



Pergamon

Glycoprotein-Inspired Materials Promote the Proteolytic Release of Cell Surface L-Selectin

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Received 28 April 1998; accepted 18 June 1998

Abstract—The proteolytic release, or shedding, of a cell surface protein can serve a regulatory role; the process liberates a soluble form of the protein into circulation while downregulating its cell surface concentration. The characteristics that render a protein susceptible to proteolytic cleavage are not known. We hypothesized that the clustering of a protein at the cell surface might target it for proteolysis. To test this hypothesis, we synthesized molecules that display multiple copies of sulfated galactose residues, termed neoglycopolymers, that are designed to mimic natural ligands for the cell adhesion protein L-selectin. We found that treatment of human neutrophils with the neoglycopolymers resulted in a dose-dependent loss of L-selectin from the cell surface, while monovalent compounds and unsulfated neoglycopolymers had no effect. Because L-selectin is an important mediator in the inflammatory response, such compounds could lead to novel antiinflammatory drugs. Moreover, molecules that control receptor shedding can be used to alter cellular responsiveness to specific ligands or to promote responses at distal sites; consequently, these results have broad implications for regulating the location and presentation of important biomolecules. © 1998 Elsevier Science Ltd. All rights reserved.

Introduction

Cells can alter the display of proteins on their cell surfaces by several different mechanisms, allowing them to respond to either intracellular or extracellular stimuli (Fig. 1). For example, transcription and translation are two intracellular processes that are used to control the production of cellular proteins, including cell surface molecules. Other mechanisms include the internalization of a protein through endocytosis, external release through vesicle shedding, or recycling through exocytosis. In addition, the proteolytic release of the ecto-domain of a target protein has recently emerged as a newly discovered process by which cells can simultaneously alter the concentration of proteins on their surfaces and in circulation.^{1–4}

Chemical strategies that manipulate one or more of these physiological processes could be used to direct the

display of proteins on the cell surface. Such methods could be applied to control cellular responses to the external environment, to block or enhance the release of signalling molecules from the cell surface, or to modulate the levels of a specific receptor in circulation. Here, we describe the design and characterization of molecules that can downregulate a target protein, L-selectin, from the cell surface.

L-Selectin is an important target because it mediates leukocyte rolling, the initial step in leukocyte recruitment to the endothelium during the inflammatory response and lymphocyte homing.^{5–7} The protein functions by binding highly glycosylated, mucin-like proteins, displayed on opposing cell surfaces.⁸ In addition to the transmembrane form that functions as a cell adhesion molecule, a soluble form of L-selectin can be generated by release or ‘shedding’ from the cell surface.⁹ This shedding event occurs when a protease cleaves L-selectin at an extracellular site that is adjacent to the membrane-spanning domain.¹⁰ Intriguingly, increased levels of soluble L-selectin are associated with some inflammatory diseases.^{11–15} Moreover, cells that have shed L-selectin can no longer function in the

Key words: Carbohydrates; glycopeptides/glycoproteins; polymers; antiinflammatories; receptors.

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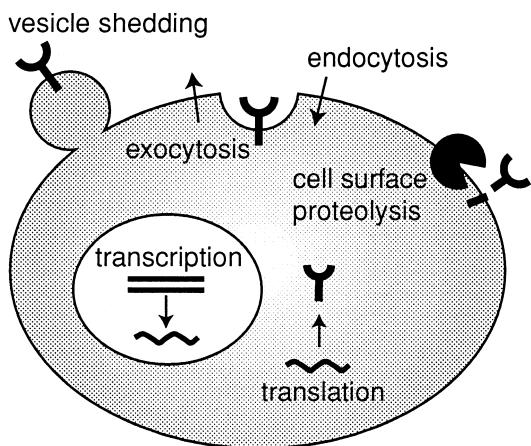


Figure 1. Mechanisms for the regulation of proteins on the cell surface.

