Biochemical and Biophysical Research Communications 445 (2014) 180–183

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Ultra structure analysis of cell–cell interactions between pericytes and neutrophils *in vitro*

Christian Pieper, Hans-Joachim Galla *

Institute of Biochemistry, Westfälische Wilhelms-Universität Münster, Wilhelm-Klemm-Str. 2, 48149 Münster, Germany

ARTICLE INFO

Article history:
Received 16 January 2014
Available online 1 February 2014

Keywords:
Neutrophils
Pericytes
Cell–cell adhesion
Scanning force microscopy (SFM)
Ultrastructure

ABSTRACT

Neutrophils' adhesion to the endothelium during inflammatory is a well-known processes. In contrast the interaction of neutrophils with cells of the neurovascular unit after they have been transmigrated into the brain is less clear. Recently, lymphocyte function-associated antigen-1 (LFA-1) dependent subendothelial crawling of neutrophils has been observed *in vivo*. This is mediated by intracellular adhesion molecule-1 (ICAM-1), which is expressed on the cell surface of pericytes. In our work we demonstrated *in vitro* a cell–cell interaction between porcine brain capillary pericytes (PBCPs) and neutrophils, with further characterization of the initial contact between these cells. PBCPs increase ICAM-1 protein expression in response to the cytokine tumor necrosis factor-alpha (TNF-α). Furthermore, an increase in neutrophil adhesion to PBCPs was determined by immunofluorescence staining. By means of scanning force microscopy (SFM), we could additionally show that pericytes as well as neutrophils form cell extensions towards the neighboring cell. Interestingly, these extensions differ for different cell types.

1. Introduction

Neutrophil transmigration is crucially dependent on the expression of adhesion molecules on the cell surface of endothelial cells as well as on immune cells. Furthermore, different adhesion molecules can be associated to different functions and time steps during neutrophil diapedesis [1]. Intracellular adhesion molecule-1 (ICAM-1) is one of the most important and best characterized adhesion molecules in the whole process [2]. ICAM-1 especially plays a role during neutrophils' crawling along the vessel wall and the trans- or paracellular cell penetration [3]. Although the process of the transmigration through the endothelium is understood quite well, the interaction of neutrophils with other cells of the neurovascular unit in the brain has poorly been investigated. Proebstl et al. were the first who reported an ICAM-1–lymphocyte function-associated antigen-1 (LFA-1) depending interaction between neutrophils and pericytes after neutrophils have penetrated the endothelium [4]. Before continuing their diapedesis, neutrophils crawl in the subendothelium and leave it through enlarged pericyte gaps as reported [4]. In one of our previous studies we demonstrated in accordance to Proebstl et al. an increase in neutrophil adhesion to pericytes *in vitro* which is massively dependent on matrix metalloproteinases [5]. As we could show that pericytes also secrete interleukin-8 (IL-8), which is the predominant chemo attractor for neutrophil recruitment to the inflamed tissue, we hypothesized that pericytes play an important role during the process of neutrophil transmigration. Up to now, nothing is known about the cellular structures of the initial interaction between these cells. In this study we investigated the expression of ICAM-1 by Western blot analysis in porcine brain capillary pericytes (PBCPs) in response to tumor necrosis factor-alpha (TNF-α) treatment *in vitro*. Furthermore, we confirmed the adhesion between neutrophils and pericytes by means of immunofluorescent staining. The main focus of this work was set to examine changes in pericytes' and neutrophils' cell morphology during the initial contact of both cells. The present study will help to understand the cell–cell interaction between neutrophils and pericytes within the brain.

2. Materials and methods

2.1. Preparation and cultivation of PBCPs

PBCPs were isolated, cultured and cryoconserved as previously described [6–8]. Briefly, cerebra of freshly slaughtered adult pigs were mechanically homogenized and stepwise digested by two proteases, followed by further purification steps. The cells were seeded on collagen G-coated culture flasks (Nunc) and were washed with phosphate-buffered saline (PBS) containing 1 mM

* Corresponding author. Fax: +49 251 8333206.
E-mail address: gallah@uni-muenster.de (H.-J. Galla).

