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Galectin-3 type-C self-association on neutrophil surfaces; The carbohydrate recognition domain regulates cell function

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Summary sentence: Galectin-3 is cleaved by both neutrophils and bacteria and the residual galectin-3C inhibits galectin-3-induced neutrophil activity but potentiates galectin-3 cell-surface binding.

Abstract

Galectin-3 is an endogenous β -galactoside-binding lectin comprising a carbohydrate recognition domain (CRD) linked to a collagen-like N-domain. Both domains are required for galectin-3 to induce cellular effects; a C-terminal fragment of galectin-3, galectin-3C, containing the CRD but lacking the N-domain, binds cell surface glycoconjugates but does not induce cellular effects since cross-linking promoted by the N-domain is thought to be required. Instead, galectin-3C is proposed to antagonize the effects of galectin-3 by competing for binding sites. The aim of this study was to investigate the effects of galectin-3C on galectin-3 interactions with human neutrophils. Recombinant galectin-3C inhibited galectin-3-induced production of reactive oxygen species in primed neutrophils. Surprisingly, this inhibition was not due to competitive inhibition of galectin-3 binding to the cells. In contrast, galectin-3C potentiated galectin-3 binding, in line with emerging evidence that galectin-3 can aggregate not only through the N-domain but also through the CRD. The cell surface interaction between galectin-3C and galectin-3 was corroborated by colocalization of fluorescently labeled galectin-3 and galectin-3C. Galectin-3C can be generated in vivo through cleavage of galectin-3 by proteases. Indeed, in circulation, galectin-3 and galectin-3C were both attached to the cell surface of neutrophils, which displayed great capacity to bind additional galectin-3 and galectin-3C. In conclusion, galectin-3C enhances galectin-3 binding to neutrophils by nonactivating type-C self-association, in parallel to inhibiting neutrophil activation by galectin-3 (induced by type-N self-association). This implicates type-C self-association as a termination system for galectin-3-induced cell activation, with the purpose of avoiding oxidant-dependent tissue damage.

KEYWORDS

carbohydrate recognition domain, galectin, neutrophils, priming, ROS production

1 | INTRODUCTION

Human neutrophils play a key role in the innate immune response to infection and aseptic tissue damage. These phagocytic cells circulate in a quiescent state in the blood stream until being recruited to sites of

inflammation or infection in the tissue. During transmigration from the circulation into tissue, the neutrophil phenotype changes into a preactivated, primed state, associated with increased numbers of receptors on the cell surface and thus a greater capacity to respond to stimuli.^{1,2} One priming-dependent neutrophil activator is galectin-3, an endogenous lectin with the ability to stimulate production of reactive oxygen species (ROS) in neutrophils that have extravasated from blood into the tissue, but not in resting, circulating cells.³⁻⁵

Galectins are a family of carbohydrate-binding proteins characterized by their affinity for β -galactoside-containing glycans. They are produced as cytosolic proteins and can be secreted extracellularly by nonclassical (non-ER-Golgi) pathways.⁶ To date, 15 galectins have been

Abbreviations: CRD, carbohydrate recognition domain aa 114–250; ecROS, extracellularly released ROS; Fura 2-AM, fura-2-acetoxymethyl ester; galectin-3C, C-terminal fragment of galectin-3 containing the CRD but lacking the N-domain; icROS, intracellularly produced ROS; KRG, Krebs-Ringer phosphate buffer; MFI, geometric mean fluorescence intensity; MMPs, matrix metalloproteinases; NADPH-oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; PFA, paraformaldehyde; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; RT, room temperature (~20°C); SOD, superoxide dismutase

identified in mammals, defined by their sequence similarities in the 135 amino acid carbohydrate recognition domain (CRD).⁷ One of the best characterized galectins is galectin-3, a unique member of the family being the only galectin composed of a CRD linked to a collagen-like, nonlectin N-domain.^{7,8} Galectin-3 has been implicated in a variety of physiological and pathological processes. In inflammation, galectin-3 has evolved as an important regulatory mediator,⁹ shown by its capacity to promote cell adhesion,^{10–13} enhance phagocytosis,^{14,15} and induce production of NADPH-oxidase-derived ROS in human neutrophils.^{3–5,16}

The N-domain of galectin-3 has been shown to take part in aggregation and cooperative binding of galectin-3 to glycoconjugates,^{17,18} functions that are proposed to be crucial for galectin-3-induced cell activation to occur.^{19,20} However, the collagen-like feature of the N-domain renders this part of the molecule sensitive to protease degradation. Nieminen et al. have shown that proteases, including elastase, released by primed neutrophils are able to cleave galectin-3 into truncated fragments containing the CRD but lacking the N-domain,²¹ designated galectin-3C. The property to cleave galectin-3 is displayed also by matrix metalloproteinases (MMPs)^{22–24} and bacterial collagenases.^{17,19,25} Galectin-3C is unable to induce cell activity^{4,21} and has been described as a dominant-negative form of galectin-3,^{26,27} since it can inhibit galectin-3-induced angiogenesis,²⁸ affect galectin-3 maintenance of the glycocalyx barrier function at the ocular surface,²⁹ suppress the contribution of galectin-3 to platelet-induced COX-2 overexpression in HT29 cells,³⁰ suppress breast cancer in vivo,²⁷ and attenuate galectin-3-induced osteoclastogenesis.³¹ The dominant negative galectin-3C has also been shown to reduce motility and invasion in ovarian cancer cells.³² So far, the inhibitory functions of galectin-3C have not been investigated with respect to neutrophil activation, but its presence in inflamed/infected human tissue is inferred, based on that galectin-3 increases during inflammation,^{33,34} neutrophils release (galectin-3-cleaving) proteases during inflammatory processes²¹ and protease-positive bacteria release proteases during infection.

We studied the interaction of galectin-3C with neutrophils, both by measuring its effect on galectin-3-induced NADPH-oxidase activation and its effect on binding of galectin-3 to the cell surface. Further, we investigated the amount of galectin-3 and galectin-3C attached to the surfaces of circulating leukocytes ex vivo. Our data demonstrate that galectin-3C indeed has the ability to inhibit galectin-3-induced ROS production in primed neutrophils. Surprisingly, however, galectin-3C potentiated the binding of galectin-3 to the surface of neutrophils, suggesting association between the CRD parts of galectin-3 and galectin-3C (so-called C-type self-association^{35–37}).

2 | MATERIALS AND METHODS

2.1 | Reagents

PD10 columns were from Pharmacia (Stockholm, Sweden) and Ficoll-Paque gradient were from Fischer Scientific GTF AB (Gothenburg, Sweden). FITC, TNF- α , lactose, isoluminol, luminol, gelatine from

porcine skin and PMA were from Sigma-Aldrich (St Louis, MO, USA). Fura-2-acetoxymethyl ester (Fura 2-AM), Alexa Fluor 647-conjugated goat anti-mouse IgG antibody, and the Alexa Fluor 488 and Alexa Fluor 647 microscale protein labeling kits were from Molecular Probes/Invitrogen (Grand Island, NY, USA). Dextran was purchased from Pharmacosmos (Holbaek, Denmark) and HRP, superoxide dismutase (SOD), and catalase were from Boehringer Mannheim (Mannheim, Germany). BSA and paraformaldehyde (PFA) were from Roche Diagnostics (Mannheim, Germany), and the RPMI medium was from PAA Laboratories (Gothenburg, Sweden). The peroxidase substrate and SDS was from Bio-Rad Laboratories (Sundbyberg, Sweden) and the phycoerythrin (PE) -labeled anti-CD11b monoclonal antibody and FACS Lysing solution was from Becton Dickinson AB (San Jose, CA, USA). The unlabeled and Alexa Fluor 647-conjugated chicken anti-CRD polyclonal antibody, the Alexa Fluor 647-conjugated chicken pre-immune IgY antibody, and the chicken sera were from Capra Science (Ängelholm, Sweden). The PE-conjugated rat anti-galectin-3 monoclonal antibody (clone M3/38) and the PE-conjugated rat IgG2a, κ antibody were from Biolegend (San Diego, CA, USA). The allophycocyanin (APC)- and fluorescein (FITC)-conjugated anti-CD45 antibody and the anti-chicken-HRP antibodies were from Abcam (Cambridge, United Kingdom). The anti-rat-HRP antibody was from DAKO (Stockholm, Sweden), and the FITC-conjugated mouse anti-human CD16 antibody was from EuroBioSciences (Friesoythe, Germany).