inflammatory response.⁹ Thus, the design of molecules that can cause L-selectin proteolysis is an attractive goal. In this context, L-selectin functions as an object for testing mechanisms by which proteins might be proteolytically released and a target for the development of new strategies for treating inflammation. The protease that cleaves L-selectin, however, is not known nor is it clear how this enzyme is regulated. Using insights obtained from cell biological and physiological studies of L-selectin, we postulated that L-selectin proteolysis is activated when the protein binds its glycoprotein ligands. In support of this hypothesis, we showed that when cell surface L-selectin was presented with multidentate, sulfated trisaccharide epitopes, the protein was shed.¹⁶ These multivalent molecules, but not their monovalent counterparts, promote the proteolytic release of L-selectin. Compounds that induce L-selectin cleavage from the cell can function as antiinflammatory agents by this new mechanism. Here, we report that multivalent ligands, with simple sulfated saccharide epitopes, can induce L-selectin shedding.

Results

To design simple molecules that could cause the proteolytic release of L-selectin, we considered the molecular mechanisms involved in leukocyte recruitment during inflammation. The increased concentrations of soluble L-selectin found in the circulatory systems of individuals afflicted with inflammatory conditions^{11–15} may be related to an increased level of L-selectin-mediated cell rolling,¹⁷ which would involve L-selectin-ligand interactions. Engagement of cell surface L-selectin by its ligands would be an attractive means by which to regulate L-selectin release, as leukocytes would be able to

survey the endothelium for signs of activation for a controlled period of time. Therefore, the strategy devised for creating molecules that activate the proteolytic release of L-selectin was predicated on our studies of L-selectin recognition.

We planned to design molecules that mimic the features of natural L-selectin ligands. In physiological settings, L-selectin binds extracellular, highly O-glycosylated proteins, including CD34,¹⁸ GlyCAM-1,¹⁹ and PSGL-1²⁰ that present clusters of sulfated, O-linked saccharides. Because physiologic ligands for L-selectin are multivalent, we postulated that the clustering of L-selectin, as a consequence of ligand binding, might promote its proteolytic release. Although it had not been shown that clustering of cell surface receptors can promote the shedding of a protein ectodomain, dimerization or clustering of specific cell surface receptors can trigger signal transduction pathways.²¹ Thus, clustering of cell surface proteins could result in these different responses, depending on the target protein and its cellular function.

The molecular structures of physiological L-selectin ligands revealed that sulfation of the displayed saccharide residues is important for high affinity interactions.⁸ These results suggest that the sulfation of particular saccharide sequences may lead to high affinity binding. Various sulfated derivatives of the tetrasaccharide sialyl Lewis x (sLe^x) terminate the saccharide chains of the L-selectin-binding protein, GlyCAM-1. These epitopes were proposed to be ligands for L-selectin (Fig. 2(A)),^{22–24} and recent binding studies reveal that 6-sulfo sLe^x and its analogues are more effective L-selectin inhibitors (approximately threefold more potent) than sLe^x.^{25–27} Moreover, we recently demonstrated that a molecule presenting a multivalent display of the 3',6-disulfo Le^x recognition unit (Fig. 2(B)) can induce the proteolytic cleavage of L-selectin.¹⁶ L-selectin, however, also binds to simpler, anionic saccharide-containing molecules, including heparin,²⁸ fucoidin,²⁹ and sulfatides³⁰ (Fig. 2(C)), although these molecules may bind L-selectin in a different mode than that used by sLe^x. Despite the potential differences, a common structural feature in many of the known L-selectin ligands is a polyvalent display of sulfated saccharides. Therefore, we designed neoglycopolymers that present a multivalent display of a simple, sulfated galactose residue. The potential for multivalent binding inherent in the structure of the neoglycopolymers allowed us to examine whether treatment of leukocytes with the neoglycopolymers would lead to L-selectin shedding.

