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Cell–specific Variation in E-selectin Ligand Expression Among Human Peripheral Blood Mononuclear Cells: Implications for Immunosurveillance and Pathobiology

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Abstract

Both host defense and immunopathology are shaped by the ordered recruitment of circulating leukocytes to affected sites, a process initiated by binding of blood-borne cells to E-selectin displayed at target endothelial beds. Accordingly, knowledge of the expression and function of leukocyte E-selectin ligands is key to understanding the tempo and specificity of immunoreactivity. Here, we performed E-selectin adherence assays under hemodynamic flow conditions coupled with flow cytometry and western blot analysis to elucidate the function and structural biology of glycoprotein E-selectin ligands expressed on human peripheral blood mononuclear cells (PBMCs). Circulating monocytes uniformly express high levels of the canonical E-selectin binding determinant sLe^X and display markedly greater adhesive interactions with E-selectin than do circulating lymphocytes, which exhibit variable E-selectin binding among $CD4^+$ and $CD8^+$ T-cells but no binding by B-cells. Monocytes prominently present sLe^X decorations on an array of protein scaffolds including PSGL-1, CD43, and CD44 (rendering the E-selectin ligands CLA, CD43E, and HCELL, respectively), and B-cells altogether lack E-selectin ligands. Quantitative PCR gene expression studies of glycosyltransferases that regulate display of sLe^X reveal high transcript levels among circulating monocytes and low levels among circulating B-cells, and, commensurately, cell surface α(1,3)-fucosylation reveals that acceptor sialyllactosaminyl glycans convertible into sLe^X are abundantly expressed on human monocytes

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Disclosures

According to the National Institutes of Health policies and procedures, the Brigham & Women's Hospital has assigned intellectual property rights regarding HCELL to the inventor (R.S.), who may benefit financially if the technology is licensed. R.S.'s ownership interests were reviewed and are managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policy. All other authors declare no competing financial interests.

yet are relatively deficient on B-cells. Collectively, these findings unveil distinct cell-specific patterns of E-selectin ligand expression among human PBMCs, indicating that circulating monocytes are specialized to engage E-selectin and providing key insights into the molecular effectors mediating recruitment of these cells at inflammatory sites.

Introduction

The ability of patrolling blood-borne effector cells to converge efficiently at discrete anatomic sites drives both immunosurveillance and the immunobiology of inflammatory reactions (1). Under physiologic blood flow conditions, circulating leukocytes extravasate at inflammatory sites via a multistep process that is initiated by cellular tethering and rolling contacts on affected endothelium (2). These “Step 1” initial adhesive interactions are principally dictated by engagement of vascular selectins, E- and P-selectin, which are Ca^{2+}-dependent lectins that bind glycosylated co-receptors (ligands) expressed on blood-borne cells, resulting in shear-resistant adherence of the circulating cells to target endothelial beds (3, 4). Importantly, the expression of E- and P-selectin critically varies among mammals: in rodents, the inflammatory cytokines IL-1β and TNF-α each upregulate transcription of mRNA encoding P-selectin and E-selectin, however, in primates, the P-selectin promoter lacks the pertinent response elements for these cytokines and only E-selectin is inducibly expressed (5). Thus, in human immunobiology, endothelial expression of E-selectin dominates in mediating recruitment of leukocytes at inflammatory sites, and, accordingly, those cells possessing the most potent E-selectin ligands serve as primary sentinels of host defense and principal effectors of both immediate and sustained inflammatory processes.

E-selectin binds to sialofucosylated glycan determinants decorating specific glycoproteins and glycolipids. These glycans contain an α(2,3)-linked sialic acid substitution on galactose and an α(1,3)-linked fucose modification on *N*-acetylglucosamine, prototypically displayed as the terminal lactosaminyl tetrasacharide known as sialyl Lewis X (sLe^X; CD15s) (3, 6–8). Although both glycoproteins and glycolipids can display sLe^X, glycoproteins, by virtue of their extended configuration beyond the cell membrane, mediate more efficient primary contacts between flowing cells and the endothelium and thereby play a predominant role in initial binding to endothelial E-selectin under hemodynamic shear stress (9). On human cells, three principal E-selectin ligands have been identified: Cutaneous Lymphocyte Antigen (CLA), Hematopoietic Cell E-/L-selectin ligand (HCELL), and CD43E, which are heavily sLe^X-decorated glycoforms of P-selectin Glycoprotein Ligand-1 (PSGL-1; CD162), CD44, and CD43, respectively (10).