http://dx.doi.org/10.1016/j.bbrc.2014.01.159
0006-291X/

Ca^{2+} and 0.5 mM Mg^{2+} 24 h after initial plating. Since pericytes attach more firmly to the culture substrate than endothelial cells, selective trypsination of endothelial cells yielded pure pericyte cultures which were grown in pericyte medium (Dulbecco's modified Eagle's medium Ham's F-12, 2 mM L-glutamine (Biochrom), 100 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% NCS) for 14 days, before they were frozen and stored in liquid nitrogen. Pericytes were characterized by their branched morphology, large size, positive immunostaining for alpha smooth muscle actin (αSMA), desmin, Thy 1.1 and NG-2 proteoglycan, and negative staining for factor-VIII-related antigen and glial fibrillary acidic protein (GFAP). Pericytes were seeded in collagen G coated culture flasks (72,000 cells per 25 cm^2) with serum containing media (Dulbecco's modified Eagle's medium Ham's F-12, 10% serum, 1% Penicillin/Strep, 1% L-Glu, 1% Gentamycin) for 2 days. For differentiation of the pericytes to an alpha smooth muscle actin negative ($\alpha SMA^{neg.}$) phenotype, the conditions were changed to serum-free media containing 20 ng/ml bFGF for 4 days. Afterwards the stimulation (2 ng/ml TNF-α) was applied for 22–24 h.

2.2. Isolation of neutrophils

Isolation of neutrophils was done as described previously [9]. Briefly, porcine peripheral blood neutrophils were isolated from fresh venous blood, allowed to sediment in 0.6% dextran (162,000 MW, Sigma–Aldrich, Steinheim, Germany) for 1 h, centrifuged for 10 min at 350*g* at room temperature, followed by further erythrocyte purification by resuspending the pellet in 2 ml ice cold water for 20 s and immediate addition of 40 ml Hank's buffered salt solution (HBSS, Biochrom, Berlin, Germany). This step was repeated twice (centrifugation: 7 min at 350*g*, RT) and afterwards, 3 ml of the cell suspension was centrifuged for 30 min over a gradient of 6 ml Histopaque 1083 (Sigma Aldrich, Steinheim, Germany) to separate neutrophils from leucocytes. The pellet was washed twice in HBSS and finally resuspended in chemically defined medium (CDM: Dulbecco's modified Eagle's medium Ham's F-12, 4 mM L-glutamine (Biochrom, Berlin, Germany), 100 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml).

2.3. Western blot

Cell lysate from pericytes was obtained by washing cells 3 times with PBS and adding 350 μl lysis buffer per 25 cm^2 culture flask. After 30 s cells were scraped from the bottom of the flask with the help of a Cell Scraper (Biologix). The lysate was centrifuged (14,000*g*, 30 min, 4 °C) and the supernatant was collected. The concentration was determined by the bicinchoninic acid test (BCA test). Western blot analysis was performed with an equal amount of cell lysate mixed with an equal volume of 2 × Laemmli-buffer (0.4 M Tris–HCl, 8% SDS, 4 mM EDTA, 40% glycerol, pH 6.8 containing 2% β-mercaptoethanol). Proteins were denaturated at 90 °C for 10 min and then separated by 10% SDS–PAGE. Afterwards the proteins were transferred to a nitrocellulose membrane (Amersham). The ICAM-1 proteins were detected by application of primary antibody mouse anti ICAM-1 (0.5 μg/ml AbD Serotec) followed by the secondary antibody, anti-mouse IgG horseradish peroxidase conjugate (0.5 μg/ml, MoBiTec). The detection was carried out with the enhanced chemiluminescence kit (Amersham), following the manufacturer's instructions. Densitometrical analysis was performed by ImageJ 1.45 (NIH).

2.4. Immunofluorescence staining

For immunofluorescent staining cells were grown on gelatin coated cover slips. The cover slips were washed with PBS twice and fixed for 10 min with 4% paraformaldehyde in PBS containing Ca^{2+} and Mg^{2+} (PBS^{++}). After PBS washing (three times), the cells were permeabilized with 0.2% Triton-X-100 in PBS^{++} for 10 min, washed three times with PBS^{++} and blocked with 3% BSA in PBS^{++} for 20 min. After a wash step with PBS^{++}, the first antibody (2.5 μg/ml mouse anti MA, Sigma–Aldrich) was applied for 60 min at 37 °C. After rinsing with PBS^{++} three times, the cells were incubated with the secondary antibody (2 μg/ml goat anti-mouse Alexa Fluor 488, Invitrogen) for 60 min at 37 °C and protected from light. The cells were rinsed with PBS^{++} three times and DAPI was applied. Finally, the cells were embedded in Aqua Polymount (Polyscience) and stored at 4 °C for microscopy evaluation.

