

RESEARCH

Open Access

Microglia use multiple mechanisms to mediate interactions with vitronectin; non-essential roles for the highly-expressed $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins

Jennifer V Welser-Alves, Amin Boroujerdi, Ulrich Tigges and Richard Milner*

Abstract

Background: As the primary resident immune cells, microglia play a central role in regulating inflammatory processes in the CNS. The extracellular matrix (ECM) protein vitronectin promotes microglial activation, switching microglia into an activated phenotype. We have shown previously that microglia express two vitronectin receptors, $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins. As these integrins have well-defined roles in activation and phagocytic processes in other cell types, the purpose of the current study was to investigate the contribution of these two integrins in microglial activation.

Methods: Microglial cells were prepared from wild-type, $\beta 3$ integrin knockout (KO), $\beta 5$ integrin KO or $\beta 3/\beta 5$ integrin DKO mice, and their interactions and activation responses to vitronectin examined in a battery of assays, including adhesion, expression of activation markers, MMP-9 expression, and phagocytosis. Expression of other αv integrins was examined by flow cytometry and immunoprecipitation.

Results: Surprisingly, when cultured on vitronectin, microglia from the different knockout strains showed no obvious defects in adhesion, activation marker expression, MMP-9 induction, or phagocytosis of vitronectin-coated beads. To investigate the reason for this lack of effect, we examined the expression of other αv integrins. Flow cytometry showed that $\beta 3/\beta 5$ integrin DKO microglia expressed residual αv integrin at the cell surface, and immunoprecipitation confirmed this finding by revealing the presence of low levels of the $\alpha v \beta 1$ and $\alpha v \beta 8$ integrins. $\beta 1$ integrin blockade had no impact on adhesion of $\beta 3/\beta 5$ integrin DKO microglia to vitronectin, suggesting that in addition to $\alpha v \beta 1$, $\alpha v \beta 3$, and $\alpha v \beta 5$, $\alpha v \beta 8$ also serves as a functional vitronectin receptor on microglia.

Conclusions: Taken together, this demonstrates that the $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins are not essential for mediating microglial activation responses to vitronectin, but that microglia use multiple redundant receptors to mediate interactions with this ECM protein.

Keywords: microglia, extracellular matrix, vitronectin, integrin, adhesion, MMP-9

Background

Microglia are immune effector cells resident in the central nervous system (CNS), whose main role is to orchestrate immunological responses following cerebral insults [1-3]. In the resting CNS, microglia occupy a basal surveillance state, but after activation by pro-inflammatory cytokines or microorganisms, they transform into metabolically active phagocytic cells, upregulating expression of cytokines and chemokines, and

migrating to the inflammatory focus. As well as playing a protective role, recent evidence suggests that in some diseases, including multiple sclerosis (MS), microglia may become inappropriately stimulated, leading to autoimmune destruction of host tissue [4-7].

To understand why microglia may become inappropriately activated in the early stages of MS, we have focused our attention on the function of certain ECM proteins present in blood at high concentrations, including fibronectin and vitronectin [8,9]. We have demonstrated that the plasma proteins vitronectin and fibronectin promote microglial activation *in vitro*

* Correspondence: rmilner@scripps.edu
Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

[10,11]. Taken together with the observation that blood-brain barrier (BBB) breakdown is an early event in the pathogenesis of MS we proposed that leakage of these two proteins into brain parenchymal tissue pre-disposes to microglial activation and myelin damage. Results obtained with the experimental autoimmune encephalomyelitis (EAE) model demonstrated that BBB breakdown was closely associated with fibronectin and vitronectin deposits in the CNS, which closely correlated with microglial activation and expression of the matrix metalloproteinase, MMP-9 [11]. Combined with the evidence from other groups demonstrating vitronectin and fibronectin deposition in demyelinated lesions in the brains of MS patients [12-14] and EAE mice [15], this supports the hypothesis that fibronectin and vitronectin promote microglial activation *in vivo*.

A major question yet to be fully answered is: which microglial receptors mediate the activation response to vitronectin? Our prior work has shown that microglia express the two vitronectin receptors, $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, and that the microglial response to vitronectin is largely mediated by αv integrins [11]. As the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins have well-defined roles in activation and phagocytic processes in other cell types, the purpose of the current study was to investigate the contribution of these two integrins in this process, and thereby test our hypothesis that absence of both $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins would render microglia unresponsive to vitronectin. To examine these events, microglial cells were prepared from wild-type, $\beta 3$ integrin KO, $\beta 5$ integrin KO and $\beta 3/\beta 5$ integrin DKO mice, and the behavior of these microglia evaluated in a battery of assays including cell adhesion, expression of activation markers and MMP-9, and phagocytosis of vitronectin-coated beads.

Methods

Animals

The studies described have been reviewed and approved by The Scripps Research Institute Institutional Animal Care and Use Committee. $\beta 3$ integrin KO and $\beta 5$ integrin KO mice (backcrossed > 10 times on the C57Bl/6 background) were maintained under pathogen-free conditions in the closed breeding colony of The Scripps Research Institute (TSRI). $\beta 3$ integrin KO and $\beta 5$ integrin KO mice were bred and offspring genotyped using previously described protocols [16-19] to generate homozygous $\beta 3$ integrin KO ($\beta 3^{-/-}$), homozygous $\beta 5$ integrin KO ($\beta 5^{-/-}$), and double-knockout (DKO) mice, homozygous ($\beta 3^{-/-}$, $\beta 5^{-/-}$). In all experiments, littermate wild-type mice were used as controls.

Cell culture

Pure cultures of mouse microglia were obtained as described previously [20], with cultures from the

different strains of mice being established at the same time in parallel. Briefly, 7-10 day old mixed glial cultures were shaken for 30 minutes and the supernatant containing detached microglia was collected. Microglia were counted by hemocytometer and plated at a density of 2×10^5 cells/well in six-well plates (Nunc, Naperville, IL) previously coated for two hours at 37°C with a 10 $\mu\text{g/ml}$ solution of vitronectin (Sigma). Cells were grown overnight in the mixed glial culture media, and then switched to N1 serum-free media (DMEM supplemented with N1 (Sigma)). The purity of these microglial cultures was > 99% as determined by Mac-1 positivity in flow cytometry.

Cell adhesion assays

Adhesion assays were performed as previously described [20]. Briefly, substrates were prepared by coating the central area of 24 well plates (Nunc) with 25 μl of ECM solution (10 $\mu\text{g/ml}$ of vitronectin or fibronectin, both from Sigma) for 2 hours at 37°C. Substrates were washed twice before addition of cells. Microglia were prepared as described above, centrifuged, re-suspended in N1 serum-free media, and 2000 microglia applied to the substrates in a 25 μl drop and then incubated at 37°C for 15 or 30 minutes. In the function-blocking experiments, the anti- αv monoclonal antibody (RMV-7), anti- $\beta 1$ monoclonal antibody (Ha2/5) or control antibodies were included at a concentration of 5 $\mu\text{g/ml}$. The assay was stopped by adding 1 ml of DMEM and washing off any loosely attached cells. The attached cells were fixed in 4% paraformaldehyde in PBS for 20 minutes, and stored in PBS. Adhesion was quantified under phase microscopy by counting all attached cells within 5 fields of view per condition. Within each experiment each condition/time-point was performed in duplicate; the results represent the mean \pm SEM of three experiments. Statistical significance was assessed by using the Student's paired t test, in which $p < 0.05$ was defined as statistically significant.