Among human leukocytes, neutrophils and monocytes are the earliest cells to colonize inflammatory sites (11, 12), with later entry of T-cells ($CD4^+$ prior to $CD8^+$ cells) (13) and, conspicuously, a relative paucity of B-cells in exudates (14, 15). While there is extensive information on the robust expression of E-selectin ligands on neutrophils (16), our understanding of the expression of these structures on circulating peripheral blood mononuclear cells (PBMCs) is incomplete. As effectors of both innate and adaptive immunity, circulating monocytes and other PBMCs serve a fundamental role in the genesis of inflammation, and their recruitment into target tissues impacts the nature and kinetics of

host defense and immunopathology. Thus, a greater understanding of E-selectin ligands expressed on native human monocytes, T-cells and B-cells is fundamental to elucidating the immunobiology of inflammation.

In this study, using complementary functional and biochemical approaches, we investigated the E-selectin ligands expressed on native human PBMCs. Adherence assays under hemodynamic shear conditions reveal that there is an extremely diverse spectrum of E-selectin ligand activity among these cells, with human monocytes uniformly binding E-selectin robustly, but variable binding among T-cells and, strikingly, absence of E-selectin binding by B-cells. Consistent with these findings, we found that whereas monocytes display a plethora of E-selectin ligands, B-cells conspicuously lack these structures. Flow cytometry analysis and gene expression studies indicate that binding to E-selectin is principally dictated by the discrete expression of glycosyltransferases that direct sLe^X decorations of the glycoconjugate core protein(s). Our studies thus reveal remarkable variety in the structural biology of E-selectin ligands expressed on human PBMCs, broadening our understanding of the molecular effectors that confer immunosurveillance capacity and providing key mechanistic insights into the heightened capacity of monocytes to infiltrate inflammatory sites.

Materials and Methods

Isolation of blood cell subsets and cell culture

Human PBMCs were isolated by Ficoll-Paque (Sigma-Aldrich) density gradient centrifugation of peripheral blood from healthy donors obtained under protocols approved by the Institutional Review Board of Brigham & Women's Hospital (BWH), with informed consent provided as per the Declaration of Helsinki. Monocytes were isolated by positive selection using anti-CD14 coated magnetic beads (Miltenyi) (17). $CD4^+$ and $CD8^+$ T-cells and B-cells were isolated by negative selection using RosetteSep Human $CD4^+$, $CD8^+$ and B-cell enrichment cocktail, according to the manufacturer's instructions (StemCell Technologies). Cell suspensions were evaluated by flow cytometry for purity, which was routinely >90%.

Human umbilical vein endothelial cells (HUVECs) were obtained from the BWH Pathology Core facility and cultured as described (18, 19). Chinese hamster ovary (CHO) cells transfected with full-length human E-selectin cDNA (CHO-E) and mock-transfected CHO cells (CHO-M) were cultured in modified Eagle's medium (MEM) supplemented with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate and 1% penicillin-streptomycin.

Antibodies and reagents used for flow cytometry

Cell surface expression of sLe^X, CD44, PSGL-1, and CD43 were assessed using mAbs HECA-452 (Biolegend), 2C5 (R&D Systems), KPL-1 and 1G10 (BD Biosciences), respectively. To evaluate cell purity post-selection, flow cytometry of monocytes, $CD4^+$ T-cells, $CD8^+$ T-cells, and B-cells was performed using, respectively, FITC-conjugated-anti-human CD14 (Southern Biotech), APC-conjugated-anti-human CD4, FITC-conjugated-anti-human CD8, and cychrome-conjugated-anti-human CD19 mAbs (from Pharmingen). To

investigate sLe^X expression among circulating human monocyte subsets, isolated PBMCs were stained with the following antibodies: FITC-conjugated-anti-human/mouse CLA, PE-conjugated-anti-human CD14, APC-conjugated-anti-human CD16 and PECy7-conjugated-anti-human HLA-DR (all from Biolegend). Monocytes were identified by their characteristic FSC/SSC proprieties and HLADR expression; subsets of monocytes were defined as classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) (Supplemental Figure S1) (20).

