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Biochemical and Biophysical Research Communications 354 (2007) 84–89
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S100A9 mediates neutrophil adhesion to fibronectin through activation of β2 integrins

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Received 11 December 2006
Available online 8 January 2007

Abstract

Neutrophil migration from the blood to inflammatory sites follows a cascade of events, in which adhesion to endothelial cells and extracellular matrix proteins is essential. S100A8, S100A9, and S100A12 are small abundant proteins found in human neutrophil cytosol and presumed to be involved in leukocyte migration. Here we investigated the S100 proteins' activities in neutrophil tissue migration by evaluating their effects on neutrophil adhesion to certain extracellular matrix proteins. S100A9 induced adhesion only to fibronectin and was the only S100 protein that stimulated neutrophil adhesion to this extracellular matrix protein. Experiments with blocking antibodies revealed that neither β1 nor β3 integrins were strongly involved in neutrophil adhesion to fibronectin, contrary to what the literature predicted. In contrast, neutrophil adhesion to fibronectin was completely inhibited by anti-β2 integrins, suggesting that S100A9-induced specific activation of β2 integrin is essential to neutrophil adhesion.

Keywords: S100 proteins; Adhesion molecules; Extracellular matrix; Inflammation; Integrins

The S100 proteins S100A8, S100A9, and S100A12 are small calcium-binding proteins constitutively expressed in the cytosol of neutrophils [1]. They exist as nonconvalent dimers, either in homodimers or in a S100A8/A9 heterodimer [2]. These proteins are found at elevated levels in the serum and at extravascular sites in people affected with diverse infections and inflammatory pathologies [3–5]. Once in the extracellular environment, S100A8, S100A9, and S100A12 show proinflammatory activities; for example, in vivo studies indicated that S100A8 and S100A9 induce the release of neutrophils from the bone marrow and are important for neutrophil accumulation in response to LPS [6]. In addition, S100A8/A9 promotes monocyte transendothelial migration, probably by decreasing tight junction protein expression and up-regulating ICAM-1 surface expression [7,8]. Moreover, S100A8, S100A9, and S100A12 induce neutrophil chemotaxis [9,10]. Thus, several reports support that S100 proteins are involved in leukocyte migration.

Neutrophil migration from the blood to the inflammatory site unfolds through a tightly regulated series of events [11], of which neutrophil adhesion to endothelial cells and extracellular matrix components is essential. Most of these interactions are mediated by integrins, a family of widely expressed cell surface adhesion molecules. The β2 integrins subset (αLβ2 (CD11a/CD18), αMβ2 (CD11b/CD18), αXβ2 (CD11c/CD18), and αDβ2 (CD11d/CD18)) is defined by leukocyte-restricted expression. β2 integrins are generally involved in cell–cell interactions, while β1 and β3 integrins mainly mediate cell–extracellular matrix protein interactions [12]. For example, most of β1 and β3 integrins expressed on leukocytes—including α2β1 (CD49b/CD29), α4β1 (CD49d/CD29), α5β1 (CD49e/CD29), α6β1 (CD49f/CD29), and αVβ3 (CD51/CD61)—are receptors for extracellular matrix proteins such as laminin and collagen, which are present in the basement membrane [13]; or fibronectin and vitronectin, which are two major components of the interstitial space [14].

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.12.203

Regulation of integrin activation is essential because cell adhesion must be controlled in time and space in processes such as diapedesis and tissue migration. Integrins of the resting neutrophil are maintained in an inactive conformation, cell stimulation is required to activate integrins and increase their ligand binding capacity. Lipid mediators, cytokines and chemotactic factors are known activators of integrins that work by inducing integrin clustering (avidity) or conformational change (affinity), or both [15]. Neutrophils must thus integrate signals originating from a complex mix of chemoattractants to regulate their adhesive state [16].

