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Analysis of sialoadhesin expression on mouse alveolar macrophages

Julie Ducreux[a], Paul R. Crocker[b], Rita Vanbever[a,*]

[a] Department of Pharmaceutical Technology, Université catholique de Louvain, 1200 Brussels, Belgium
[b] Wellcome Trust Biocentre, College of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom

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ABSTRACT

Sialoadhesin (Sn) is a macrophage-restricted receptor that was first characterised on mouse resident bone marrow macrophages as a receptor that mediates the binding, without ingestion, of sheep erythrocytes. Sn is highly conserved in mammals but its expression on tissue macrophages is heterogeneous. In the mouse, high levels of erythrocytes binding are shown on macrophages from lymphoid tissues but a low erythrocytes binding activity is detectable on macrophages isolated from the broncho-alveolar space. Yet, Sn expression has been demonstrated on human, rat and pig alveolar macrophages (AM) using methods of molecular biology. Therefore, the present study aimed to investigate the expression of Sn on mouse AM in order to confirm the presence of the protein on this population of murine macrophages. Using cytometrical analyses, we showed that Sn was expressed on mouse AM surface. Following desialylation, AM largely bound erythrocytes and this binding was inhibited by 3D6, an anti-mouse Sn monoclonal antibody, in a dose-dependent manner. This indicates that Sn is expressed on mouse AM but that the sialic acid binding activity mediated by this molecule is naturally masked by endogenous sialic acid within the glycocalyx on the cell surface.

1. Introduction

Sialoadhesin (Sn, Siglec-1, CD169) is a macrophage-restricted receptor that mediates sialic acid-dependent adhesion to a range of cell types including lymphoid and myeloid cells [1]. Sn was originally described on murine resident bone marrow macrophages as a sheep erythrocyte receptor which mediates binding but not phagocytosis of erythrocytes via recognition of sialylated glycoconjugates [2]. In addition to bone marrow macrophages, erythrocytes binding or rosetting activity of Sn has been demonstrated in other murine macrophage populations, especially those isolated from lymph nodes and spleen [3]. In contrast, macrophages isolated by lavage from the bronchoalveolar space and the peritoneal cavity show low although detectable levels of rosetting activity [2]. This activity can be induced by a still unidentified factor present in mouse serum, but that is absent from a range of other species sera [4]. The production of anti-Sn monoclonal antibody (mAb) enabled the purification and characterisation of Sn as a 185 kDa member of the Siglec family of sialic acid binding immunoglobulin (Ig)-like lectins that preferentially recognises sialoglycoconjugates containing the terminal oligosaccharide Neu5Acα2,3Galβ1,3GalNAc [3,5,6].

Sn expression was initially demonstrated by the ability of macrophages to mediate erythrocytes binding. It was later confirmed on resident bone marrow macrophages and isolated from the spleen and lymph nodes using more specific methods. Among them, competitive haemagglutination assay, immunoprecipitation, western blot analysis and immunocytochemistry showed Sn expression on these macrophages [3,5]. Once molecular cloning of murine Sn was performed, northern blot analysis was also carried out and showed high levels of Sn on these macrophages [6]. Northern blot analysis showed low amounts of Sn mRNA detectable in the lung [6]. Yet, Sn expression on murine AM had not been further investigated as the low efficiency of these cells to naturally agglutinate erythrocytes, even when induced with mouse serum [4], suggested a low expression of the receptor.

However, Sn expression on AM has been shown in human, rat and pig using more sensitive methods than rosetting assays [7–9]. The aim of the present study was therefore to confirm or refute the expression of Sn on mouse AM using flow cytometry and competitive rosetting assays.

2. Experimental

2.1. Animals

Wild-type female C57BL6 and NMRI mice were bred at the Faculty of Medicine animal facilities of the Université catholique de Louvain (Belgium) and used between 7 and 11 weeks of age. Animals had free access to tap water and laboratory diet during the experimental period. All experimental protocols with mice were approved by the Institutional Animal Care and Use Committee of the Faculty

* Corresponding author. Tel.: +32 2 764 73 25; fax: +32 2 764 73 98.
E-mail address: rita.vanbever@uclouvain.be (R. Vanbever).