E-selectin reactivity was determined using recombinant mouse E-selectin-human Fc Ig chimera (E-Ig) (R&D Systems) in Dubelcco's phosphate-buffured saline (DPBS) containing Ca^{2+}/Mg^{2+}, as previously described (10), with addition of 5 mM EDTA (calcium chelation) serving as staining control. The cell surface display of glycans containing terminal sialic acid-α(2,6)-linked to galactose was assessed by lectin staining with biotin-conjugated *Sambucus nigra* lectin (SNA; Vector Laboratories) at 1:500 dilution for 20 min at 4°C. Cells were then washed and incubated with APC-conjugated streptavidin (10 μg/mL, Biolegend) for 15 min at 4°C.

Flow cytometry was performed using Cytomics FC 500 MPL (Beckman Coulter) or BD FACSCanto (Becton Dickinson) flow cytometers, with data analysis using FlowJo version 10.0.6 (TreeStar).

Preparation of whole cell lysates

Cells were suspended in 150 mM NaCl, 50 mM Tris-HCI (pH 7.4), 0.02% NaN_3, 20 μg/mL PMSF and protease inhibitor cocktail (Roche), sonicated, and then solubilized in 2% Nonidet P-40 (NP-40). For lysates undergoing immunoprecipitation with E-Ig, 2mM $CaCl_2$ was added to lysate solution.

Western blot analysis

Protein samples were boiled in reducing Laemmli loading buffer (Boston BioProducts) and then resolved on 7.5% SDS-PAGE electrophoresis gels (Bio-Rad). Resolved proteins were transferred to PVDF membranes (Bio-Rad) and blocked with 10% milk and 0.1% Tween20 in TBS. Western blots were probed with primary antibodies (1 μg/mL), followed by incubation with HRP-conjugated secondary antibodies (Southern Biotech). Expression of sLe^X, CD44, PSGL-1, CD43, MPO and L-selectin were assessed using the mAbs HECA-452 (Biolegend), 2C5 (R&D Systems), KPL-1 (BD Biosciences), 1G10 (BD Biosciences), 2C7 (Abcam) and LAM1-116 (Santa Cruz Biotechnology), respectively. For E-Ig blotting, membranes were incubated with E-Ig (1 μg/mL) suspended in TBS 0.1% Tween20 containing 2 mM $CaCl_2$, followed by incubation with rat anti-mouse CD62E mAb (R&D Systems) and then goat anti-rat IgG-HRP (Southern Biotech). Antigens were detected by chemiluminescence using Lumi-Light Western blotting substrate (Roche).

Immunoprecipitation studies

Cell lysates were precleared with protein G-agarose (Invitrogen), followed by incubation with antibodies (anti-PSGL-1, anti-CD43 (each from BD Biosciences), anti-CD44 (R&D Systems), anti-myeloperoxidase mAb (clone 03D03, Abcam)) or E-Ig. Immunoprecipitates

were then collected with protein G beads, beads were boiled, and released proteins were subjected to SDS-PAGE and western blotting.

Cell Surface biotinylation

Cells were incubated with Sulfo-NHS-SS-Biotin according to the manufacturer's instructions (Pierce Biotechnology). After cell lysis, biotinylated proteins were isolated with streptavidin beads (Invitrogen).

Blot rolling assay

The blot rolling assay was performed as described previously (21). Briefly, western blots of CD44 immunoprecipitates were stained using HECA-452 mAb and rendered translucent by immersion in HBSS with 10% glycerol, 10 mM HEPES (pH 7.4) and 5 mM $CaCl_2$. Blots were then placed in a parallel-plate flow chamber, and CHO-E cells were perfused over blots at shear stress of 0.17 dynes/cm^2. Perfusion of CHO-M cells served as control to assess binding specificity.

Microfluidic adhesion assay

HUVEC monolayers were grown to confluence in microfluidic channels (Bioflux, Fluxion Biosciences). To stimulate expression of E-selectin, HUVECs were incubated with 40 ng/mL of TNF-α (R&D Systems) for 4 hours at 37°C. PBMC subsets were perfused through channels at an initial shear rate of 0.5 dynes/cm^2, with step-wise increments in shear rate up to 8 dynes/cm^2. Number of rolling and arrested cells per mm^2 of HUVEC area was quantified at each shear rate, averaged across three different fields of view and normalized to 1×10^6 cells/mL of infusate. Rolling velocities of individual cells were calculated by dividing the distance traveled by the tracking time at each shear stress. Binding specificity was assessed by incubating HUVECs with function-blocking anti-E-selectin mAb (clone 68-5H11, BD Pharmingen) (22). Additionally, negative controls were performed by treating PBMCs with sialidase or by using unstimulated HUVECs (i.e., not activated with TNF-α).