The neoglycopolymers were synthesized by a procedure similar to that reported previously.³¹ To create molecules of defined length, however, we performed the polymerization reaction under living conditions using

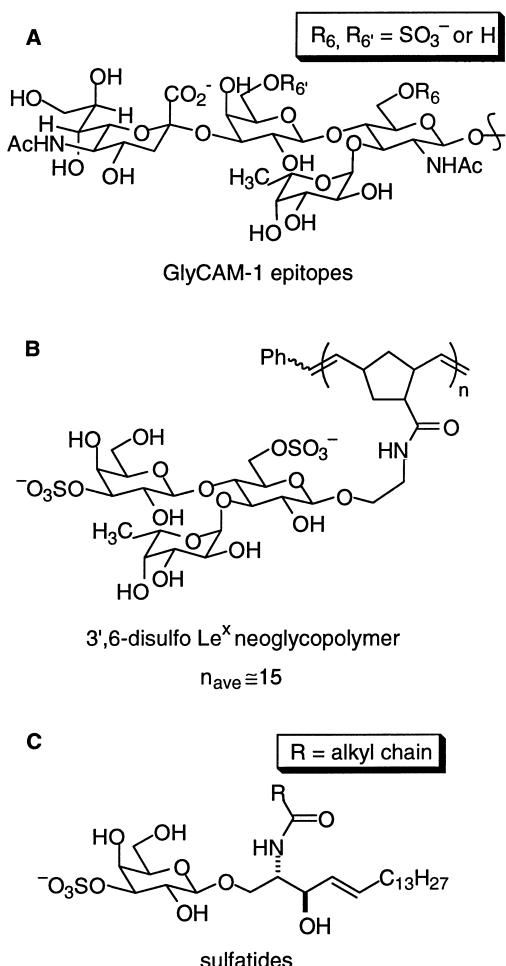


Figure 2. Chemical structures of L-selectin ligands. (A) Sulfated derivatives of sialyl Lewis X present in GlyCAM-1, including sulfation at the 6-position of glucosamine and/or the 6-position of galactose. (B) Synthetic neoglycopolymers that promotes L-selectin shedding. (C) Sulfatides are glycolipids containing 3-sulfo galactose residues.

the defined ruthenium catalyst $[(Cy)_3P]_2Cl_2Ru=CHPh$.^{32,33} Living polymerizations are those in which the rate of polymerization exceeds that of termination.³⁴ In living processes when the rate of initiation is fast relative to the rate of propagation, polymer length can be controlled by varying the ratio of monomer to catalyst. Therefore, we constructed the neoglycopolymers with an average length of 15 monomer units per molecule simply by using a monomer to catalyst ratio of 15:1 (Scheme 1).

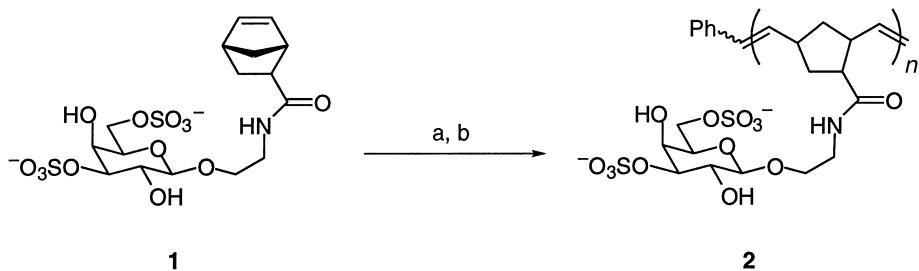
The sulfated galactose-substituted neoglycopolymers were tested for their ability to promote L-selectin shedding from the surface of human neutrophils. Cell surface levels of L-selectin were monitored by flow cytometry

using a fluorescein isothiocyanate (FITC)-conjugated anti-L-selectin antibody. By comparing treated and untreated cells, the ability of the neoglycopolymers to downregulate cell surface L-selectin could be determined. In addition, the soluble form of L-selectin could be detected in the supernatant using a commercially available enzyme-linked immunosorbent assay (ELISA). In this assay, supernatant from untreated cells or cells treated with the neoglycopolymers was added to microtiter plates containing an antibody to L-selectin adsorbed to the wells, allowing for the capture of L-selectin. Addition of a second antibody to L-selectin that is conjugated to horseradish peroxidase, followed by a colorimetric substrate for the enzyme, allowed for detection of L-selectin (data not shown). Significantly, neutrophils treated with neoglycopolymers **2** were subject to loss of L-selectin from the cell surface (Fig. 3(A)).

