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Endoglycan, a member of the CD34 family of sialomucins, is a ligand for the vascular selectins

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Abstract

The interactions of the selectin family of adhesion molecules with their ligands are essential for the initial rolling stage of leukocyte trafficking. Under inflammatory conditions, the vascular selectins, E- and P-selectin, are expressed on activated vessels and interact with carbohydrate-based ligands on the leukocyte surface. While several ligands have been characterized on human T cells, monocytes and neutrophils, there is limited information concerning ligands on B cells. Endoglycan (EG) together with CD34 and podocalyxin comprise the CD34 family of sialomucins. We found that EG, previously implicated as an L-selectin ligand on endothelial cells, was present on human B cells, T cells and peripheral blood monocytes. Upon activation of B cells, EG increased with a concurrent decrease in PSGL-1. Expression of EG on T cells remained constant under the same conditions. We further found that native EG from several sources (a B-cell line, a monocyte line and human tonsils) was reactive with HECA-452, a mAb that recognizes sialyl Lewis X (sLex) and related structures. Moreover, immunopurified EG from these sources was able to bind to P-selectin and where tested E-selectin. This interaction was divalent cation-dependent and required sialylation of EG. Finally, an EG construct supported slow rolling of E- and P-selectin bearing cells in a sialic acid and fucose dependent manner, and the introduction of intact EG into a B cell line facilitated rolling interactions on a P-selectin substratum. These in vitro findings indicate that EG can function as a ligand for the vascular selectins.

Keywords

Adhesion molecules; Cell trafficking; B cells

Introduction

The leukocyte adhesion cascade is a complex multi-step process essential for immune surveillance (reviewed in (1)). The initial steps are the tethering of leukocytes to the endothelium and subsequent rolling of the leukocyte along the vessel wall under blood flow. This rolling step is mediated by catch and slip bonds formed between members of the selectin family of adhesion molecules and their ligands (2). The selectin family is comprised of 3 members, each acting as a lectin-like receptor, in a calcium dependent manner, via an amino-terminal C-type lectin domain (reviewed in (3)). L-selectin on lymphocytes interacts with ligands expressed on specialized high endothelial venules (HEVs) in secondary lymphoid organs during the process of lymphocyte homing (reviewed in (4)). In contrast, P-selectin and

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E-selectin are both expressed on vascular endothelium with P-selectin also expressed on platelets. P-selectin is stored in α-granules in platelets and in Weibel-Palade bodies in endothelial cells, where it is rapidly mobilized to the cell surface upon activation (3). E-selectin is induced on the surface of endothelium by activation with inflammatory mediators including TNF-α and LPS (3).

The selectins bind to oligosaccharide structures, usually presented on protein scaffolds. sLex and related structures, containing α2,3 sialylation and α1,3 fucosylation, are key recognition determinants in many selectin ligands (3,5). Most of the selectin ligands identified thus far are sialomucins (3,6); that is, proteins possessing large segments with extensive O-linked glycosylation. Carbohydrate recognition determinants can occur on O-glycans, N-glycans or both (7-9). Sialomucin ligands include GlyCAM-1, PSGL-1, CD43, CD44, endomucin, nepmucin, and members of the CD34 family (reviewed in (10). A family of HEV-expressed sialomucins, known as the peripheral lymph node addressin (PNAd), serve as adhesive ligands for L-selectin dependent rolling (10). For PNAd, 6-sulfo sLex (containing GlcNAc-6-SO_4) is a key recognition determinant (11,12). L-selectin can also mediate secondary tethering interactions between leukocytes through binding of L-selectin to PSGL-1 on adherent leukocytes (13).

PSGL-1 was first identified as a major ligand for P-selectin (reviewed in (14)). It is a sialomucin and is found on all types of circulating myeloid cells, dendritic cells, all subsets of T cells and $CD34^+$ progenitor cells (15,16). However, PSGL-1 expression on human B cells is at low levels (15,17). Recently, PSGL-1 was found on cultured endothelial cells and on certain venules *in situ* (18,19). PSGL-1 supports rolling of neutrophils, T cells and monocytes via interactions with P-selectin (20-23), and PSGL-1 null mice show severe defects in leukocyte rolling on P-selectin expressing vessels (24). PSGL-1 also interacts with E-selectin (20-22,25). However, analysis of PSGL-1 null mice indicates that there are alternative ligands for E-selectin on neutrophils (24,25) and Th1 T cells (21,22). Recently, CD43 was identified as an E-selectin ligand on activated murine T cells (26-28) with evidence that it can act in concert with PSGL-1 in Th1 cell homing to the skin (29). CD44 is also an E-selectin ligand on neutrophils (30) with a recent study assigning the complete E-selectin ligand activity on neutrophils to PSGL-1, CD44 and ESL-1 (31).

Although PSGL-1 has a broad expression pattern, the functionality of the molecule is dependent on the proper post-translational modifications. Furthermore, each of the selectins has different requirements as to which post-translational modifications are optimal for binding. Binding of PSGL-1 to L-selectin and P-selectin requires the sLex determinant attached to a Core-2 O-linked glycan on Thr-57 (8,32). Tyrosine sulfation also contributes to PSGL-1 binding to both L-selectin and P-selectin with Tyr-51 playing the predominant role in L-selectin binding, while Tyr-48 is most important for P-selectin binding (32-34). Although E-selectin also recognizes sLex-related determinants within PSGL-1, there are alternative O-glycosylation sites to Thr-57 involved and no absolute requirement for tyrosine sulfation (33,35,36). Additionally, PSGL-1 when expressed on subsets of skin homing T cells in human, carries the cutaneous lymphocyte antigen (CLA), as defined by reactivity with HECA-452. This sLex-related determinant is correlated with binding to E-selectin but not necessarily to P-selectin (21,37,38). Therefore, the binding activity and selectin preferences of PSGL-1 depend on the nature of the modifications that decorate the molecule.

As yet there have been no molecularly-defined selectin ligands on human B cells, although B cells can express carbohydrate modifications which promote selectin binding. PMA-activated but not resting B cells bind to recombinant E-selectin and P-selectin under static conditions through induction of sLex-related determinants defined by the CSLEX and HECA-452 mAbs (39). Rott *et al.* observed that populations of circulating memory B cells react with HECA-452

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and exhibit E-selectin binding ability (40). Additional, as yet undefined, recognition determinants exist on human B cell lines, since some of these cells are able to bind to E-selectin despite expressing little or no sLex, as measured by HECA-452 and other antibodies. Binding of these cells was attributed to novel sialylated structures with a requirement for fucosyltransferase (FT) activity, specifically from FTVII and/or FTIV (41). Montoya *et al.* found that B cells with a memory phenotype rolled on E-selectin in a sialic acid-dependent manner (42). Armerding *et al.* (43) detected selectin ligand activity on tonsillar B-cells based on the observation that these cells rolled on both E-selectin and P-selectin substrata. Enzymatic treatments of the cells demonstrated distinct ligand activities; a sialidase and O-sialoglycopeptidase (OSGE) sensitive E-selectin ligand and a sulfation-dependent P-selectin ligand. Biochemical analyses of HECA-452 reactive proteins from B-cells have revealed a complex pattern of components (39,43). The molecular identities of these various ligand candidates are as yet unknown; however, it is clear that none corresponds to PSGL-1.

