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Research paper

Monoclonal antibody binding to the macrophage-specific receptor sialoadhesin alters the phagocytic properties of human and mouse macrophages

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ABSTRACT

Sialoadhesin (Sn) is a surface receptor expressed on macrophages in steady state conditions, but during inflammation, Sn can be upregulated both on macrophages and on circulating monocytes. It was shown for different species that Sn becomes internalized after binding with monoclonal antibodies. These features suggest that Sn is a potential target for immunotherapies. In this study, human and mouse macrophages were treated with anti-Sn monoclonal antibodies or F(ab′)$_2$ fragments and the effect of their binding to Sn on phagocytosis was analyzed. Binding of antibodies to Sn resulted in delayed and reduced phagocytosis of fluorescent beads. No effect was observed on Fc-mediated phagocytosis or phagocytosis of bacteria by human macrophages. In contrast, an enhanced phagocytosis of bacteria by mouse macrophages was detected. These results showed that stimulation of Sn could have different effects on macrophage phagocytosis, depending both on the type of phagocytosis and cellular background.

1. Introduction

Macrophages are a type of phagocytes that play a role in both the innate and adaptive immune system. Along with neutrophils, macrophages are the first defensive cells to meet intruding microorganisms. Upon recognition through a set of cell specific receptors, phagocytic cells are able to engulf the microorganisms to prevent further spread in a process called phagocytosis. Phagocytosis is often associated with the release of cytokines and chemokines and unlike neutrophils, the more specialized macrophages can present antigens to T cells, activating the adaptive immune system [1,2]. In some cases, pathogens like *Legionella* and *Mycobacterium*, abuse phagocytosis by macrophages to evade the immune system while preventing phagosome maturation by inhibiting phagosome-lysosome fusion [2]. To that respect, modulation of phagocytosis could be interesting in some diseases, for instance in chronic obstructive pulmonary disease and hemophagocytic syndromes. In chronic obstructive pulmonary disease, a reduced bacterial phagocytosis was observed in macrophages [3,4]. In contrast, upregulated phagocytosis of erythrocytes and platelets was observed in hemophagocytic syndromes [5]. Modulation of phagocytosis could aid in these conditions to obtain an elevated or reduced phagocytosis rate in the macrophages. Indeed, modulation of phagocytosis was shown by targeting transmembrane proteins with monoclonal antibodies (mAbs) leading to reduced phagocytosis of apoptotic cells [6,7]. It was shown that treating porcine sialoadhesin (Sn; Siglec-1, CD169), a macrophage-specific receptor, with anti-Sn mAbs also reduced phagocytosis of fluorescent beads in porcine primary macrophages [8].

Sn is expressed on a subset of tissue resident macrophages in steady state conditions, but during inflammation Sn is upregulated on macrophages, monocytes and possibly also on dendritic cells

Abbreviations: BHI, Brain Heart Infusion; DAP12, DNAX-activating protein of 12 kDa; DAPI, 4′,6-diamidino-2-phenylindole; IFN-α, interferon-α; mAb, monoclonal antibody; MDMs, monocyte-derived macrophages; Siglec, sialic acid-binding Ig-like lectin; Sn, sialoadhesin; TLR, Toll-like receptor.

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[9]. For instance, during arthritis, the expression of Sn was shown to increase with the severity of the disease [10]. Sn is part of the Siglec family (sialic acid-binding Ig-like lectin). Other Siglecs, CD22 and CD33, are able to internalize after mAb binding, moreover, antibodies are being used for the development of immunotoxins for anti-cancer immunotherapy [11,12]. Studies with the porcine *Arterivirus* and HIV-1, show that Sn can mediate uptake of virus into early endosomes and in the case of HIV-1 can also mediate trans-infection [13–18]. Further research then showed that in different species, anti-Sn mAbs are able to induce internalization of Sn [18–21]. Interestingly, unlike most other Siglecs, no known signalization motif has yet been identified for Sn, even more, the short cytoplasmic tail of Sn is not conserved between species [22]. However recently, the interaction of mouse Sn (mSn) has been observed with DNAX-activating protein of 12 kDa (DAP12), suggesting Sn could contain a still unknown signalization motif [23]. Sn, described as a non-phagocytic receptor, is like other Siglecs able to bind with sialic acids, which are terminal nine-carbon sugar molecules found on vertebrates and pathogens [24,25]. It was shown that pathogens containing sialic acids are able to bind with Sn, as observed for *Campylobacter jejuni* and *Neisseria meningitides*. Moreover, these bacteria were more easily phagocytosed after binding with Sn in a sialic acid-dependent manner [26,27]. Group B *Streptococcus* could also bind to Sn in a sialic acid-dependent manner, moreover, it was shown that in primary mouse macrophages, Sn contributes to the bactericidal activity of macrophages against group B *Streptococcus* [28].