Enzymatic treatments

To remove *N*-glycans, CD44 immunoprecipitates were treated with Peptide-*N*-Glycosidase (PNGase-F, New England Biolabs) according to the manufacturer's instructions. Sialic acid residues were removed by treatment with 200 mU/mL *Vibrio Cholerae* sialidase (Roche Molecular Biochemicals) at 37°C for 1 hour. Cell surface proteins were cleaved with 0.1% bromelain (Sigma-Aldrich) for 1 hour at 37°C, and efficiency was assessed by flow cytometry staining for residual CD44 expression. For α(1,3)-exofucosylation, cells were treated with 0.07 mg/mL of fucosyltransferase VII (FTVII) (R&D systems) in HBSS buffer containing 10 mM HEPES, 0.1% human serum albumin and 1 mM GDP-fucose (Sigma-Aldrich) for 60 minutes at 37°C. As controls, cells were suspended in the same HBSS buffer but without addition of FTVII (buffer-treated).

Isolation of RNA and Quantitative Real-Time PCR

Total RNA from each sample was isolated using the RNeasy Micro Kit (Qiagen) and reverse-transcribed using the SuperScript VILO cDNA Synthesis kit (Thermo Fisher

Scientific). Quantitative real-time PCR (qRT-PCR) reaction was performed using SYBR Select Master Mix (Applied Biosystems). Primer sequences used for qRT-PCR are listed in Supplemental Table I and gene expression was normalized to *GAPDH* (endogenous control) expression. The relative mRNA levels were calculated by adapting the $2^{-\Delta Ct} \times 100$ formula (23).

Statistical analysis

Student's *t* tests were performed in order to test the statistical difference between the groups of our experimental study. Differences were considered to be significant when $p < 0.05$ (*p<0.05; **p<0.01; ***p<0.001). The obtained data were analyzed by GraphPad Prism 4 (GraphPad Software, Inc).

Results

Differential capacity of PBMCs to engage E-selectin

To assess binding of human PBMCs to vascular E-selectin under hemodynamic flow conditions, we used a microfluidics chamber to examine tethering/rolling adhesive interactions of monocytes, $CD4^+$ and $CD8^+$ T-cells and B-cells on TNF-α-stimulated HUVEC (Figures 1a and 1b). Within the physiologic fluid shear stress range of 1–4 dynes/cm^2, monocytes uniformly exhibited the highest tethering/rolling capacity and, notably, rolling adhesive interactions of monocytes on HUVEC were also uniformly maintained at shear stress levels exceeding the typical post-capillary venule shear stress range (i.e., at upwards of 5 dynes/cm^2). Moreover, the greater ability of circulating monocytes to efficiently bind E-selectin under shear stress conditions was further demonstrated in rolling velocity analysis, as rolling velocity of monocytes was consistently lower than that of $CD4^+$ T-cells and much lower than that of $CD8^+$ T and B-cells (Figure 1b). A fraction of $CD4^+$ and, to a lesser extent, $CD8^+$ T-cells showed endothelial interactions most prominently at shear stress of 1–2 dynes/cm^2, whereas B-cells exhibited only transient tethering interactions (mostly at shear stress of 0.5–1 dynes/cm^2) and displayed the highest rolling velocities (Figures 1a and 1b). PBMC/HUVEC interactions under shear stress were E-selectin–dependent, as confirmed by abrogation of binding in the presence of a function-blocking anti–E-selectin mAb. Additionally, controls for specificity were performed using HUVECs not stimulated with TNF-α and by pre-treating the PBMC subsets with sialidase, which in each case dramatically reduced rolling interactions (data not shown).

To directly determine whether the observed variations in adhesive interactions between PBMCs and endothelium were secondary to differential expression of E-selectin ligands, flow cytometry was performed using E-Ig chimera to measure the E-selectin binding activity of each PBMC subset and using mAb HECA-452 to quantify the expression of the canonical selectin-binding determinant sLe^X. Blood monocytes exhibited robust reactivity with E-Ig and HECA-452 mAb (Figure 1c). Among lymphocytes, $CD4^+$ T-cells exhibited greater E-Ig and HECA-452 reactivity than $CD8^+$ T-cells, whereas B-cells generally lacked reactivity (Figure 1c). For each PBMC subset, levels of HECA-452 staining and E-Ig-reactivity by flow cytometry each correlated with observed E-selectin binding under hemodynamic shear

stress, underscoring the functional significance of surface sLe^X/E-Ig staining levels in predicting E-selectin ligand activity.