2.2 | Production and fluorescence labeling of recombinant galectin-3 and galectin-3C

Recombinant human galectin-3 and galectin-3C were produced in *Escherichia coli* and purified as described.¹⁷ Briefly, after isolation of the recombinant proteins, galectin-3 and galectin-3C were kept at 4°C in PBS (pH 7.2) containing lactose (150 mM) until applied to a gel filtration column (PD10) to remove lactose. The proteins were diluted in Krebs-Ringer phosphate buffer (KRG: pH 7.3) supplemented with Ca²⁺ (1 mM) and stored at –80°C. For some experiments, recombinant galectin-3 and galectin-3C were labeled with FITC essentially as described by Patnaik et al.³⁸ or with Alexa Fluor 488, or Alexa Fluor 647 microscale protein labeling kit according to the manufacturer's protocol (Molecular Probes/Invitrogen, Grand Island, NY, USA).

In solution, both galectin-3 and galectin-3C are monomeric, but they can become multimeric when encountering ligands.³⁷

2.3 | Isolation of neutrophils

Neutrophils from healthy blood donors were isolated from peripheral blood or buffy coats, by a standard technique based on dextran sedimentation and Ficoll-Paque gradient centrifugation.³⁹ After hypotonic lysis of remaining erythrocytes, the neutrophils were diluted to 5×10^6 cells/ml in KRG with Ca²⁺ and stored on ice.

For some experiments, the neutrophils were pretreated with TNF- α (10 ng/ml, 20 min, 37°C) that induces degranulation with a concomitant upregulation of receptors from gelatinase and specific granules¹⁶ including the galectin-3 binding proteins CD66a (CEACAM1) and CD66b (CEACAM8).⁴⁷

2.4 | Production of ROS

Neutrophil NADPH-oxidase-derived ROS was measured using a luminol/isoluminol-amplified chemiluminescence system.⁴⁰ Briefly, neutrophils (5×10^5 cells/ml, either resting or TNF- α primed) in KRG with Ca^{2+} were equilibrated in a six channel Biolumat LB 9505 instrument or a Mithras LB940 plate reader (both from Berthold Technologies, Bad Wildbad, Germany) at 37°C for 5 min (with or without recombinant galectin-3C or galectin-3 inhibitors lactose and 3,3'-dideoxy-3,3'-bis-[4-(3-fluorophenyl)-1H-1,2,3-triazol-1-yl]-1,1'-sulfanediyl-di- β -D-galactopyranoside (Compound 1, synthesized in the authors' laboratories as described)^{41,42} in the presence of isoluminol (5×10^{-5} M) and HRP (4 U/ml) to detect release of extracellular ROS (ecROS), or in the presence of luminol (5×10^{-5} M), SOD (50 U/mL), and catalase (1000 U/mL) to detect intracellular production of ROS (icROS). Cells were then stimulated with galectin-3, galectin-3C, or PMA, and the light emission, reflecting superoxide production, was recorded over time. The levels of ROS are expressed as mega counts per minute (Mcpm) or relative light units (RLU).

2.5 | Measurement of intracellular Ca^{2+}

TNF- α -primed neutrophils (see above, 5×10^6 cells/ml) in KRG supplemented with BSA (0.1%) were loaded with Fura 2-AM (2 μM), for 30 min at RT, washed in RPMI medium without phenol red, suspended in KRG with Ca^{2+} and kept on ice until use. Prior to stimulation, the cells (400 μL , 2×10^6 neutrophils/ml) were equilibrated in a Perkin Elmer fluorescence spectrophotometer (LC50, Perkin Elmer, Waltham, MA, USA), at 37°C for 5 min in the absence or presence of Compound 1 (10 μM). Neutrophils were then stimulated with recombinant galectin-3 or galectin-3C, and the fluorescence was recorded (excitation wavelengths 340 and 380 nm and emission wavelength 509 nm). The transient rise in intracellular Ca^{2+} is presented as a ratio of the fluorescence change (340 nm/380 nm).⁴³

2.6 | SDS-PAGE and Western blotting

Samples were diluted 1:1 in nonreducing sample buffer, boiled for 5 min and applied to SDS-PAGE using 12% gels. After electrophoresis, the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes using a Tris-glycine buffer system.⁴⁴ The membranes were then blocked by incubation in PBS containing Tween (0.05%) supplemented with gelatine (1%, prior to incubation with the anti-galectin-3 antibody) or BSA (1%, prior to incubation with the anti-CRD antibody) at RT for 30 min, or overnight at 4°C. After blocking, the membranes were washed with PBS containing Tween (0.05%) and incubated for 2 h at RT, with either rat anti-human monoclonal galectin-3 antibody (culture supernatant of the hybridoma M3/38; diluted 1:100 in blocking buffer) which binds to the N-terminal domain of galectin-3,²¹ or with affinity-purified chicken polyclonal anti-human CRD antibody (2.2 $\mu\text{g}/\text{ml}$ in blocking buffer; produced by Capra Science, Ängelholm, Sweden). The polyclonal anti-CRD antibody was produced in chicken due to the fact that mammals show highly conserved sequences, and thus low immunogenicity, between

their CRDs.⁷ After incubation with antibodies, the membranes were washed and incubated for 2 h in RT with HRP-labeled anti-rat-IgG antibody (1.3 $\mu\text{g}/\text{ml}$ in blocking buffer) or HRP-labeled anti-chicken-IgG antibody (diluted 1:5000 in blocking buffer), respectively. After additional washing, peroxidase substrate was added according to manufacturer's instructions, and luminescence was analyzed in a Molecular Imager ChemiDoc XRS by Quantity One Software (Bio-Rad Laboratories, Sundbyberg, Sweden). The specificities of the antibodies are demonstrated in Figure 3 and Supplemental Figure 1.

2.7 | Proteolytic cleavage of galectin-3

Recombinant galectin-3 (4 μM) was incubated with TNF- α -primed neutrophils (5×10^6 cells/ml in KRG) or *Staphylococcus aureus* (model laboratory strain 8325-4; harvested from horse blood agar plates and suspended in tryptic soy broth) at 37°C for 2 or 18 h, respectively. Cell- and bacteria-free supernatants were obtained by centrifugation (15 s at 16,100 g or 1 min at 9300 g, respectively) and analyzed by Western blotting for galectin-3 and fragments thereof.