2.5. Scanning force microscopy

For scanning force microscopy (SFM) images cells were cultured on 12.57 cm^2 collagen G coated petri dishes, stimulated for 22 h with TNF-α and incubated for 6 h with neutrophils. After washing the cells with PBS twice, they were fixed with 2% glutardialdehyde for 45 s and additionally with 4% PFA for 20 min. Then the cells were washed 5 times with PBS and analyzed by a NanoWizard II SFM (JPK Instruments) operating in contact mode in PBS. A silicon nitride tip (Bruker SFM probes) with a spring constant of 0.01 Nm^{-1} and a resonance frequency of 7 ± 3 kHz was used. The images were obtained at 512 × 512 pixel resolution with a scan rate of 0.3–1.0 Hz. The data analysis was carried out by JPK image processing software.

2.6. Statistic analysis

If not stated otherwise, the results are expressed as a mean ± standard error (SD). Statistical analysis was carried out by Student's *t*-test, *P*-values <0.05 were considered significant.

3. Results

The ICAM-1 protein expression in pericytes was analyzed by Western blot analysis and densitometrical measurements of the band intensity after they have been treated with 2 ng/ml TNF-α or stimulant free media (Fig. 1). TNF-α increased the ICAM-1 expression significantly to 1.80 ± 0.12-fold in comparison to the unstimulated control (set to 1.0 ± 0.24-fold). In addition, a minor expression of ICAM-1 in unstimulated pericytes is visible in Western blot analysis. Immunofluorescent analysis revealed that neutrophils attach to pericytes, showing the functionality of the increased adhesion molecule expression on pericytes' cell surface to adhere neutrophils after TNF-α treatment (Fig. 2). The initial contact between pericytes and neutrophils was examined by scanning force microscopy. It is shown that pericytes as well as

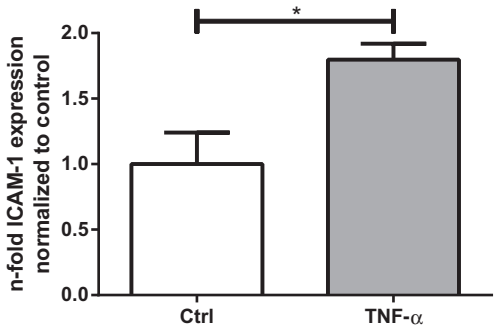

Fig. 1. Densitometrical Western blot analysis of ICAM-1 protein performed 24 h after the addition of TNF-α or stimulant free media. Values of treated samples were normalized to the control (untreated) which was set to 1, $N = 3$.

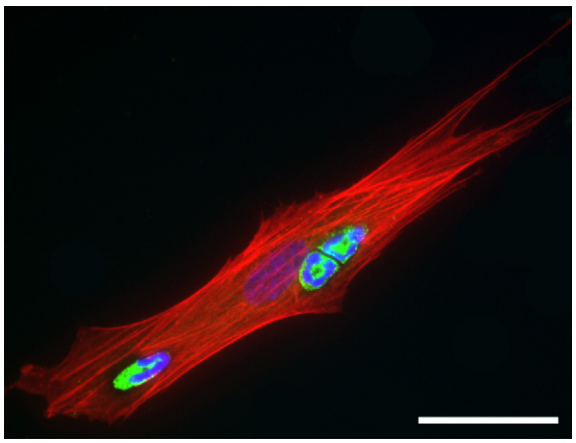

Fig. 2. Immunofluorescent staining of neutrophils adhered to a pericyte on collagen G coated cover slides performed 24 h after stimulation with TNF-α and 6 h after application of neutrophils. The actin skeleton of the PBCPs was stained with Phalloidin (red), neutrophils were stained by an antibody against granulocytes (green) and the nuclei were stained with DAPI (4′ 6-diamino-2-phenylindole) (blue). Scale bar represents 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

neutrophils developed extensions towards the other cell and spread on the substrate. Pericytes are characterized by broad extensions with a maximum height of around 250 nm (Fig. 3A, red arrow and line). As opposed to this, neutrophils' initial extensions are long and thin with a maximum height of 80 nm and a width of 1 μm (Fig. 3B, blue arrow and line). After contact with the pericyte, neutrophils' extensions thicken to 240 nm in height and 2 μm in width (Fig. 3B green arrow and line). For both cell types, these extensions are only visible when a neighboring cell is in close distance to the extension building cell.