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of Medicine of the Université catholique de Louvain. Sn-deficient C57BL6 mice, generated as described [10] were kindly provided by the Wellcome Trust Biocentre (University of Dundee, UK).

2.2. Preparation of cells

Alveolar macrophages (AM) were harvested by bronchoalveolar lavage (BAL) using Hank's Buffered Salt Solution (HBSS) without Ca^{2+}, Mg^{2+} and phenol red (Invitrogen, Carlsbad, CA, USA). The BAL was performed by a slow injection of 1 mL HBSS into the trachea of euthanised animals following by the withdrawing of the liquid from the lungs. The lavage procedure was repeated once with 1 mL HBSS. About 10^5 cells, of which more than 90% were macrophages, were recovered per mouse [11].

2.3. Flow cytometry

AM, recovered by BAL, were resuspended in PBA (PBS + 1% BSA + 0.05% sodium azide) and incubated with rat anti-mouse CD16/32 IgG2b (clone 2.4G2, 1:150; BD Biosciences, San Diego, CA, USA) for 20 min at 4 °C to block Fc receptors. After washing with PBA, cells were incubated with biotinylated 3D6 mAb (rat anti-mouse Sn IgG2a, 10 μg/mL; prepared as described [12]) and subsequently labeled with phycoerythrin-conjugated streptavidin (1:300) and FITC-conjugated rat anti-mouse F4/80 mAb (1:100) for 30 min at 4 °C. Cells were finally stained with 7-amino actinomycin D to exclude dead cells and analysed using a FACScan (Becton Dickinson) and CellQuest software.

2.4. Red blood cells (RBC) binding assay

AM, recovered by BAL from NMRI mice, were resuspended in RPMI 1640 supplemented with 2 mM L-glutamine and 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA). A total of 50 μL suspensions of AM (2×10^6 cells/mL) were seeded in 96-wells tissue culture plate in warm medium. Non-adherent cells were removed by washing with serum-free RPMI medium after 40 min of incubation in a humidified atmosphere at 37 °C in 5% CO_2. Macrophages were then incubated in 100 μL serum-free RPMI + 0.1% BSA and 100 μL of freshly prepared human erythrocytes (from healthy volunteers) were added directly to the wells at 0.5% vol/vol and incubated at 37 °C for 30 min. Unbound red blood cells were removed by repeated washing with medium. Cells were then fixed by addition of 1% glutaraldehyde and the percentage of macrophage binding > 4 red blood cells was determined by microscopy (100 cells counted; Axiovert S100, Zeiss, Minneapolis, MN, USA) [3]. Sialidase pretreatments of erythrocytes or AM were carried out by incubating cells with *Vibrio cholerae* neuraminidase for 1 h at 37 °C (Calbiochem, La Jolla, CA, USA; 1/20 diluted in serum-free RPMI supplemented with 0.1% BSA).

To assess the effect of anti-Sn mAb on RBC binding, AM were previously treated with sialidase for 1 h at 37 °C. Cells were then precooled at 4 °C in serum-free RPMI supplemented with 0.1% BSA and subsequently incubated at 4 °C with rat anti-mouse 2.4G2 mAb for 20 min and 100 μL threefold serial dilutions of 3D6 (10 μg/mL) for 45 min. Human RBC were then directly added to cells as described above.

3. Results

3.1. Sn expression on mouse AM

To assess the expression of Sn on AM from C57BL6 mice, flow cytometry was performed on cells isolated from wild-type animals and compared to Sn-deficient mice. BAL of wild-type and Sn-deficient mice recovered a large majority of macrophages as showed by F4/80 positive cells (Fig. 1, inset). The anti-Sn mAb 3D6 stained AM recovered from wild-type mice whereas no staining was observed on AM from Sn-deficient mice (Fig. 1).