Antibodies

The following monoclonal antibodies were obtained from BD Pharmingen (La Jolla, CA): rat monoclonal antibodies reactive for MHC class I (M1/42.3.9.8), the integrin subunits $\alpha 4$ (MFR4.B), $\alpha 5$ (5H10-27 (MFR5)), αv (RMV-7), αM (M1/70), and the isotype control antibody, rat anti-KLH (A110-2), and the hamster monoclonal antibodies reactive for the $\beta 1$ (Ha2/5; function-blocking antibody) and $\beta 3$ (2C9.G2) integrin subunits and isotype control (G235-1). Rabbit polyclonal antibodies specific for the αv integrin subunit were obtained from Chemicon (Temecula, CA). The anti- $\beta 8$ integrin polyclonal antibody was a kind gift from Dr. Joseph McCarty, M.D. Anderson Cancer Center, Houston, TX.

Cell surface labeling and immunoprecipitation

Microglial cell surface molecules were labeled with biotin as previously described [10,21]. Briefly, microglial cell cultures were incubated with NHS-LC-biotin (Pierce, Rockford, IL) for 30 minutes, washed in TRIS-containing cell wash buffer (CWB), and then removed from tissue culture plates and centrifuged. Cells were lysed in 0.5% Triton-X100 in CWB that contained a cocktail of protease inhibitors (Invitrogen, Carlsbad, CA). After 30 minutes on ice, the lysate was centrifuged to remove the insoluble fraction. The supernatants were pre-cleared for one hour with 30 μ l of protein A sepharose or protein G sepharose per ml of cell lysate. Immunoprecipitations were performed overnight at 4°C on a rotating platform using the polyclonal anti- α v or anti- β 8 integrin antibodies at 1:250 dilution in a tube containing 30 μ l protein A sepharose. Beads were washed 5 times in immunoprecipitation wash buffer, as previously described and the integrin immune complexes were separated by boiling the beads in non-reducing sample buffer for 5 minutes before being analysed by 8% SDS-PAGE (Invitrogen) under non-reducing conditions. Proteins were electro-blotted for 1.5 hours onto nitrocellulose membranes (Invitrogen), blocked overnight in 3% BSA in TBS containing 0.1% Tween-20 (Sigma) and probed with streptavidin-HRP conjugate (Pierce) for one hour, before being extensively washed. Protein bands were visualised with the SuperSignal WestFemto ECL detection system (Pierce) according to the manufacturers' instructions.

Microglial phagocytosis of vitronectin-coated beads

Microglial cells were plated at a density of 2×10^5 cells/well in six-well plates. After one day of culture, 2.5 μ l of a suspension of yellow-green fluorescent beads (Molecular Probes, Eugene, OR) previously coated in a 100 μ g/ml vitronectin solution for 2 hours at 37°C, was added to the microglia, and thoroughly mixed with the tissue culture media to distribute the beads throughout the culture. After a further 24 hours, cultures were visualised for microglial uptake of beads, and microglia collected and phagocytic uptake of fluorescent beads analyzed by flow cytometry, with 10,000 events recorded for each condition. The phagocytic index of microglia was quantified and expressed as the mean fluorescent intensity of the cell population. Each experiment was repeated a minimum number of four times and the data expressed as mean \pm SD. Statistical significance was assessed by using the Student's paired t test, in which $p < 0.05$ was defined as statistically significant.

Flow cytometry

Microglia, isolated from the four different strains of mice, were cultured in vitronectin-coated 6-well plates

under serum-free conditions. After 2 days, microglia were removed from the culture plates and cell surface expression of MHC class I and the integrins α 4, α 5, α v or Mac-1 analyzed by flow cytometry using direct fluorescent-conjugated monoclonal antibodies, as described previously [20]. The fluorescent intensity of the labeled cells was analyzed with a Becton Dickinson FACScan machine (San Diego, CA), with 10,000 events recorded for each condition. For each experimental condition, the mean fluorescent intensity was compared with the control state and expressed as the percentage change relative to the control condition. Each experiment was repeated a minimum number of four times and the data expressed as mean \pm SD. Statistical significance was assessed by using the Student's paired t test, in which $p < 0.05$ was defined as statistically significant.

Gel zymography

Gelatin zymography was used to detect MMP-9 activity as previously described [11,22]. Microglial cells were plated at a density of 2×10^5 cells/well in six-well plates that were either left uncoated, or coated with vitronectin or fibronectin. After 2 days culture, microglial supernatants were collected and analyzed for gelatinolytic activity. Positive controls for MMP-9 and MMP-2 (obtained from R&D) were included. For quantification, gels were scanned using a Bio-Rad VersaDoc imaging system (Hercules, CA) and band intensities quantified using the NIH Image program. Each experiment was repeated a minimum number of four times and the data expressed as mean \pm SD. Statistical significance was assessed by using the Student's paired t test, in which $p < 0.05$ was defined as statistically significant.

