utrecht, the Netherlands) and 10 ng/ml *Escherichia coli* LPS (026:B6; Sigma-Aldrich). Using the Griess assay the nitric oxide (NO) production was measured to confirm the CA phenotype. The AA phenotype was generated by two day exposure to 10 ng/ml IL-4 ⁴⁰ or 10ng/ml IL-4 and IL-10 (Invitrogen). The AA phenotype was assessed using FACS analysis for mannose receptor expression, a typical marker for alternative activation. IL-4 and IL-10 stimulated macrophages showed the highest expression of mannose receptor and were therefore used as AA macrophages. Control macrophages were cultured in complete macrophage medium alone.

To harvest the macrophages, they were incubated with lidocaine (4 mg/ml in PBS, Sigma-Aldrich) for 15 min at 37°C. Afterwards the cells were scraped, centrifuged and brought to a concentration of 1×10^6 cells/ml.

For the neuronal antigen degradation experiments, macrophages, at a concentration of 6×10^5 cells/ml, were transferred to 24-wells plate containing coverslip glasses and stimulated for 48 h with either IFN- γ and LPS or IL-4 and IL-10 to generate the different phenotypes.

Phagocytosis

To determine whether macrophages phagocytose primary neurons (calcein labeled), neuronal particles (Dil labeled), , latex beads (FITC labeled) and myelin (Dil labeled) differently we analysed the phagocytosis rate by fluorescence activated cell sorting (FACS). We adapted a protocol described previously for myelin phagocytosis ⁴¹.

For the phagocytosis of primary neurons, the concentration of calcein labeled primary neurons was adjusted to 20×10^6 cells/ml and 10 μ l of the cell suspension was added to 3×10^5 macrophages for 4 h at 37°C. Macrophages were washed three times and harvested using 4 mg/ml lidocain (Sigma-Aldrich) for 15 min at

37°C. The percentage of macrophages positive for calcein, indicating neurons, was determined using the FACS Calibur and the Cellquest Pro software as described above. Phagocytosis was displayed in percentages relative to control macrophages.

The macrophages were washed twice and incubated with fluorescently labeled beads, myelin (10 or 20 µg/ml) or neuronal particles for 1.5 h at 37°C. Macrophages were washed three times and harvested using 4 mg/ml lidocain (Sigma-Aldrich) for 15 min at 37°C. The macrophages were washed and analyzed using flow cytometry and Cellquest Pro software. For comparison, phagocytosis was displayed in percentages relative to control macrophages.

Degradation of neuronal antigens

To determine the degradation of neuronal antigens in macrophages, the macrophages were incubated with unlabeled neuronal particles for 1.5, 4, 24 or 48 h at 37°C. Afterwards, the macrophages were fixed by incubation for 30 min with paraformaldehyde (4% in PBS).

The macrophages were washed twice with PBS, exposed to 0.1% Triton X-100 in PBS followed by normal goat or donkey serum. The cells were exposed overnight at 4°C to the primary antibody (for list see Table 1) in 0.1% saponine, 0.1% BSA in PBS. After washing, the secondary antibody was added for 1h. The cells were washed with PBS again and incubated with Hoechst (Invitrogen). Omission of the primary antibody was the negative control.

Images were acquired on a Leica DM6000 (Leica LAS AF software, Leica Microsystems, Bensheim, Germany). The images were processed using Adobe Photoshop 6.0 (San Jose, USA). For quantification of the percentage of macrophages positive for neuronal antigens, at least 10 random positions were analyzed per well from 3 to 5 separate experiments. The number of macrophages that were positive for neuronal antigens per field were counted using the image analysis program AnalySIS software (Soft Imaging System GmbH, Münster, Germany).

| Primary antibody | Subtype | Dilution | Secondary antibody | Dilution |
|--|---------|----------|-----------------------|----------|
| β-tubulin-III (Tuj1) (Covance, Uden, the Netherlands) | IgG2A | 1:400 | Anti-mouse Alexa 488 | 1:400 |
| MAP-2 (Sigma, Zwijndrecht, the Netherlands) | IgG1 | 1:1000 | Anti-mouse Alexa 488 | 1:400 |
| NF (Sigma, Zwijndrecht, the Netherlands) | IgG1 | 1:100 | Anti-rabbit Alexa-488 | 1:400 |

Table 1: Primary antibodies and dilutions used in immunohistochemical stainings

Statistical analysis

All data are expressed as mean ± SEM of 3 to 4 separate experiments performed in duplicate. Statistics were performed in SPSS (15.0, Chicago, USA). The data were analyzed using one-way ANOVA with Bonferoni correction. A p-value of <0.05 was considered significant.