EG was originally characterized as a member of the CD34 family of transmembrane sialomucins in human (44) and subsequently found to be highly conserved in mouse (45). EG along with the other two members of the family (CD34 and podocalyxin) share a similar domain structure and genomic organization and appear to have arisen from a common precursor (46). In distinction from the other two family members, EG has an amino-terminal acidic region appended to its sialomucin domain (47). EG exists as a disulfide-linked dimer with a subunit molecular weight of 120-160 kD depending on the level of substitution with chondroitin sulfate (CS) glycosaminoglycan chains, a modification also unique to this member of the CD34 family (47,48). EG has a broad expression pattern, including vascular endothelium (the basis of its naming), hematopoietic precursors, and smooth muscle (47). In contrast to podocalyxin and CD34, EG has not been detected in the PNAd complex (48), although a recombinant form exhibits ligand activity for L-selectin (48,49). EG has a significant structural similarity to PSGL-1 including juxtaposed sites of tyrosine sulfation and an O-glycan with sLex-related determinants in its acidic region. In fact, EG interacts with L-selectin using a mechanism similar to that used by PSGL-1 (48).

In this report, we report the expression of EG on human B cells, T cells and monocytes. In view of this expression pattern and the existence of undefined selectin ligands on leukocytes (in particular on B-cells), we investigated the ability of native and recombinant EG to interact with the vascular selectins.

Materials and Methods

Immunohistochemistry of tonsil sections

Specimens of human tonsils were obtained after routine tonsillectomy from the Department of Pathology, University of California, San Francisco and the Department of Ambulatory Surgery, California Pacific Medical Center and frozen in O.C.T embedding medium (Sakura, Torrance CA). 10 μM sections were cut and fixed for 20 min in PBS containing 1% paraformaldehyde. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 20 min. Slides were blocked in 5% human serum plus 3% BSA in PBS (staining buffer) and incubated with MECA-79 and the anti-EG antibody PCLP-2 (47) at 10 μg/ml for 1 hour in staining buffer. Bound antibodies were detected using Cy-3 conjugated anti-rat IgM (Invitrogen, Carlsbad CA) or biotinylated anti-rabbit IgG (Jackson Immunoresearch, West Grove PA) at 1.5 μg/ml in staining buffer. EG staining was visualized using streptavidin-HRP (Invitrogen) at 1 μg/ml and NovaRED substrate (Vector laboratories, Burlingame CA). Normal rat IgM (Invitrogen) or rabbit IgG (Pharmingen, San Jose CA) were used as staining controls. All studies were approved by the UCSF Committee on Human Research.

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Purification of human B cells, T cells and monocytes

Peripheral blood was obtained by venipuncture and peripheral blood mononuclear cells (PBMC) were isolated using a ficoll-hypaque gradient (GE Healthcare, Piscataway NJ) and washed twice with PBS before resuspension in PBS, 0.5% BSA, 5 mM EDTA. Monocytes were purified using magnetic bead negative selection with the monocyte isolation kit II (Miltenyi Biotech, Auburn, CA). B cells were purified from PBMC using negative selection with the B cell isolation kit II (Miltenyi Biotech). T cells were purified from PBMC using negative selection with the Pan T cell isolation kit II (Miltenyi Biotech). To obtain cells from tonsil, specimens were teased apart over nitex mesh to release lymphocytes and washed twice with PBS. B and T cells were then purified as described above. In each case the purity of the preparation was >97% as shown by flow cytometry staining with anti-CD14, anti-CD19 and anti-CD3 (Invitrogen).

Flow cytometry

Leukocyte populations purified as described above were either stained fresh or cultured overnight in RPMI-1640, 10% FBS (Invitrogen), 2 mM glutamine, 100 μg/ml sodium pyruvate, 100 μg/ml penicillin, 100 μg/ml streptomycin. Monocyte lines (U937 and THP-1) were cultured in RPMI-1640, 10% FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin. B cell lines (Daudi, RPMI-8226 and U266) were cultured in RPMI-1640, 10% FBS, 2 mM glutamine, 100 μg/ml sodium pyruvate, 100 μg/ml penicillin, 100 μg/ml streptomycin. All cells were washed twice with PBS prior to staining. All staining steps were performed at 4°C, using 5×10^5 cells/sample. Cells were blocked with 10% human serum in PBS, 0.25% BSA, 0.02% Sodium azide (PBA) for 1 hr. Anti-EG antibodies; rabbit pAb (PCLP-2) (47) goat pAb (AF1524, R&D systems, Minneapolis MN) or mouse mAb (clone 211816, R&D systems) were incubated with the cells at 10 μg/ml in PBA for 1 hr. Cells were washed with PBA and positive staining was detected with 10 μg/ml FITC-conjugated anti-rabbit IgG (Invitrogen), anti-goat IgG (Invitrogen) or anti-mouse IgG (Invitrogen) in PBA. Cells were washed again in PBA and fixed in 2% formaldehyde in PBS for 20 min at room temperature. Normal rabbit IgG (Pharmingen), goat IgG (Invitrogen) or mouse IgG1 (Invitrogen) were used as controls. For dual staining of T cells, cells were labeled with anti-EG antibodies as described above but after incubation with secondary antibodies, cells were incubated with CD4-PE or CD8-PE (Invitrogen) for 30 min at 10 μg/ml in PBA, washed with PBA and fixed as described above. PE-conjugated mouse IgG2a (Invitrogen) was used as a control. For PMA activation, lymphocytes were purified and then cultured overnight with 100 ng/ml PMA (Sigma, St Louis MO). Cells were stained as described previously with anti-EG antibodies or anti-PSGL-1 antibodies PL-1 (Abcam, Cambridge MA), PL-2 (Abcam) or KPL-1 (Pharmingen). Control staining was performed with mouse IgG1 (Invitrogen). For 3-color staining of B cells, cells were purified from human tonsils as described above and stained with anti-EG pAb PCLP-2 as above. Positive staining was detected using an APC-conjugated anti-rabbit IgG (Jackson). Cells were washed and labeled with PE-conjugated anti-IgD (Pharmingen) and FITC-conjugated anti-CD38 (Pharmingen) at 10 μg/ml in PBA for 30 min prior to washing and fixation. Controls were rabbit IgG (Pharmingen), mouse IgG2a-PE (Invitrogen) or mouse IgG1-FITC (Invitrogen). All analyses were performed on a Becton Dickinson FACSort (Becton Dickinson, Franklin Lakes NJ) with Cytek 4 color upgrade (Cytek, Fremont CA).

Immunoprecipitation and immunoblotting

EG peptides A (CSSLDLGPTADYVFPDLTEK) and B (CSKPSEKEQHLLMTLVGEQG) were used to immunize a goat and the serum was recovered, hereafter referred to as anti-EG goat serum (peptide antibodies were produced by ProSci Inc. Poway CA). Lysates from whole human tonsils were prepared by homogenizing 10 g human tonsil in 50 ml 1% Triton X-100 in TBS, 5 mM EDTA, protease inhibitor cocktail (Roche, Indianapolis IN). Lysates were

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prepared from cell lines or purified primary lymphocytes as follows. Cells were washed with PBS and lysed at 5×10^7 cells/ml with 1% Triton X-100 in TBS, 5 mM EDTA, protease inhibitor cocktail (Roche). For immunoprecipitation, lysate derived from 1×10^8 cells or 1 ml of tonsil lysate was pre-cleared with protein A agarose (Repligen, Waltham MA) for rabbit Abs or protein-G Sepharose (Invitrogen) for goat Abs. Lysates were then incubated with 5 μg of PCLP-2 or normal rabbit IgG (Pharmingen) coupled to protein A agarose or 20 μl of anti-EG goat serum or pre-immune goat serum coupled to protein G Sepharose overnight at 4°C. The beads were washed and eluted by boiling with sample buffer under reducing conditions before fractionation by SDS PAGE (7.5%). The proteins were transferred to ProBlott PVDF membranes (Applied Biosystems, Foster City CA) which were blocked with 5% dried milk powder in TBS, 0.1% Tween 20 (TBS-T). Blots were reacted with primary antibodies: NTX (47), anti-EG pAb (R&D systems) or HECA-452 (Pharmingen) at 1 μg/ml in 5% milk in TBS-T for 2 hr at room temperature. Positive reactivity was detected using HRP-conjugated antibodies: anti-rabbit IgG (1/10000, Jackson), anti-goat IgG (1/3000, Invitrogen) or anti-rat IgM (1/10000, Jackson) in TBS-T for 1 hr at room temperature. Blots were then washed in TBS-T and developed using ECL-Plus (GE Healthcare). For digestion with chondroitinase ABC or heparitinase, the immunoprecipitated protein bound to protein G beads was washed and resuspended in PBS, 0.1% Triton X-100 then digested with 30 mU chondroitinase ABC (Seikagaku, Japan) or mock treated for 2 hr at 37°C. Alternatively the bound protein was digested with 10 mU each of heparitinase I, II and III (Seikagaku) for 2 hr at 37°C. The beads were washed, eluted and analyzed as described previously.