Several different lines of evidence suggest the process is specific. First, the amount of L-selectin released from the cell depended on the concentration of neoglycopolymers added (Fig. 3(B)). Second, treatment of neutrophils with the corresponding neoglycopolymers containing unsulfated galactose residues did not result in L-selectin shedding, even at the highest concentrations tested (Fig. 3(C)). As was observed with the tri-saccharide-substituted neoglycopolymers reported previously,¹⁶ only the multivalent derivative was able to promote the proteolytic release of L-selectin; the monovalent compound **1** had no effect (Fig. 3(D)). Additionally, the ligand-induced shedding occurs by a mechanism that appears to be conserved across cell types. Specifically, lymphocytes, which also possess cell surface L-selectin, similarly shed the target protein upon neoglycopolymers exposure (data not shown).

Discussion

Prior chemical and biological studies aimed at elucidating the recognition properties of the cell adhesion protein L-selectin provided a basis for devising and implementing a new strategy for controlling L-selectin function. In these investigations, we demonstrate that simple, synthetic ligands can be constructed that induce the proteolytic release of a target protein, L-selectin, from a cell surface. The ligands were designed to mimic the important features of the natural glycoproteins that bind L-selectin. The ring-opening metathesis polymerization (ROMP) provides a means to rapidly assemble these materials in a single synthetic operation from readily accessed building blocks. In addition to its applications in generating potent inhibitors of biological processes,^{31,35–38} our results indicate that ROMP can be used to synthesize molecules that activate specific biological processes.



Scheme 1. Synthesis of the 3,6-disulfo galactose neoglycopolymers. Reagents and conditions: (a) $[(\text{Cy})_3\text{P}]_2\text{Cl}_2\text{Ru}=\text{CHPh}$, dodecyltrimethylammonium bromide (DTAB), bis-Tris buffer (pH 5.9), dichloroethane (DCE), 45°C , 24 h; (b) ethyl vinyl ether.

