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CHAPTER

3

Classically and alternatively activated macrophages differ in migratory properties in the CNS

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Manuscript in preparation



ABSTRACT

Macrophages play an important role in multiple sclerosis (MS), being involved in both damage and repair during lesion formation. The divergent effects of macrophages might be explained by the differences in activation status: classically activated (CA/M1), pro-inflammatory, macrophages and alternatively activated (AA/M2), growth promoting, macrophages. Little is known about the actions of these subpopulations of macrophages in the central nervous system (CNS). The aim of this study is determining the functional characteristics of these phenotypically different macrophages, generated by two day exposure to either IFN- γ and LPS (CA) or IL-4 (AA), in the context of the CNS, with respect to migration, motility and adhesion.

The results show that CA and AA macrophages behave differently under the influence of CNS cells. By studying migration towards conditioned medium it was found that AA macrophages were preferentially attracted towards neuronal conditioned medium (NCM) and the chemokine CXCL12, while CA macrophages were attracted in higher numbers towards astrocyte- and oligodendrocyte conditioned medium. Intrinsic motility was higher in AA macrophages, while adhesion, for example to extracellular matrix molecules (ECM), was higher in CA macrophages.

In conclusion, since AA macrophages are more motile and are attracted by NCM, they probably migrate easily towards neurons in the CNS and have a growth promoting effect. Due to the lower motility and strong adhesion to ECM of CA macrophages, the area of bystander damage induced by reactive oxygen species (ROS) and pro-inflammatory cytokines could be limited. In MS this might indicate that AA macrophages play a role in neuronal repair. The restricted migration and motility of CA macrophages might limit lesion expansion.

INTRODUCTION


Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) and the most common cause of neurological disability in young adults. Major neuropathological hallmarks of MS are inflammatory demyelinating lesions with perivascular infiltrates containing macrophages and lymphocytes. MS lesions are further associated with oligodendrocyte death, blood-brain barrier disturbance, axonal damage and loss, and glial scar formation due to astrogliosis^{1,2}.

It is widely accepted that macrophages play an important role during MS pathogenesis and both detrimental and beneficial effects were found during MS and experimental autoimmune encephalomyelitis (EAE), an animal model for MS. Evidence for a detrimental role for macrophages in MS was found in several studies. First, a correlation was found in MS between location and amount of axonal damage and loss, and cellular infiltrates containing macrophages³⁻⁵. Secondly, in MS lesions a decrease in expression of macrophage inhibitory molecules, CD200 and CD47, was found indicating that hampered inhibition of macrophages could play a role in lesion formation during MS⁶. Furthermore, matrix metalloproteinases (MMPs), produced by macrophages in MS lesions⁷, could contribute to axonal damage and loss seen in MS lesions⁸. Several studies have also implicated macrophages in disease progression of EAE. Elimination of infiltrating macrophages by clodronate liposomes suppressed the clinical signs of EAE^{9,10}. Blocking the signal of CD200/CD200R between neurons and macrophages led to an aggravated EAE course¹¹, indicating a direct link between reduced inhibition of macrophages and increased severity of clinical course of EAE.

All these studies suggest that macrophages play a detrimental role, but also neuroprotective effects have been reported for macrophages as well. Phagocytosis of myelin debris is important for axonal repair/regrowth and remyelination, since myelin debris is found to be growth inhibiting^{12,13}. Furthermore, activated macrophages have been found in areas with increased growth associated protein-43 (GAP-43), a marker for axonal growth, expression in MS lesions indicating that macrophages could affect axonal growth¹⁴. In MS lesions, activated macrophages/microglia are found to be sources of neurotrophins and growth factors, like brain derived neurotrophic factor (BDNF)^{15,16}, that actively promote remyelination, axonal repair and regrowth. Elimination of infiltrating macrophages reduced remyelination in demyelinating models¹⁷. Finally, *in vitro*, foamy, myelin-laden macrophages express anti-inflammatory cytokines and inhibit the response to pro-inflammatory stimuli¹⁸. In MS lesions foamy macrophages express anti-inflammatory molecules¹⁸, indicating that foamy macrophages could be involved in limiting lesion expansion.