Precipitation with selectins

The anti-EG pAb NTX was coupled to CNBr-activated Sepharose 4B as described in (50). To immunopurify EG, lysate from 2×10^8 cells, prepared as above, or 20 ml of tonsil lysate was passed over the column, washed with 10 mM CHAPS in PBS and the bound protein was eluted with 0.1 M glycine in PBS with 10 mM CHAPS into 1/10th vol 1 M Tris/Cl pH 8.8. Eluted protein was concentrated and buffer exchanged into 10 mM CHAPS in PBS using a Centricon-30 concentrator (Millipore, Billerica MA). 50 μg of L-selectin-IgG, E-selectin-IgG or P-selectin-IgG (R&D systems) or human IgG (Invitrogen) was coupled to 10 μl protein A agarose and incubated with immunopurified EG for 4 hr at 4°C in the presence or absence of 10 mM EDTA. The beads were washed with 10 mM CHAPS in PBS and eluted with 10 mM EDTA in PBS with 10 mM CHAPS for 1 hr at room temperature before being subjected to immunoblotting analysis as described above. For sialidase digestion, the immunopurified EG in PBS with 10 mM CHAPS, was treated with 200 mU sialidase (*Arthrobacter ureafaciens*, Roche) or mock treated for 30 min at room temperature prior to precipitation by selectins.

Generation of EG-BJAB cells

An EG expression construct was created by excising the EG insert from EG-pCMV6-XL4 (Origene, Rockville MD) by Not I digestion and subcloning into the Not I site of pCEP4 (Invitrogen). BJAB cells stably expressing FTVII and β-1,6-GlcNAc transferase (Core2 GnT) (51) (termed parental cells) were electroporated using Gene Pulser Xcell at 200 V, 950 μF and selected in 400 μg/ml hygromycin B to produce EG-BJAB cells.

Laminar flow assays

The EG fusion proteins were produced and laminar flow assays carried out as previously described (48). Briefly, the amount of purified protein was determined by Bradford assay and coating density of the proteins was determined by ELISA as described in (48). AD-Ig proteins (AD-FT, AD-no FT,) were coated at equivalent densities onto 35 mm dishes overnight in TBS, pH 9.0. Plates were washed with PBS and blocked with 3% BSA in PBS for 2 hr at room temperature. 300-19 cells, stably transfected with L-selectin or E-selectin (52), grown in

RPMI-1640, 10% FCS, 100 μg/ml penicillin, 100 μg/ml streptomycin, 55 μM 2-mercaptoethanol or CHO cells expressing P-selectin (51), cultured in HAM'S F12, 10% FCS, 100 μg/ml penicillin, 100 μg/ml streptomycin, were washed once with RPMI-1640 with 25mM HEPES (300-19) or HAM'S F12 with 25mM HEPES (CHO) and resuspended at 10^6 cells/ml. For inhibition studies E-selectin and P-selectin expressing cells were incubated with 20 μg/ml BB1 (R&D systems), control mouse IgG (Invitrogen) or 10 mM EDTA for 20 min at room temperature prior to rolling. For sialidase treatments, coated substrates were incubated with 10 mU sialidase (*Vibrio cholerae*, GLYKO inc.) in 50 mM sodium acetate, 4 mM $CaCl_2$, 0.1% BSA, pH 5.5 for 1 hr or buffer alone as a control. Cells were perfused through the flow chamber and counts and velocities for rolling cells were determined over a range of shear stresses (1-8 dyne/cm^2). Cells were analyzed with 2 substrate preparations, 2 times each. Values shown are shown for one representative experiment of at least 3 independent experiments, each employing a separate protein preparation. Cell counts and velocities were measured using the NIH Image J program. To examine rolling of primary human lymphocytes, B and T cells were purified from human tonsil as described above and treated with KPL-1 (anti-PSGL-1, Pharmingen) or isotype control mouse IgG (Invitrogen) at 20 μg/ml for 20 min at room temperature prior to rolling. Recombinant P-selectin IgG (R&D systems) was coated at 20 μg/ml and the cells were perfused through the flow chamber and analyzed as described above. In experiments with BJAB cells, recombinant P-selectin (extracellular domain, R&D systems) was coated at 3 μg/ml in PBS and blocked as above. Parental BJAB and EG-BJAB were rolled over a range of shear stresses (0.5-6 dyne/cm^2) and analyzed as above. For PSGL-1 blocking experiments, cells were incubated with KPL-1 or control mouse IgG as above. Statistical significance was determined using the Student t-test.

Results

Expression of endoglycan on lymphocytes and monocytes

As EG is expressed on vascular endothelium, we wanted to determine whether it was detectable on HEVs in a lymphoid organ. We performed a histological analysis of human tonsil sections, using double staining with an EG antibody and MECA-79 as a marker of HEVs through its recognition of the PNAd complex (10). We found that EG was expressed on a subset of MECA-79$^+$ vessels in human tonsils. Approximately 25% of the HEVs were dual stained (Fig. 1A). In addition to HEV staining, we observed strong staining of EG on all germinal centers and of occasional cells in the stroma (Fig. 1B). Germinal center staining was also observed in mouse PLN and MLN, although HEV staining could not be detected in these mouse lymphoid organs (data not shown).

In order to investigate EG expression on leukocytes, we isolated B and T cells from peripheral blood and human tonsils and monocytes from peripheral blood, using magnetic bead selection. The cells were reacted an anti-EG antibody and analyzed by flow cytometry. As shown in Fig. 2A, EG was found at moderate levels on tonsillar B cells and T cells and at low levels on monocytes. Using purified T cells, we performed two-color analysis using antibodies against EG and CD4 or CD8. EG was expressed on both subsets of T cells at similar levels (Fig. 2B). EG was found at comparable levels on lymphocytes isolated from tonsils and peripheral blood (data not shown). We also screened a number of cell lines for EG staining. Three out of six B-cell lines (RPMI-8226, Daudi and U266) and two of three monocyte lines (U937, THP-1) (Fig. 2C) were positive. However, none of the T-cell lines screened (Jurkat, Hut-78, and Molt-4) showed expression. EG expression or the lack thereof was verified with at least 2 independent antibodies in each case mentioned above.

To determine whether EG on lymphocytes was affected by activation, we treated B and T cells with PMA overnight at 100 ng/ml. Flow cytometry demonstrated a 10-fold increase in EG on

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B cells, while the level on T cells remained constant (Fig. 3A). EG was also increased to a similar extent on B cells after activation with anti-CD40 and anti-IgM (data not shown).

Using three independent antibodies, we confirmed that PSGL-1 was weakly expressed on B-cells and was prominent on T-cells (15,17,43). In contrast to EG, PMA activation reduced PSGL-1 on B-cells to a barely detectable level (Fig. 3A). PSGL-1 on T cells also decreased but still remained at a relatively high level (Fig. 3A). Short-term exposure of B-cells to PMA did not increase EG, suggesting that transcriptional activation was required for the overnight PMA induction (data not shown). As is the case for PSGL-1 (53), EG was decreased on monocytes after short-term exposure to PMA, suggestive of shedding (data not shown).