4. Discussion

In this study we could show that pericytes increase the expression of ICAM-1 in response to TNF-α stimulation after 24 h. For our experiments we used αSMA$^{neg.}$ pericytes which represent the situation *in vivo* the most [10–12]. The expression of proteins and the induction of the blood brain barrier integrity by pericytes are dependent on pericytes' differentiation stage which again is regulated by the cell culture conditions [6,13,14]. In our work we figured out that αSMA$^{neg.}$ PBCPs constitutively express ICAM-1 as seen in the Western blot analysis. These findings are in accordance to the heterogeneous results of others where pericytes express

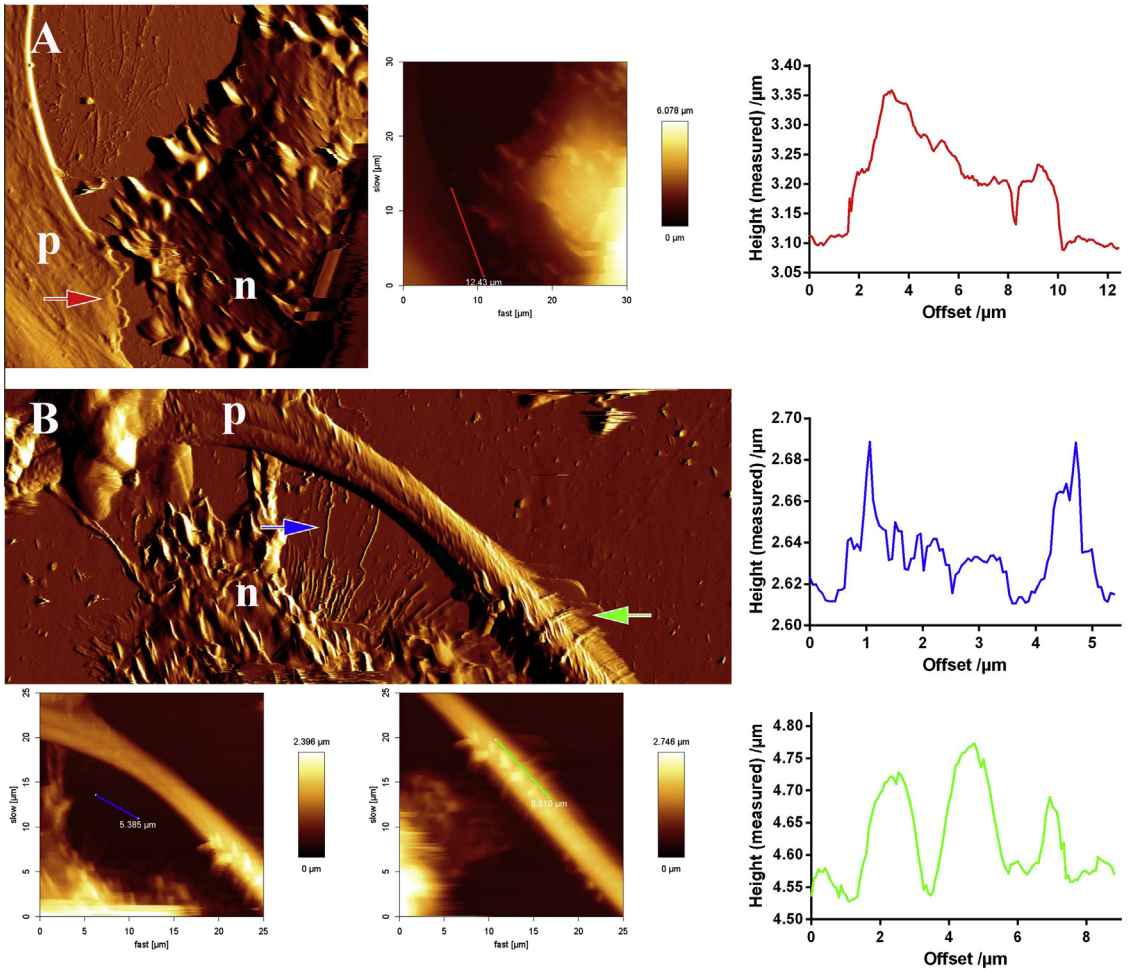

Fig. 3. Nanoscale morphologies of the cell–cell contact between pericytes (p) and neutrophils (n) after the stimulation with TNF-α (24 h) and the application of neutrophils (6 h). (A) Pericyte's extension (red) towards a neutrophil in deflection image (left), the SFM height image (middle) and the analyzed line profile (right). (B) Neutrophil's extensions towards a pericyte in a merged deflection image (upper left), two corresponding SFM height images (lower left, middle) and the analyzed line profiles (upper right, lower right) of initial contact extensions (blue) and matured contact extension (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

constitutively low amounts of ICAM-1 or not, depending on their differentiation stage and origin [4,15]. ICAM-1 is one of the most important adhesion molecules during neutrophil transmigration across the blood vessel [2]. The dependency and importance of ICAM-1 during the process of transmigration was emphasized by inhibition experiments, where the blocking of the adhesion molecule leads to severe consequences in immunological processes [16–18]. Furthermore, an ICAM-1–LFA-1 depending cell crawling between pericytes and neutrophils was figured out, indicating the importance of the subendothelial region during the process of immune cell diapedesis into the brain [4]. Next to the cell