Macrophages are not a homogeneous cell population, several phenotypes exist dependent on their activation status^{19,20}. The two phenotypes that are considered to be the most extreme are classically activated (CA/M1) pro-inflammatory macrophages and alternatively activated (AA/M2) or growth promoting macrophages. In tissues, the micro-environment of the macrophages is thought to determine the phenotype that develops²⁰. *In vitro*, cytokines and other stimuli induce these activation phenotypes. In culture, CA macrophages are induced by interferon-





gamma (IFN- γ) and lipopolysaccharide (LPS), while AA macrophages are induced *in vitro* by interleukin (IL)-4/IL-13^{19,21}. These subtypes have different functions in tissue repair and inflammation. The CA macrophages produce nitric oxide (NO) and reactive oxygen species (ROS) making them cytotoxic^{19,22}. Furthermore, they secrete high amounts of pro-inflammatory cytokines, for example IL-12, promoting inflammation. On the other hand, AA macrophages produce the anti-inflammatory cytokine IL-10^{19,20}, thereby reducing inflammation. AA macrophages have a higher angiogenic potential compared to CA macrophages²³. They also produce growth factors, such as platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)²³⁻²⁵. AA macrophages can induce tumour progression and metastasis^{24,26,27}. In mice, AA macrophages express high levels of arginase and the activity of this enzyme is also increased. Due to the activation of arginase, arginine is converted to ornithine, a precursor for polyamines and collagen, which contributes to the production of extracellular matrix (ECM)²⁸⁻³¹ and promotes cell growth^{30,32}. Together these features determine the wound healing and growth promoting phenotype of AA macrophages^{19,20}.

Little research has been done on AA and CA macrophages in relation to the pathogenesis of MS. Some researchers have found evidence for CA macrophages in MS lesions, since inducible nitric oxide synthetase (iNOS) positive macrophages were present at the active rim of the lesion³³. Mannose receptor (MR) positive macrophages, which are considered to be AA due to the expression of anti-inflammatory molecules, were present in the center of the lesion¹⁸. Due to the differences in localization in MS lesions, we expected that CA and AA macrophages migrated differently and could be attracted by distinct cell types in the CNS. Nothing is known about these migratory characteristics of these macrophages in the CNS.

The aim of the current study was to determine the functional characteristics of CA and AA macrophages, with respect to migration, motility and adhesion, in the context of the CNS. We demonstrate for the first time that CA and AA macrophages behave differently under influence of the conditioned media of CNS cells. By studying migration towards conditioned medium it was found that AA macrophages were more attracted towards neuronal conditioned medium, while CA macrophages were more attracted towards astrocyte- and oligodendrocyte (precursor)- conditioned medium. The chemokine CXCL12 was found to attract AA macrophages significantly more compared to CA macrophages. AA macrophages were more motile and adhered less compared to CA macrophages. The CA macrophages strongly adhered to the ECM. In neurodegenerative diseases, the restricted migration of CA macrophages may limit the amount of damage induced by CA macrophages. The higher migratory capacity of AA macrophages and attraction by neurons may contribute to increased neuronal repair.

MATERIALS AND METHODS

Animals

For neuronal and astrocyte cultures timed pregnant C57BL/6 mice were obtained from Charles River (Maastricht, The Netherlands). For isolation of bone

marrow, adult C57BL/6 mice were used from Charles River.

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).

Macrophages

Bone marrow derived macrophages were generated as described previously³⁴. Bone marrow was flushed from femurs and tibias of C57/BL6 mice and cultured for 1 week in complete macrophage medium (Dulbecco modified Eagle's minimal essential medium (DMEM) (Invitrogen, Breda, the Netherlands) supplemented with 10% foetal calf serum (FCS) (Invitrogen), 15% conditioned medium from macrophage-colony stimulating factor-secreting L929 fibroblasts and 2% penicillin/streptomycin-glutamine (Lonza, Breda, the Netherlands) at 37°C. After 7-10 days in culture adherent cells were approximately 95% pure macrophages and cells were used for experiments.

The CA phenotype was induced by exposing macrophages for two days to 5×10^3 U/ml IFN- γ (U-Cytech, the Netherlands) and 10 ng/ml *Escherichia coli* LPS (O26:B6; Sigma-Aldrich, Zwijndrecht, the Netherlands) in the culture medium. AA macrophages were prepared by exposure to 10 ng/ml IL-4 (Invitrogen) in the culture medium^{21,35}. Control macrophages were cultured for the same period in medium alone.