To determine whether EG varied with B-cell differentiation in a more physiological context, we investigated EG on naïve, germinal center, and memory subsets of tonsillar B cells. These subsets were defined based on expression of IgD and CD38 (54). As shown in Fig. 3B, EG was present at the lowest level on naïve cells, with an increased amount on germinal center cells and the highest level on memory B cells. This elevated expression of EG on these subsets of differentiated B-cells is likely to be the basis for the germinal center staining (Fig. 1).

Posttranslational modifications of natively-expressed endoglycan

EG has the potential to carry several post-translational modifications including chondroitin sulfate chains, tyrosine sulfation and sLex-related modifications on O-glycans (47,48). To determine which of these posttranslational modifications was present on native EG, we analyzed EG that was immunoprecipitated from detergent lysates of cell lines, purified lymphocytes and whole tonsils. As shown in Fig. 4A, immunoblotting of tonsillar EG after SDS-PAGE revealed two bands of ≈120 and ≈160 kD. The broad nature of the bands was consistent with the highly glycosylated nature of the molecule (47). EG derived from B cells or T cells was detected only as the 120 kD species (Fig. 4A). To exclude the possibility that subsets of B or T cells carrying high molecular weight forms of EG were lost during purification, we performed the same analysis on an unfractionated lymphocyte-rich population from tonsils. Although there was a strong band at 120 kD, we still could not detect a band at 160 kD (Fig. 4B). A monocyte cell line, U937, showed both the 120 kD and 160 kD forms of EG (see Figure 5 below). Treatment of tonsillar EG with chondroitinase ABC, which degrades chondroitin sulfate chains, markedly decreased the intensity of the 160 kD band with concomitant increase in the level of the 120 kD species (Fig. 4C). Treatment with heparitinase did not alter the pattern of the bands. These results indicate that a subset of tonsil-derived EG is modified with chondroitin sulfate chains but not heparan sulfate chains. However, the lack of a detectable 160 kD band from lymphocytes suggests that the chondroitin sulfate modification is not normally present on EG in B and T cells. The 160Kd form of EG has previously been detected in human umbilical vein endothelial cells (HUVEC) (47). Preliminary data from $CD31^{+}$ cells isolated from human tonsil also showed expression of high molecular weight EG (data not shown), suggesting that endothelial cells were one source of the 160Kd form of EG.

sLex and related modifications can be detected using the monoclonal antibody HECA-452 (55,56). HECA-452 reactivity has been employed as a reporter of potential selectin ligand activity in many studies (55-59). We previously showed that recombinant EG generated in FTVII-transfected COS cells carries the HECA-452 epitope (48). To determine whether native EG also expressed this determinant, we affinity purified EG from detergent lysates of U937 cells, RPMI-8226 cells (a B-cell line) and whole tonsils, performed SDS-PAGE and immunoblotted with HECA-452. We detected HECA-452 reactivity in the 120 kD component from each of these sources (Fig. 5). For tonsils, a strong signal was present in a high molecular weight species.

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Endoglycan as a ligand for E-selectin and P-selectin

EG has been characterized as a ligand for L-selectin (48,49). Our finding that EG was expressed on human B-cells and the lack of molecularly defined selectin ligands on these cells, prompted us to investigate whether EG had ligand activity for E-selectin and P-selectin. To address this issue, we immunopurified EG from several native sources (U937, RPMI-8226 and tonsils) and tested for interactions with selectin chimeras coupled to protein-A Sepharose. The chimeras were treated with EDTA to release the EG that was bound via the C-type lectin domains. As shown in Fig. 6A, EG from U937 cells was precipitated by all three selectins. The 120 and 160 kD forms were both detected. As the binding of EG to P-selectin was the strongest, we concentrated our further biochemical studies on this selectin. EG from the B cell line RPMI-8226 also interacted strongly with P-selectin (Fig. 6B). As shown in Fig. 6C, treatment of U937-derived EG with sialidase completely abrogated P-selectin binding, establishing a sialic acid requirement for its ligand activity. EG derived from whole tonsil lysate was also able to interact with P-selectin, with both the 120 and 160 kD forms exhibiting ligand activity (Fig. 6D).

Selectin-mediated rolling on endoglycan

Previously, we showed that EG can mediate rolling of L-selectin expressing cells under shear stress in a parallel plate flow chamber (48). We wanted to determine whether the interaction of P-selectin and E-selectin with EG could also support rolling of cells. Our earlier work established that the acidic domain of EG bearing appropriate posttranslational modifications is sufficient for L-selectin ligand activity (48). Adopting our previous approach, we produced a recombinant form of EG, termed AD-Ig, consisting of the acidic domain of EG (residues 1-161) fused to the Fc region of human IgG (48). COS-7 cells were transfected with this construct with or without a cDNA encoding FTVII to provide the α1,3 fucosylation modification. Purified AD-Ig was coated onto dishes, which were then incorporated as the bottom plates of a parallel plate flow chamber. Cells were then allowed to interact with the substrata over a range of sheer stresses between 1-8 dyne/cm^2. For the selectin-bearing cells, we used transfected 300-19 cells for L-selectin and E-selectin, and transfected CHO cells for P-selectin. As shown in Fig. 7A, FTVII modified AD-Ig (AD-FT) supported tethering and rolling interactions for all 3 selectins. The result for 300/19 L-selectin cells confirmed earlier findings obtained with Jurkat T-cells (48). AD-FT supported slow rolling of E-selectin and P-selectin transfected cells with an average velocity of ≈10 um/s at 1 dyne/cm^2 for both, as compared to 67 um/s for L-selectin transfected cells (Fig. 7B).

We investigated requirements that were established for the L-selectin interaction (48). Ligand activity was dependent on sialylation, since treatment of AD-FT with sialidase completely abrogated E-selectin dependent rolling and reduced P-selectin rolling by 90%. Fucosylation was also an absolute requirement in that AD-Ig generated in the absence of FTVII (AD-no FT) exhibited no ligand activity for E-selectin or P-selectin. Specificity of the interactions was confirmed through the use of blocking antibodies against the selectins and treatment with 10 mM EDTA. All of these treatments reduced rolling by 90-100% (Fig. 7A).

Rolling of an endoglycan expressing B-cell line on a P-selectin substratum

To investigate the contribution of EG to B-cell rolling on P-selectin, we first verified that tonsillar B-cells could roll on a substratum of P-selectin at physiologic shear stresses (43). The frequency of rolling cells was less than for T-cells isolated from the same tonsils (Fig. 8A). In the presence of a KPL-1, a function-blocking antibody to PSGL-1 (51), the rolling interactions of T-cells were greatly blunted but not eliminated, whereas B-cells rolling was marginally inhibited only at the highest shear stresses. These results support the existence of a major component of PSGL-1 independent ligands on B-cells and a relatively minor component on T-cells. The lack of function-blocking antibodies to EG precluded direct testing of its ligand

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activity on these primary cells. As an alternative approach, we engineered a B-cell line to express full-length EG with the proper posttranslational modifications and tested the rolling interactions of these cells on immobilized P-selectin. We used BJAB cells (a human Burkitt's lymphoma-derived cell line) which stably express Core-2 GnT and FTVII (parental BJAB) and correspondingly react with HECA-452 (51). These cells were previously shown to roll on P-selectin when a suitable ligand (i.e. PSGL-1) was introduced (51). The parental BJAB cells showed negligible PSGL-1 and a low but detectable level of EG (Fig. 8B). We transfected EG into the parental cells and produced a stable line (termed EG BJAB) expressing EG at levels similar to those found on activated B-cells (Fig. 8B, Fig. 3). There was no change in PSGL-1 or HECA-452 staining (data not shown) between the parental and EG BJAB cells.