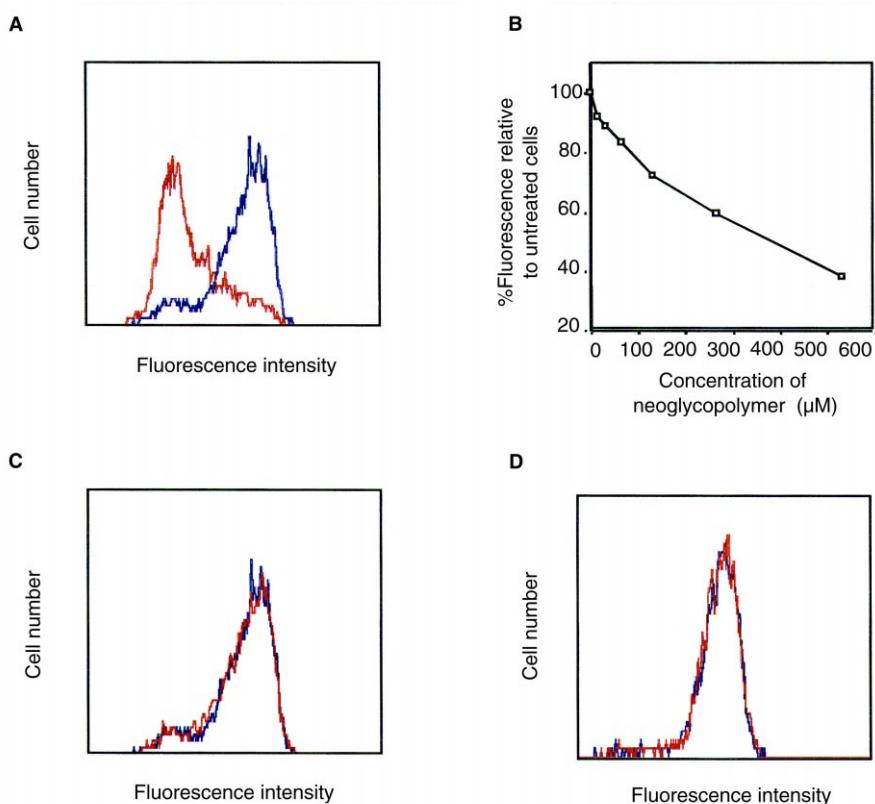


Figure 3. Neoglycopolymers-induced shedding of L-selectin from neutrophils. (A) Histogram representation of untreated cells (blue) and cells treated with the 3,6-disulfo galactose neoglycopolymer (4 mM on a per saccharide basis) (red). (B) Dose-dependent shedding of L-selectin from the surface of human neutrophils by the 3,6-disulfo galactose neoglycopolymer. (C) Histogram representation of untreated cells (blue) and cells treated with an unsulfated galactose neoglycopolymer (4 mM on a per saccharide basis) (red). (D) Histogram representation of untreated cells (blue) and cells treated with the 3,6-disulfo galactose monomer (4 mM) (red).

These studies provide support for the hypothesis that L-selectin clustering induces the proteolytic release of the protein's ectodomain (Fig. 4). We recently reported that synthetic molecules designed to present disulfated, trisaccharide derivatives of sLe^x could promote the proteolytic cleavage of L-selectin from the surface of human leukocytes.¹⁶ Here, we show that structurally less complex molecules, but molecules that have features of glycoproteins, can also promote L-selectin

shedding. The sulfated galactose-substituted neoglycopolymers could bind to L-selectin through interactions that differ somewhat from those used by physiological ligands such as GlyCAM-1, but both the glycoprotein mucin and the synthetic analogues have the ability to engage in multivalent interactions. Monovalent ligands do not have the capacity to effect this process, suggesting that L-selectin clustering induces protein shedding.

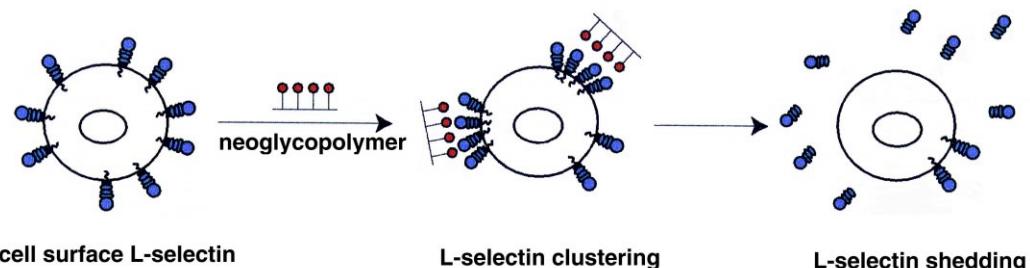


Figure 4. Schematic depiction of neoglycopolymers-induced shedding of L-selectin from the surface of leukocytes.

The protease that cleaves L-selectin has not been identified, but it does appear to be membrane-associated.^{39,40} Several mechanisms by which L-selectin clustering might lead to its activation can be envisioned. For example, L-selectin clustering could result in transmission of a signal, which leads to protease activation (e.g. through phosphorylation). There is some evidence that L-selectin can transduce signals.^{41–46} Alternatively, L-selectin could associate with another protein that blocks an active protease from cleaving it. Aggregation of L-selectin may cause the inhibitor protein to dissociate, rendering L-selectin susceptible to cleavage.⁴⁷ If the protease that cleaves L-selectin exists as a homodimer, homotetramer, or some other specific oligomer, it may prefer the clustered substrate.⁴⁸ Another scenario is that L-selectin binding to its ligands results in a change in the accessibility of the cleavage site. Molecules, such as the neoglycopolymers, that selectively cause L-selectin shedding may be used to uncover the molecular interactions that result in shedding. Moreover, these agents can be used to identify the enzyme that facilitates this cleavage event; such studies are ongoing.