RESULTS

Phagocytosis of neuronal particles and damaged neurons

During development neurons undergoing the first, still reversible stages of apoptosis are engulfed and phagocytosed by macrophages²³. We determined whether differently activated macrophages show altered phagocytosis of primary neurons. The neurons were labeled with calcein and after 4 h phagocytosis of the neurons was assessed using FACS analysis (Figure 1). The neurons were phagocytosed by AA, CA and control macrophages. The CA macrophages phagocytosed significantly more neurons compared to both AA and control macrophages.

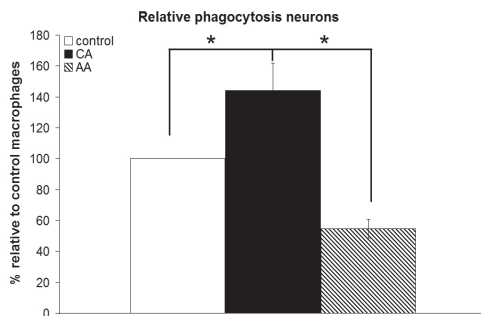


Figure 1: Phagocytosis of primary neurons. Phagocytosis of neurons by macrophages was established by FACS analysis after 4 h exposure to calcein labeled neurons. Phagocytosis, as determined by the amount of fluorescence per cell (mean fluorescence index (MFI)), was expressed as a percentage of the MFI obtained using control macrophages. Data are the mean of 4 separate experiments and expressed as percentage \pm SEM. $*=p<0.05$. CA macrophages phagocytosed significantly more neurons compared to both AA and control macrophages.

Next we determined whether macrophages could phagocytose neuronal particles, as a model for neuronal debris in MS lesions. The results showed that neuronal particles were phagocytosed by AA, CA and control macrophages. The CA macrophages phagocytosed significantly more neuronal particles compared to both AA and control macrophages (Figure 2A). Interestingly, no difference could be observed in the percentage of macrophages that had phagocytosed neuronal particles between the subtypes (Figure 2B).

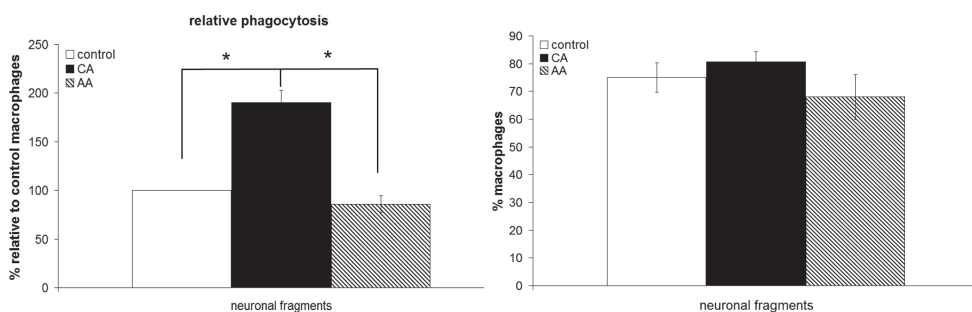


Figure 2: Phagocytosis of neuronal particles. Macrophages were exposed to fluorescently labeled neuronal particles for 1.5 h before phagocytosis was assessed by FACS analysis. The data is expressed as percentage of MFI obtained using control macrophages. The data are the mean of 6 separate experiments (n=6) and are expressed as percentage \pm SEM. $*=p<0.05$. A) The mean MFI of CA macrophages was significantly higher compared to control and AA macrophages. B) The percentage of macrophages positive in the FACS analysis is equal for all phenotypes.

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Phagocytosis of beads and myelin

Next, we investigated whether the increased phagocytic capacity of CA macrophages was a characteristic feature of CA macrophages. Therefore we determined phagocytosis of myelin and latex beads in control, CA and AA macrophages.

For the highest concentration of latex beads, phagocytosis by CA and AA macrophages was similar (see Figure 3). The phagocytosis of beads by CA macrophages was significantly higher compared to AA macrophages at lower concentration of beads. Phagocytosis of myelin by CA macrophages was significantly higher compared to AA macrophages when 10 μg myelin was added (Figure 3). Adding 20 μg myelin led to increased phagocytosis in all subtypes of macrophages. However, CA macrophages still phagocytosed significantly more myelin compared to AA.