Circulating human monocytes are a heterogeneous population of peripheral blood cells, comprising three different functional and migratory subsets. The expression levels of the lipopolysaccharide receptor (CD14) and of FcγIIIR (CD16) distinguishes these subsets, which are classified as classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) (24). Classical monocytes are short lived-cells, with high phagocytic and myeloperoxidase activities that are recruited preferentially to inflamed lesions. By contrast, the non-classical monocytes are longer lived-cells, with low phagocytic activity that are known to patrol healthy tissues (25, 26). Intermediate monocytes have been proposed to serve as a transition subset from classical to non-classical and are characterized by enhanced production of TNF-α and IL-1β (26). The differential migratory behaviors between these monocyte subsets prompted us to assess the expression levels of sLe^X among circulating human monocytes. As shown in Supplemental Figure S1, classical monocytes ($CD14^{++}CD16^{-}$) consistently show the highest sLe^X reactivity among circulating monocytes, followed by the intermediate subset ($CD14^{++}CD16^{+}$), whereas non-classical monocytes ($CD14^{+}CD16^{++}$) express low to no amounts of sialofucosylated determinants.

To assess the relative contribution of glycoproteins versus glycolipids as E-selectin ligands, the different PBMC subsets were treated with the broad-specificity protease bromelain. All subsets showed a substantial reduction of HECA-452 reactivity after bromelain treatment (Figure 1d), indicating that E-selectin-binding determinants are preferentially expressed on proteins rather than lipids.

Glycoforms of CD43, CD44, PSGL-1 and a ~70 kDa protein are E-selectin ligands on human monocytes

To identify the molecular effectors of E-selectin binding by monocytes, E-Ig was used as a probe in Western blot analysis. As shown in Figure 2a, under reduced SDS-PAGE conditions, three prominent E-Ig-reactive bands were observed at ~120–130 kDa, ~80–90 kDa and ~65–70 kDa. The monomer (i.e., reduced homodimer) of CLA characteristically migrates at ~120–130 kDa (27), and flow cytometry indicated consistent surface expression of PSGL-1 among monocytes (Figure 2b). To confirm the expression of CLA (i.e., the PSGL-1 glycoform that binds E-selectin), E-selectin ligands were immunoprecipitated from monocyte lysates using E-Ig chimera, and analyzed by Western blot using anti-PSGL-1 antibody as a probe. As shown in Figure 2c, staining with anti-PSGL-1 mAb was observed at ~120–130 kDa, providing direct evidence of CLA expression.

A specialized sialofucosylated CD43 glycoform, known as “CD43E”, has a molecular mass similar to CLA (~130 kDa) and functions as an E-selectin ligand in human lymphocytes (28) and hematopoietic stem/progenitor cells (HSPCs) (10). Flow cytometry confirmed the surface expression of CD43 on human monocytes (Figure 2b). The E-Ig immunoprecipitate also stained with anti-CD43 antibody, confirming that the ~120–130 kDa E-Ig-reactive band is comprised of CD43E in addition to CLA (Figure 2c).

HCELL, the E-selectin-binding glycovariant of CD44, migrates in reduced SDS-PAGE conditions as an ~80–90 kDa band (29). We observed high CD44 expression on monocytes by flow cytometry (Figure 2b). Accordingly, E-Ig immunoprecipitates of monocyte lysates were probed with anti-CD44 antibody on Western blots, and anti-CD44 mAb staining showed that the ~80–90 kDa band is HCELL (Figure 2c).

The E-Ig-reactive ~65–70 kDa glycoprotein did not stain with anti-PSGL-1, anti-CD43 or anti-CD44 mAbs and it is expressed on the cell surface of monocytes, as assessed by monocyte surface-biotinylation followed by streptavidin pull-down of cell lysate material (Figure 2d). A recent report suggested that G-CSF induces expression of an E-selectin-reactive glycoform of myeloperoxidase (designated “MPO-EL”) with molecular mass ~65 kDa (30), however, MPO immunoprecipitates from monocyte lysates did not stain with E-Ig, indicating that the E-Ig-reactive ~65–70 kDa protein is not MPO-EL (Figure 2e). In addition, some studies have reported that L-selectin (~70–90 kDa) can serve as an E-selectin ligand on human neutrophils (31–33) and on cultured human lymphoblasts (34). However, immunoprecipitates of E-selectin ligands from circulating human monocytes using E-Ig chimera did not stain with L-selectin, indicating that L-selectin does not have E-selectin binding capacity on human monocytes (Figure 2f).