In vitro studies have shown that S100A8, S100A9, and S100A12 affect neutrophils both by stimulating their adhesion to fibrinogen in a β2 integrin-dependent manner and by acting as chemoattractants [9,17]. However, the roles of S100 proteins in tissue migration of neutrophils remain unknown. In this study, we investigated the functions of S100A8, S100A9, and S100A12 in neutrophil tissue migration by evaluating their effects on neutrophil adhesion to extracellular matrix proteins.

Materials and methods

Reagents and cells. Vitronectin was obtained from BD Biosciences (Mississauga, ON). Laminin and collagen type IV were purchased from Sigma–Aldrich (Oakville, ON). Purified human fibronectin was a generous gift from Dr. André Beaulieu (CRCHUQ, QC). The nomenclature of the different integrins investigated here, as well as the antibodies directed against each of them are provided in Table 1. Isotype control Abs were purchased from Sigma–Aldrich (St. Louis, MO). ALEXA 488-conjugated goat anti-mouse IgG Ab was obtained from Molecular Probes (Eugene, OR). Neutrophils, as well as recombinant S100A8, S100A9, and S100A12 were purified as previously described [9,17].

Table 1
Monoclonal antibodies used in this study

Integrin	Clone	Ig subclass	Use
α2 (CD49b)	AK-7[a]	IgG1	FC, B
α4 (CD49d)	9F10[a]	IgG1	FC, B
α5 (CD49e)	IIA1[a]	IgG1	FC, B
β1 (CD29)	P4C10[b]	IgG1	FC, B
β1 (CD29) activated	HUTS-4[b]	IgG2b	FC
αV (CD51)	M9[b]	IgG1	FC, B
αVβ3 (CD51/CD61)	LM609[b]	IgG1	FC, B
αL (CD11a)	MEM-25[c]	IgG1	FC, B
αM (CD11b)	ICRF44[d]	IgG1	FC, B
αX (CD11c)	3.9[e]	IgG1	FC, B
αD (CD11d)	236L[f]	IgG1	FC, B
β2 (CD18)	IB4[g]	IgG2a	FC, B
β2 (CD18) activated	mAb24[h]	IgG1	FC, B

[a] BD Biosciences Pharmingen (Mississauga, ON).
[b] Chemicon International (Temecula, CA).
[c] Exbio Praha (Vestec, Czech Republic).
[d] Sigma–Aldrich (St. Louis, MO).
[e] Chemicon Europe (Hampshire, UK).
[f] Generous gift from Dr. D. Allison (ICOS, Bothell, WA).
[g] Generous gift from Dr. P.H. Naccache (CRCHUQ, QC, Canada).
[h] Generous gift from Dr. N. Hogg (Cancer Research UK, London, UK).
FC, flow cytometry; B, Blocking.

Adhesion to extracellular matrix proteins. Ninety-six-well immunoassay plates (Corning Inc., Oneonta, NY) were coated and left overnight at 4 °C with 50 μL of different extracellular matrix protein solutions in $NaHCO_3$ 0.1 M, pH 9.6. The concentrations used were 50 μg/mL for fibronectin, 5 μg/mL for vitronectin, 10 μg/mL for laminin, and 1 μg/mL for collagen type IV. The plates were washed three times with 100 μL M199 culture medium (Wisent, St. Bruno, QC) before use. Fifty microliters of S100 proteins diluted in M199 medium at 20 μg/mL were added to the wells. To obtain the complex S100A8/SA9, equimolar amounts of S100A8 and S100A9 were mixed and incubated 15 min at 37 °C. M199 medium and fMLF 10^{-6} M were used as a negative and positive controls, respectively. Neutrophils were resuspended at 5×10^6 cells/mL in M199 medium and labelled with 5 μM intracellular fluorescent dye calcein-AM (Calbiochem, San Diego, CA) for 30 min at 37 °C. After washing, 10 μL of the cell suspension was added to the wells. Neutrophils were let to adhere for 30 min at 37 °C before being washed 3 times by immersion in cold PBS. The adherent cells were lysed by adding deionized distilled water, and fluorescence was measured at $\lambda_{ex} = 485$ nm and $\lambda_{em} = 530$ nm using a 96-well plate fluorescence reader. In the blocking experiments, the neutrophils were preincubated for 20 min at 37 °C with 30 μg/mL of Abs directed against the different subunits of β1, β2, and β3 integrins, or their isotype controls before being added to the wells. The inhibition coefficient was calculated as followed: $100 - ([N - N_{ctl}]/[N_{iso} - N_{ctl}] \times 100)$, where N = stimulated cells with blocking antibody, N_{ctl} = unstimulated cells, and N_{iso} = stimulated cells with isotype control antibody.