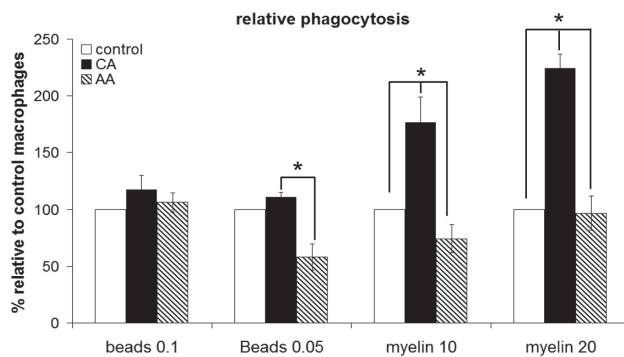


Figure 3: Phagocytosis of latex beads and myelin. Phagocytosis was determined by FACS analysis of macrophages after 1.5h exposure to fluorescently labeled beads and myelin. To compare, phagocytosis was expressed as percentage of MFI obtained using control macrophages. The data are the mean of 4 separate experiments (n=4) and are expressed as percentage \pm SEM. * $p < 0.05$. 0.05

$\mu\text{g/ml}$ beads led to a significantly higher phagocytosis of latex beads by CA macrophages compared to AA macrophages. Addition of both 10 and 20 $\mu\text{g/ml}$ myelin led to significantly higher phagocytosis by CA macrophages compared to both control and AA macrophages.

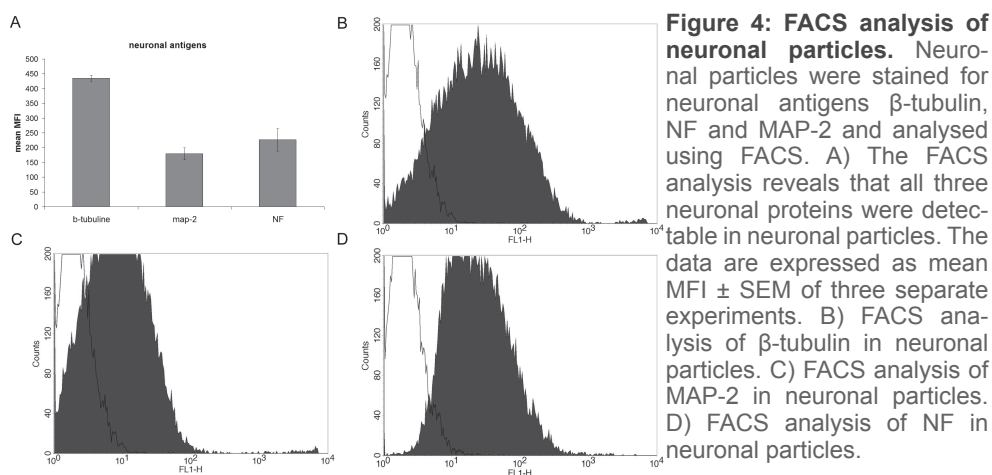
Degradation of neuronal antigens

Next, we determined the time line of degradation of neuronal antigens. First, the presence of neuronal proteins in the neuronal particles was assessed by FACS analysis. The neuronal particles contained high amounts of β -tubulin and lower amounts of MAP-2 and NF (Figure 4).