Human CD4$^+$ T-cells natively express CLA, CD43E and HCELL, whereas CD8$^+$ T-cells express only CLA and CD43E, and B-cells lack E-selectin ligands

To identify E-selectin ligands expressed on subsets of circulating lymphocytes, Western blots of CD4$^+$ T-cell, CD8$^+$ T-cell and B-cell lysates were performed using E-Ig as a probe. As shown in Figure 3a, E-Ig staining of CD4$^+$ T-cell lysates revealed two distinct bands at ~120–130 kDa and ~80–90 kDa (with more prominent staining at ~120–130 kDa), whereas staining of CD8$^+$ T-cell lysates showed one band at ~120–130 kDa and B-cell lysates had no E-Ig reactivity (Figure 3a).

Given the typical molecular weight of E-selectin-binding glycoforms of PSGL-1 and CD43 at ~120–130 kDa, and of CD44 at ~80–90 kDa, we performed flow cytometry to assess whether these proteins are expressed among CD4$^+$ T-cells, CD8$^+$ T-cells and B-cells. As shown in Figure 3b, circulating CD4$^+$ and CD8$^+$ T-cells uniformly express PSGL-1, CD43 and CD44 at similarly high levels. In contrast, B-cells generally lack PSGL-1 expression and only a small proportion (~5%) of cells express CD43, whereas CD44 is characteristically expressed but at levels slightly below that of T-cells (Figure 3b).

Among CD4$^+$ T-cells, PSGL-1 and CD43 immunoprecipitated from whole cell lysates showed equivalently robust staining with E-Ig, thus indicating significant expression of both CLA and CD43E (Figure 3c). Immunoprecipitated CD44 showed nominal reactivity with E-Ig, indicating that there is low level HCELL expression in CD4$^+$ T-cells (Figure 3c). In lysates of CD8$^+$ T-cells, though PSGL-1 and CD43 immunoprecipitates each showed reactivity with E-Ig, CLA is the more prominent E-selectin ligand, and, in further contrast to CD4$^+$ T-cells, CD44 immunoprecipitates were not E-Ig-reactive, indicating absence of HCELL expression (Figure 3c).

E-selectin binding determinants of HCELL are displayed on *O*-glycans on monocytes and *N*-glycans on CD4$^+$ T-cells

HCELL expression has previously been reported on human HSPCs (29) and in certain types of human tumors (22). Structurally, the HCELL glycoform identified on human HSPCs consists of sLe^X motifs decorating *N*-glycans of "standard" CD44 (i.e., CD44 lacking splice exons) (35), whereas the HCELL glycoform found in solid malignancies displays sLe^X decorations on *O*-glycans of peptides encoded by splice exons (i.e., splice-variant CD44 isoforms) (22, 36). Western blot reveals that monocytes and T-cells each express CD44 of ~80–90 kDa (i.e., standard CD44) and can also display splice isoforms of ~120 kDa and ~150 kDa (Figure 4a). However, in each case, HCELL-specific sLe^X decorations are predominantly present on the standard CD44 protein scaffold (Figures 2c, 3c, and 4b).

To determine whether the E-selectin-binding determinants of HCELL on human monocytes and CD4$^+$ T-cells are displayed on *N*- or *O*-glycans, CD44 was immunoprecipitated and treated with PNGase-F, which removes *N*-glycans from the protein backbone. As shown in Figure 4b, E-Ig reactivity of CD44 immunoprecipitates from CD4$^+$ T-cells was eliminated following PNGase-F treatment (Figure 4b, left panel), indicating that HCELL in these cells exclusively displays sLe^X on *N*-glycans, similar to human HSPCs. Surprisingly, although PNGase-F treatment was effective in trimming *N*-glycans of monocyte CD44 (as evidenced by reduction in CD44 molecular weight), the intensity of E-Ig reactivity by western blot was not affected (Figure 4b, right panel). Notably, these data are the first to reveal that a primary, native human cell type expresses HCELL displaying sLe^X on PNGase-F-resistant glycans, suggesting that *O*-glycans of the standard CD44 isoform can be modified by sLe^X.