Macrophages were harvested by 15 min incubation at 37°C with lidocaine (4 mg/ml in PBS; Sigma-Aldrich). Macrophages were washed and centrifuged 5 min at 170 g. These macrophages were used in different experiments.

Conditioned media

Neuronally conditioned medium (NCM) was derived from neurons of C57/BL6 primary mouse CNS. Embryonic day 19 mouse pups were sacrificed and the brain was isolated. For neuronal cultures, the cortex was isolated and incubated with trypsin containing 0.1 mg/ml DNase for 15 min at 37°C. The cell suspension was extensively washed and the neurons were triturated to create a single cell suspension. Neurons were cultured in complete neurobasal medium, consisting of incomplete neurobasal medium with 1% glutamax, 2% B27 and 0.01% gentamycin (all obtained from Invitrogen), at a concentration of 1×10^5 cells/ml. Beta-tubulin (Covance, the Netherlands) staining was performed and cultures were found to be approximately 90% pure (data not shown). After 2 days in culture NCM was harvested.

For astrocyte conditioned medium (ACM), the forebrain cortex was isolated and single cell suspension was generated. Cells were cultured for 1 week in complete medium which consisted of DMEM with high glucose, supplemented with 1% glutamax, 10% FCS and 0.01% gentamycin. After 1 week the culture was washed and new medium was added. Cultures were characterized using glial fibrillary acidic protein (GFAP) staining and were found to be approximately 85% pure. After 2 days ACM was harvested.



Oligodendrocyte conditioned medium (OCM) was harvested during oligodendrocyte development. Primary rat oligodendrocytes were cultured as described previously ³⁶. Briefly, oligodendrocyte precursors were cultured on poly-L-lysine coated cell culture plates for 2 days in a defined SATO medium ³⁷ containing platelet derived growth factor-AA (PDGF-AA) and fibroblast growth factor-2 (FGF-2) in order to synchronize precursors to the bipolar oligodendrocyte-type II astrocyte (O2A) stage. Differentiation was induced by replacing the growth factors with 0.5% FCS in SATO medium. Medium was harvested from: (i) cells in the O2A stadium (designated as O2A medium); (ii) cells that had differentiated from O2A to the galactocerebroside (GC) stage (3 days differentiation, GC medium); (iii) cells that differentiated from GC to myelin basic protein (MBP) positive stage (7 days differentiation, MBP medium); finally from cells that had developed from MBP to MBP+ stage (10 days differentiation, designated MBP+ medium).

Fractioning of the conditioned media

In order to determine the range of molecular weight of the factors responsible for attraction of macrophages, the conditioned media were fractioned based on molecular weight. Aliquots of the conditioned media were filtered using 10, 50 and 100 K Amicon Ultra centrifugal filter units as described by the manufacturer (Millipore, Amsterdam, the Netherlands). The aliquots were first filtered using the 10kD filter, subsequently 50 and 100 kD, creating three fractions: one containing low molecular weight (< 10 kD), one with intermediate molecular weight (between 10 and 50 kD) and a fraction from 50 to 100 kD. The fractions were reconstituted in half of the original volume.

Migration: blind well chamber

The migratory capacity of CA and AA macrophages was studied using a 48-wells micro chemotaxis chamber (Neuro Probe, Gaithersburg, USA) as described previously ³⁸ with some modifications. In the bottom well, 25 μ l of the conditioned media, control medium, monocyte chemotactic peptide-1 (MCP-1) (20 ng/ml; Peprotech inc, London, UK), formyl methionineleucyl-phenylalanine (fMLP) (10 nM; Sigma-Aldrich), CXCL12 (300 ng/ml; R&D systems, Abingdon, UK) or CCL5 (1ng/ml; R&D systems) was added. A filter with a pore size of 10 μ m was used. In the upper chamber differently activated macrophages were added. The macrophages were left to migrate for 6 h. The side of the filter in direct contact with the upper chamber was washed and scraped clean of cells and the filter was subsequently stained using Coomassie blue. The number of migrated cells was counted per 0.1 mm² using a scored eyepiece. From this the total number of migrated cells was calculated.

Motility

CA and AA macrophages, after harvesting, were seeded in culture plastic 96 wells plate (Greiner Bio One, Alphen a/d Rijn, the Netherlands) and cultured for 1 h. The 96-wells plate was placed in a time-lapse videomicroscope. Per well the

macrophages were followed for 15 min and images were taken every 20 seconds at a 40 times magnification.