Recombinant P-selectin was coated onto the bottom plate of the flow chamber and the BJAB cells were perfused through the chamber over a range of shear stresses (0.5-6 dyn/cm^2). Both parental BJAB and EG BJAB cells rolled on P-selectin (Fig. 8C). However, EG BJAB cells exhibited a markedly higher frequency of rolling interactions and slower rolling velocities than the parental cells (Figs. 8C and 8D). There was no change in the number of rolling cells or the velocity of EG BJAB cells after incubation with KPL-1, demonstrating that PSGL-1 did not contribute to the interaction of these cells with P-selectin (data not shown). Specificity of the interaction of EG BJAB cells with P-selectin was shown by the complete abrogation of rolling after treatment with 10 mM EDTA. Endogenous EG could also be the basis for the rolling of the parental cells, as KPL-1 treatment did not significantly alter the rolling behavior of these cells.

Discussion

In the present study, we present evidence that EG is expressed on several populations of mature mononuclear leukocytes and that it is a novel ligand for E-selectin and P-selectin. EG was previously characterized as the third member of the CD34 family of sialomucins (47). Podocalyxin and EG, like CD34, are expressed on early stages of hematopoietic lineages but have been less studied in this capacity than CD34 (46). All three members have potential signaling functions, yet little is known about how these molecules transduce signals and the functional consequences of signaling. Most of the interest in the function of the CD34 family has focused on their extracellular regions. The negatively charged, highly extended sialomucin domains common to these proteins are consistent with possible anti-adhesive functions, and such activities are clearly indicated for CD34 and podocalyxin in several cellular contexts (45,60-63). However, CD34 and podocalyxin, when appropriately modified with sulfated glycans, function pro-adhesively as HEV-expressed ligands for L-selectin (10,44,64,65). EG was also shown to be a ligand for L-selectin (48) but employs a different strategy, which closely resembles that used by PSGL-1 (48,49). Further evidence for the distinctive features of EG came when we performed immunohistochemistry on human tonsils and found that only 25% of the HEVs were positive for EG. In contrast, CD34 is found on almost all HEVs and podocalyxin occurs on the majority of these vessels (44).

The strong EG staining on germinal center cells (Fig. 1) prompted us to investigate the expression of this protein on human leukocytes. EG was detected on most peripheral blood lymphocytes and monocytes, although the expression levels varied greatly. This distribution is in marked contrast to that of CD34 and podocalyxin, which are absent on all classes of circulating mature leukocytes (46). As is the case for PSGL-1, the complex nature of the post-translational modifications required for endoglycan ligand function (48), needs to be taken into account in evaluating expression data. Thus, although endoglycan was detected at a moderate level on T cells (Fig. 2), it is likely that the endoglycan on naïve T cells lacks selectin ligand activity due to the absence of HECA-452 reactivity on this population (66). T cell populations exhibiting HECA-452 staining are comprised of memory T cells and skin-homing cells (58,

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66). Cultured HECA-452^{+} T cells have been shown to carry most, if not all, of their HECA-452 reactivity on two protein scaffolds: PSGL-1 and CD43 (27,37). Therefore, it is doubtful that endoglycan has a primary role as a selectin ligand on T cells. A similar argument applies to monocytes where PSGL-1 is the main carrier of HECA-452 reactivity (67).

The possibility that EG serves selectin ligand functions on B-cells is intriguing. Activated B-cells can exit lymphoid organs and migrate through the blood to bone marrow and epithelial surfaces, but there is very limited information about selectin ligands on B-cells at different stages of activation (68,69). We found that B-cells and 3 out of 6 B-cell lines expressed considerable levels of EG (Fig. 2) with BJAB cells expressing a low but measurable level (Fig. 8B). Strikingly, the expression of EG on B cells increased upon PMA activation (Fig. 3A), which was mirrored by an increase of HECA-452 staining (not shown). In contrast, PMA activation decreased PSGL-1 expression to a negligible level (Fig. 3A). Consistent with the PMA findings, EG expression was higher on memory and germinal center cells than on naïve B-cells (Fig. 3B), likely explaining the germinal center staining. As reviewed in the **Introduction**, a number of studies observed selectin ligand activity and/or HECA-452 reactivity on memory/activated B-cells. Studies from Postigo *et al.* identified a HECA-452 reactive band at approximately 160 kD from PMA-activated B cells (39), while a band of similar size was precipitated by E-selectin from lysates of human tonsillar B cells (42). The molecular weights of these molecules are consistent with those for tonsillar EG (Fig. 4). Both of the earlier studies also identified a ligand of 240 kD, which could be an incompletely reduced homodimer of the 120 kD subunit of EG (39,42).

To directly explore the potential selectin ligand functions of EG, we embarked on a biochemical analysis, taking advantage of EG-specific antibodies. We established that the HECA-452 epitope was present on EG when immunoprecipitated from several sources: tonsils, unfractionated tonsillar lymphocytes, U937 cells, and RPMI cells (a B cell line). Consistent with the expression of this sLex-like determinant, we found that EG from tonsils, U937 cells, and RPMI cells was precipitated by selectin chimeras in a divalentcation dependent manner. For U937 EG, we established this interaction for all three selectins. For the other two sources of EG, we focused on the P-selectin interaction. Importantly, the interaction of EG with P-selectin required sialylation. It should be noted that our previous work found that recombinant EG can interact with L-selectin (48). However, the present findings are the first to demonstrate an interaction between L-selectin and a native form of EG. These findings suggest a possible role for EG in L-selectin dependent secondary tethering, heretofore a function demonstrated only for PSGL-1 (70).

To evaluate EG interactions with vascular selectins under more physiological conditions, we carried out parallel plate flow experiments in two different formats. First, we found that a recombinant form of EG supported tethering and rolling of E and P-selectin-bearing cells under physiologic shear stresses. Secondly, when full-length EG was transfected into a B-cell line (BJAB), rolling interactions on a P-selectin substratum were greatly enhanced. We therefore conclude that EG can support tethering and rolling of leukocytes via the vascular selectins. Rolling interactions are known to depend on specialized dynamic bonds between selectins and their ligands. PSGL-1 and EG were previously shown to share a catch and slip binding mechanism in their interactions with L-selectin (49), and it is likely that bonds between EG and the vascular selectins will have similar properties. In our previous study, we found that juxtaposed tyrosine sulfates and an sLex-bearing O-glycan in the acidic domain of EG were needed for optimal L-selectin binding to EG in rolling assays (48). We predict similar requirements for the interaction of EG with P-selectin.

Multiple ligands on leukocytes or vascular endothelium are known for E- and L-selectin, yet only PSGL-1 has been established as a major ligand for P-selectin (3,14). An important finding

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of the present study is that appropriately modified EG can serve as a P-selectin ligand. For the reasons detailed, differentiated B-cells are predicted to be one leukocyte population in which the selectin ligand function of EG is exerted. In vivo validation of EG as a physiologically relevant ligand in these cells and others awaits further tools such as function-blocking antibodies and gene-targeted mice.

PSGL-1 has recently been shown to facilitate homing of naïve T-cells to lymph nodes and Peyer's patches independently of selectin binding (71). This occurs through the ability of PSGL-1 to bind homing chemokines (CCL19 and CCL21) and to augment the responses of T-cells to these chemokines (71). Tyrosine sulfates are implicated in these chemokine interactions (72). A "pass-on" mechanism has been proposed in which PSGL-1 first traps the chemokine and transfers it to the chemokine receptor (e.g., CCR7) for signal transduction (71). It is reasonable to consider an analogous function for EG. In addition to possessing tyrosine sulfates, some forms of EG are decorated with chondroitin sulfate chains. A number of studies have demonstrated cytokine and chemokine interactions with chondroitin sulfate chains (73,74). Preliminary observations indicate that recombinant endoglycan is capable of binding to the homing chemokines CCL21, CXCL12 and CXCL13 (S Kerr, unpublished). The interactions of EG with chemokines is a subject for future investigation.