interaction between neutrophils and pericytes, the assumption of cell adhesion towards other cells of the NVU has been proven by the adhesion between the immune cells and astrocytes [19]. Although, no adhesion molecule has been investigated in this study, due to the expression of ICAM-1 on the astrocytes surface it can be speculated that this interaction is mediated by the same molecule.

The functionality of the adhesion between neutrophils and pericytes was confirmed in this work by immunofluorescent cell staining. We observed neutrophils adhered to pericytes which is in accordance to our previous study where neutrophils' adhesion to pericytes was further strengthened by using an inhibitor against matrix metalloproteinases [5]. The immunofluorescent staining is a fast, specific and visible method to determine the cell adhesion. However, with this method no high resolution cell morphology images according to the initial cell contact can be obtained. To investigate this, we analyzed the first initial contact between the cells by SFM. We could show that both cell types are able to perform cell extensions towards the other cell type. Cell extensions of migrating cells like macrophages had been shown before by SFM but regardless their dimensions [20]. Here, we could show for the first time that neutrophils and pericytes express initial cell extensions which differ in their structure according to whether the cells belong to adherent or suspensory cells. The adherent pericytes are characterized by wide and relatively high initial extensions (8 μm width, 250 nm high). In contrast, neutrophils as an example of suspensory cells perform relatively thin and plain extensions (1 μm width, 80 nm high). After having contact to pericytes theses structures thicken more to 2 μm in width and up to 250 nm in height. This indicates that pericytes may provide structures supporting neutrophils' migration.

Acknowledgments

The authors are very grateful to Sabine Hüwel and Patrick Seelheim for their continuous help and the constructive discussions.