2.8 | Flow cytometry analysis of cell-bound galectin-3 and galectin-3C using antibodies

Galectin-3 binding to leukocytes in blood circulation was investigated by immunostaining and flow cytometry analysis. Briefly, anticoagulated blood was incubated in the presence or absence of TNF- α (10 ng/ml, 20 min, 37°C) and treated with FACS Lysing Solution (Becton Dickinson) that fixes the leukocytes and lyses erythrocytes simultaneously, according to the manufacturer's instructions. Cells were then washed with PBS, and duplicate samples were labeled with antibodies for 60 min in darkness at 4°C. Galectin-3 was stained by PE-conjugated rat anti-galectin-3 monoclonal antibody (clone M3/38) with the corresponding isotype control (PE-conjugated rat IgG2a, κ antibody; 2.5 $\mu\text{g}/\text{ml}$ in PBS supplemented with 10% human serum). Galectin-3C was stained by Alexa Fluor 647-conjugated chicken anti-CRD polyclonal antibody with the corresponding control (Alexa Fluor 647-conjugated chicken pre-immune IgY antibody; 0.5 $\mu\text{g}/\text{ml}$ in PBS supplemented with 10% chicken serum). The priming state of neutrophils was investigated as CD11b exposure by staining with PE-labeled anti-CD11b monoclonal antibody (1:20 dilution). To ensure proper identification of neutrophils, monocytes, and lymphocytes during flow cytometry analysis, the cells were colabeled with mouse anti-human CD45 antibody conjugated with APC (for anti-galectin-3 or anti-CD11b stained cells) or FITC (for anti-CRD stained cells).⁴⁵ The CD45 staining allows for clear gating between the different white blood cells as demonstrated in Supplemental Figure 2. Samples were then washed with PBS and analyzed with an Accuri C6 flow cytometer (Becton Dickinson, San Jose, CA, USA) using FlowJo software (Tree Star Inc, Ashland, Oregon, USA). For some experiments, the blood was treated with Compound 1 (30 min, RT) and/or recombinant lectin (15 min, RT) prior to fixation and immunostaining. Results are shown as geometric mean fluorescence intensity (MFI) after subtraction of the isotype control MFI value.

2.9 | Flow cytometry analysis of cell-bound galectin-3 and galectin-3C using fluorescently labeled lectins

Isolated neutrophils (5×10^6 neutrophils/ml), resting or TNF- α -primed, were incubated for 15 min on ice with buffer, Compound 1, recombinant unlabeled galectin-3 or galectin-3C, followed by incubation for an additional 15 min with FITC-labeled recombinant galectin-3 or galectin-3C. Thereafter the neutrophils were fixed with PFA (2%, 20 min on ice), washed with PBS, and analyzed by flow cytometry and FlowJo software.

2.10 | Imaging flow cytometry analysis of galectin-3 and galectin-3C colocalization on the neutrophil cell surface using fluorescently labeled lectins

Isolated neutrophils (5×10^6 neutrophils/ml), kept on ice or primed with TNF- α , were cocubated with recombinant FITC-conjugated galectin-3 (1 μ M) and Alexa Fluor 647-conjugated galectin-3C (1 μ M) on ice for 15 min. Thereafter the neutrophils were fixed with PFA (2%, 20 min on ice), washed with PBS, and analyzed using an imaging flow cytometer (ImageStreamX, Amnis, Seattle, WA, USA). As a positive control for colocalization, neutrophils labeled with FITC-conjugated mouse anti-human CD16 antibody (diluted 1:20) followed by Alexa Fluor 647-conjugated goat anti-mouse IgG antibody (2.5 μ g/ml) were used. As a negative control for colocalization, neutrophils colabeled with Alexa Fluor 647-conjugated galectin-3C and FITC-conjugated mouse anti-human CD16 antibody were used. Methodological details, gating strategy, colocalization analysis method, and representative images are given in Supplemental Figure 3. In order to analyze colocalization, the colocalization wizard in the Ideas v.5.0 software (Amnis, Seattle, WA, USA) was used, as described by Beum et al.⁴⁶

2.11 | Statistical analysis

Statistical calculations were performed in GraphPad Prism 6.0a (Graphpad Software, San Diego, CA, USA). The specific statistical tests used for different sets of experiments are stated in the respective figure legend. A *P*-value < 0.05 was regarded as statistically significant and is indicated by **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

3 | RESULTS

3.1 | Galectin-3, but not galectin-3C, activates neutrophils

As previously shown by us and others, galectin-3 activates the NADPH-oxidase in primed, but not resting neutrophils,^{3,4,16,47,48} generating ROS released extracellularly (ecROS; Figure 1A) as well as ROS produced and retained intracellularly (icROS; Figure 1B). The N-terminal domain of galectin-3, described to be responsible for aggregation and cooperative binding of galectin-3 to glycoconjugates,^{17,18}

was necessary for NADPH-oxidase activity to be induced, as no ROS was produced when cells were exposed to recombinant galectin-3C alone (Figure 1A, B). Also the β -galactoside-binding activity was essential for activation with galectin-3, as the presence of Compound 1, a synthetic inhibitor of the galectin-3 carbohydrate-binding activity,^{41,42} completely abolished galectin-3-induced ROS production (Figure 1A, B) with an IC₅₀ value of 400–1000 times less than the traditionally used inhibitor lactose (Figure 1C, D). This difference in inhibitory concentration in this cell assay is less than the 6000-fold difference in galectin-3 affinity between lactose (*K_d* 93 μ M) and Compound 1 (*K_d* 14 nM),^{41,42,49} mainly because more of Compound 1 than simply correlated with its *K_d* is required to inhibit at least 50% of the added galectin-3 (0.8 μ M). This is mathematically explained in Supplemental Figure 4.

The galectin-3-induced ROS production was paralleled by a rise in intracellular Ca²⁺ in primed neutrophils (Figure 1E), in agreement with a previous study by Stowell et al.⁵⁰ In line with the galectin-3-induced ROS production, the Ca²⁺ response was reliant on intact galectin-3 molecules, as no Ca²⁺ signal was achieved when stimulating with recombinant galectin-3C and the response was abrogated in the presence of Compound 1 (Figure 1E).

3.2 | Galectin-3C inhibits galectin-3-induced neutrophil ROS production

Galectin-3C is by itself usually inert in biological processes, including activation of neutrophils,^{4,17–20} but has been found to inhibit effects induced by galectin-3.⁵¹ To investigate whether this is true also for galectin-3-induced activation of neutrophils, we tested whether the presence of recombinant galectin-3C could affect galectin-3-induced NADPH-oxidase activation in primed human neutrophils. TNF- α -primed cells were pre-incubated with galectin-3C at different concentrations for 5 min at 37°C prior to stimulation with galectin-3 (0.4 μ M). Indeed, the galectin-3-induced NADPH-oxidase response was inhibited by galectin-3C in a concentration-dependent manner (Figure 2A, B, D), at concentrations equimolar to or higher than that of galectin-3 (Figure 2D). This inhibitory effect was specific for galectin-3-induced ROS production and was not seen when neutrophils were stimulated with the protein kinase C agonist PMA, a well-established NADPH-oxidase activator (Figure 2C, D). Thus, we confirm that galectin-3C can inhibit galectin-3-induced biological effects and add that this may influence neutrophil activation by galectin-3.

3.3 | Galectin-3 is cleaved by neutrophil proteases as well as by *S. aureus* proteases, producing truncated fragments containing the CRD

The presence of galectin-3C in vivo has not been thoroughly investigated. However, Nieminen et al. have demonstrated that primed neutrophils are able to cleave galectin-3 into truncated fragments containing the CRD but lacking the N-terminal domain,²¹ suggesting that galectin-3C may be present at inflammatory sites in vivo. Further, certain species of bacteria express collagenases that cleave galectin-3 into truncated fragments,^{17,19,25} in a similar fashion as primed neutrophils. To be able to investigate such galectin-3 processing by human