In light of the evidence indicating that human CD4$^+$ T-cells and monocytes exhibit structurally distinct glycoforms of HCELL (i.e., CD44 bearing *N*- versus *O*-glycan-sialofucosylated determinants), we sought to assess the capacity of these two types of HCELL to support E-selectin-dependent cell rolling under physiological shear conditions. To this end, blot-rolling assays (21, 37) were performed and results showed that HCELL immunoprecipitated from both monocytes and CD4$^+$ T-cells are each capable of supporting rolling adhesive interactions of CHO-E cells (Figure 4c). These data indicate that though the structural biology of the core glycosylation of standard CD44 that display terminal sLe^X decorations may differ on monocytes compared to other human hematopoietic cells, the monocyte HCELL glycovariant efficiently engages E-selectin under fluid shear conditions.

qRT-PCR analysis of glycan-modifying enzymes that affect lactosaminyl glycan display on monocytes and B-cells

The biosynthesis of sialofucosylated determinants involves a series of enzymatic steps mediated by several well-established glycosyltransferases and glycosidases (Supplemental Figure S2). To gain further mechanistic insight(s) into the molecular basis of cell type-specific variations in E-selectin binding, we performed qRT-PCR studies of relevant glycosyltransferases that create sialic acid and fucose modifications of terminal Type 2 lactosamines among the PBMC subsets that express the lowest and highest levels of sLe^X, B-cells and monocytes, respectively. There are 6 fucosyltransferases (FT) that can add fucose in α(1,3)-linkage to *N*-acetylglucosamine present within a terminal lactosamine unit:

FTIII, FTIV, FTV, FTVI, FTVII and FTIX. Of these, FTVII can only create sLe^X because it is specialized to only fucosylate a sialylated Type 2 lactosamine, and FTIX is incapable of creating sLe^X because it is specialized to only fucosylate an unsialylated lactosamine (thus it makes only Le^X (CD15)) (3). As shown in Figure 5, with the exception of FTV, monocytes highly express all of the α(1,3)-fucosyltransferases including, of particular significance, FTIX (which as noted above directs CD15 expression, a well-recognized marker of human myeloid cells). B-cells lack expression of most of the α(1,3)-fucosyltransferases, but express low levels of FTIV transcripts and, though completely lacking sLe^X display, express FTVII at transcript levels similar to that of monocytes. Among the three α(2,3)-sialyltransferases that place terminal sialic acid modifications in α(2,3)-linkage of Type 2 lactosamine units (3), ST3GalIII and ST3GalIV were expressed by both monocytes and B-cells, whereas ST3GalVI transcripts were only observed in monocytes. The surprising finding that enzymes that direct synthesis of sialofucosylations of terminal Type 2 lactosamines are present on B-cells prompted us to evaluate the expression of key enzymes that can influence expression of sLe^X by either competing for common acceptors (ST3GalI, ST6GalI, ST6GalNAcII) (38–40), are requisite to create core glycans carrying terminal sialyllactosaminyl glycans (C2GnT1, MGAT1) (38, 41), or are “trimming” glycosidases (Neu1, Neu 3, and Fuc1) (42, 43) that can remove sialic acid or fucose modifications from sLe^X (see Supplemental Figure S2 for details). Additionally, we probed for expression of the nucleotide-fucose transporter (GDP-FucT1) that controls the Golgi availability of the GDP-fucose donor sugar indispensable for fucosylation (44). The transcript levels of glycosyltransferase ST6GalNacII and the glycosidases Neu3 and Fuc1 were found to be expressed at similarly low levels in both monocytes and B-cells (Figure 5), and Neu1 and GDP-FucT1 transcript levels were comparable in both cell types. However, we observed that there were significant differences in expression of ST6GalI, ST3GalI, C2GnT1 and MGAT1 among B-cells and monocytes. As shown in Figure 5, increased gene expression of both ST6GalI and ST3GalI were observed in B-cells compared to monocytes. ST6GalI is responsible for α(2,6)-sialylation of terminal lactosamines of *N*-glycans (competing with α(2,3)-sialyltransferases) and its higher expression would result in greater display of sialic acid-α(2,6)-galactose glycans on B-cells (39). Commensurately, as measured by SNA-lectin staining, B-cells consistently displayed greater α(2,6)-sialylation compared to monocytes (see Supplemental Figure S3). The observed higher expression of ST3GalI on B-cells would be associated with the formation of the sialyl-T (sT) antigen, thereby blocking lactosaminyl glycan synthesis on *O*-glycans; thus, although transcript levels of C2GnT1 are higher among B-cells compared to monocytes, this enzyme cannot modify an sT acceptor glycan (i.e., bearing an ST3Gal1-sialylated Core 1 backbone), and the blockade of core-2 formation would thereby prevent sLe^X display on *O*-glycans (45). Moreover, with regards to *N*-glycans, the observed low expression of MGAT1 on B-cells would also limit sLe^X display by blunting creation of lactosaminyl scaffolds (46). Collectively, this pattern of glycosyltransferase gene expression among B-cells, in addition to the relatively low expression of proteins that serve as core backbones for sLe^X decorations (Figure 3b), provides mechanistic insights into the observed paucity of E-selectin ligand expression on B-cells.