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Abbreviations used in this paper

EG, Endoglycan
PSGL-1, P-selectin glycoprotein ligand-1
HEV, high endothelial venule
sLex, sialyl lewis(x)
PNAd, peripheral node addressin
GlcNAc, N-acetyl glucosamine
ESL-1, E-selectin ligand-1
CLA, cutaneous lymphocyte antigen
FTIV, fucosyltransferase IV
FTVII, fucosyltransferase VII
OSGE, O-sialyl glycoprotein endopeptidase
CS, chondroitin sulfate
pAb, polyclonal antibody
Core-2 GnT, β-1-6-N-acetyl glucosaminyltransferase
AD-Ig, Acidic domain-Fc fusion protein of endoglycan
PLN, peripheral lymph node
MLN, mesenteric lymph node

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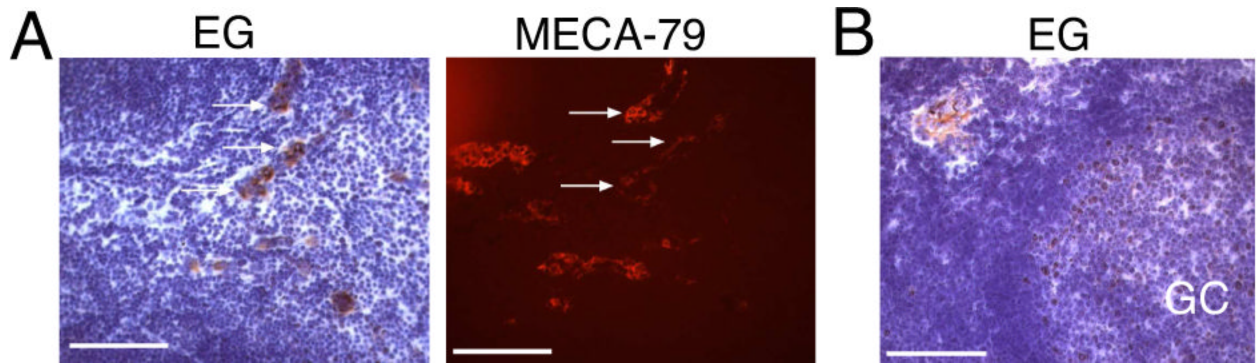

Figure 1. Endoglycan expression in human tonsil

A shows a tonsil section simultaneously stained with the anti-endoglycan rabbit pAb PCLP-2 and MECA-79. The secondary antibodies were respectively an anti-rabbit HRP in conjunction with nova red substrate (brown) and anti-rat IgM Cy3 (red). No reactivity was detected using isotype matched control antibodies. The arrows denote MECA-79^{+} vessels which are also positive for EG. **B** shows a secondary follicle containing a germinal center (GC). Staining for EG (brown) was performed as above. The stained structure in the upper left of the field is an HEV. The scale bars denote 100 μM. These results are representative of 4 experiments.

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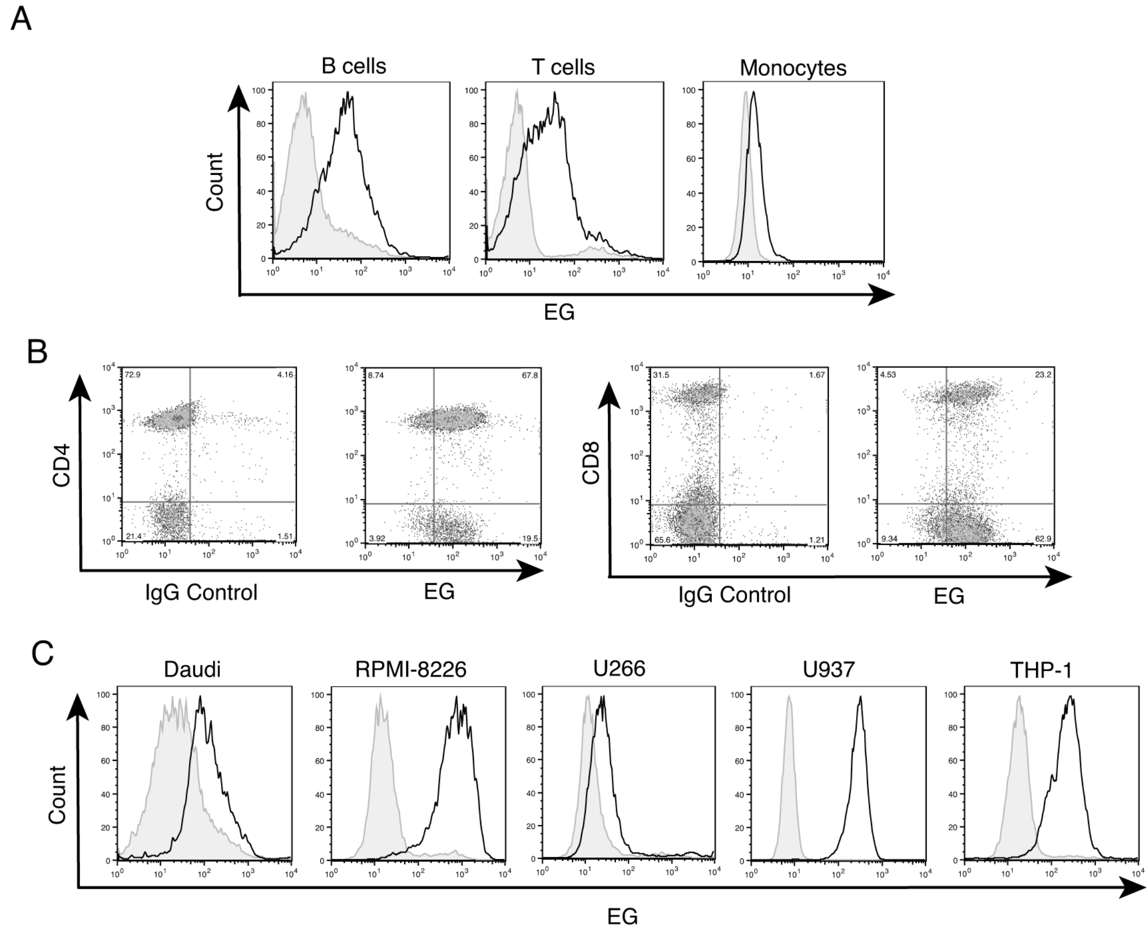

Figure 2. Endoglycan expression on B cells, T cells and monocytes

B cells and T cells were purified from tonsils and monocytes were isolated from peripheral blood. **A** shows cells stained with anti-EG Ab (PCLP-2, black line) or rabbit IgG (grey filled profile) and anti-rabbit FITC. **B** shows purified T cells dual-stained with anti-EG or isotype control, detected with anti-rabbit FITC and either CD4-PE or CD8-PE. **C** shows hematopoietic cell lines stained with anti-EG Ab (black line) or isotype control (grey filled profile), detected with anti-rabbit FITC. B cell lines are Daudi (Burkitts lymphoma), RPMI-8226 (multiple myeloma) and U266 (myeloma). Monocyte cell lines are U937 (histiocytic lymphoma) and THP-1 (acute monocytic leukemia).