The software program Track-It® was used to determine motility. All the cells present in the field were tracked manually.

Adhesion

Macrophage adhesion to plastic and several extracellular matrix molecules (ECM) was determined as described previously³⁹. CA and AA macrophages were harvested and labelled with 1 μ M BCECF-AM (Invitrogen) for 15 min at 37°C. After labelling, cells were washed and 100 μ l of 1×10^6 macrophages were seeded in the 96 wells plates. Macrophages were left to adhere for 2 h at 37°C and 5% CO₂. After incubation, non-adherent cells were removed by washing 3 times in PBS and the remaining adherent cells were lysed with 0.1 N NaOH. Fluorescence was measured in a Fluostar24 (BMG labtechnologies, Offenburg, Germany). A standard curve with various cell concentrations ranging from 5×10^3 to 1×10^6 cells/ml was used to determine the percentage of adhering cells.

To determine the adherence of differently activated macrophages to different ECM molecules, wells of a 96-well culture plate were precoated with either collagen (type I from calf skin, Sigma-Aldrich), for 1 h at room temperature, or fibronectin (derived from human plasma; Roche, Almere, the Netherlands), for 1 h at 37°C.

Actin cytoskeleton

The actin cytoskeleton of the differently activated macrophages was visualized using rhodamine phalloidin. The macrophages were cultured on glass coverslips and stimulated in order to generate the different phenotypes. After 2 days macrophages were fixed by incubation for 30 min with paraformaldehyde (4% in PBS). The macrophages were washed twice with PBS and exposed to rhodamine phalloidin (1:300, Sigma-Aldrich) in PBS. To visualize the nuclei, the cells were counterstained with Hoechst (Invitrogen) and embedded in mounting medium.

Statistical analysis

The data are expressed as mean of 3 to 4 separate experiments performed in duplo (\pm SEM). Statistics were performed in SPSS (15.0.0, Chicago, USA). The motility, migration and adhesion experiments were analysed using one-way ANOVA with Bonferroni correction. A p-value of less than 0.05 was considered significant.

RESULTS

CA and AA macrophage migration towards conditioned media from CNS cells

To investigate whether CA and AA macrophages migrate towards different CNS cell types, a blind well chamber was used. Macrophages migrated over a

filter in response to conditioned medium from neurons, astrocytes and several developmental stages of oligodendrocytes.

AA macrophages migrated in significantly higher numbers towards NCM compared to the control neurobasal medium and CA macrophages (Figure 1A). Migration of CA macrophages was significantly enhanced towards ACM compared to control medium (Figure 1B). AA macrophages did not migrate in higher numbers towards ACM compared to control medium. No difference was observed in the number of AA and CA macrophages migrating towards ACM.

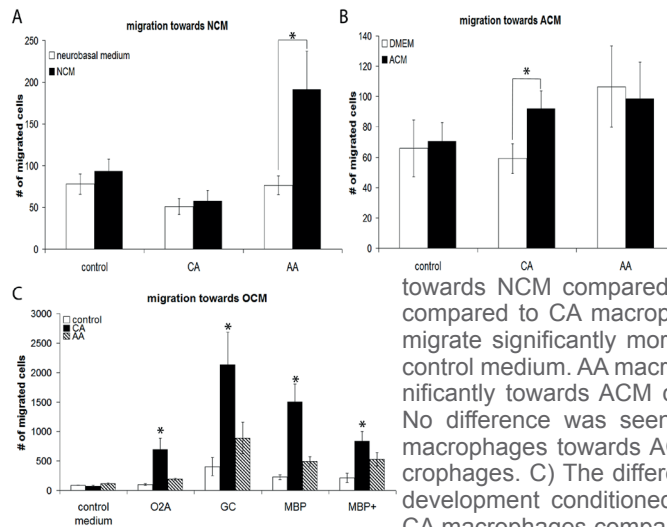


Figure 1: Migration of macrophages towards conditioned medium from several CNS cell types. The data are expressed as the mean of 4 separate experiments (n=4) ± SEM. * = p < 0.05. A) AA macrophages migrate significantly more towards NCM compared to control medium and also compared to CA macrophages. B) CA macrophages migrate significantly more towards ACM compared to control medium. AA macrophages are not attracted significantly towards ACM compared to control medium. No difference was seen in the number of migrated macrophages towards ACM between CA and AA macrophages. C) The different stages of oligodendrocyte development conditioned medium significantly attract CA macrophages compared to oligodendrocyte

medium. AA macrophages also migrate significantly towards oligodendrocyte conditioned medium, however CA migrate significantly more compared to AA and control macrophages. The asterisk signifies the difference in migration between CA and AA macrophages.