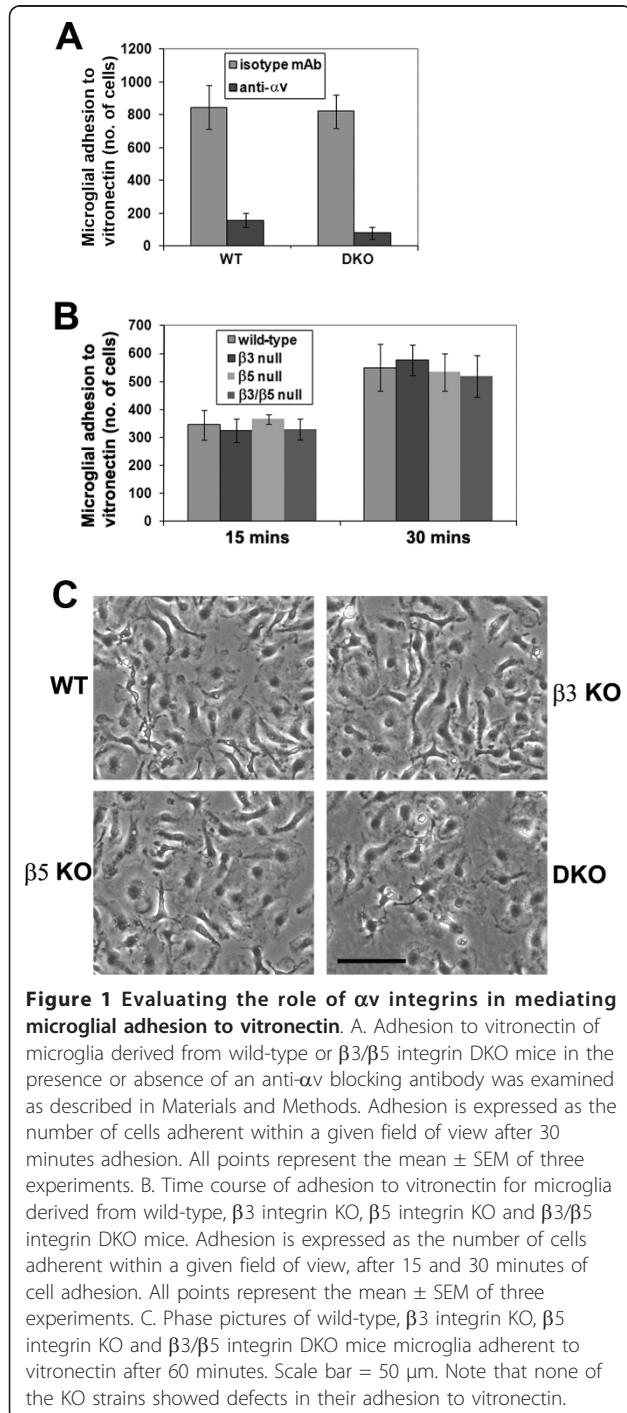
Results

Absence of α v β 3, α v β 5, or both integrins does not diminish microglial adhesion to vitronectin

Vitronectin is a strong inducer of microglial activation, and antibody-blocking studies have demonstrated that this effect is mediated primarily via α v integrins [11]. Microglia express high levels of the two vitronectin receptors, α v β 3 and α v β 5 integrins [23], which have well-defined roles in activation and phagocytic processes in other cell types [24,25]. The purpose of the current study was to investigate the contributions of the α v β 3 and α v β 5 integrins to this process, and test our hypothesis that absence of both these integrins would render microglia unresponsive to vitronectin. To examine these events, mixed glial cultures (MGC) were established from postnatal brains of four different strains of mice: wild-type, β 3 integrin KO, β 5 integrin KO and β 3/ β 5 integrin DKO. In the first set of experiments, we examined the role of α v integrins in mediating microglial adhesion to vitronectin. In 30-minute adhesion assays,

an αv integrin function-blocking antibody significantly inhibited the adhesion of both wild-type microglia (from 847 ± 134 cells under control conditions to 157 ± 44 cells with αv antibody, $p < 0.005$) and $\beta 3/\beta 5$ integrin DKO microglia (from 822 ± 101 cells under control conditions to 78 ± 39 cells with αv antibody, $p < 0.005$) to vitronectin (Figure 1A). This demonstrates that αv integrins are the major class of vitronectin receptors

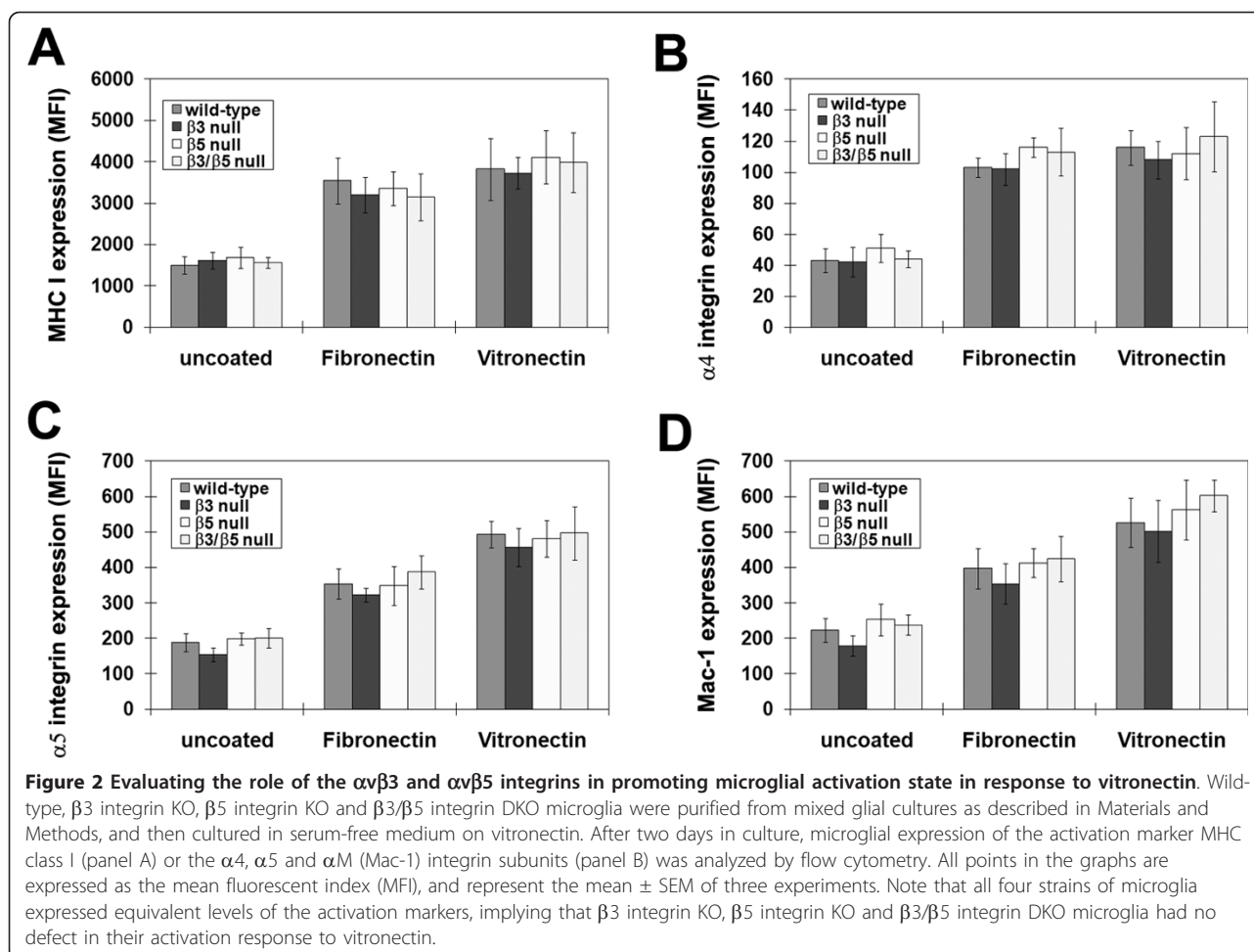
that mediate microglial adhesion to vitronectin, confirming the findings from previous studies [11,20]. Next, we examined whether microglia lacking the $\alpha v\beta 3$ or $\alpha v\beta 5$ integrins can attach to vitronectin. In short term adhesion assays lasting 15 or 30 minutes, we detected no defects in the attachment of any of the knockout strains of microglia compared to wild-type cells (Figure 1B). In addition, after 60 minutes of adhesion, there were no obvious differences in the spreading characteristics or morphology of the different strains (Figure 1C).