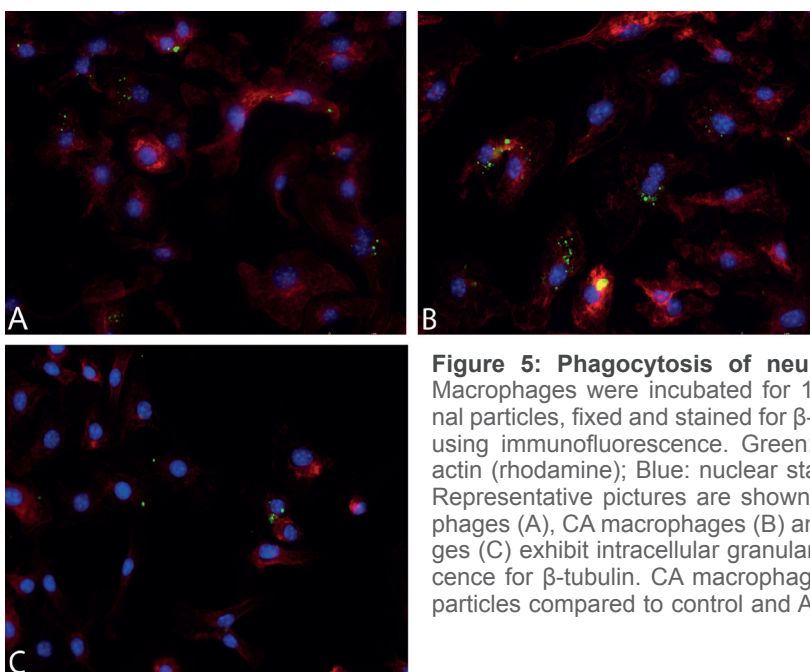
To determine the time frame of neuronal antigen degradation macrophages were incubated with 10 μg of unlabeled neuronal particles for 1.5, 4, 24 and 48h. Using immunofluorescent staining for β -tubulin, NF and MAP-2 the degradation of these neuronal antigens was established. Representative images are shown of macrophages with immunoreactivity for the β -tubulin antibody at 1.5h of incubation with neuronal particles. The presence of β -tubulin particles inside the macrophages, confirms that macrophages have phagocytosed neuronal particles (Figure 5A-C).

The percentage of macrophages positive for the neuronal antigen β -tubulin showed a similar pattern over time in all the macrophage subtypes (Figure 6A). Similar percentages of macrophages were positive for β -tubulin in all macrophage subtypes. Immunoreactivity for β -tubulin was present after 1.5 h and a higher

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percentage of macrophages was positive for β -tubulin after 4 h. After 24 h a significant reduction of approximately 75% in the percentage of macrophages positive for β -tubulin was observed. After 48 h almost no macrophages were positive for β -tubulin. Similarly in the first 4 h the NF reactivity increased. The NF reactivity was significantly reduced, approximately 70%, after 24 h and almost no macrophages positive for NF were present after 48 h (Figure 6B). The degradation of MAP-2 seemed to be a little slower, in that a significant decrease, of approximately 70%, in the percentage of macrophages positive for MAP-2 could only be observed after 48 h (Figure 6C).



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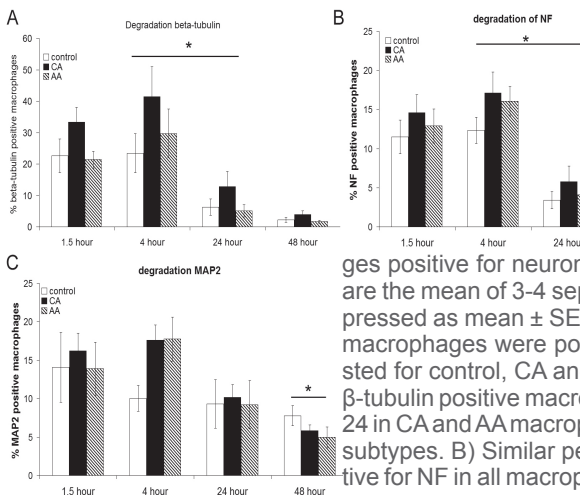


Figure 6: Degradation of neuronal antigens. Macrophages on coverslips were incubated with neuronal particles for 1.5, 4, 24 or 48 h. Afterwards, the macrophages were fixed and stained with either β -tubulin, NF or MAP-2. The percentage of macrophages

positive for neuronal antigens was determined. The data are the mean of 3-4 separate experiments ($n=3-4$) and are expressed as mean \pm SEM. $*=p<0.05$. A) Similar percentages of macrophages were positive for β -tubulin at all time points tested for control, CA and AA macrophages. The percentage of β -tubulin positive macrophages was significantly reduced after 24 h in CA and AA macrophages and after 48 h in all macrophage subtypes. B) Similar percentages of macrophages were positive for NF in all macrophage subtypes at all time points tested.

After 24h the percentage of macrophages positive for NF was significantly reduced after 24 h and 48 h in all macrophage subtypes. C) The percentage of macrophages positive for MAP-2 was similar for all macrophage subtypes at all time points tested. Only after 48h of incubation a significant decrease in the percentage of macrophages positive for MAP-2 was observed.