The conditioned media of different stages of oligodendrocyte development were tested, from precursors to mature oligodendrocytes. Conditioned media from all stages of oligodendrocyte development significantly attracted all subtypes of macrophages compared to the control medium (Figure 1C). CA macrophages were attracted in significantly higher numbers compared to both control and AA macrophages.

Size fractioning of NCM and ACM

In order to determine the molecular weight range of the factors that could attract the macrophages, NCM and ACM were fractioned according to molecular weight. The NCM fraction containing proteins smaller than 10 kD attracted AA macrophages significantly more compared to the neurobasal control medium. This fraction of the NCM attracted AA macrophages significantly more compared to CA macrophages. Both 50 and 100 kD fractions attracted CA and AA macrophages in comparable numbers and did not attract macrophages in higher numbers compared to control neurobasal medium. Similar results were observed for ACM. The ACM fraction

Migration of CA and AA macrophages in the CNS

containing molecules smaller than 10 kD attracted CA macrophages significantly more compared to control DMEM medium and AA macrophages (Figure 2). Fractions containing larger proteins than 10 kD did not significantly attract more macrophages than medium alone.

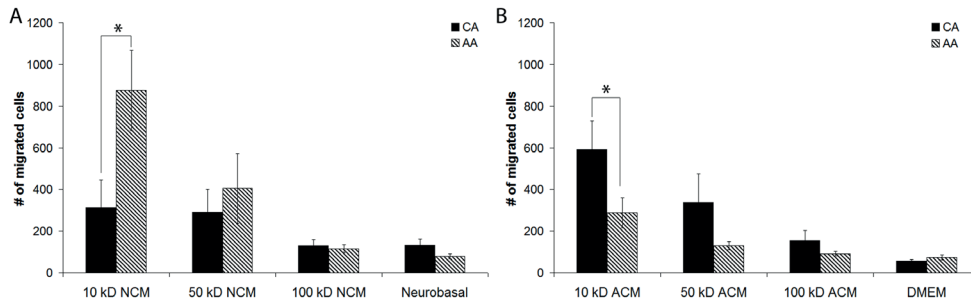


Figure 2: Fractionation of the conditioned media. The data are expressed as the mean of 4 separate experiments ($n=4$) \pm SEM. A) The fraction of the neuronal conditioned medium containing proteins smaller than 10 kD attracted AA macrophages significantly more compared to CA macrophages. Both the fractions containing proteins smaller than 10 kD and between 10 and 50 kD significantly attracted AA macrophages more compared to control medium. B) The fraction of the ACM containing proteins smaller than 10 kD significantly attracted the CA macrophages and attracted CA more compared to AA macrophages.

Attraction by chemokines

The fact that smaller fractions of the conditioned medium attracted the macrophages indicated that chemokines might play a role. We therefore tested whether CXCL12, a chemokine produced by neurons in the normal CNS, and CCL5, upregulated during MS, would attract CA and AA macrophages. CCL5 attracted AA macrophages significantly more compared to CA macrophages (Figure 3). However, the macrophages were not attracted significantly more towards CCL5 compared to the control medium. A significantly higher number of AA macrophages was attracted by CXCL12 compared to CA macrophages. AA macrophages were attracted significantly more towards CXCL12 compared to the control medium.

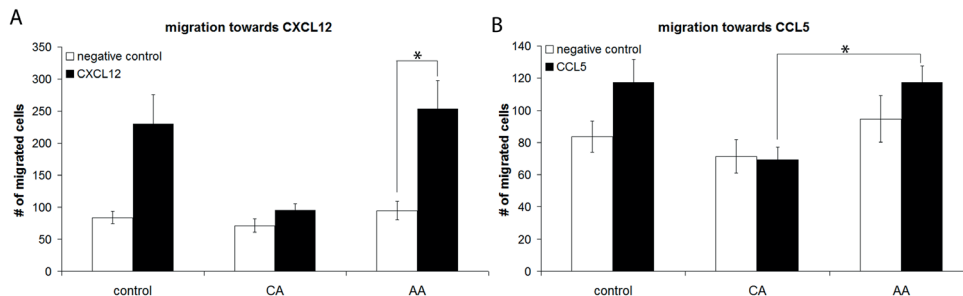


Figure 3: Migration towards chemokines. The data are expressed as the mean (4 separate experiments ($n=4$)) \pm SEM. $p < 0.05$. A) The chemokine CXCL12 attracted AA macrophages significantly more compared to both control medium and CA macrophages. B) The chemokine CCL5 did not induce migration significantly in both AA and CA macrophages.