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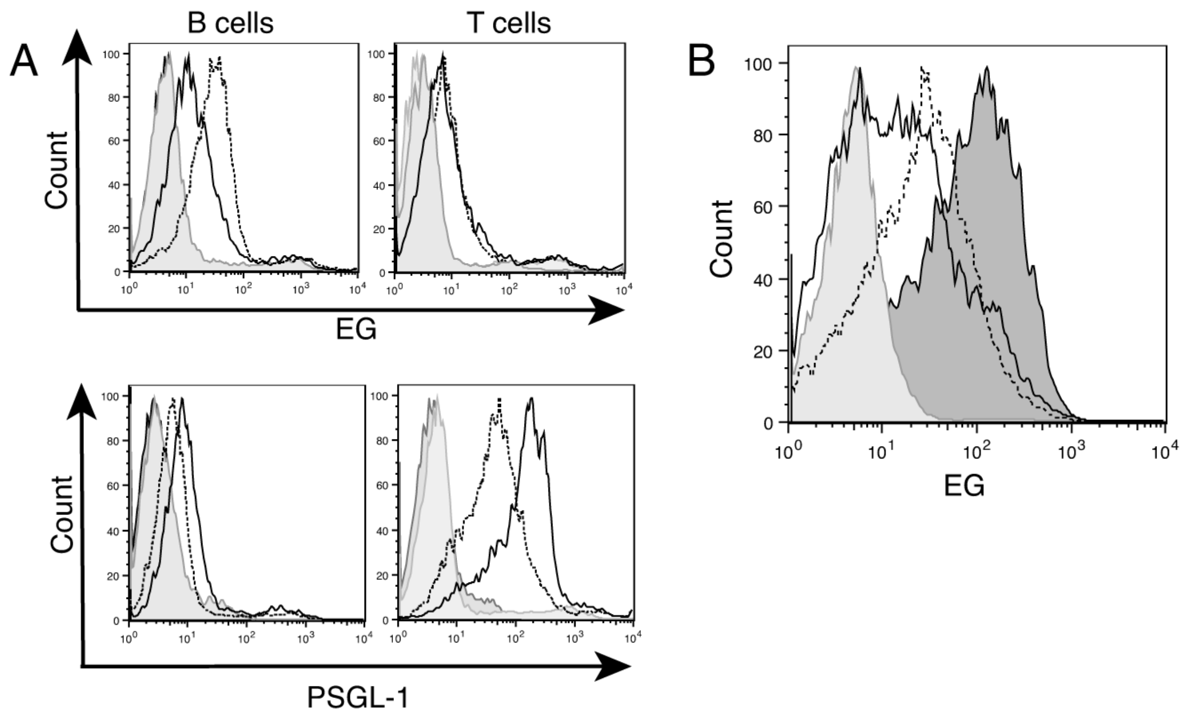

Figure 3. Effects of PMA activation on endoglycan expression by lymphocytes

A, B and T cells derived from tonsil were cultured overnight in the presence or absence of 100 ng/ml PMA. Cells were stained using anti-EG Ab (PCLP-2) or anti-PSGL-1 mAb (KPL-1) or isotype controls (grey filled profiles). Antibody binding was detected with anti-rabbit FITC or anti-mouse FITC. The dashed lines show staining of PMA-treated cells and solid lines show untreated cells. Cell activation by PMA was confirmed by staining with anti-CD69 and CD86. **B**, Tonsillar B cells were purified and stained with anti IgD-PE, anti CD38-FITC (or the appropriate controls, data not shown) and anti EG (PCLP-2) or rabbit IgG detected with anti-rabbit APC. Cells were gated as naïve (IgD^{+}, $CD38^{-}$), germinal center (IgD^{-}, $CD38^{+}$) or memory (IgD, $CD38^{-}$). The profiles show staining for EG on naïve (black solid line), germinal center (black dotted line) and memory (dark grey filled profile) populations. The light grey shaded profile shows isotype control staining of memory B cells, which was representative of staining with an isotype control on naïve and GC populations.

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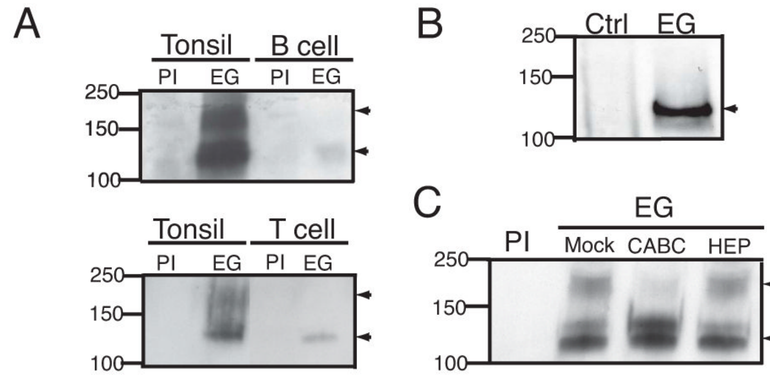

Figure 4. Expression of glycoforms of endoglycan

A, Detergent lysates from whole tonsils and tonsillar-derived B and T cells were immunoprecipitated with pre-immune (PI) or anti-endoglycan (EG) goat serum, separated by SDS-PAGE and immunoblotted with anti-EG (NTX). **B**, A detergent lysate of lymphocytes (obtained by mechanical disruption of a tonsil) was immunoprecipitated with rabbit IgG (control) or anti EG (PCLP-2), separated by SDS-PAGE and immunoblotted with anti EG (PCLP-2). **C**, A detergent lysate of tonsils was immunoprecipitated with pre-immune (PI) or anti-endoglycan (EG) goat serum, mock treated (mock) or treated with chondroitinase ABC (CABC) or a mixture of heparitinases I, II and III (HEP) then separated by SDS-PAGE and immunoblotted with anti EG (NTX). The bands did not react with isotype control rabbit IgG (data not shown). The arrowheads indicate the 120 kD and 160 kD components.

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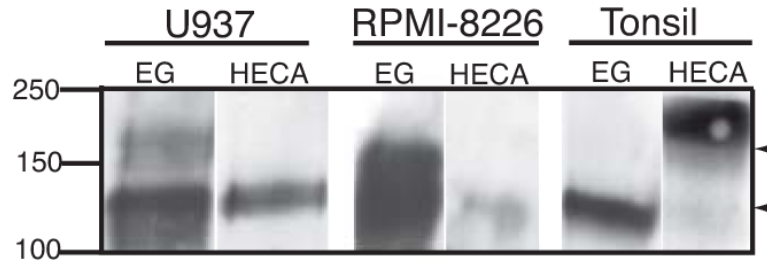

Figure 5. sLex-related determinants on native endoglycan

Anti EG (NTX) was coupled to CNBr activated Sepharose and used to purify EG from U937, RPMI-8226 and human tonsils. Purified EG was immunoblotted with either NTX or HECA-452 after SDS-PAGE fractionation. No reactivity was detected with rabbit IgG or rat IgM class matched isotype controls (data not shown). The arrowheads indicate the 120 kD and 160 kD components. EG from RPMI-8226 cells ran as a broad smear extending from 120-150 KD, of which only the 120 kD component reacted with HECA-452. The 160 kD band was variably isolated on the NTX affinity column, explaining its absence from tonsillar EG in the experiment shown. When EG was detected in tonsils by combined immunoprecipitation and immunoblotting (as in Fig 4), the 120 and 160 kD components were always present.