Flow cytometry analysis. Neutrophils (10×10^6 cells/mL) were stimulated with S100A9 (20 μg/mL) or HBSS at 37 °C for 30 min and then washed with HBSS-0.1% autologous serum. To detect β1, β2, β3 integrin expression, neutrophils (1×10^6 cells/well) were incubated at the end of the stimulation period with 1 μg of Abs directed against the different subunits of β1, β2, and β3 integrins, or their isotype controls on ice for 25 min. To detect CD18 activation, the neutrophils were incubated with 1 μg of mAb24 at 37 °C 10 min before the end of the stimulation time. After 2 washes in PBS/0.2% BSA/0.1% azide, the neutrophils were incubated with ALEXA 488-conjugated goat anti-mouse IgG Ab for 25 min on ice, washed twice and fixed in PBS containing 2% formaldehyde. Detection was done with an EPICS XL cytometer (Beckman Coulter, Mississauga, ON).

Results and discussion

Effect of S100 proteins on neutrophil adhesion to extracellular matrix proteins

S100A8, S100A9, and S100A12 have been shown to stimulate neutrophil adhesion to fibrinogen [9,17]. To find out whether S100 proteins also stimulated adhesion to other extracellular matrix proteins, neutrophils were let to adhere onto extracellular matrix proteins in presence of the different S100 proteins. Fibronectin, vitronectin, laminin, and collagen were chosen as representative proteins of the extracellular matrix; fMLF, a powerful neutrophil agonist, induced neutrophil adhesion to all proteins tested (Fig. 1). As shown in Fig. 1A, only S100A9 and S100A8/A9 induced neutrophil adhesion to fibronectin. S100A9 and S100A8/A9 also consistently induced adhesion to vitronectin, but not with statistical significance (Fig. 1B). Neutrophils from most donors adhered to these matrixes, although some failed to respond (data not shown). That neutrophil adhesion to fibronectin and vitronectin was not affected by S100A8 nor by S100A8/A9 any more than S100A9 alone suggests that the adhesion seen in these experiments is mediated by S100A9. Interestingly,