The interesting expression pattern of Sn during inflammation, the interaction of Sn with sialic acids and the capacity of anti-Sn mAbs to induce internalization of Sn, makes Sn a potential target for immunotherapy. Some possibilities for targeting Sn have already been explored for porcine Sn (pSn). PSn was targeted with immunotoxins, which resulted in killing of primary porcine macrophages [16] or with fusion proteins containing virus epitopes, which resulted in the induction of virus-specific antibodies [29]. MSn expressed on mouse splenic macrophages was targeted using anti-Sn mAbs for antigen targeting, activating both cellular and humoral immune responses [30]. Not only anti-Sn mAbs were used, mSn and hSn have also been targeted using liposomal nanoparticles coated with glycan ligands [20,31,32]. Nevertheless, using mAbs in immunotherapy could induce undesirable side effects on the host cell [33].

Previous research showed that targeting Sn with specific mAbs also blocks phagocytosis, which can be considered as an unwanted side effect [8]. However, this was only shown in porcine macrophages, and due to the non-conserved amino acid sequence and the lack of a known signalization motif in the cytoplasmic tail of Sn amongst different species [22], the outcome could be different in mouse and human macrophages. Moreover, for porcine macrophages the effect on phagocytosis was only investigated with polystyrene beads and not with more relevant assays such as Fc-mediated phagocytosis or phagocytosis of bacteria. Therefore, in this study, the effect of stimulation with anti-hSn mAbs and anti-mSn mAbs on different types of phagocytosis was analyzed on human and mouse macrophages. Our results show that the effect of anti-Sn mAbs on phagocytosis drastically differs between the types of phagocytosis. While a substantial reduction is seen in the phagocytosis of fluorescent beads, the Fc-mediated phagocytosis and phagocytosis of bacteria are unaffected and sometimes even slightly increased.

2. Material and methods

All products were purchased from Thermo Scientific, unless otherwise stated.

2.1. Ethical statement

The Ethical Committee for Animals of the University of Antwerp authorized experiments regarding BALB/c mice; permit number 2014-17. The Ethical Committee of the Antwerp University Hospital and the University of Antwerp authorized the use of human monocytes; permit number 16108.

2.2. Primary macrophages

Human monocytes were obtained from buffy coats of healthy volunteers from the Belgian Red Cross-Flanders. The mononuclear cells were isolated from the buffy coats using Ficoll (Sigma-Aldrich). $CD14^+$ monocytes were isolated using MACS separation following manufacturer's instructions (Miltenyi Biotec). The monocytes were seeded in RPMI with 10% inactivated human serum, 1% non-essential amino acids, 1% sodium pyruvate and 1% glutamine. After two days in culture, human IFN-α was added to the cells during three days to obtain hSn-positive monocyte-derived macrophages (MDMs).

BALB/c mice were sacrificed and their primary bone marrow cells were collected in RPMI medium. Red blood cells were removed using an ammonium-chloride-potassium lysis buffer (VWR and Janssen Chimica). Macrophages were seeded on coverslips in a 24-well plate in RPMI with 10% inactive fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, 1% glutamine and with addition of L929 supernatant. L929 supernatant was retrieved from L929 cells, kindly provided by dr. C. Uyttenhove (Ludwig Institute for Cancer Research, Brussels, Belgium). After three days in culture, mouse interferon-alpha (IFN-α) was added to the cells, to increase the number of Sn-positive macrophages.

2.3. Production of antibodies and $F(ab')_2$ fragments

Anti-hSn mAb 12A4 and anti-mSn mAb SySy94 were produced and purified as previously published [21]. $F(ab')_2$ fragments were produced as previously described [34]. Briefly, PNGase F (New England Biolabs) was added to purified mAbs to allow proteolytic cleavage following manufacturer's instructions. Pepsin agarose beads were added to cleave the mAbs after the deglycosylation step. The $F(ab')_2$ fragments were purified using a Zeba spin column with a 40 kDa cut off. Control antibodies were used for hSn and mSn using respectively 40-1a and normal rat IgG control (R&D). The hybridoma 40-1a developed by Joshua Sanes was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.

2.4. Phagocytosis assay

Macrophages were treated with anti-Sn mAbs or $F(ab')_2$ fragments 12A4 or SySy94 during different times, from 1 to 24 h. Control antibodies were added as control for the binding of the Fc domain of the mAbs with Fc receptors. Antibodies were added at a concentration of 2,5 μg/ml. Carboxylate-modified polystyrene yellow-green fluorescent beads (Sigma-Aldrich) were added to the macrophages and cells were kept at 37 °C to induce phagocytosis during 1 or 6 h. As a negative control for the phagocytosis, macrophages were kept at 4 °C during the experiment. Finally, macrophages were washed to remove unphagocytosed beads, fixed with 4% paraformaldehyde (Merck) and permeabilized using 0,05% Triton X-100 (Sigma-Aldrich). Macrophages were further stained with Texas Red®-X phalloidin to stain F-actin, located just beneath the plasma membrane and cell nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Each experiment