DISCUSSION

Next to demyelination, axonal damage plays an important role in MS, leading to the presence of axonal debris. Little research has been performed on the phagocytosis of neurons and neuronal debris by macrophages. The aim of this study was to determine whether the phagocytosis and subsequent degradation of neurons and neuronal particles differed between CA and AA macrophage subtypes.

First we showed that phagocytosis of whole neurons was significantly higher in CA macrophages compared to AA macrophages. Parnaik et al.²⁴ showed that microglial cells phagocytose apparently healthy cerebellar granule neurons, suggesting that microglial cells can recognize dying cerebellar granule neurons, before these neurons show the characteristic morphological features of apoptosis. Increased recognition of neurons by macrophages may have occurred in our experiments, since the primary neurons were harvested using trypsin leading to cleavage of surface molecules. This alters the molecular pattern recognized by macrophage receptors. Also a lack of synaptic transmission in neurons can lead to degeneration and changes in cell surface molecule expression⁴²⁻⁴⁴.

We observed that CA macrophages were most efficient in phagocytosing neuronal particles. In line with our data, systemic injection of LPS, a classical activator of macrophages, has been shown to enhance phagocytic activity of macrophages during Wallerian degeneration⁴⁵ and clearance of axonal debris by microglial cells²¹.

Similarly to neuronal particles, CA macrophages phagocytosed myelin in higher amounts compared to AA and control macrophages. This might be due to the fact that macrophages stimulated with IFN- γ and LPS increase the expression of complement receptor 3 (CR3) also called Mac-1⁴⁶, which has been shown to be

involved in myelin phagocytosis⁴⁷. The increased phagocytic capacity could also be a characteristic feature of CA macrophages. The intrinsic phagocytic ability, as determined using latex beads, was found to be greater in CA macrophages compared to AA macrophages, however, no difference was observed when comparing control and CA macrophages using latex beads. Using biological substrates, such as myelin and neuronal particles, the difference in phagocytic ability was much more evident since CA macrophages phagocytosed significantly more myelin and neuronal particles compared to both AA and control macrophages. The percentage of macrophages that phagocytosed neuronal particles was similar between macrophage subtypes, however CA macrophages ingested significantly more particles per macrophage. This again indicates that CA macrophages had a higher phagocytic ability compared to AA macrophages. In line with our data, stimulating macrophages with IFN- γ and LPS was found to induce a higher level of phagocytosis compared to macrophages stimulated with IL-4^{36,48}.

Alternative ways to induce the AA phenotype, than used in our study, are by adding glucocorticoids and IL-10, which led to increased phagocytosis^{35,49}. These contrasting results with the AA phenotype suggest that there are different types of AA macrophages depending on the stimulation protocol. The AA macrophages have been grouped into 2 subsets: (i) wound healing macrophage, generated by IL-4 or IL-13 and (ii) the regulatory macrophage, generated by exposure to glucocorticoids or IL-10²⁵, which have been found to be functionally distinct²⁵.

We also determined the time-frame of neuronal antigen degradation using FACS analysis. Macrophages positive for β -tubulin and NF staining were observed after 1.5 and 4 h of incubation. After 24 h of incubation the number of positive macrophages was significantly decreased, indicating that β -tubulin and NF degradation took place within 24 h of ingestion. Likewise, myelin oligodendrocyte glycoprotein (MOG) staining disappears within 24 h after phagocytosis⁵⁰, which makes it a useful marker to detect recent myelin phagocytosis. Based on our current data, the intracellular staining of β -tubulin or NF indicates recent axonal debris ingestion. In the cervical lymph nodes of MS patients NF heavy staining has been observed in macrophages²². Our data suggest that these macrophages have ingested the axonal debris less than 24 h prior to arriving in the cervical lymph node. This can be explained by the fact that MS patients with active lesions were chosen specifically for that study²². The degradation of MAP-2 was slower than for β -tubulin and NF, since a significant decrease in percentage of macrophages positive for MAP-2 was observed only after 48 h. Altogether, these data indicate that neuronal antigens are degraded rapidly within macrophages and no difference was observed in degradation between activation phenotypes.

The results of this study suggest that CA macrophages are more effective in phagocytosing intact primary neurons *in vitro* than AA macrophages. This may indicate that classical activation in combination with minor damage to neurons could be a factor contributing to increased neuronal damage. On the other hand effective removal of dead material, such as neuronal and myelin debris which can inhibit axonal regeneration^{21,51}, may contribute to regeneration.



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