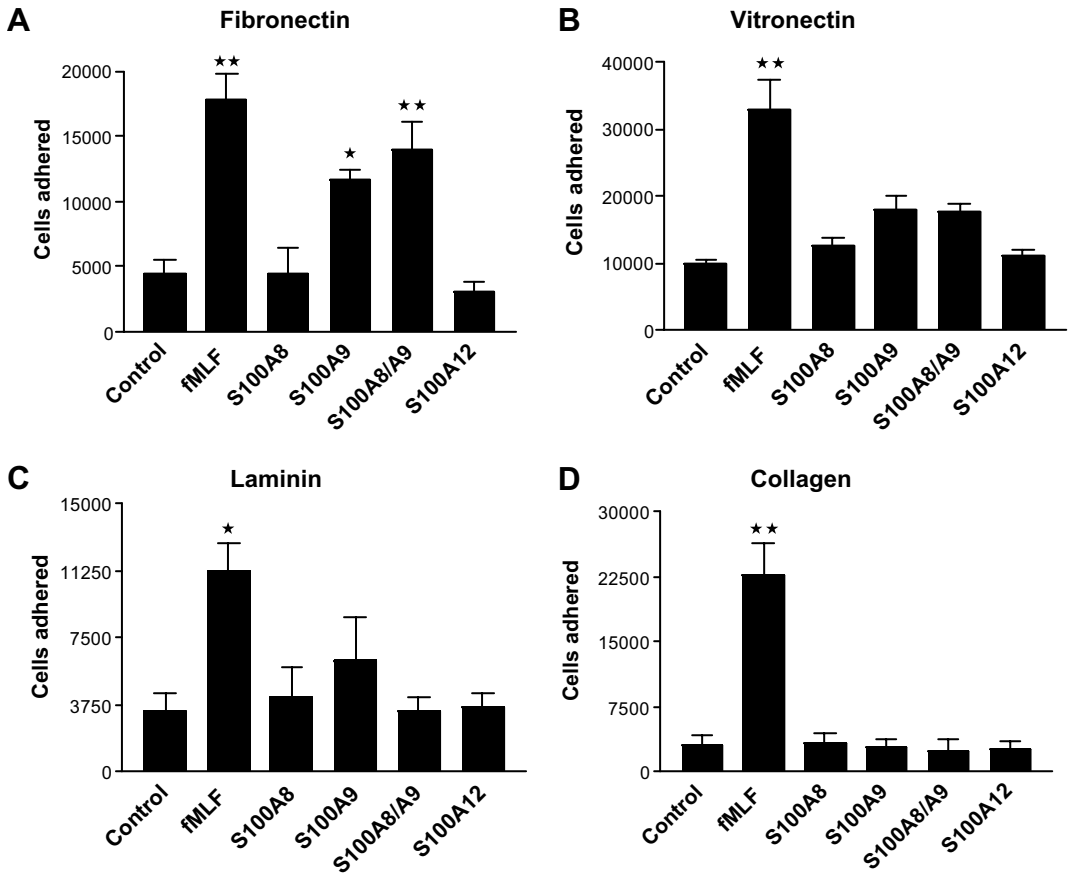

Fig. 1. S100A9 induces neutrophil adhesion to fibronectin. Neutrophils (5×10^4 cells/well) were incubated with S100A8, S100A9, S100A8/A9 or S100A12 (20 μg/mL), fMLF 10^{-6} M (positive control) or M199 (negative control) and let to adhere on fibronectin (A), vitronectin (B), laminin (C), and collagen (D) for 30 min at 37 °C. The number of adhered neutrophils was determined as described in Materials and methods. Data shown represent means + SEM of $\geqslant 3$ experiments performed on neutrophils from different donors. $^{\star}p < 0.05$, $^{\star\star}p < 0.01$, one-way ANOVA, Dunnett multiple comparison test (compared with control).

none of the S100 proteins induced neutrophil adhesion to laminin or collagen (Fig. 1C and D). These results show that S100A9 only stimulates neutrophil adhesion to the extracellular matrix proteins fibronectin, and at a lesser degree, vitronectin. Thus, S100 proteins have distinct functions during neutrophil migration within the inflamed tissue that arise from differential stimulation of adhesion to extracellular matrix proteins.

The importance of S100A9 and S100A8/A9 to leukocyte migration across endothelial cells has been shown in reports indicating that they enhance monocyte transendothelial migration possibly by decreasing tight junction protein expression and up-regulating ICAM-1 (CD54) surface expression on endothelial cells [7,8]. Our results show that S100A9 also stimulates neutrophil adhesion to fibronectin. The glycoproteins fibronectin and vitronectin are present in the interstitial space and play a major role in cell adherence to the tissue [14], whereas laminin and collagen type IV are found only in the basement membrane, a specialized matrix which surrounds endothelial cells [13]. This suggests that S100A9 also regulates neutrophil migration within the tissue after the neutrophils leave the vicinity of endothelial cells.