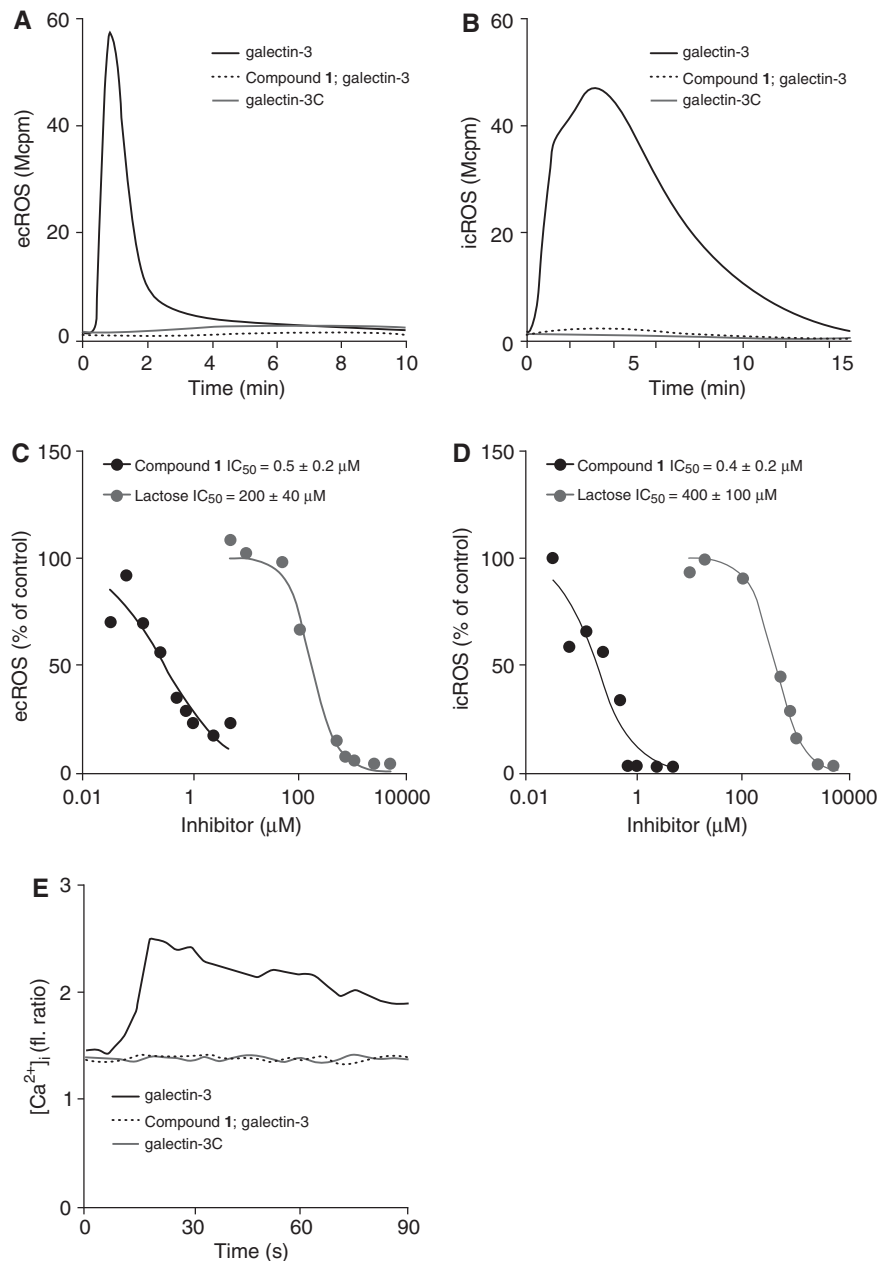


FIGURE 1 Galectin-3, but not galectin-3C, activates neutrophils. (A–D) TNF- α -primed neutrophils were incubated for 5 min at 37°C in the presence or absence of Compound 1 or lactose prior to stimulation with recombinant galectin-3 (0.8 μ M) or galectin-3C (0.8 μ M) and measurement of ROS production over time was done by chemiluminescence. (A, B) Representative traces are of (A) ecROS production ($n = 3$) and (B) icROS production ($n = 5$) by cells stimulated with galectin-3 (after preincubation in presence or absence of 10 μ M Compound 1) or galectin-3C are shown. (C, D) Representative graphs of the dose responses for inhibition of the galectin-3-induced (C) ecROS production (D) icROS production by lactose or Compound 1 are shown. The half maximal inhibitory concentrations (IC_{50}) calculated from four (lactose) or three (Compound 1) experiments, respectively, are given in each graph. (E) Fura-2 loaded TNF- α -primed neutrophils were incubated for 5 min at 37°C in the presence or absence of Compound 1 (10 μ M) prior to stimulation with galectin-3 (1.6 μ M) or galectin-3C (1.6 μ M) and intracellular calcium mobilization (shown as the fluorescence ratio; 340 nm / 380 nm; $n = 3$) was measured over time

and bacterial proteases in detail, we first developed a chicken anti-human CRD antibody for proper detection of the produced fragments (see the section Materials and Methods).

The specificity of the anti-CRD antibody was tested and compared to the broadly used anti-galectin-3 antibody clone M3/38. In Western blots of recombinant galectin-3 and galectin-3C, the anti-galectin-3 antibody detected only full-length galectin-3 (Figure 3A and Supple-

mental Figure 1) while the anti-CRD antibody detected both full-length galectin-3 and galectin-3C (Figure 3B and Supplemental Figure. 1), as was to be expected. Neither of the antibodies detected the unrelated proteins galectin-1 or wheat germ agglutinin (Supplemental Figure 1).

Using the anti-CRD antibody, we could confirm the finding by Nieminen et al,²¹ showing that primed neutrophils cleave galectin-3 into truncated fragments containing CRD (Figure 3C). Furthermore, we

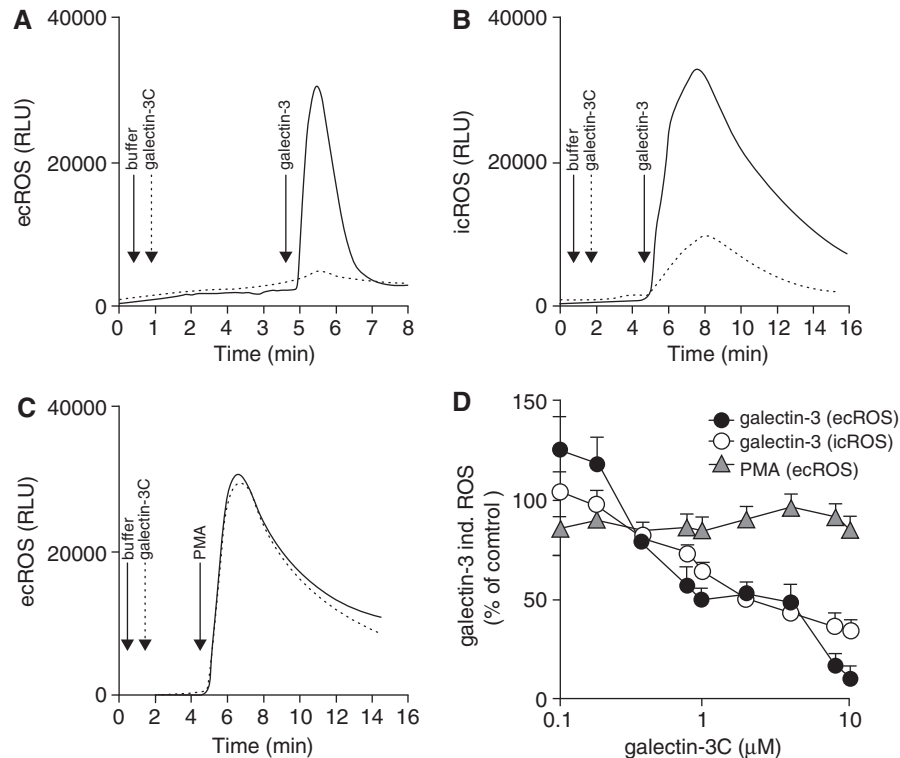


FIGURE 2 Galectin-3C inhibits galectin-3-induced ROS production. (A–D) Neutrophils were pretreated in the absence (buffer; solid line) or presence (dotted line) of recombinant galectin-3C for 5 min at 37°C prior to stimulation and measurement of ROS production by chemiluminescence. (A, B) TNF- α -primed neutrophils were, after pretreatment with or without galectin-3C (10 μ M; left arrow), stimulated with galectin-3 (0.4 μ M; right arrow) to induce ROS production. Representative trace of (A) ecROS production ($n = 5$) and (B) icROS production; $n = 5$) are shown. (C) Representative traces of ecROS production ($n = 5$) in neutrophils pretreated with galectin-3C (10 μ M; left arrow) and stimulated with PMA (5×10^{-8} M; right arrow) are shown. (D) The graph shows the % inhibition of galectin-3-induced ecROS production (black circles) and icROS production (white circles), and PMA-induced ecROS production (grey triangles) after pretreatment with different concentrations of galectin-3C. Data are given as mean % of control + SEM ($n = 5$)