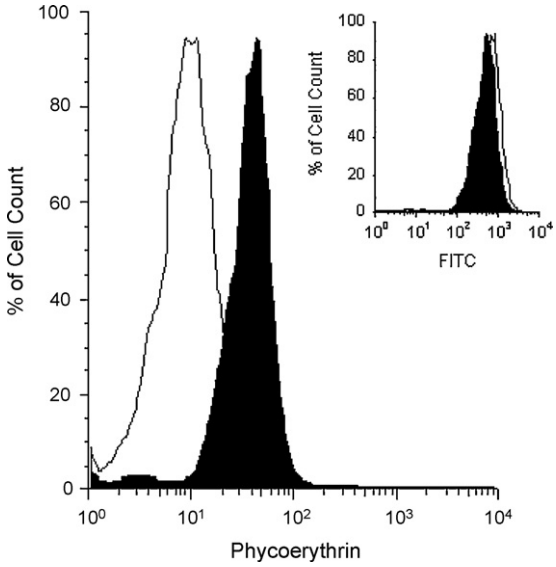

Fig. 1. Representative flow cytometrical analysis of freshly isolated alveolar macrophages (AM) from C57BL6 mice. Data showed binding of biotin-labeled 3D6 mAb on AM recovered from wild-type C57BL6 mice (shaded) in comparison to AM recovered from Sn-deficient C57BL6 mice (open). Inset, binding of anti-F4/80 mAb showed that all cells analysed were macrophages. This result is representative of 2 independent experiments.

In order to study the expression of Sn on AM from NMRI mice, competitive labeling of cells was performed using unlabeled 3D6 mAb (Fig. 2). Cells were first incubated with unlabeled 3D6 mAb for 1 h at 4 °C prior to the incubation with biotin-labeled 3D6. The pre-incubation with unlabeled mAb reduced the binding of biotinylated 3D6 mAb by approximately 70%.

3.2. Rosetting activity of mouse AM

Human red blood cells strongly bind Sn in a sialic acid dependent manner and this provides a useful model to study the adhesion mediated by Sn [5]. When freshly recovered AM were incubated

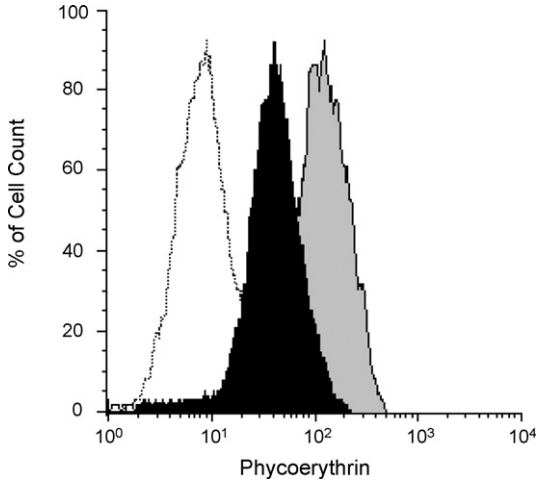

Fig. 2. Representative flow cytometrical analysis of freshly isolated AM from NMRI mice. Data showed binding of biotin-labeled 3D6 mAb on AM following their incubation with (black filled) or without (grey filled) unlabeled 3D6 mAbs. The fluorescence background (autofluorescence of unstained cells) is shown in dotted. This result is representative of 2 independent experiments.