References

[1] A. Woodfin, M.B. Voisin, S. Nourshargh, Recent developments and complexities in neutrophil transmigration, Curr. Opin. Hematol. 17 (2010) 9–17.
[2] K. Ley, C. Laudanna, M.I. Cybulsky, S. Nourshargh, Getting to the site of inflammation: the leukocyte adhesion cascade updated, Nat. Rev. Immunol. 7 (2007) 678–689.
[3] M. Phillipson, B. Heit, P. Colarusso, L. Liu, C.M. Ballantyne, P. Kubes, Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade, J. Exp. Med. 203 (2006) 2569–2575.
[4] D. Proebstl, M.B. Voisin, A. Woodfin, J. Whiteford, F. D'Acquisto, G.E. Jones, D. Rowe, S. Nourshargh, Pericytes support neutrophil subendothelial cell crawling and breaching of venular walls in vivo, J. Exp. Med. 209 (2012) 1219–1234.
[5] C. Pieper, P. Pieloch, H.J. Galla, Pericytes support neutrophil transmigration via Interleukin-8 across a porcine co-culture model of the blood–brain barrier, Brain Res. 1524 (2013) 1–11, http://dx.doi.org/10.1016/j.brainres.2013.05.047.
[6] G. Thanabalasundaram, C. Pieper, M. Lischper, H.J. Galla, Regulation of the blood–brain barrier integrity by pericytes via matrix metalloproteinases mediated activation of vascular endothelial growth factor in vitro, Brain Res. 1347C (2010) 1–10.
[7] S. Kroll, J. El-Gindi, G. Thanabalasundaram, P. Panpumthong, S. Schrot, C. Hartmann, H.J. Galla, Control of the blood–brain barrier by glucocorticoids and the cells of the neurovascular unit, Ann. N. Y. Acad. Sci. 1165 (2009) 228–239.
[8] H. Franke, H. Galla, C.T. Beuckmann, Primary cultures of brain microvessel endothelial cells: a valid and flexible model to study drug transport through the blood–brain barrier in vitro, Brain Res. Brain Res. Protoc. 5 (2000) 248–256.
[9] M. von Wedel-Parlow, S. Schrot, J. Lemmen, L. Treeratanapiboon, J. Wegener, H.J. Galla, Neutrophils cross the BBB primarily on transcellular pathways: an in vitro study, Brain Res. 1367 (2011) 62–76.
[10] V. Nehls, D. Drenckhahn, Heterogeneity of microvascular pericytes for smooth muscle type alpha-actin, J. Cell Biol. 113 (1991) 147–154.
[11] V. Nehls, D. Drenckhahn, The versatility of microvascular pericytes: from mesenchyme to smooth muscle?, Histochemistry 99 (1993) 1–12
[12] M.M. Verbeek, I. Otte-Holler, P. Wesseling, D.J. Ruiter, R.M. de Waal, Induction of alpha-smooth muscle actin expression in cultured human brain pericytes by transforming growth factor-beta 1, Am. J. Pathol. 144 (1994) 372–382.
[13] P. Dore-Duffy, A. Katychev, X. Wang, E. Van Buren, CNS microvascular pericytes exhibit multipotential stem cell activity, J. Cereb. Blood Flow Metab. 26 (2006) 613–624.
[14] G. Thanabalasundaram, J. Schneidewind, C. Pieper, H.J. Galla, The impact of pericytes on the blood–brain barrier integrity depends critically on the pericyte differentiation stage, Int. J. Biochem. Cell Biol. 43 (2011) 1284–1293.
[15] K. Stark, A. Eckart, S. Haidari, A. Tirniceriu, M. Lorenz, M.L. von Bruhl, F. Gartner, A.G. Khandoga, K.R. Legate, R. Pless, I. Hepper, K. Lauber, B. Walzog, S. Massberg, Capillary and arteriolar pericytes attract innate leukocytes exiting through venules and 'instruct' them with pattern-recognition and motility programs, Nat. Immunol. 14 (2013) 41–51.
[16] F. Vanky, P. Wang, M. Patarroyo, E. Klein, Expression of the adhesion molecule ICAM-1 and major histocompatibility complex class I antigens on human tumor cells is required for their interaction with autologous lymphocytes in vitro, Cancer Immunol. Immunother. 31 (1990) 19–27.
[17] S.K. Lo, G.A. Van Seventer, S.M. Levin, S.D. Wright, Two leukocyte receptors (CD11a/CD18 and CD11b/CD18) mediate transient adhesion to endothelium by binding to different ligands, J. Immunol. 143 (1989) 3325–3329.
[18] M.W. Makgoba, M.E. Sanders, G.E. Ginther Luce, E.A. Gugel, M.L. Dustin, T.A. Springer, S. Shaw, Functional evidence that intercellular adhesion molecule-1 (ICAM-1) is a ligand for LFA-1-dependent adhesion in T cell-mediated cytotoxicity, Eur. J. Immunol. 18 (1988) 637–640.
[19] L. Xie, E.C. Poteet, W. Li, A.E. Scott, R. Liu, Y. Wen, A. Ghorpade, J.W. Simpkins, S.H. Yang, Modulation of polymorphonuclear neutrophil functions by astrocytes, J. Neuroinflammation 7 (2010) 53.
[20] M. Li, L. Liu, N. Xi, Y. Wang, X. Xiao, W. Zhang, Imaging and measuring the biophysical properties of Fc gamma receptors on single macrophages using atomic force microscopy, Biochem. Biophys. Res. Commun. 438 (2013) 709–714.