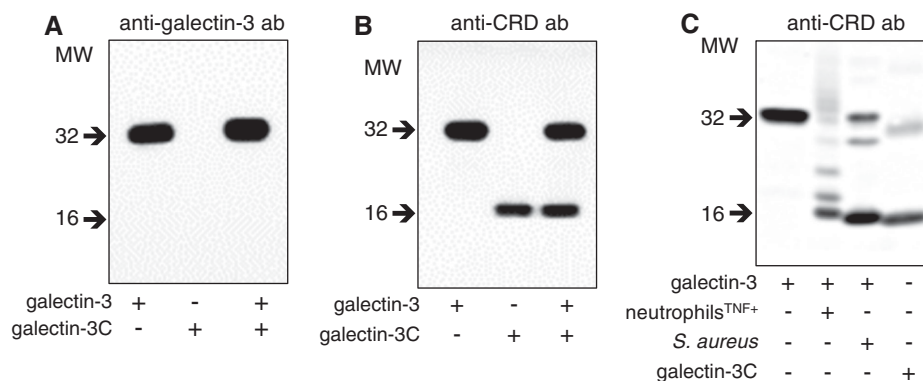


FIGURE 3 Galectin-3 is cleaved by TNF- α -primed neutrophils and *S. aureus* strain 8325-4. (A, B) Recombinant galectin-3 (20 nM) and galectin-3C (20 nM) were subjected to SDS-PAGE followed by Western blotting using (A) an anti-galectin-3 antibody (detecting only full-length galectin-3) or (B) an anti-CRD antibody (detecting both full-length galectin-3 and galectin-3C). (C) Galectin-3 (4 μ M) was incubated with TNF- α -primed neutrophils for 2 h, or with *S. aureus* for 18 h, at 37°C, after which the cell-free supernatants were analyzed by SDS-PAGE/Western blotting using the anti-CRD antibody. A representative blot from 3 to 5 separate experiments is shown

show that incubation of galectin-3 with the proteolytically competent *S. aureus* strain 8325-4 also resulted in truncated fragments containing the CRD (Figure 3C), although with a somewhat different pattern. In conclusion, galectin-3 fragments, including the bare CRD domain, may tentatively be present in vivo.

3.4 | Endogenous galectin-3 and galectin-3C are attached to leukocytes in circulation

Galectin-3 was first discovered as a cell surface molecule on macrophages,⁵² reflecting the adsorption of circulating galectin-3

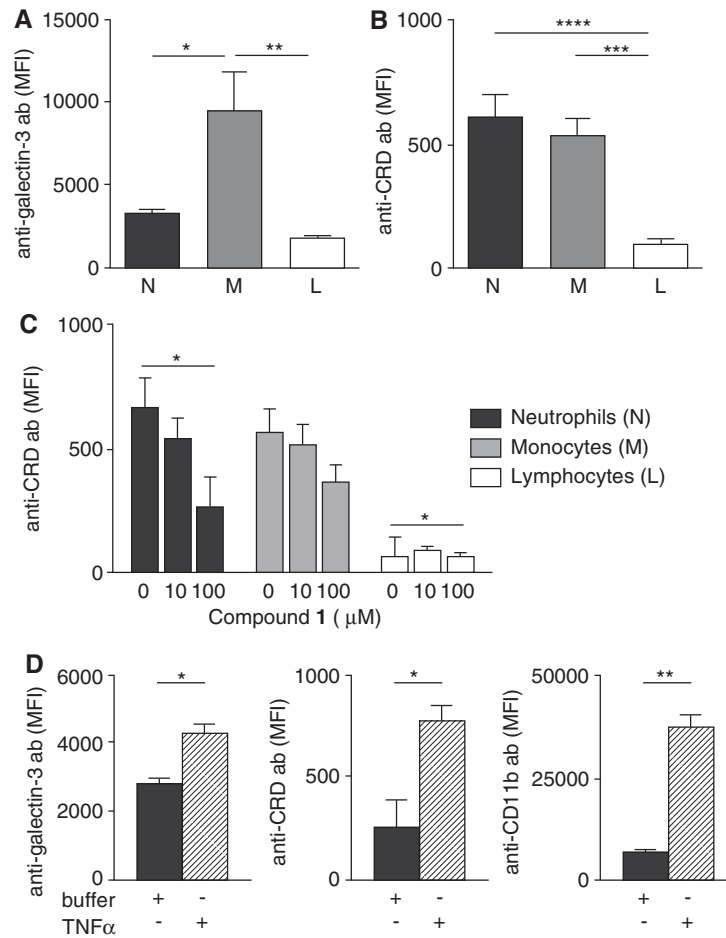


FIGURE 4 Both galectin-3 and galectin-3C are attached to leukocytes in blood circulation. Cell-surface attachment of endogenous galectin-3 and/or galectin-3C was analyzed on neutrophils (N; black), monocytes (M; grey), lymphocytes (L; white), and TNF- α -primed neutrophils (dashed) in whole blood of healthy humans by flow cytometry. (A, B) Leukocyte attachment of galectin-3 and galectin-3C was investigated after immunostaining with (A) an anti-galectin-3 antibody (detecting only full-length galectin-3; $n = 6$) and (B) an anti-CRD antibody (detecting both full-length galectin-3 and galectin-3C; $n = 6$). (C) Human blood was incubated for 15 min at room temperature with buffer or different concentrations of Compound 1, before immunostaining with the anti-CRD antibody ($n = 5$). (D) Leukocyte attachment to TNF- α -primed neutrophils as compared to resting neutrophils was investigated by staining of blood cells treated in the presence or absence of TNF- α for 20 min ($n = 3$), using anti-galectin-3, anti-CRD, or anti-CD11b antibodies. Data are shown as mean + SEM. Statistical analysis in A and B was performed using a one-way ANOVA followed by Tukey's multiple comparison test. In C, paired Student's *t*-test was used to compare cells treated with 100 μ M Compound 1 to untreated cells. In D, TNF- α -treated neutrophils are compared to untreated cells using paired Student's *t*-test.

to glycoconjugate receptors on the white blood cells. We hypothesized that not only full-length galectin-3, but also the truncated galectin-3, can be attached to white blood cells in circulation. We evaluated this by staining fixated peripheral blood leukocytes with anti-galectin-3 or the anti-CRD antibody, and analysis by flow cytometry.

The level of surface bound galectin-3 detected with the anti-galectin-3 antibody (M3/38) was highest for monocytes followed by the neutrophils and the lymphocytes (Figure 4A and Supplemental Figure 5A). The level of anti-CRD antibody staining, detecting both full-length galectin-3 and galectin-3C, was, however, quite different, with monocytes and neutrophils displaying equivalent levels of binding, much higher than for lymphocytes (Figure 4B and Supplemental Figure 5B). The differences in binding patterns for the two antibodies indicate that full-length galectin-3 and galectin-3C are both attached to circulating leukocytes but at different ratios, with monocytes binding

the highest amount of galectin-3 while neutrophils are the most prone to bind truncated forms. The data were verified using a commercially available anti-human CRD antibody (clone Gal397, Biolegend, San Diego, CA, USA, Supplemental Figure 5C). Treatment with the inhibitor Compound 1 before immunostaining resulted in decreased binding of both the anti-CRD antibody (Figure 4C) and the anti-galectin-3 antibody (Supplemental Figure 5D), suggesting that galectin-3/galectin-3C is attached to cell surfaces in circulation by carbohydrate-dependent interactions. Of note, a statistically significant decrease in lectin binding was found when comparing untreated neutrophils to neutrophils treated with 100 μ M Compound 1. No such difference was seen for monocytes, suggesting that some lectin attached to the monocytes might be binding through a carbohydrate-independent mechanism that is insensitive to Compound 1.