Involvement of integrins in S100A9-induced neutrophil adhesion to fibronectin

The β1 and β3 integrins are major cell-extracellular matrix adhesion proteins found on the surface of leukocytes. To evaluate their roles in S100A9-induced neutrophil adhesion to fibronectin, we examined the surface expression of neutrophil integrins involved in fibronectin recognition. The integrins α2β1 (CD49b/CD29), α4β1 (CD49d/CD29), α5β1(CD49e/CD29), and αVβ3 (CD51/CD61) are known to bind fibronectin [18]. Neutrophils were stimulated with S100A9 incubated with anti-β1 and -β3 integrins Abs, and analyzed by flow cytometry. All integrins tested were weakly expressed on the surface of resting neutrophils. Unexpectedly, S100A9 did not increase the expression of these integrins on neutrophil surface (Fig. 2A and B). In contrast, significant increases in αL (CD11a), αM (CD11b), αX (CD11c), and β2 (CD18) subunit surface expression, particularly in its activated form, were observed following stimulation with S100A9 (Fig. 2C). These results suggested that β2 integrins might play a role in neutrophil adhesion to fibronectin.

To investigate the role of β1, β2, and β3 integrins in neutrophil adhesion to fibronectin, neutrophils were incubated

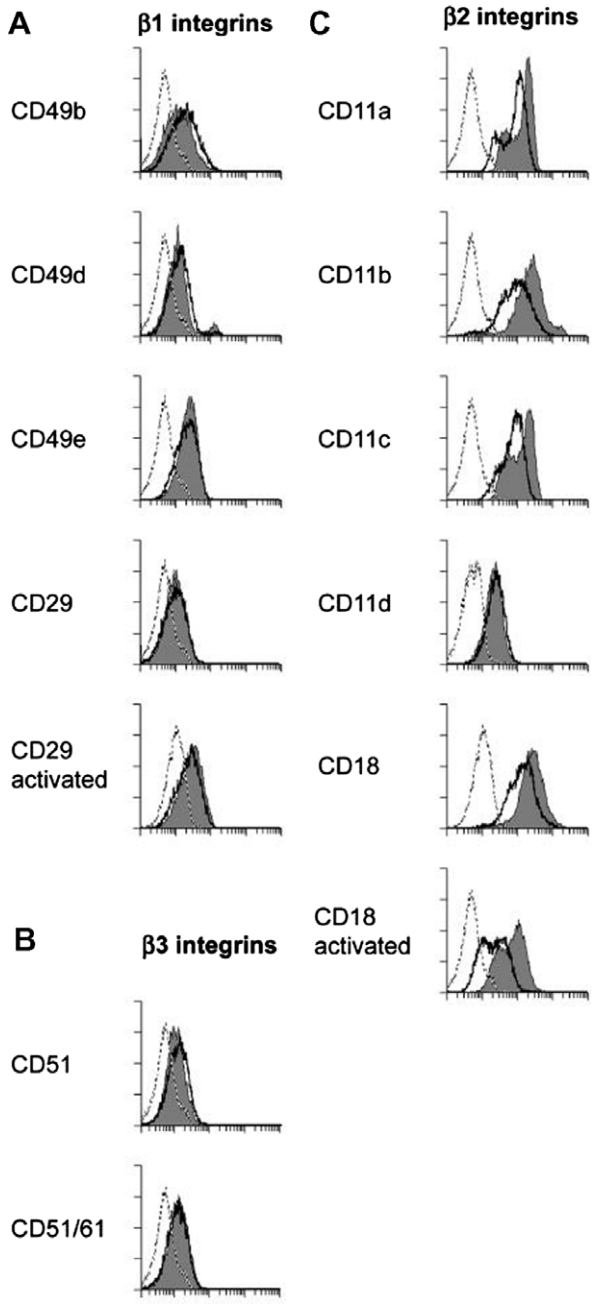

Fig. 2. S100A9 has no effect on β1 and β3 integrins, but upregulates β2 integrins surface expression. Expression of β1, β2, and β3 integrins was analyzed on S100A9-stimulated neutrophils by flow cytometry analysis. Neutrophils were stimulated with S100A9 (20 μg/mL) for 30 min at 37 °C and incubated with specific Abs directed against the different subunits of β1, β2, and β3 integrins. Histograms of S100A9-stimulated neutrophils are illustrated in gray, while unstimulated neutrophils (HBSS) appear in open histograms. Nonspecific stainings are illustrated by a dotted line. Data shown are from an experiment representative of 2 others done on cells from different donors.