Beyond the applications for study of L-selectin release,^{17,49} this approach can be used to investigate other shedding processes. Protein shedding is emerging as a common mechanism for the regulation of cell surface receptors. Receptor shedding in response to clustering could be a general mechanism that cells use to remove hyperactive or unproductive proteins from their surface. Many proteins, including growth factors, growth factor receptors, cytokines, cytokine receptors, G-protein coupled receptors, and cell adhesion molecules are shed from the surface of cells in response to a variety of stimuli.^{1–4,50,51} However, as with L-selectin, the pathways and enzymes involved in the regulation of protein shedding must be elucidated. The ability to control L-selectin shedding by ligand binding opens the exciting possibility that the release of other proteins from the cell surface might be controlled by similar mechanisms. With these results, the issue of whether shedding following protein clustering is a common

mechanism that cells widely employ to modulate their cell surfaces or whether this mechanism is unique to L-selectin can be addressed.

The demonstration that designed synthetic ligands can promote L-selectin shedding suggests new approaches for the creation of therapeutic agents. For example, molecules that cause L-selectin downregulation will diminish L-selectin-dependent leukocyte rolling and the transendothelial migration that can follow. At the same time, these agents can cause an increase in the concentration of soluble L-selectin, which can act as an inhibitor of the rolling process. The involvement of the selectins in the inflammatory cascade has prompted efforts to generate synthetic molecules that compete with natural ligands for selectin binding.⁵² The multivalent neoglycopolymers share this property with those ligands, yet by also provoking L-selectin release, they can function by a more efficient mechanism of action. Specifically, the neoglycopolymers appropriate an endogenous enzyme to cause a change in covalent bonding, unlike conventional inhibitors that act through non-covalent bond association. In addition, when receptors are shed from the cell surface the affinity of the soluble receptors for the multivalent ligand should decrease, allowing dissociation and rebinding of the ligand to L-selectin-positive cells in circulation. Molecules that promote L-selectin proteolysis, therefore, can function at a sub-stoichiometric levels to promote an irreversible, proteolytic event.

The many functions associated with proteins that are regulated in part by protein shedding suggest that the ability to manipulate the proteolytic release of these proteins could have a wide variety of therapeutic applications. Our results indicate that multivalent molecules can be designed not only to target specific cell surface receptors but to selectively promote their shedding. Understanding and manipulating protein shedding at the molecular level merits intense investigation, and innovative molecular tools that can regulate this process can illuminate the critical molecular features.

Conclusion

The ability to design molecules capable of performing explicit biological functions has tremendous potential for elucidating and controlling biological events. We have developed a flexible strategy to create molecules capable of engaging L-selectin in multivalent interactions. These molecules not only act as inhibitors of L-selectin binding, but they also promote the proteolytic release of L-selectin from the cell surface. The results illustrate that endogenous proteases can be recruited to surgically remove a target cell surface protein. Because other proteins may undergo ligand-induced shedding, these results have broad implications for the investigation and manipulation of the cell surface and the extracellular environment.

Experimental

General methods. Reactions were carried out under a nitrogen atmosphere, except where otherwise noted. ACS grade 1,2-dichloroethane was used as received. Solvents used in polymerization reactions were deoxygenated with a minimum of three freeze-pump-thaw cycles prior to use. Analytical thin-layer chromatography (TLC) was performed on 0.25 mm precoated silica gel plates (60F-254). Visualization of TLC was done with ultraviolet light and *p*-anisaldehyde stain. ¹H and ¹³C nuclear magnetic resonance (NMR) spectra were recorded on 300 or 500 MHz spectrometers; chemical shifts are reported downfield from tetramethylsilane in parts ppm. ¹H NMR data are assumed to be first order with apparent doublets and triplets reported as d and t, respectively. Resonances that appear broad are designated as br.