Microglial activation responses to vitronectin are not affected by the absence of $\alpha v\beta 3$, $\alpha v\beta 5$ or both these integrins

Microglial activation correlates with a morphological switch from a phase-bright, process bearing cell to a phase-dark amoeboid phenotype. In light of our finding that $\beta 3$ KO, $\beta 5$ KO and $\beta 3/\beta 5$ DKO show the same morphological activation on vitronectin as wild-type cells (Figure 1B), this suggests that the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins are not essential for mediating microglial responses to vitronectin. However, to confirm changes of microglial activation at the molecular level, we also examined cell surface expression of the activation markers MHC class I, and the integrins, $\alpha 4\beta 1$, $\alpha 5\beta 1$ and $\alpha M\beta 2$ (Mac-1). Microglia were cultured on vitronectin under serum-free conditions for 2 days, and their expression levels of activation markers quantified by flow cytometry (Figure 2). Consistent with previous results [10], fibronectin and vitronectin strongly promoted microglial expression of all the cell surface markers of activation, including MHC class I and the different activation integrins. However, relative to wild-type cells, microglia lacking $\beta 3$, $\beta 5$, or both integrins showed no significant difference in their expression of the activation markers MHC class I, or the integrins, $\alpha 4\beta 1$, $\alpha 5\beta 1$, or Mac-1.

As vitronectin strongly promotes microglial expression of the matrix metalloproteinase MMP-9 [11], we also tested whether $\beta 3$ KO, $\beta 5$ KO or $\beta 3/\beta 5$ DKO microglia were deficient in their expression of MMP-9 in response to vitronectin. To quantify microglial expression of MMP-9, gelatin zymography was performed on supernatants taken from microglia cultured under serum-free conditions for three days on vitronectin. Consistent with previous results, fibronectin and vitronectin promoted strong induction of pro-MMP-9 compared to the uncoated plastic control substrate (Figures 3A and 3B) [11]. However, there were no significant differences in the level of MMP-9 induction of any of the KO strains of microglia cultured on vitronectin (or fibronectin), compared with wild-type cells. Next, we evaluated whether there were any defects in the ability of integrin-deficient microglia to phagocytose vitronectin-coated



beads. Microglia were incubated with vitronectin-coated fluorescent beads for 24 hours, and the phagocytic uptake of beads analyzed by flow cytometry. As shown in Figure 3C, there were no differences in the phagocytic activity of microglia derived from the different strains of mice.

Microglia deficient in $\beta 3$ and $\beta 5$ integrins show low expression levels of two additional αv integrins

Unexpectedly, our experiments revealed that microglia lacking the $\beta 3$, $\beta 5$ or both integrins show no defects in their activation responses to vitronectin. As previous pharmacological function-blocking experiments demonstrated that αv integrins are an important mediator of this response [11], this suggests that microglia may express additional αv integrins to mediate this effect. To test this, we performed flow cytometry on $\beta 3/\beta 5$ DKO microglia to evaluate expression of the αv integrin subunit. This showed that $\beta 3/\beta 5$ DKO microglia express the αv integrin subunit, albeit at much-reduced levels, approximately 10-15% that of wild-type cells (Figure 4A). To confirm this finding, we next examined this at the biochemical level by

performing αv immunoprecipitations on all four strains of microglia: wild-type, $\beta 3$ KO, $\beta 5$ KO and $\beta 3/\beta 5$ DKO. The advantage of this approach is that it reveals all the αv integrin heterodimers expressed by microglial cells, so as well as addressing whether αv integrin is expressed, it also allows us to identify the different β integrin subunits that associate with the αv subunit. Microglia were cultured on vitronectin under serum-free conditions for two days, then cell surface molecules biotinylated and lysates prepared. As shown in Figure 4B, wild-type microglia expressed the αv integrin subunit (140 kD) in association with high levels of the $\beta 3$ (80 kD) and $\beta 5$ (90 kD) integrin subunits. As expected, an αv immunoprecipitation of $\beta 3$ KO microglia revealed only the αv and $\beta 5$ subunits, while one of $\beta 5$ KO microglia revealed only the αv and $\beta 3$ subunits. Significantly, an αv immunoprecipitation of $\beta 3/\beta 5$ DKO microglia showed that the αv subunit was still present, albeit at very low levels, in association with two different integrin β subunits, with molecular weights of approximately 110 and 80 kD. Based on molecular weight, these can be identified as the $\beta 1$ and $\beta 8$ subunits, respectively [21,26,27]. Further immunoprecipitations with a $\beta 8$ integrin-specific