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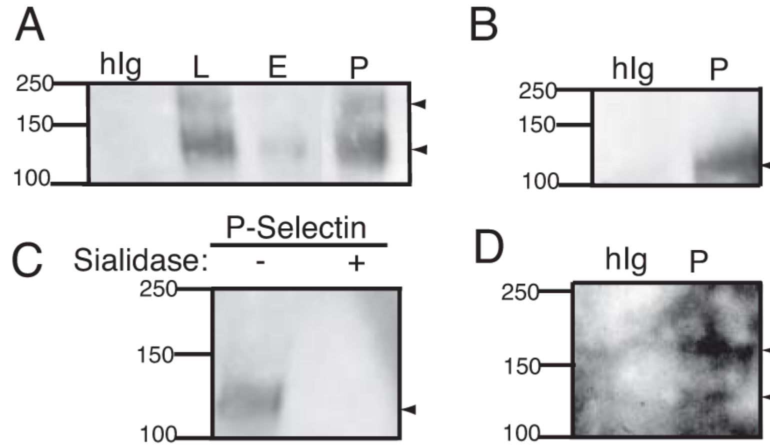

Figure 6. Interaction of native endoglycan with selectin chimeras

A, Endoglycan immunopurified from U937 as in Figure 5 was reacted with L-selectin-IgG, P-selectin IgG, E-selectin IgG, or human IgG (control) coupled to protein A beads. Bound protein was released with EDTA and analyzed by SDS-PAGE and immunoblotting. Endoglycan was detected using an anti-EG Ab (pAb, R&D systems). B, EG immunopurified from RPMI-8226 cells was reacted with P-selectin-IgG or hIgG and analyzed as above. **C**, EG immunopurified from U937 was mock treated or digested with 200 mU of sialidase (Arthrobacter) and then reacted with P-selectin-IgG as described above. **D**, EG immunopurified from lysates of whole human tonsils was reacted with P-selectin-IgG or hIgG and analyzed as above. The arrowheads indicate the 120 kD and 160 kD components.

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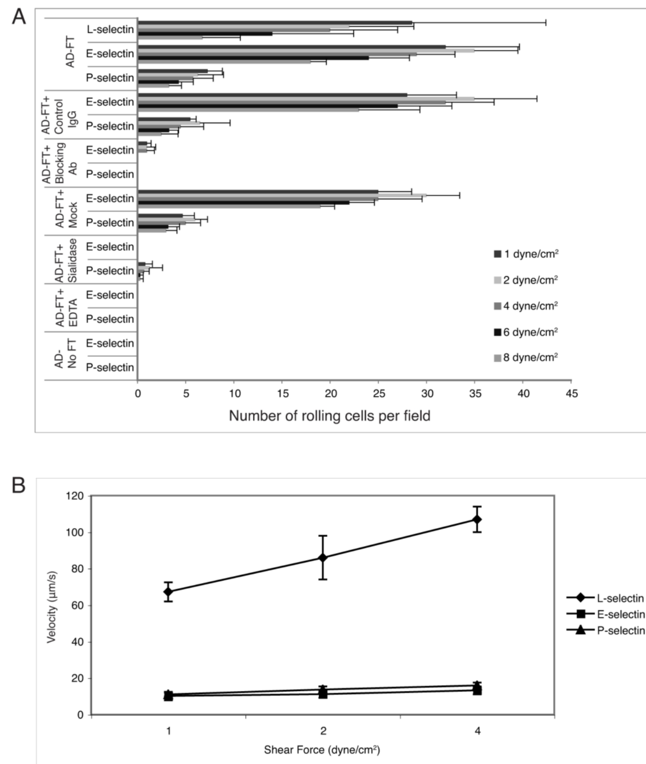

Figure 7. Rolling interactions mediated by selectin-endoglycan interactions

Recombinant endoglycan consisting of the acidic domain of EG fused to the Fc portion of human IgG (AD-Ig) was produced by COS-7 cells. AD-Ig was coated on plastic dishes, which formed the bottom chamber of a parallel plate flow chamber. 300-19 B cells expressing L-selectin or E-selectin and CHO cells expressing P-selectin were allowed to interact with the substratum with increasing increments of shear force and analyzed by video microscopy. Rolling on AD-Ig was analyzed with (AD-FT) or without α1,3 fucosylation (AD-Ig-no FT). For inhibition experiments, E-selectin 300-19 cells or P-selectin CHO cells were incubated with the E-selectin/P-selectin blocking mAb (BBA-16) or isotype control mouse IgG prior to use in the rolling chamber. For sialidase treatment, AD-FT was treated with Vibreo cholerae sialidase or buffer alone. Dependency on divalent cations was tested by adding 10 mM EDTA to the flow medium. Analyzed parameters were number of rolling cells per field (**A**) and velocity of rolling cells (**B**). Shown is one representative experiment of 5 experiments with mean values and SD's determined from at least 3 independent plates per condition.

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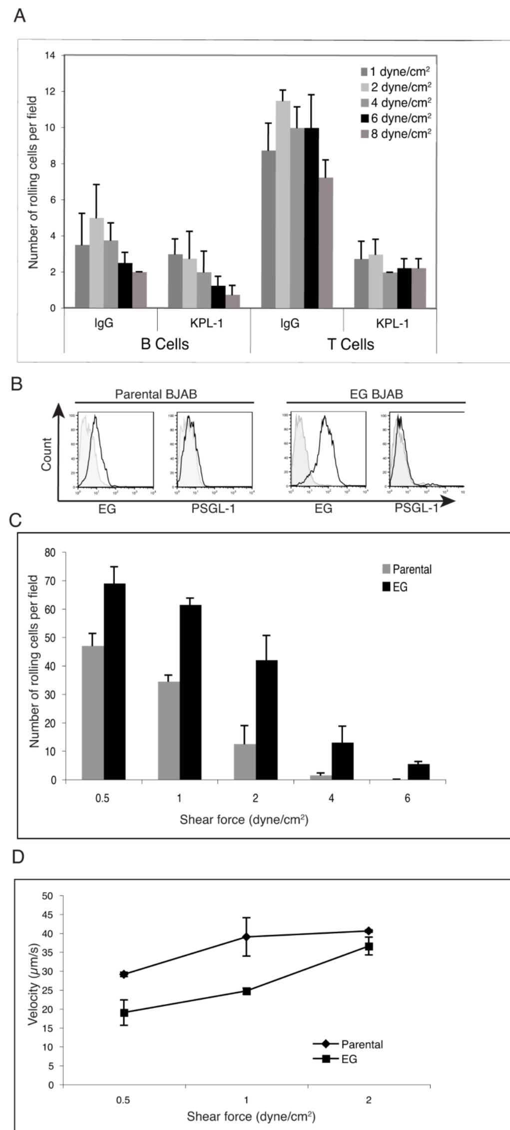

Figure 8. Rolling of lymphocytes on a P-selectin substratum

A, Recombinant P-selectin (IgG chimera) was coated onto plates that formed the bottom of a parallel plate flow chamber. Tonsillar B and T cells were allowed to interact with the substratum under varying shear in the presence of either KPL-1 or isotype control mouse IgG and analyzed. Means and SD's are shown for each shear stress (2 measurements per condition). No rolling interactions were observed when 10 mM EDTA was included or when human IgG was substituted for the P-selectin chimera. For T cells, KPL-1 produced a statistically significant decrease of rolling at all shear stresses ($p < 0.0005$ in all cases by the Student t-test). For B-cells, KPL-1 reduced rolling at only 6 and 8 dynes/cm^2, $p < 0.02$). **B**, EG was stably transfected into BJAB cells. Parental and EG BJAB cells were stained with anti-EG (R&D) or KPL-1 (black lines) or the appropriate controls (grey filled profiles) and analyzed by flow cytometry. **C**, **D**, Recombinant P-selectin (extracellular domain) was coated onto plates that formed the bottom of a parallel plate flow chamber. Parental and EG BJAB were perfused over the substratum and analyzed as above. The measured parameters were the number of rolling cells (**C**) ßand velocity of rolling cells (**D**). Means ± SD's based on two measurements are shown. No rolling events were observed in the presence of 10 mM EDTA. More EB-BJAB cells rolled

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than parental cells with the differences having p values of 0.05, 0.01, 0.06, 0.1, and 0.001 at 0.5, 1, 2, 4, and 6 dynes/cm^2, respectively. The rolling velocities of EG-BJAB cells were slower than parental cells with p values of 0.05, 0.06, and 0.1 for 0.5, 1, and 2 dynes/cm^2, respectively.