AA macrophages have a higher motility than CA macrophages

Next, motility of CA and AA macrophages was determined using a time-lapse video microscope. The motility of macrophages on culture plastic was determined. The CA macrophages appeared to move very little (Figure 4). AA macrophages were significantly more motile compared to CA macrophages.

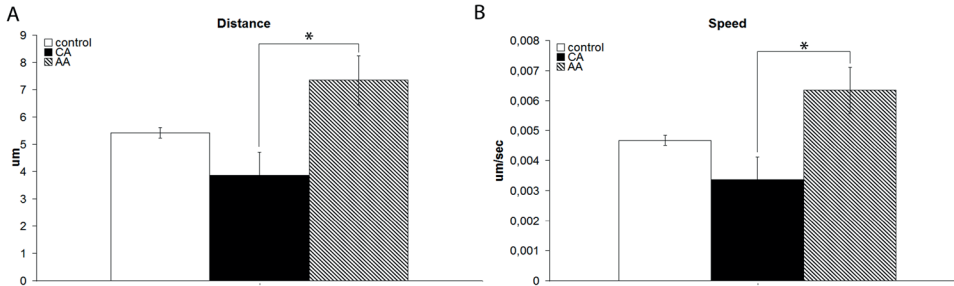


Figure 4: AA macrophages have a higher migratory capacity compared to CA macrophages. The data are the mean of 3 separate experiments (n=3) ± SEM. *= p<0.05. A) During the recorded time the average distance AA macrophages moved was significantly larger compared to CA macrophages. The distance was recorded in µm. B) AA macrophages moved at a significantly higher speed compared to CA macrophages. Speed was recorded in µm/sec.

fMLP and MCP-1 induced migration is comparable between CA and AA macrophages

Since motility was lower in CA macrophages compared to AA macrophages, we wanted to determine whether the intrinsic migratory capacity of the macrophages differed. Migration towards the potent macrophage attractors MCP-1 and fMLP, a synthetic analogue of a bacterial signal peptide, was used to determine the migratory capacity of CA and AA macrophages. As can be seen in figure 5, AA and CA macrophages migrated in comparable numbers towards fMLP and MCP-1.

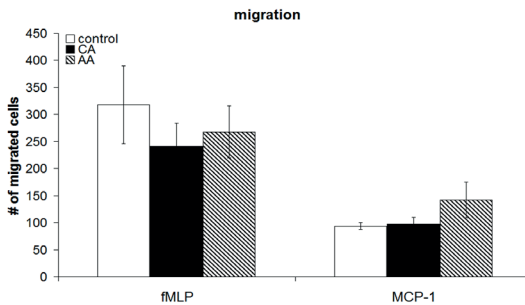


Figure 5: Migratory capacity under influence of fMLP and MCP-1. The data are the mean of 3 separate experiments (n=3) ± SEM. No difference in migration was seen between the different macrophage phenotypes when comparing migration towards either fMLP or MCP-1.

Adherence of CA macrophages is higher compared to AA

Since AA macrophages displayed a higher motility, we investigated whether this was related to decreased adherence. Therefore, the adhesion capacity of the differently activated macrophages to culture plastic and ECM molecules was measured. On culture plates, no significant difference between CA and AA macrophages was observed (figure 6). After coating the culture plates with either

Migration of CA and AA macrophages in the CNS

collagen or fibronectin, the percentage of CA macrophages that adhered was significantly higher compared to both control and AA macrophages. Fibronectin showed even higher adhesion percentages compared to collagen.