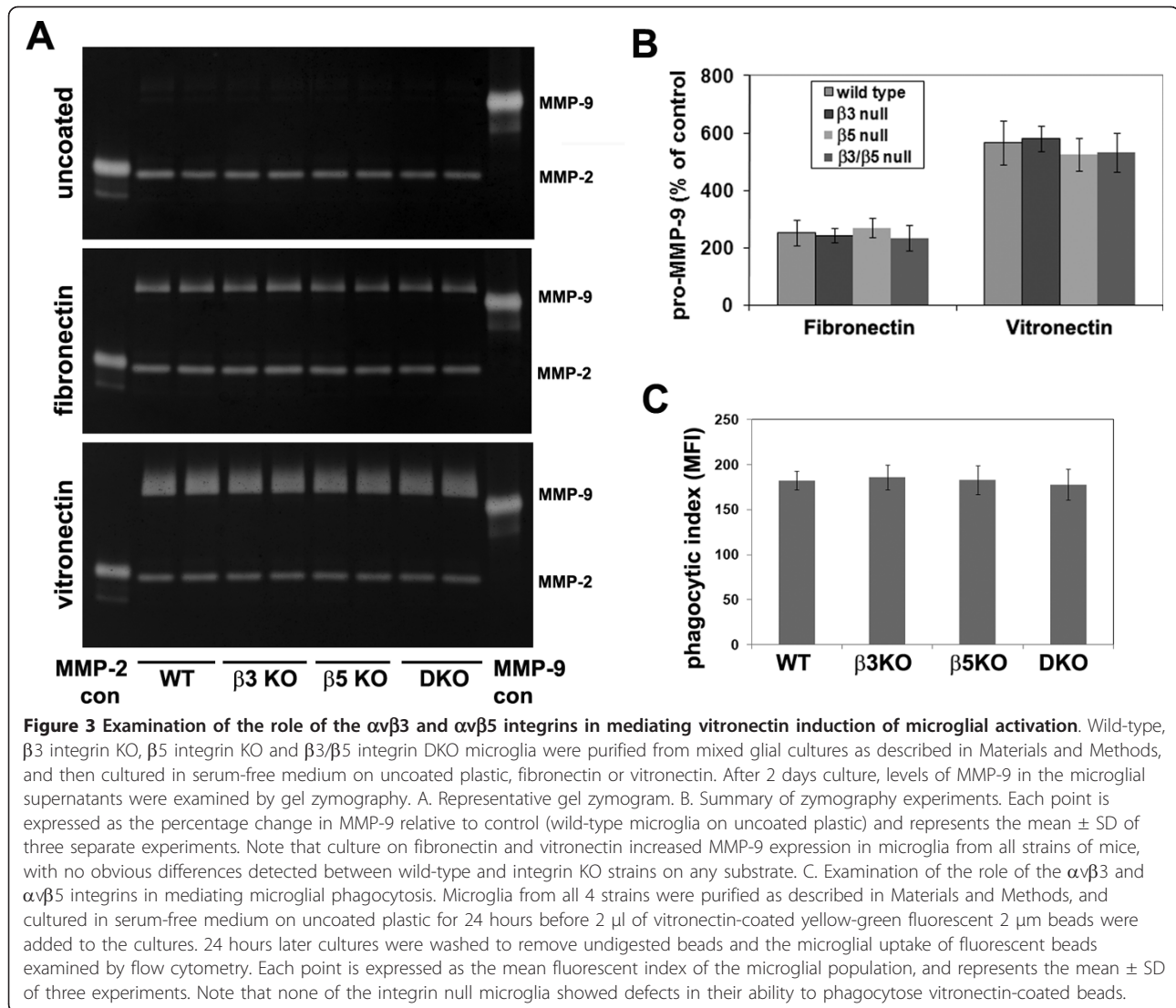


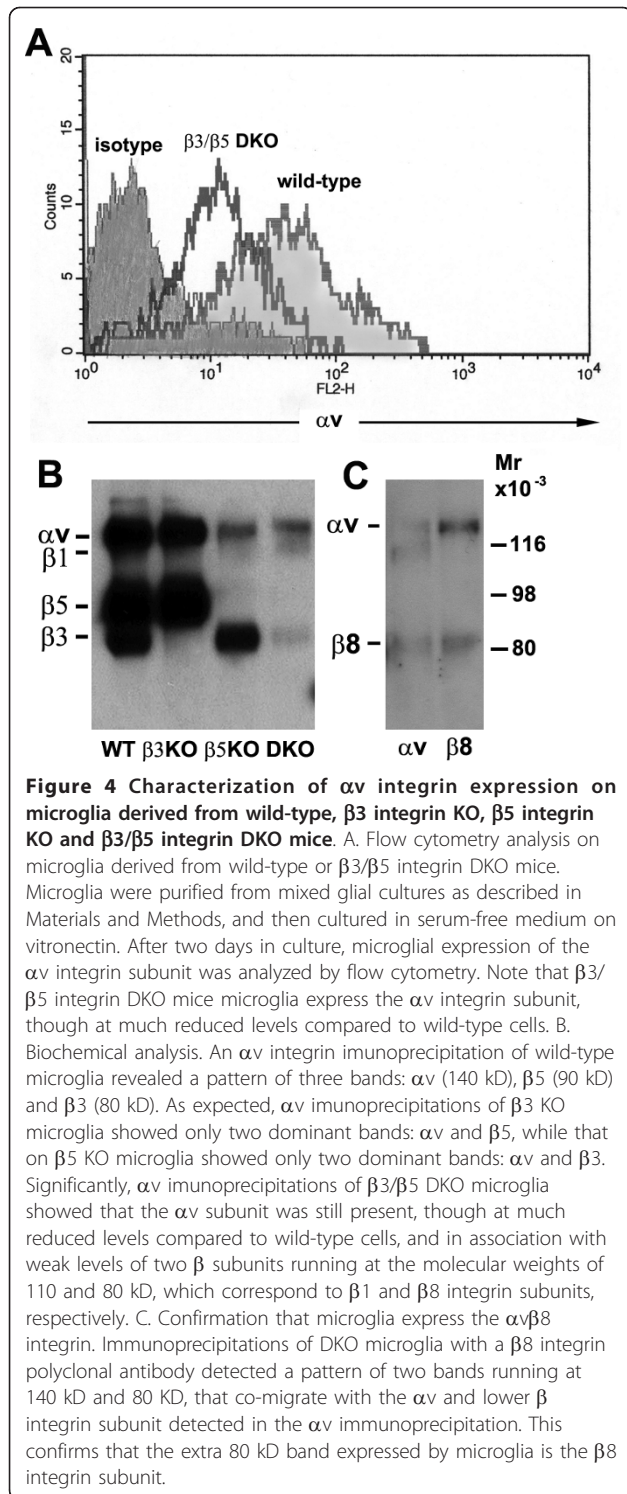
Figure 3 Examination of the role of the $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins in mediating vitronectin induction of microglial activation. Wild-type, $\beta 3$ integrin KO, $\beta 5$ integrin KO and $\beta 3/\beta 5$ integrin DKO microglia were purified from mixed glial cultures as described in Materials and Methods, and then cultured in serum-free medium on uncoated plastic, fibronectin or vitronectin. After 2 days culture, levels of MMP-9 in the microglial supernatants were examined by gel zymography. A. Representative gel zymogram. B. Summary of zymography experiments. Each point is expressed as the percentage change in MMP-9 relative to control (wild-type microglia on uncoated plastic) and represents the mean \pm SD of three separate experiments. Note that culture on fibronectin and vitronectin increased MMP-9 expression in microglia from all strains of mice, with no obvious differences detected between wild-type and integrin KO strains on any substrate. C. Examination of the role of the $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins in mediating microglial phagocytosis. Microglia from all 4 strains were purified as described in Materials and Methods, and cultured in serum-free medium on uncoated plastic for 24 hours before 2 μ l of vitronectin-coated yellow-green fluorescent 2 μ m beads were added to the cultures. 24 hours later cultures were washed to remove undigested beads and the microglial uptake of fluorescent beads examined by flow cytometry. Each point is expressed as the mean fluorescent index of the microglial population, and represents the mean \pm SD of three experiments. Note that none of the integrin null microglia showed defects in their ability to phagocytose vitronectin-coated beads.

antibody revealed a pattern of two bands, running at approximately 140 and 80 kD, corresponding to the αv and $\beta 8$ subunits respectively, confirming $\beta 8$ as the additional β subunit. Levels of microglial $\beta 8$ expression were not significantly different amongst the different strains of mice (not shown). Thus, these biochemical results support our flow cytometry observations, demonstrating that in addition to $\alpha v \beta 3$ and $\alpha v \beta 5$, microglia also express low levels of two additional αv integrins, $\alpha v \beta 1$ and $\alpha v \beta 8$.