As shown above, the prerequisite for neutrophils to be activated by galectin-3 is that the cells have been prestimulated to become primed.

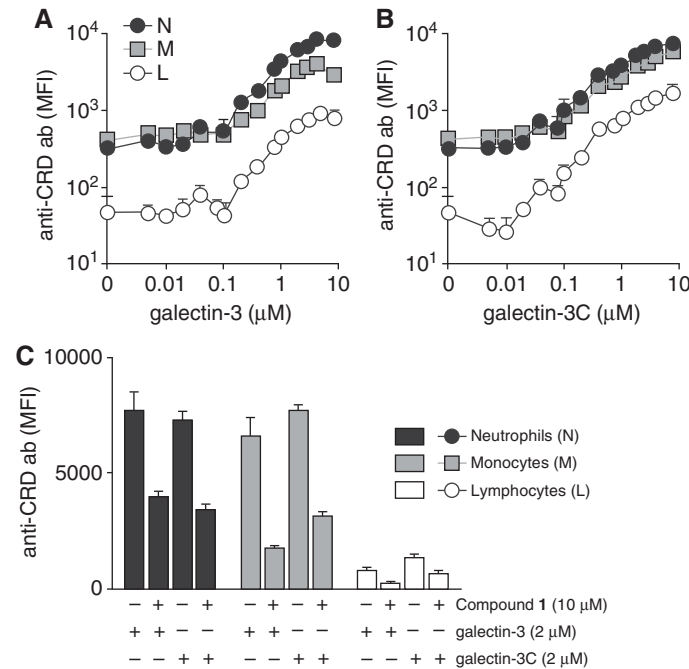


FIGURE 5 Leukocytes display great capacity to bind additional galectin-3 and galectin-3C. Cell-surface attachment of additional recombinant galectin-3 and galectin-3C was evaluated on neutrophils (N; black), monocytes (M; gray) and lymphocytes (L; white), by pretreating blood from healthy humans with recombinant lectin, before immunostaining the cells with an anti-CRD antibody and analysis by flow cytometry. (A, B) The blood was pretreated for 15 min at RT with buffer (0 μM) or different concentrations of (A) galectin-3 ($n = 3$) or (B) galectin-3C ($n = 3$) before analysis. (C) The blood was incubated with buffer (–) or Compound 1 (10 μM) at room temperature for 30 min, with addition of galectin-3 (2 μM) or galectin-3C (2 μM) for the last 15 min of incubation before analysis ($n = 3$). Data are shown as mean + SEM

We examined the association of endogenous galectin-3/galectin-3C also on neutrophils primed by TNF- α in whole blood and found the levels of antibody binding to be increased on the responsive, primed cells (Figure 4D), suggesting that they expose a higher level of galectin-3 receptors. This was in line with upregulation of the granule marker CD11b, which is seen in increased amounts on primed (degranulated) neutrophils (Figure 4D).^{47,48} Treatment of whole blood with TNF- α for 20 min may speculatively also induce release of more galectin-3 from activated monocytes, possibly contributing to the increased lectin binding to the cells.

3.5 | Leukocytes can sequester large amounts of exogenously added galectin-3 and galectin-3C

In order to investigate the extent of galectin-3/galectin-3C binding capacity on leukocytes, we next incubated blood with either recombinant galectin-3 or galectin-3C before fixation and immunostaining with the anti-CRD antibody (detecting both galectin-3 and galectin-3C). All investigated leukocytes had the ability to bind both galectin-3 (Figure 5A) and galectin-3C (Figure 5B) added extracellularly, and saturation of binding was reached at ~ 8 μM for both molecules (Figure 5A, B). Similarly to the endogenously bound galectin-3 and/or galectin-3C, binding of the recombinant equivalents was reduced in the presence of Compound 1 (Figure 5C). Leukocytes stained with the anti-galectin-3 antibody also showed saturated binding of galectin-3 at ~ 8 μM (Supplementary Figure 6A). As expected, no increased binding was seen after incubation with galectin-3C using this antibody (Supplementary Figure 6B).

3.6 | Addition of galectin-3C increases the binding of galectin-3 to neutrophil cell surfaces

Since galectin-3C was shown to inhibit the activation of neutrophils induced by galectin-3 (Figure 2), we hypothesized that it also would inhibit the binding of galectin-3 to the cell surface of neutrophils. To test this, we performed coinubation experiments at 4°C where binding of FITC-labeled galectin-3 to neutrophils was measured in the presence or absence of unlabeled galectin-3C. Surprisingly, the addition of unlabeled galectin-3C significantly enhanced the binding of FITC-labeled galectin-3 (Figure 6A), indicating that cooperative binding between the lectins was taking place.

The classic view on galectin-3 intermolecular interactions involves only the N-terminal part of the lectin (type-N self-association^{17,18,37}), leading to di- or oligomerization of the molecules. However, in some studies galectin-3 interactions have been found to involve the CRD domains.^{35–37} This so-called type-C self-association was in fact the major form of interaction when galectin-3 was exposed to glycoconjugates in solution,²⁹ although the N-terminal domain was required to enhance it. Our data that show increased binding of galectin-3 in the presence of galectin-3C indicate that such interaction occurs also in a biological setting, that is, on a cell surface.

To compare the extent to which cooperative binding occurs heterologously (galectin-3/galectin-3C), to that occurring homologously (galectin-3C/galectin-3C or galectin-3/galectin-3), we performed additional experiments. The increase in surface binding of recombinant FITC-labeled galectin-3 in the presence of unlabeled galectin-3C was similar to that of FITC-labeled galectin-3C in the presence of unlabeled

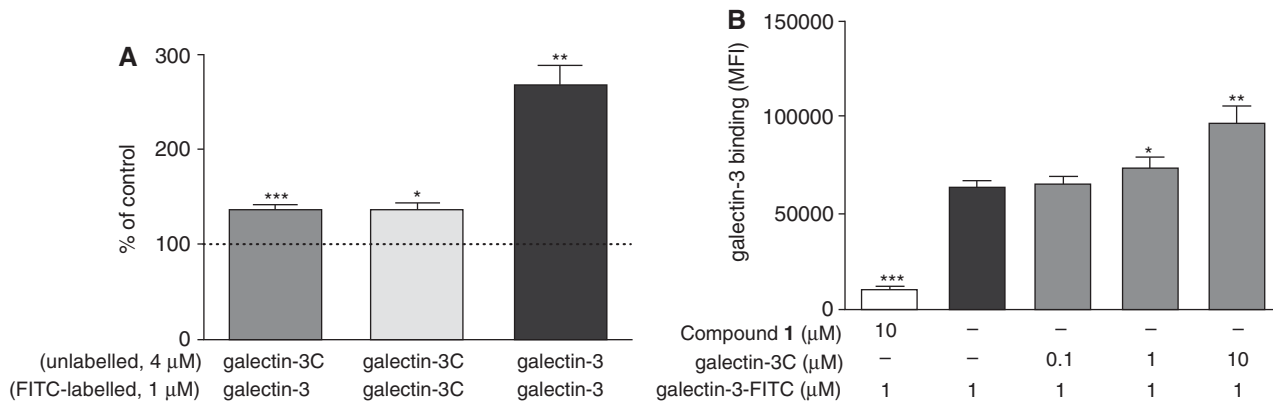


FIGURE 6 Galectin-3C potentiates galectin-3 cell surface binding through type-C self-association. TNF- α -primed neutrophils were incubated for 15 min on ice with Compound 1 (white bar), buffer (black bar), recombinant galectin-3C (gray bars), or galectin-3 (dark gray bar) and then incubated for an additional 15 min with FITC-labeled galectin-3 or galectin-3C. The cells were then fixed and analyzed for lectin attachment (FITC fluorescence) by flow cytometry. (A) The graph shows binding of FITC-labeled galectin-3C or FITC-labeled galectin-3 after pretreatment with unlabeled galectin-3C or unlabeled galectin-3 (mean % of control + SEM; $n = 4-5$). Statistical analysis was performed on sample MFI values in comparison to control, using paired Student's *t*-test. (B) The graph shows binding of FITC-labeled galectin-3 to neutrophils after pretreatment with Compound 1 or different concentrations of unlabeled galectin-3C (mean MFI + SEM, $n = 5$). Statistical analysis was performed using a repeated measurement one-way ANOVA, followed by Dunnet's multiple comparison test to neutrophils incubated with buffer followed by FITC-labeled galectin-3