with the different blocking Abs before being let to adhere onto fibronectin. The Abs directed against the β1 integrins (alone or in combinations) did not inhibit S100A9-induced neutrophil adhesion to fibronectin (Fig. 3A). However, the anti-CD49d, CD49e, and CD29 subunits Abs inhibited PMA-induced Jurkat cells adhesion to fibronectin, confirming their blocking activity (data not shown). On the other hand, although it did not reach statistical significance, the anti-β3 integrin (CD51) antibody partially inhibited S100A9-induced neutrophil adhesion to fibronectin (57% of inhibition) (Fig. 3B). It is worth noticing that variability between blood donors explained the difficulty to obtain statistical significance. As the flow cytometry analysis predicted, these results indicated that integrins other than β1 and β3 participate in neutrophil adhesion to fibronectin. To further test whether β2 integrins play a role in neutrophil adhesion to fibronectin, neutrophils were incubated with blocking Abs against the different β2 integrin subunits before being let to adhere to fibronectin. Unexpectedly, the Ab IB4 directed against the β2 (CD18) subunit completely blocked S100A9-induced neutrophil adhesion to fibronectin (Fig. 3C). The failure of anti-β2 integrin to inhibit neutrophil adhesion to plastic confirmed the specificity of the observed interaction between the β2 integrins and fibronectin (data not shown). However, although the anti-CD11b Ab slightly reduced (not statistically significant) S100A9-induced neutrophil adhesion to fibronectin (30% inhibition), none of the Abs directed against the other α subunits blocked neutrophil adhesion to fibronectin. This absence of inhibition could not be explained by the inability of the Abs to block adhesion as anti-CD11a inhibited PMA-stimulated Jurkat cell adhesion to fibronectin (data not shown).

Most of the literature reports that activation of the β1 and β3 integrins [12,18] mediates leukocyte adhesion to the extracellular matrix proteins; however our results and few recent studies report existence of other receptors. More precisely, αMβ2 (CD11b/CD18) has been shown to be involved in neutrophil adhesion to fibronectin [19]. Although it did not reach statistical significance, we also observed moderate inhibition with anti-αM, suggesting that αMβ2 (CD11b/CD18) could be used by neutrophils to bind to fibronectin. However, we could not identify the exact β2 integrin involved in neutrophil adhesion to fibronectin because all Abs against α subunit tested did not completely inhibit neutrophil adhesion. Possible explanations for this would be that the site on α subunits binding to fibronectin differs from that binding β2 integrin ligands such as ICAM-1 (CD54), LPS, and fibrinogen. Another explanation would be that the site recognized by fibronectin could lie in the β2 subunit alone. Indeed sequences other than the I-domain of the α subunit have been shown to be involved in ligand binding [20].

In conclusion, the present study shows that S100A9 induces neutrophil adhesion to fibronectin (a major constituent of the extracellular matrix), which supports its key role in neutrophil migration to the inflammatory site. S100 proteins could thus be involved in neutrophil adhesion to endothelium and extracellular matrix proteins by activating β2 integrins and in tissue migration by attracting neutrophils [9,17]. This could explain the dramatic inhibition of neutrophil migration to inflammatory sites