Synthesis of the 3,6-disulfo galactose neoglycopolymers. Deoxygenated 2,2-bis(hydroxymethyl)-2',2"-nitrilo-triethanol (bis-Tris) buffer (240 μ L, 100 mM, pH 5.9) was added to a mixture of the triethylammonium salt of the monomer (**1**, 25.8 mg, 0.0357 mmol) and the detergent dodecytrimethylammonium bromide (DTAB) (19.6 mg, 0.0571 mmol) under nitrogen. Degassed 1,2-dichloroethane (163 μ L) was added to the catalyst bis(tricyclohexylphosphine)benzylidene ruthenium(IV)dichloride (2.6 mg, 0.0032 mmol), and a portion (119 μ L, 0.0023 mmol) of the solution was added to the reaction vessel containing the monomer. After 45 min, the reaction was heated to 45 °C and stirred for 24 h. The reaction was allowed to cool to room temperature, and an excess of ethyl vinyl ether was added to terminate the polymerization. The reaction was stirred for 30 min and then opened to air. Methanol was added, and the solution was filtered through size exclusion resin (Sephacryl

S-400 HR, H₂O eluent) and cation exchange resin (Sephadex SP C-25, Na⁺ form, H₂O eluent) to remove DTAB and generate the sodium salt. The solvent was removed under reduced pressure and the resulting solid was precipitated three times with methanol. The resulting material was dialyzed against deionized water (1 L) for 48 h. The solution from the dialysis tubing was concentrated under reduced pressure to yield the final product **2** (16.8 mg, 86%). ¹H NMR (300 MHz, D₂O) δ 7.55–7.26 (0.20 H), 5.59–5.21 (2 H), 4.59–4.48 (1 H), 4.42–4.30 (2 H), 4.28–4.15 (2 H), 4.05–3.89 (2 H), 3.85–3.64 (2 H), 2.83–2.43 (3 H), 2.18–1.87 (2 H), 1.81–1.53 (1 H), 1.47–1.12 (2 H); ¹³C NMR (126 MHz, D₂O) δ Major signals: 178.5, 135.5–130.7 (multiple peaks), 102.6, 80.0, 72.3, 68.8, 66.9, 66.6, 51.4, 48.9, 41.7, 41.2, 39.2, 36.4, 35.3; Minor signals: 68.3, 67.4, 53.0, 48.6, 44.0, 43.9, 40.7, 38.8, 36.0, 35.9.

Leukocyte isolation. Human blood was collected by venipuncture from healthy volunteers and centrifuged at 200 g for 20 min. Platelet-rich plasma was removed, and the remaining solution was diluted to the original volume with Hanks' Balanced Salt Solution (HBSS) without Ca²⁺ or Mg²⁺. Red cells were removed by dextran sedimentation (1 mL of 4.5% dextran in 0.85% saline per 5 mL blood), and leukocytes were pelleted at 400 g for 10 min and washed with HBSS without Ca²⁺ or Mg²⁺ at 4 °C. The remaining red blood cells were hypotonically lysed by resuspension in 4.5 mL H₂O for 25 s followed by addition of 0.5 mL of 10×HBSS without Ca²⁺ or Mg²⁺. Cells were washed twice in HBSS without Ca²⁺ or Mg²⁺ and finally resuspended in buffer for flow cytometry analysis (PBS, 1% BSA, 0.1% NaN₃) at a concentration of 2 × 10⁶/mL. Cells were >95% viable by trypan blue exclusion and were used within 2 h of isolation.

Flow cytometry. Leukocytes (~2 × 10⁵) were incubated with the neoglycopolymers at the concentrations indicated (or left untreated) for 20 min at 37 °C. FITC-conjugated anti-L-selectin antibodies (or isotype matched controls) were added and samples were incubated at 4 °C for 30 min. Cells were washed and resuspended in ~200 μ L FACS buffer. Propidium iodide (5 μ L of 0.5 mg/mL) was added to stain dead cells, allowing for their exclusion in data analysis. Samples were analyzed on a Becton Dickinson FACScan cytometer using CELLQUEST software. Neutrophils and lymphocytes were identified by their characteristic forward and side scatter; typically, 20,000 cells were analyzed from each sample.

Acknowledgements

This research was supported in part by the NIH (GM-55984). L.L.K. acknowledges the American Cancer

Society, the NSF NYI Program, the Camille and Henry Dreyfus Teacher–Scholar Program, and the Alfred P. Sloan Foundation for support. E.J.G. thanks the Biotechnology Training Program for a predoctoral fellowship (T32GM08349). L.E.S. was supported by an NIH predoctoral fellowship (NIH GM 18750).

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