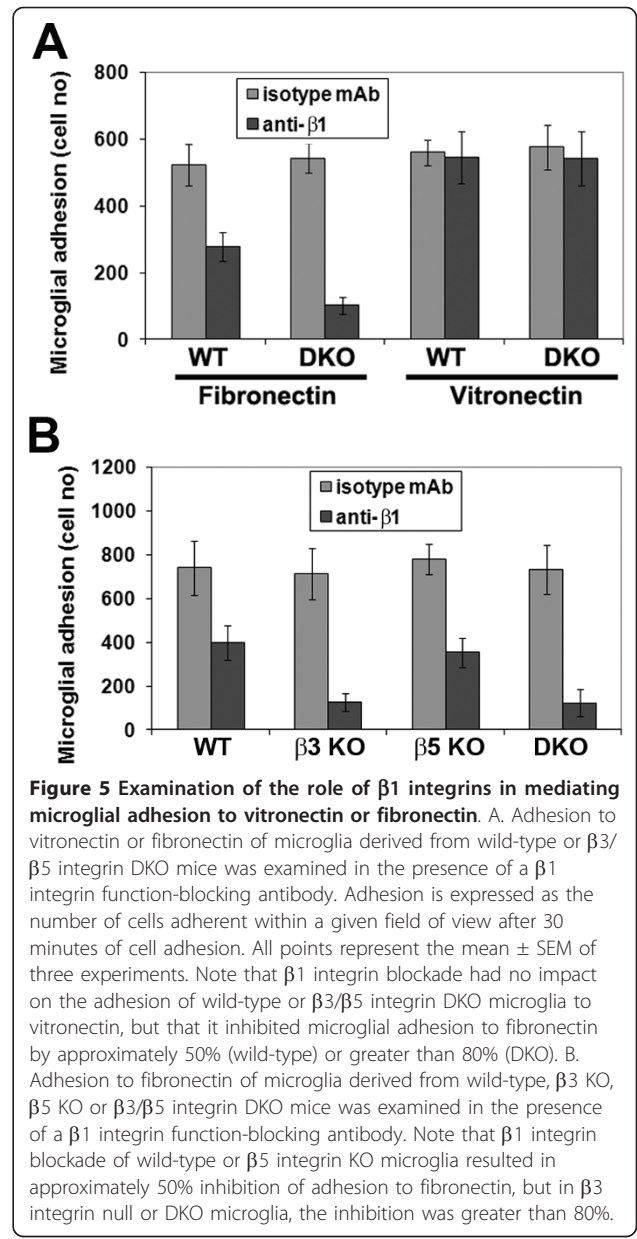
The microglial $\alpha v \beta 8$ integrin acts as a functional vitronectin receptor, and $\alpha v \beta 3$ is a functional fibronectin receptor

While $\alpha v \beta 1$ integrin is a well described functional vitronectin receptor [28], it is less clear whether the $\alpha v \beta 8$ integrin also fulfils this role. To investigate whether $\alpha v \beta 8$ is a functional vitronectin receptor in microglia,

we examined the effect of function-blocking anti- $\beta 1$ integrin antibodies on microglial adhesion to vitronectin. Under these conditions, the $\beta 3/\beta 5$ integrin DKO microglia have only one potential αv integrin available to mediate adhesion to vitronectin, namely $\alpha v \beta 8$. As shown in Figure 5, in 30 minute adhesion assays, $\beta 1$ integrin blockade had no impact on the adhesion of wild-type or $\beta 3/\beta 5$ integrin DKO microglia to vitronectin. This shows that in the absence of $\alpha v \beta 1$, $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins, microglia can still attach to vitronectin, suggesting that the $\alpha v \beta 8$ integrin serves as a functional vitronectin receptor on microglia. Interestingly, $\beta 1$ integrin blockade revealed markedly different effects on the ability of wild-type and $\beta 3/\beta 5$ integrin DKO microglia to adhere to fibronectin. While the adhesion of wild-type microglia was inhibited by approximately 50% (from 523 ± 62 cells under control conditions to $278 \pm$



43 cells with the anti- β_1 antibody, $p < 0.01$), the adhesion of β_3/β_5 integrin DKO microglia to fibronectin was inhibited by more than 80% (from 543 ± 45 cells under control conditions to 102 ± 25 cells with the anti- β_1 antibody, $p < 0.005$). Direct comparison of the effect of



β_1 integrin blockade on wild-type or β_3/β_5 integrin DKO microglia adhesion to fibronectin was found to be statistically significant ($p < 0.005$). That β_1 integrin blockade only partially inhibits WT microglial adhesion to fibronectin suggests that microglia use other receptors to adhere to this substrate. As β_1 integrin blockade is much more effective at blocking the adhesion of β_3/β_5 integrin DKO microglia to fibronectin, relative to wild-type cells, this suggests that the $\alpha_V\beta_3$ or $\alpha_V\beta_5$ integrins may also act as fibronectin receptors. To determine the role of the $\alpha_V\beta_3$ or $\alpha_V\beta_5$ integrins in microglial adhesion to fibronectin, we next examined the effect of β_1 integrin blockade on the four different strains of microglia (Figure 5B). This showed that β_1

integrin blockade resulted in approximately 50% inhibition of adhesion to fibronectin of wild-type (from 740 ± 123 cells to 398 ± 79 cells with anti- $\beta 1$ antibody, $p < 0.02$), or $\beta 5$ integrin null microglia (from 778 ± 69 cells to 355 ± 67 cells with anti- $\beta 1$ antibody, $p < 0.01$), but greater than 80% inhibition in $\beta 3$ integrin null (from 713 ± 116 cells to 126 ± 41 cells with anti- $\beta 1$ antibody, $p < 0.005$) or DKO microglia (from 733 ± 112 cells to 123 ± 61 cells with anti- $\beta 1$ antibody, $p < 0.005$). Taken together, this suggests that the $\alpha v \beta 3$ integrin, but not the $\alpha v \beta 5$ integrin, makes a significant contribution to microglial adhesion to fibronectin.

Discussion

Microglia play a critical role in the CNS by performing immune surveillance and regulating inflammatory processes [1,2], therefore defining the factors that control microglial activation state is of fundamental importance. Evidence suggests that ECM proteins play an important role in this process. In particular, vitronectin, present at high levels in plasma [8], and absent in the normal CNS, is deposited in a perivascular manner in MS tissue [13] and in the mouse model of MS, EAE [11,15]. Consistent with this, we have shown that vitronectin directly promotes microglial activation in vitro [10,11]. The next important question is to identify the microglial receptors that mediate this effect. Having shown previously that microglia express the two vitronectin receptors, $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins [10,23], the purpose of the current study was to investigate the contribution of these two integrins in microglial activation, and thereby test our hypothesis that absence of both $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins would render microglia unresponsive to vitronectin. Using microglia derived from different strains of mice (wild-type, $\beta 3$ integrin KO, $\beta 5$ integrin KO and $\beta 3/\beta 5$ integrin DKO), different aspects of microglial activation were examined. Surprisingly, when cultured on vitronectin, microglia from the knockout strains showed no obvious defects in adhesion, activation marker or MMP-9 expression, or phagocytosis of vitronectin-coated beads. To investigate the reason for this lack of effect, we examined the expression of other αv integrins. This revealed that microglia also express low levels of the alternative vitronectin receptors, $\alpha v \beta 1$ and $\alpha v \beta 8$ integrins. This demonstrates that the $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins are not essential for mediating microglial activation responses to vitronectin, but that microglia use multiple redundant receptors to mediate interactions with this ECM protein.

Non-essential roles for the $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins in microglial activation

Evidence gathered from a variety of different cell types supports a role for the $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins in promoting cellular activation and phagocytic responses. The

$\alpha v \beta 3$ integrin has been implicated in mediating phagocytosis in monocytes and peripheral macrophages, and the $\alpha v \beta 5$ integrin plays a similar role in macrophages and in retinal pigment epithelial (RPE) cells [24,25,29]. Indeed, $\beta 5$ KO mice develop accelerated age-related blindness as a result of defective phagocytic clearance of old photoreceptor cells in the retina [19]. In addition, $\alpha v \beta 3$ plays an essential role in osteoclast function, supported by the fact that osteoclasts in $\beta 3$ KO mice have defective bone resorption [30]. With this in mind, we were surprised to find that microglia lacking $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins showed normal adhesion and activation responses to vitronectin, and is further demonstration that the findings of pharmacological blockade studies are not always borne out by the use of genetic KO approaches. This is perhaps best illustrated by the case of the role of $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins in promoting angiogenesis, in which antibody blockade suggested key roles for these integrins in angiogenesis [31,32], but mice lacking these integrins display no apparent angiogenic defect [16], actually displaying an enhanced angiogenic response in tumor growth [33].