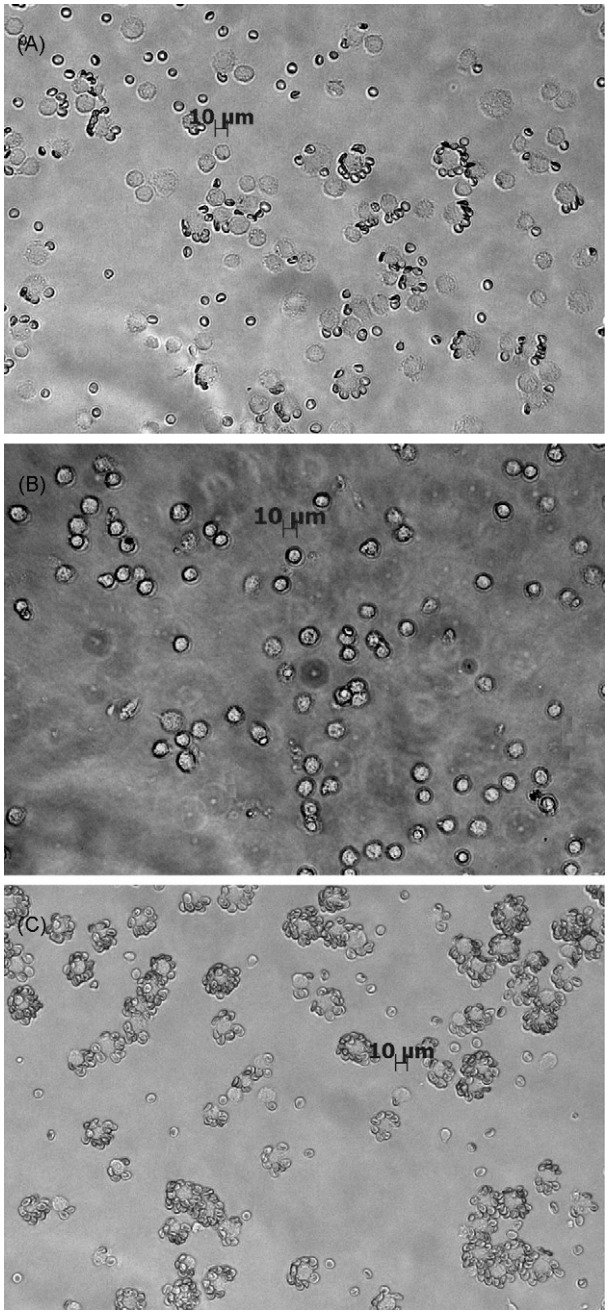

Fig. 3. Phase contrast micrographs of freshly isolated AM cultured on 96-wells plate and assayed for rosetting activity. A low level of rosetting was observed on freshly recovered AM (A) and it was completely abolished when erythrocytes were pre-treated with sialidase (B). In marked contrast, sialidase pretreatment of AM greatly increased the formation of rosettes (C).

with human RBC, a low binding was observed as only 10% of AM formed rosettes with erythrocytes (Fig. 3A). The low binding activity of AM was completely abolished when human RBC were pre-treated with sialidase (Fig. 3B). However, when AM were pre-treated with sialidase prior to RBC incubation, rosette formation increased greatly and almost 90% of AM bound 4 or more erythrocytes (Fig. 3C).

3.3. Functional inhibition of Sn

To confirm that Sn is expressed on AM and mediates erythrocyte binding, sialidase-pretreated AM were incubated with various concentrations of anti-Sn 3D6 mAb. When freshly recovered

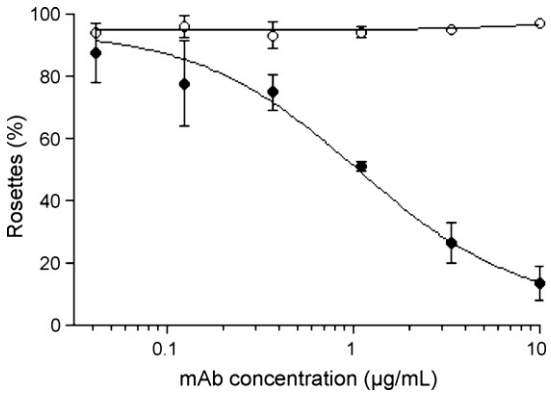

Fig. 4. Effect of 3D6 mAb on rosetting activity of desialylated AM. Freshly isolated AM were pretreated with *Vibrio cholerae* sialidase and incubated with serial dilution of 3D6 mAb (filled circles). The percentage of rosettes was normalised to the percentage of rosetting activity of desialylated AM in absence of 3D6 (considered to be equal to 100%). Experiments with irrelevant rat IgG2a isotype (open circles) did not show any inhibitory effect on rosette formation. Data are the mean ± S.D. of 2 pooled independent experiments (carried out in duplicate).

sialidase-pretreated AM were incubated with increasing concentrations of anti-Sn 3D6 mAb, the rosetting activity decreased by up to 90% (Fig. 4). Incubation of sialidase-pretreated AM with irrelevant IgG2a isotype did not show any inhibitory effect on rosette formation (Fig. 4).