galectin-3C, indicating that preincubation of the cells with galectin-3C before addition of galectin-3 appears to limit the interaction to type-C self-association (Figure 6A). The increase in binding was much higher for FITC-labeled galectin-3 in the presence of unlabeled galectin-3 (Figure 6A), indicating that the N-terminal domain of galectin-3 may enhance the type C-self association not only in solution²⁹ but also on cell surfaces, or alternatively, that the system shifts to a more pure type-N self-association.

The type-C self-association on the cell surface of neutrophils was investigated in more detail by treating the neutrophils with different concentrations of recombinant unlabeled galectin-3C prior to incubation with a fixed concentration of FITC-labeled galectin-3. At equimolar concentrations of galectin-3 and galectin-3C, a significant increase of galectin-3 binding was seen on both TNF- α primed (Figure 6B) and unprimed (Supplemental Figure 7) neutrophils. The binding of galectin-3 was even more enhanced by pretreating the neutrophils with 10 times as much galectin-3C as galectin-3 and was inhibited in the presence of Compound 1 (Figure 6B), signifying that the type-C self-association complexes are attached to the cell surface, in a carbohydrate-dependent manner.

3.7 | Galectin-3 and galectin-3C colocalize on the cell surface of leukocytes

To analyze how the associated galectin-3 and galectin-3C are distributed on the neutrophil surface, the binding pattern of recombinant fluorescently labeled galectin-3 and galectin-3C was examined using imaging flow cytometry. At equimolar concentrations, we found galectin-3 and galectin-3C to colocalize (Figure 7A), identified by a colocalization score equal to that of the positive control (Figure 7B and Supplemental Figure 3). Unprimed and TNF- α primed neutrophils displayed similar extents of colocalization for galectin-3 and galectin-3C on the cell surface, and the score was significantly increased in com-

parison to the negative control (Figure 7C and Supplemental Figure 3). This overlap (colocalization) between galectin-3 and galectin-3C on the neutrophil cell surface gives support to the notion that the lectins are associated homo- and heterologously in networks supported by both N- and C-type self-association.

4 | DISCUSSION

This study was initiated to perform an in detail investigation of the galectin-3C effect on the galectin-3-neutrophil interaction. Our data demonstrate that galectin-3C inhibits galectin-3-induced activation of neutrophil ROS production, but that this inhibition was not due to abrogated binding of galectin-3, as originally hypothesized. Instead, we found that leukocytes can bind large amounts of both galectin-3 and galectin-3C on the cell surface and that the presence of galectin-3C on neutrophil plasma membranes increases the binding of galectin-3, most probably by a process called type-C self-association, which seems to be a nonactivating process in contrast to type-N self-association, which seems to be necessary for neutrophil activation by galectin-3.

Galectin-3 is present in plasma of healthy humans at a concentration of ~ 12 ng/ml (0.5 nM),^{53,54} and diversions from this level have emerged as a diagnostic tool in different pathologies, including cancer⁵⁵⁻⁵⁷ and heart failure.^{58,59} However, the lectin was first discovered as a cell surface marker on mononuclear cells,⁵² implying that there is both a soluble and a cell-attached pool of galectin-3 in circulating blood. We found that circulating galectin-3 is attached not only to monocytes but also to neutrophils and lymphocytes. Based on the finding that both neutrophils and bacteria can cleave galectin-3 into truncated galectin-3C forms,^{17,19,21,25} and that such truncated molecules have been shown to inhibit galectin-3-induced activities,⁵¹ we also investigated the presence of endogenous galectin-3/galectin-3C attached to leukocytes in blood. By comparing the relative amount of cellular staining by an anti-CRD antibody (detecting both full-length

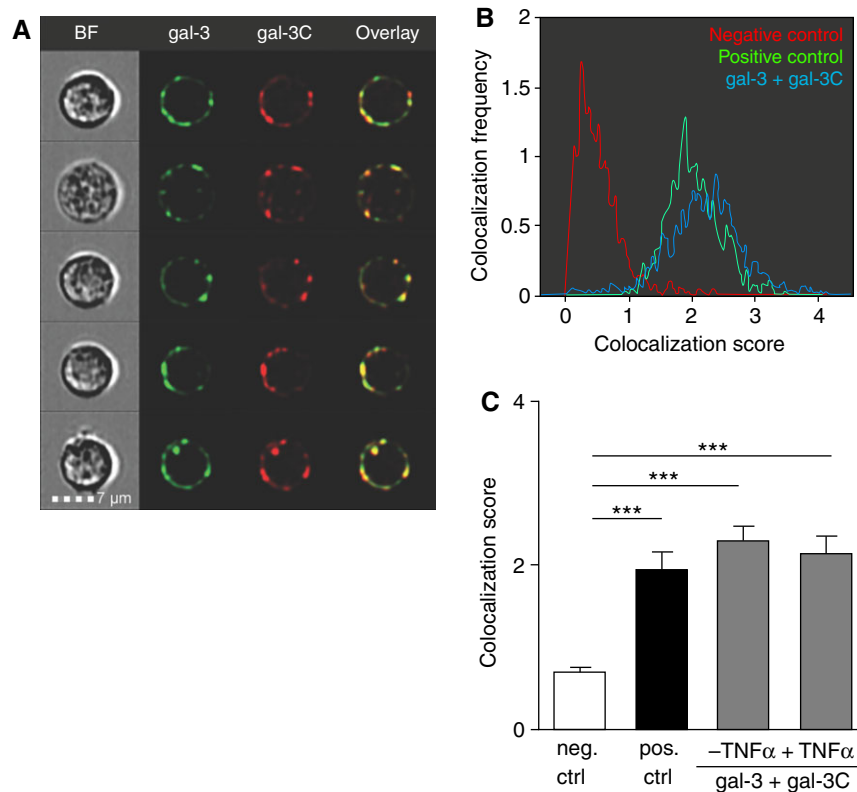


FIGURE 7 Galectin-3 and galectin-3C colocalize on the cell surface of neutrophils. Neutrophils were incubated simultaneously with FITC-labeled galectin-3 (gal-3, 1 μ M; green) and Alexa Fluor 647-conjugated galectin-3C (gal-3C, 1 μ M; red) for 15 min on ice, before fixation and analysis of colocalization using imaging flow cytometry. (A) Representative brightfield and fluorescence images of neutrophils incubated with the fluorescently labeled galectin-3 and galectin-3C. (B) A representative histogram ($n = 5$) of the colocalization score for galectin-3/galectin-3C-incubated cells (blue), positive control (FITC-labeled mouse anti-human CD16 ab, and Alexa Fluor 647-labeled goat anti-mouse IgG 2 $^{\circ}$ ab; green) and negative control (FITC-labeled mouse anti-human CD16 ab, and Alexa Fluor 647-labeled galectin-3C; red) is shown. (C) The graph shows a quantitative analysis of the colocalization scores (mean + SEM, $n = 3-5$) of unprimed ($-TNF\alpha$) and $TNF\alpha$ -primed ($+TNF\alpha$) neutrophils treated with fluorescently labeled galectin-3 and galectin-3C as compared to positive and negative controls. Statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple comparison test

galectin-3 and galectin-3C) with that of an anti-galectin-3 antibody (detecting only full-length galectin-3), we draw the conclusion that galectin-3C is in fact present and attached to neutrophils in circulation. This suggests that quantification of soluble galectin-3 in serum/plasma does not necessarily reflect the total amount of galectin-3 in blood.