Redundancy of microglial αv integrins

Our data show that the $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins comprise the major fraction of total αv integrins expressed by microglia. However, we have found that microglia also express two other vitronectin receptors, the $\alpha v \beta 1$ and $\alpha v \beta 8$ integrins, though at appreciably lower levels than $\alpha v \beta 3$ and $\alpha v \beta 5$. What is surprising about our data is that despite lacking the two abundant vitronectin receptors, $\beta 3/\beta 5$ DKO microglia show no obvious defects in adhesion to vitronectin, or in the subsequent activation responses. This is in stark contrast to our finding with brain endothelial cells, where absence of the $\alpha v \beta 3$ integrin leaves the cells totally unable to attach to vitronectin [34]. This clearly demonstrates that the $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins are not essential for mediating microglial activation responses to vitronectin, highlighting the redundancy of microglial vitronectin receptors. In this light it is informative to compare the αv integrin expression profile of microglia with other CNS cell types. Both neural stem cells and oligodendrocyte precursor cells also express the four αv integrins expressed by microglia, $\alpha v \beta 1$, $\alpha v \beta 3$, $\alpha v \beta 5$ and $\alpha v \beta 8$ [21,27,35], and it is interesting to note that all three cell types have the capacity to migrate considerable distances, even in the adult CNS. In contrast, astrocytes, which are far less motile, express only $\alpha v \beta 5$ and $\alpha v \beta 8$ [18], while brain endothelial cells express just the $\alpha v \beta 3$ integrin, and then only when actively undergoing angiogenesis [34,36,37].

Regulation of αv integrin heterodimer formation

Integrins comprise a family of $\alpha \beta$ heterodimers, composed of 11 different α and 9 different β subunits. In

light of the potential to form up to 99 potential different $\alpha\beta$ heterodimers, in reality, only 24 heterodimers have been identified [26]. We have shown that microglia express four different $\alpha\beta$ heterodimers, with high levels of $\alpha\beta 3$ and $\alpha\beta 5$, and much lower levels of $\alpha\beta 1$ and $\alpha\beta 8$. This begs the question: what regulates the coupling and abundance of each of the different $\alpha\beta$ heterodimers? It has been previously suggested that the α integrin subunit is produced in excess, and its cell surface appearance is limited by the transcription level of the associated β subunits [38]. An alternative mechanism would be that the α subunit is produced in a fixed amount, and that loss of one or more β subunits (e.g.: $\beta 3$) would automatically lead to compensatory upregulation of the other β subunits (e.g.: $\beta 8$). Our data would appear to support the first mechanism, because loss of both of the major β subunits $\beta 3$ and $\beta 5$ in microglia did not result in any compensatory upregulation of $\alpha\beta 1$ or $\alpha\beta 8$; rather the total amount of the α integrin subunit was massively reduced.

Conclusions

The aim of this study was to define the role of the $\alpha\beta 3$ and $\alpha\beta 5$ integrins in mediating microglial activation responses to vitronectin. Microglia from $\beta 3$, $\beta 5$, or $\beta 3/\beta 5$ knockout strains showed no defects in adhesion, activation marker expression, MMP-9 induction, or phagocytosis of vitronectin-coated beads. Flow cytometry and biochemical analysis revealed that microglia also express low levels of the alternative vitronectin receptors, $\alpha\beta 1$ and $\alpha\beta 8$ integrins. Taken together, we conclude that the $\alpha\beta 3$ and $\alpha\beta 5$ integrins are not essential for mediating microglial activation responses to vitronectin, but that microglia employ multiple receptor systems to mediate interactions with vitronectin. On embarking on these studies, we were hopeful that identification of a single vitronectin receptor would lead to potential therapeutic targets for blocking microglial activation. The outcome of the current study suggests that pinpoint targeting of single $\alpha\beta$ integrins will not be productive, but rather a broad-spectrum blockade aimed at targeting all $\alpha\beta$ integrins is more likely to be successful.

Acknowledgements

This work was supported by the National Multiple Sclerosis Society: by a Harry Weaver Neuroscience Scholar Award to RM (JF 2125A1/1), and by a Post-Doctoral Fellowship to JW (FG 1879-A-1). This is manuscript number 21399 from The Scripps Research Institute.

Authors' contributions

JW genotyped the KO mice strains, prepared the cell cultures, and contributed to drafting the manuscript. AB genotyped the mice, ran the gel zymography and contributed to drafting the manuscript. UT performed the flow cytometry and contributed to drafting the manuscript. RM conceived of the study, performed the biochemical analysis, and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 5 August 2011 Accepted: 10 November 2011