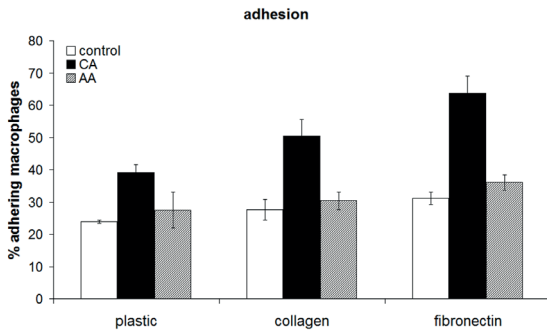


Figure 6: CA macrophages adhere more compared to AA macrophages. The data are the mean of 3 separate experiments ($n=3$) \pm SEM. $*=p<0.05$. After 2 h of incubation 24% (± 1) of control macrophages adhere to plastic, 39% (± 3) of CA and 29% (± 6) of AA macrophages. Using extracellular matrix molecules, such as collagen and fibronectin, adherence of CA macrophages was significantly higher compared to either control or AA macrophages. Control macrophages

adhered 28% (± 3) to collagen and 31% (± 2) to fibronectin, whereas CA macrophages adhered 50% (± 5) to collagen and 64% (± 5) to fibronectin. AA macrophages show an adherence of 30% (± 3) to collagen and 36% (± 2) to fibronectin.

Actin cytoskeleton of CA and AA macrophages

Migration, motility and adhesion are all linked to the cytoskeletal organization of cells. Therefore we tested if differences in the cytoskeleton could be observed between the different activation subtypes. To examine the cytoskeleton and morphology of CA and AA macrophages, they were cultured on coverslips and stained using rhodamine phalloidin (figure 7). CA macrophages appeared spread out, flattened and rounded. AA macrophages were elongated and appeared stretched. Control macrophages had adopted a morphology in between the two extremes. Some cells were elongated and spindle shaped, while others appeared more rounded. The rhodamine phalloidin staining showed clustering of the cytoskeleton around the nucleus in CA macrophages. This clustering of actin around the nucleus was not observed as frequently in AA macrophages. In AA macrophages the rhodamine staining was more prominent at the border of the cell.

DISCUSSION

In this study we focused on migration characteristics of CA and AA macrophages in the CNS. In the periphery different activation phenotypes of macrophages have different effects in inflammation²⁰. CA macrophages are considered to be pro-inflammatory, due to the secretion of pro-inflammatory cytokines and production of NO and ROS¹⁹. The anti-inflammatory actions of AA macrophages have been described in an excellent review³⁵. The AA macrophages produce anti-inflammatory cytokines^{19,35}, growth factors^{15,32} and do not produce pro-inflammatory cytokines¹⁹. The functional consequences of differential activation of macrophages for cytoskeleton-associated functions such as migration, adhesion and motility were unknown until now. These properties may have considerable impact on the local effects of macrophage subtypes during MS.

This study shows that AA and CA macrophages differ in the CNS cell types they



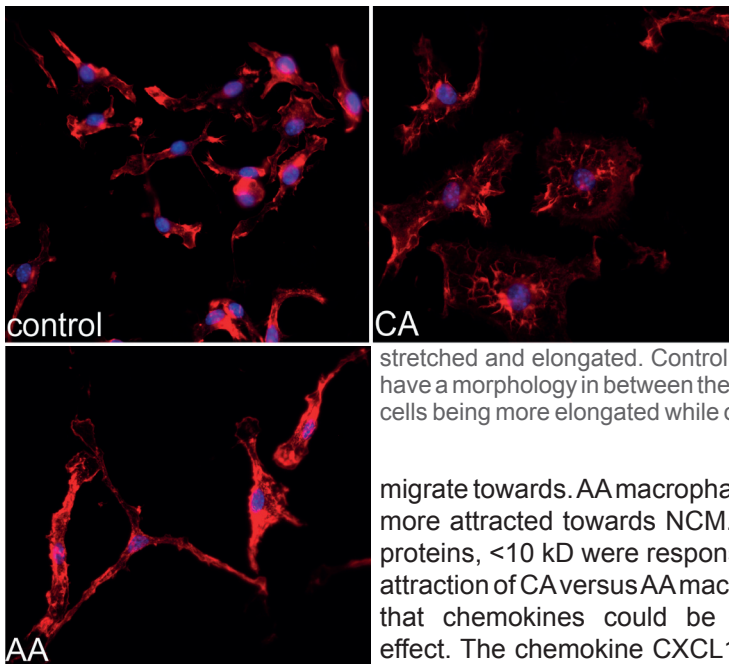


Figure 7: Morphology of differently activated macrophages. Red: actin (rhodamine phalloidin); Blue: nuclear staining (Hoechst). Representative pictures are shown. CA macrophages appear flattened and spread out. They are rounded. AA macrophages are more stretched and elongated. Control macrophages appear to have a morphology in between the two extremes, with some cells being more elongated while others are more rounded.