4. Discussion

Whereas Sn expression has been demonstrated by different assays on several macrophage populations [2,3,5,6], Sn expression on murine AM was only evaluated by rosetting assay and these cells have been considered to be weakly positive for the expression of this molecule when compared to macrophages from the spleen or lymph nodes. However, Sn is highly conserved in mammals [13] and was showed to be expressed on human, rat and porcine AM [7–9]. To clarify the expression of Sn on murine AM, flow cytometry was performed. Flow cytometry is a method that enables a sensitive analysis of Sn expression using 3D6 mAb, a rat anti-mouse Sn mAb directed to domain 1 of the receptor [14]. Sn was shown to be expressed on murine AM, independently of the mouse strain (Figs. 1 and 2). To evaluate the functional activity of Sn expressed on AM surface, rosetting assays were performed. As previously described [2], erythrocytes poorly bound to mouse AM. The efficacy of AM to agglutinate human RBC was low as 10% rosettes were formed (Fig. 3A). In addition, erythrocytes binding to mouse AM was shown to be sialic acid dependent as sialidase treatment of both cells greatly influenced rosetting activity. Desialylation of erythrocytes completely abolished rosette formation (Fig. 3B), whereas removing sialic acid from AM surface greatly increased, from 10% to 90%, the proportion of AM forming rosettes with RBC (Fig. 3C). These results suggest that mouse AM bind erythrocytes in a sialic acid dependent manner and that the receptor on AM surface is naturally blocked by *cis*-interactions with endogenous sialic acid exposed on the cell surface. An additional effect of sialidase treatment is to remove negative surface charges and hence the increase in RBC binding could also be in part explained by reduced repulsion following sialidase treatment. Whereas Siglecs are naturally masked by low affinity *cis*-interactions with sialo-oligosaccharides within the glycocalyx, Sn is assumed to extend its sialic acid binding site away from the glycocalyx on the cell surface in order to mediate macrophage interactions [15]. However, as rosetting activity of AM is induced after removing α2-3,6,8-linked sialic acids on AM surface, our study suggests that Sn might be involved in RBC binding to AM but is naturally regulated

by *cis*-interactions at the AM surface. Accordingly, we showed that RBC binding to sialidase-pretreated AM was inhibited by 3D6 mAb in a dose-dependent manner and the abolition attained 90% at a concentration of 10 μg/mL (Fig. 4). This indicates that the binding activity detected on desialylated AM is indeed mediated by Sn. Sialoadhesin has similarly been shown to be partially masked by endogenous sialoglycoconjugates on mouse as well as rat splenic macrophages [16,17]. In contrast, Sn is unmasked on rat lymph node macrophages and this latter case could be explained by the absence or poor accessibility of endogenous ligands, or by the presence of a large excess of Sn over endogenous ligands [17]. Therefore, it is noteworthy that the exceptional length of Sn, compared to the other Siglecs, does not prevent but reduces cis-inhibition.

Delputte and Nauwynck have suggested that Sn on pig AM functions as a virus receptor [18] and it is then possible that Sn expressed on murine AM could be involved in endocytosis of sialylated pathogens, including viruses, in the lung. However, this would probably involve synergy with endocytic receptors, given that mouse Sn is not endocytic on its own [2].

In conclusion, the present study suggests that Sn is expressed on mouse AM but that its sialic acid binding activity is naturally masked by endogenous sialic acid within the glycocalyx on the cell surface. The likely presence of a large excess of endogenous ligands over Sn on AM enables the control of the lectin activity and cell binding properties.

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