Published: 10 November 2011

References

1. Carson MJ: Microglia as liaisons between the immune and central nervous systems: functional implications for multiple sclerosis. *Glia* 2002, **40**:218-231.
2. Hanisch UK, Kettenmann H: Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* 2007, **10**:1387-1394.
3. Raivich G, Bohatschek M, Kloss CU, Werner A, Jones LL, Kreutzberg GW: Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Res Brain Res Rev* 1999, **30**:77-105.
4. Barnett MH, Prineas JW: Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. *Ann Neurol* 2004, **55**:458-468.
5. Hickey WF: The pathology of multiple sclerosis: a historical perspective. *J Neuroimmunol* 1999, **98**:37-44.
6. Ransohoff RM: Mechanisms of inflammation in MS tissue: adhesion molecules and chemokines. *J Neuroimmunol* 1999, **98**:57-68.
7. Trapp BD, Bo L, Mork S, Chang A: Pathogenesis of tissue injury in MS lesions. *J Neuroimmunol* 1999, **98**:49-56.
8. Felding-Habermann B, Cheresh DA: Vitronectin and its receptors. *Curr Opin Cell Biol* 1993, **5**:864-868.
9. Purves LR, Brown G, Linton N: Fibronectin levels in normal subjects and patients admitted to a respiratory intensive care unit. *S Afr Med J* 1982, **61**:733-736.
10. Milner R, Campbell IL: The extracellular matrix and cytokines regulate microglial integrin expression and activation. *J Immunol* 2003, **170**:3850-3858.
11. Milner R, Crocker SJ, Hung S, Wang X, Frausto RF, Del Zoppo GJ: Fibronectin- and Vitronectin-Induced Microglial Activation and Matrix Metalloproteinase-9 Expression Is Mediated by Integrins $\alpha 5 \beta 1$ and $\alpha v \beta 5$. *J Immunol* 2007, **178**:8158-8167.
12. Han MH, Hwang SI, Roy DB, Lundgren DH, Price JV, Ousman SS, Fernald GH, Gerlitz B, Robinson WH, Baranzini SE, et al: Proteomic analysis of active multiple sclerosis lesions reveals therapeutic targets. *Nature* 2008, **451**:1076-1081.
13. Sobel R, Chen M, Maeda A, Hinojoza J: Vitronectin and integrin vitronectin receptor localisation in multiple sclerosis lesions. *J Neuropathol Exp Neurol* 1995, **54**:202-213.
14. Sobel RA, Mitchell ME: Fibronectin in multiple sclerosis lesions. *Am J Pathol* 1989, **135**:161-169.
15. Teesalu T, Hinkkanen AE, Vaeheri A: Coordinated induction of extracellular proteolysis systems during experimental autoimmune encephalomyelitis in mice. *Am J Pathol* 2001, **159**:2227-2237.
16. Hodivala-Dilke KM, McHugh KP, Tsakiris DA, Rayburn H, Crowley D, Ullman-Cullere M, Ross FP, Collier BS, Teitelbaum S, Hynes RO: Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *J Clin Invest* 1999, **103**:229-238.
17. Huang X, Griffiths M, Wu J, Farese RV, Sheppard D: Normal development, wound healing, and adenovirus susceptibility in beta5-deficient mice. *Mol Cell Biol* 2000, **20**:755-759.
18. Milner R, Huang X, Wu J, Nishimura S, Pytela R, Sheppard D, Ffrench-Constant C: Distinct roles for astrocyte $\alpha v \beta 5$ and $\alpha v \beta 8$ integrins in adhesion and migration. *J Cell Sci* 1999, **112**:4271-4279.
19. Nandrot EF, Kim Y, Brodie SE, Huang X, Sheppard D, Finnemann SC: Loss of synchronized retinal phagocytosis and age-related blindness in mice lacking $\alpha v \beta 5$ integrin. *J Exp Med* 2004, **200**:1539-1545.
20. Milner R, Campbell IL: Cytokines regulate microglial adhesion to laminin and astrocyte extracellular matrix via protein kinase C-dependent activation of the $\alpha 6 \beta 1$ integrin. *J Neurosci* 2002, **22**:1562-1572.
21. Milner R, Ffrench-Constant C: A developmental analysis of oligodendroglial integrins in primary cells: changes in αv -associated β subunits during differentiation. *Development* 1994, **120**:3497-3506.
22. Heo JH, Lucero J, Abumiya T, Koziol JA, Copeland BR, del Zoppo GJ: Matrix metalloproteinases increase very early during experimental focal cerebral ischemia. *J Cereb Blood Flow Metab* 1999, **19**:624-633.

23. Milner R: **Microglial expression of alphavbeta3 and alphavbeta5 integrins is regulated by cytokines and the extracellular matrix: beta5 integrin null microglia show no defects in adhesion or MMP-9 expression on vitronectin.** *Glia* 2009, **57**:714-723.
24. Finnemann SC, Bonilha VL, Marmorstein AD, Rodriguez-Boulan E: **Phagocytosis of rod outer segments by retinal pigment epithelial cells requires alpha v beta 5 integrin for binding but not for internalization.** *Proc Natl Acad Sci USA* 1997, **94**:12932-12937.
25. Finnemann SC, Rodriguez-Boulan E: **Macrophage and retinal pigment epithelium phagocytosis: apoptotic cells and photoreceptors compete for alpha v beta 3 and alpha v beta 5 integrins, and protein kinase C regulates alpha v beta 5 binding and cytoskeletal linkage.** *J Exp Med* 1999, **190**:861-874.
26. Hemler ME: **VLA proteins in the integrin family: Structures, functions, and their role on leucocytes.** *Ann Rev Immunol* 1990, **8**:365-400.
27. Milner R, Frost EE, Nishimura S, Delcommenne M, Streuli C, Pytela R, ffrench-Constant C: **Expression of $\alpha v \beta 3$ and $\alpha v \beta 8$ integrins during oligodendrocyte precursor differentiation in the presence and absence of axons.** *Glia* 1997, **21**:350-360.
28. Bodary SC, McLean JW: **The integrin $\beta 1$ subunit associates with the vitronectin receptor αv subunit to form a novel vitronectin receptor in a human embryonic kidney cell line.** *J Biol Chemistry* 1990, **265**:5938-5941.
29. Miceli MV, Newsome DA, Tate DJJ: **Vitronectin is responsible for serum-stimulated uptake of rod outer segments by cultured retinal pigment epithelial cells.** *Invest Ophthalmol Vis Sci* 1997, **38**:1588-1597.
30. McHugh KP, HodiVala-Dilke K, Zheng MH, Namba N, Lam J, Novack D, Feng X, Ross FP, Hynes RO, Teitelbaum SL: **Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts.** *J Clin Invest* 2000, **105**:433-440.
31. Brooks P, Clark RAF, Cheresh DA: **Requirement for vascular integrin $\alpha v \beta 3$ for angiogenesis.** *Science* 1994, **264**:569-571.
32. Friedlander M, Brooks P, Shaffer RW, Kincaid CM, Varner JA, Cheresh DA: **Definition of two angiogenic pathways by distinct αv integrins.** *Science* 1995, **270**:1500-1502.
33. Reynolds LE, Wyder L, Lively JC, Taverna D, Robinson SD, Huang X, Sheppard D, Hynes RO, HodiVala-Dilke K: **Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins.** *Nat Med* 2002, **8**:27-34.
34. Li L, Welser JV, Milner R: **Absence of the $\alpha v \beta 3$ integrin dictates the time-course of angiogenesis in the hypoxic central nervous system: accelerated endothelial proliferation correlates with compensatory increases in $\alpha 5 \beta 1$ integrin expression.** *J Cereb Blood Flow Metab* 2010, **30**:1031-1043.
35. Jacques TS, Relvas JB, Nishimura S, Pytela R, Edwards GM, Streuli CH, ffrench-Constant C: **Neural precursor chain migration and division are regulated through different $\beta 1$ integrins.** *Development* 1998, **125**:3167-3177.
36. Okada Y, Copeland BR, Hamann GF, Koziol JA, Cheresh DA, del Zoppo GJ: **Integrin $\alpha v \beta 3$ is expressed in selective microvessels following focal cerebral ischemia.** *Am J Pathol* 1996, **149**:37-44.
37. Wei L, Erinjeri JP, Rovainen CM, Woolsey TA: **Collateral growth and angiogenesis around cortical stroke.** *Stroke* 2001, **32**:2179-2184.
38. Koistinen P, Heino J: **The selective regulation of $\alpha v \beta 1$ integrin expression is based on the hierarchical formation of αv -containing heterodimers.** *J Biol Chem* 2002, **277**:24835-24841.

doi:10.1186/1742-2094-8-157

Cite this article as: Welser-Alves et al.: Microglia use multiple mechanisms to mediate interactions with vitronectin; non-essential roles for the highly-expressed $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins. *Journal of Neuroinflammation* 2011 **8**:157.

**Submit your next manuscript to BioMed Central
and take full advantage of:**

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