migrate towards. AA macrophages were significantly more attracted towards NCM. Especially the small proteins, <10 kD were responsible for this selective attraction of CA versus AA macrophages, suggesting that chemokines could be responsible for this effect. The chemokine CXCL12, is expressed both in cultured neurons⁴⁰ and in the normal brain⁴¹. In

the present study, CXCL12 was found to attract AA macrophages significantly more compared to CA macrophages, indicating that this chemokine could be involved in the differential attraction towards NCM. These findings indicate that chemokine receptor expression could be different between the different types of macrophages. In human macrophages differences in chemokine receptor expression have been found between CA and AA macrophages. The AA macrophages have increased expression levels of CXCR4, the receptor for CXCL12, while CA macrophages have higher expression levels of CCR7⁴². The CA macrophages were significantly attracted towards ACM and oligodendrocyte conditioned medium. Using the ACM, again, small proteins, <10 kD, in the conditioned media attracted the macrophages, suggesting that astrocytes release cytokines or chemokines that attract macrophages. Since CCL5 is a chemokine that is upregulated in astrocytes and endothelium in the brain during MS⁴³, we determined the chemotactic potential of CCL5 on CA and AA macrophages. In our experiments, CCL5 did not significantly enhance migration of either CA or AA macrophages, therefore we concluded that CCL5 is not the chemokine in ACM that attracts CA macrophages.

Our results showed that the differences in migration were not due to intrinsic differences in migratory capacity, since fMLP and MCP-1 attracted both types of macrophages in comparable levels. Both fMLP and MCP-1 are very potent chemoattractants^{44;45}. Subtle differences in chemoattraction, due to differences in chemokine receptor expression, might be masked due to high potency of these molecules.

In addition to chemotactic migration we investigated differences in morphology, cytoskeletal arrangement and spontaneous motility. Motility was found to be

significantly higher in AA macrophages compared to CA macrophages. The morphology of AA macrophages appeared to be more elongated, whereas CA macrophages were more rounded. This is in line with findings in human M1 and M2 macrophages. In human M2 macrophages, analogous to AA macrophages, were found to appear stretched with a spindle-like morphology, while human M1, analogous to CA macrophages, macrophages appeared rounder with a “fried-egg” morphology ⁴⁶. The cytoskeletal organization differed in that CA macrophages showed clustering of actin cytoskeleton around the nucleus, while the actin cytoskeleton of AA macrophages was more prominent at the border of the cell. These results indicate that during the transition to AA macrophages become increasingly stretched and motile allowing migration into tissues, while transition to the CA phenotype leads to decreased motility and a round morphology, probably limiting migration.

Another factor important for the extent of migration, is the adhesive capacity of macrophages. Adhesion could also be influenced by the cytoskeletal organization ^{47,48}. Here we showed that CA macrophages adhered to a higher extent to culture plates and ECM molecules compared to AA macrophages, confirming that this might also have an impact on the increased motility and migration towards NCM of AA macrophages. Our findings are in line with a previous study where it was found that glucocorticoid stimulated murine monocytes, which display some features of AA macrophages, adhered less and were more motile than control cells ⁴⁹. These differences might be due to altered expression of adhesion receptors, since activation with LPS and IFN- γ increase the expression of adhesion molecules such as lymphocyte function associated antigen-1 (LFA-1), MAC1 and intercellular adhesion molecule-1 (ICAM1) ^{50,51}.

CA macrophages have a lower ability to migrate, since they adhere strongly to the ECM and are generally less motile, limiting the amount of bystander damage due to the ROS and NO secreted by the CA macrophages. Due to the fact that AA macrophages are more motile, adhere less to the ECM and are attracted by NCM, they can migrate towards neurons. They may then locally release growth factors where they are most needed. AA macrophages are considered to be growth promoting and can indeed secrete neurotrophic factors ¹⁵. Therefore, skewing macrophages towards an AA phenotype could be a great avenue for the development of new therapeutical strategies.

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