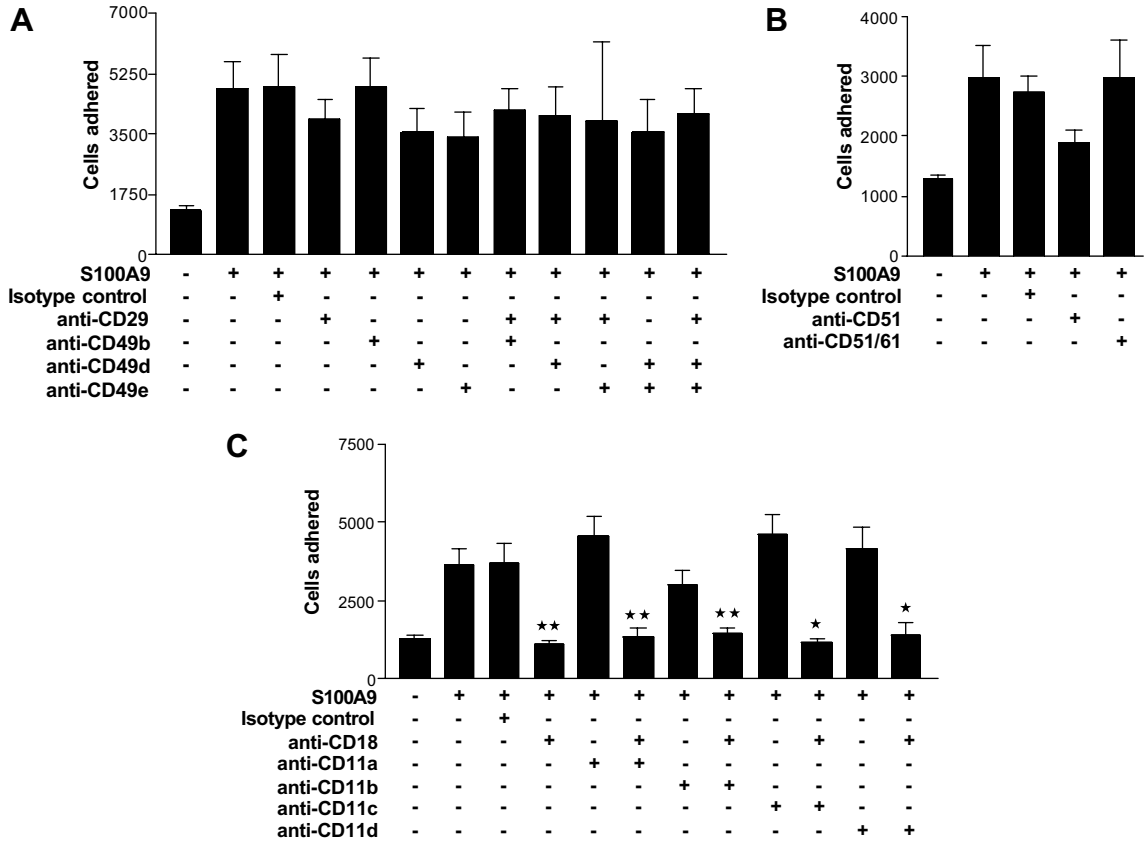

Fig. 3. S100A9 stimulates neutrophil adhesion to fibronectin via β2, but not β1 nor β3 integrins. Neutrophils were incubated with blocking Abs directed against (A) β1 integrins, (B) β3 integrins, or (C) β2 integrins and their adhesion to fibronectin was stimulated with S100A9 (20 μg/mL) or M199 (negative control) for 30 min at 37 °C. Data shown represent means + SEM of ⩾3 experiments performed on neutrophils from different donors. $^{\star}p < 0.05$, $^{\star\star}p < 0.01$, one-way ANOVA, Dunnett multiple comparison test (compared with isotype control).

observed in vivo using blocking antibodies against S100A8 and S100A9 [6,21].

Acknowledgments

We thank Dr. A. Beaulieu (CRCHUQ, QC) for providing fibronectin, as well as Dr. D. Allison (ICOS, Bothell, WA), Dr. N. Hogg (Cancer Research UK, London, UK) and Dr. P.H. Naccache (CRCHUQ, QC) for providing antibodies. Funding for this research project was provided by grants from the Canadian Institutes for Health Research (57777) and the National Institutes of Health (AR052614). P.A.T. holds a scholarship from the Fonds de la Recherche en Santé du Québec.

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