The presence of endogenous galectin-3C in circulation suggests that a continuous cleavage process of galectin-3 takes place in (the vicinity of) the blood of healthy humans. There are several neutrophil proteases that may be responsible for this processing. In the study by Nieminen et al., it was suggested that elastase is responsible for the cleavage of galectin-3 into galectin-3C by primed neutrophils, as evidenced by inhibited cleavage in the presence of the elastase-inhibitor Eglin C,²¹ but neutrophils also contain MMP-9 that has shown to cleave galectin-3 into truncated fragments lacking parts of the N-domain, just as MMP2 and MMP7.²²⁻²⁴ To this background, the identity of the protease(s) responsible for the galectin-3C found on leukocytes (primarily neutrophils; 4B) in circulation is uncertain; most probably cleavage is achieved by a combination of different proteases, for example, serine proteases and MMPs. Neutrophil-derived proteases could be present in circulation provided that the cells have degranulated, that is, become primed, or undergone violent cell death

such as necrosis; however, these are processes that most probably does not occur in the blood of healthy humans. Another possibility would be that galectin-3 is cleaved in extravascular tissue and that galectin-3C subsequently diffuses into circulation. The investigation of galectin-3C presence in tissue is however difficult, since the use of CRD-specific antibodies detects both galectin-3C and its molecule of origin, galectin-3. Only in a total absence of galectin-3 (and thus absence of staining with an N-terminal specific antibody) could the presence of galectin-3C be proven (with a CRD-specific antibody). Attempts to determine the amount of soluble galectin-3C in circulation has failed in our hands. The commercially available ELISAs cannot be used for this purpose, since they utilize antibodies binding to the N-terminal domain of galectin-3, thus not detecting the CRD domain. Instead we have used Western blots of serum samples with our anti-CRD antibody, but due to the insensitivity of this technique together with that the levels of galectin-3C probably are very low (the galectin-3 levels are in the lower nanometer range), this has so far not been successful (data not shown) for determining soluble galectin-3C in circulation.

Since inflammatory activation of macrophages results in production of large amounts of galectin-3,⁶⁰ we found it valuable to

investigate the capacity of different leukocytes to bind additional lectin, through the use of recombinant protein. All examined leukocyte populations demonstrated great capacity to bind additional galectin-3, in a dose-dependent manner. We also found, for the first time, that the leukocytes all had the ability to bind large amounts of recombinant galectin-3C. This indicates that, in situations where increased amounts of galectin-3C are present due to cleavage of galectin-3, for example, in the presence of primed neutrophils, high levels of galectin-3C may actually bind to the surface of neutrophils. Whether this has a down-regulating effect on the primed neutrophil response to galectin-3, shown as a decrease in production of ROS, can at this point only be speculated upon. The presence of primed neutrophils in circulation has been demonstrated, by us and others, for diseases such as systemic inflammatory response syndrome (SIRS),^{61,62} autoinflammatory diseases,⁴⁸ and anti-neutrophil cytoplasmic autoantibody (ANCA) associated vasculitis.⁶³ Possibly, a down-regulation of galectin-3-induced NADPH-oxidase activation could be achieved by galectin-3C in these primed cells, resulting from activity of proteases released from the same cells, a function that could be of protective value to the host.

In this study, we show that galectin-3 and galectin-3C are attached to resting neutrophils in circulation, as well as and in increased amounts to TNF- α primed neutrophils. We and others have earlier shown that activation of neutrophils by galectin-3 only occurs if the cells are primed,^{4,16,47,48} either through in vivo transmigration⁴ or in vitro prestimulation.^{3-5,16,48} The need of priming of the cells for activation with galectin-3 is most probably due to the upregulation of (specific) receptors on primed as compared to unprimed/resting neutrophils, and earlier we have tentatively identified possible receptor candidates to be CD66a and CD66b.⁴⁷ The attachment of galectin-3 and galectin-3C to the surface of resting cells, not resulting in any cellular activation, is however intriguing. Whether these attached lectins play any role is unknown, but it has been suggested that galectin-3 takes part in neutrophil adhesion to extracellular matrix proteins and other cells.^{11,14,64,65} Possibly, these lectins could also be functional as blockers or enhancers of receptor signaling influencing cell activation/inactivation in circulation.

Our data on attachment of endogenous galectin-3 to the cell surface of leukocytes are in contrast to the study by Baseras et al.,⁶⁶ showing no attachment of endogenous galectin-3 on neutrophils. The reason for the contradiction between our results could, for example, be the use of different antibodies, as our experience is that the specificity of different anti-galectin-3 antibodies varies considerably. Another possibility is that differences in experimental protocols may lead to different extents of galectin-3 dissociation from the cell surface during the experimental assays. However, Baseras et al.⁶⁶ showed that neutrophils display capacity to bind additional galectin-3, which agrees with our results.

As galectin-3C was found to inhibit the galectin-3-induced activation of neutrophils, we hypothesized that galectin-3C would competitively inhibit the binding of galectin-3 to neutrophils. However, galectin-3C instead potentiated the binding of galectin-3. The original dogma that galectin-3 molecules form oligomers through interactions involving the N-domain (type-N self-association^{17,18,37}) has

been complemented with another galectin-3-organizational model by data demonstrating that galectin-3 molecules apart from type-N self-association also can form oligomers by interactions involving the CRD part of the molecules.³⁵⁻³⁷ This novel type of association was recently named type-C self-association,³⁷ to distinguish it from the classical type-N self-association. The biological relevance of type-C self-association has so far not been elucidated, but our data demonstrating that the presence of galectin-3C increases binding of both itself and galectin-3, indicate that this mechanism not only occurs homologously but also heterologously and not only in soluble systems but also on cell surfaces.

The increase in galectin-3 binding by galectin-3C occurring in parallel to galectin-3C inhibition of galectin-3-induced neutrophil activation indicates that the mechanism of inhibition is not simply blockade of activation receptors. However, even though the net binding of galectin-3 increases, the binding to the activation receptors may be blocked, either through specific interaction with galectin-3C, or through the alteration of lattices' structural organization covering the cell surface. Lattice formation has been shown to form on neutrophils¹⁸ and may play important roles in regulation of cellular function, why alteration of its structural organization influenced by varying levels of galectin-3C may be important.

In summary, galectin-3 and galectin-3C interact on the surface of neutrophils in that the attachment of one is increased by presence of the other, that is, we provide proof of principle for type-C self-association to occur not only in solution but also on cell surfaces. Since this increase in binding is paralleled by galectin-3C-mediated inhibition of the galectin-3-induced ROS production, type-C self-association appears to be a nonactivating process in contrast to type-N self-association, which is necessary for neutrophil activation by galectin-3. We speculate that type-C self-association is a "turn-off" system for galectin-3-induced ROS, with the purpose of avoiding overproduction of ROS that may cause extensive tissue damage.

AUTHORSHIP

M.S., A.K., J.B., and A.W. designed the research. M.S., A.W., J.E., and V.O. performed the experiments. M.S., A.K., J.B., A.W., J.E., U.J.N., and H.L. interpreted the data, and M.S. and A.K. wrote the manuscript. All authors revised and approved the final manuscript.

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DISCLOSURE

U.J.N. and H.L. are cofounders of and shareholders in the company Galecto Biotech AB, Sweden, which develops galectin-targeting drugs.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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