Analysis of cell surface sialylated lactosaminyl glycan expression on monocytes and B-cells via FTVII-mediated α(1,3)-exofucosylation

Based on results of qRT-PCR studies, we sought to directly assess the relative levels of sialylated lactosaminyl glycans displayed by monocytes and B-cells. To this end, we performed cell surface α(1,3)-exofucosylation as a probe for such structures: i.e., the α(1,3)-fucosylation reaction can yield increased expression of sLe^X (10, 47), but only if, *a priori,* target cells display (unfucosylated) sialylated lactosaminyl glycans that can be converted to sLe^X determinants (3). Accordingly, we treated cells with the enzyme fucosyltransferase VII (FTVII), a glycosyltransferase whose only function (as stated above) is to create sLe^X via stereoselective α(1,3)-fucosylation of α(2,3)-sialylated type 2 lactosaminyl glycans (48).

FTVII-treated monocytes showed dramatically increased expression of many HECA-452-reactive glycoproteins, including bands corresponding to CLA, CD43E, HCELL and the ~65–70kDa molecule (Figure 6a). To assess which of the protein scaffolds are preferentially targeted by exofucosylation, E-Ig immunoprecipitates from lysates of buffer-treated and FTVII-treated monocytes were resolved by SDS-PAGE and blotted with anti-PSGL-1, anti-CD44 and anti-CD43 antibodies. CD43E expression was dramatically increased following FTVII treatment, whereas the expression of CLA and HCELL were also increased but to a lesser extent (Figure 6a). These studies indicate that each of these cell surface proteins are decorated with “underfucosylated” sialylated lactosamines, with CD43 having the greatest amount of such structures. Interestingly, following exhaustive immunoprecipitation of CD44 (Figure 6b), we observed persistent E-Ig reactivity in the residual (cleared) lysates at ~90 kDa, suggesting the presence of another protein within the molecular weight range of CD44 that does not natively express sLe^X, but that possesses requisite underfucosylated sialylated lactosaminyl glycans that can serve as acceptor for α(1,3)-fucosylation thereby creating sLe^X. Notably, in B-cells, α(1,3)-exofucosylation engendered only modest amounts of CD43E, indicating that, consistent with the data predicted from qRT-PCR studies, there is a general deficit in expression of sialylated type 2 lactosaminyl glycans within B-cells (Figure 6c). Paradoxically, although B-cells express much more CD44 than CD43, exofucosylation yielded expression of only CD43E, indicating that CD43 is the sole carrier of sialylated lactosaminyl glycans on the B-cell surface.

To analyze the impact of α(1,3)-exofucosylation on E-selectin binding of monocyte and B-cell populations, we compared the ability of buffer-treated and FTVII-treated cells to engage with E-selectin under physiological shear stress conditions. Exofucosylation engendered a ~2-fold increase in the number of monocytes engaging E-selectin and, though the number of tethering/rolling events at equivalent input cell numbers is modest in comparison to monocytes, exofucosylation rendered a significant increase in E-selectin binding of B-cells (Figure 6d).

Discussion

The efficient recruitment of leukocytes into tissue(s) is critical to protective immunity and to the pathobiology of all acute and chronic inflammatory conditions (1, 49). The temporal sequence of leukocyte infiltration at inflammatory sites has been well-characterized, but the molecular basis of this process is incompletely understood. Monocytes and neutrophils are

the first host defense cells to arrive at initiation of an inflammatory event (11, 50–52). As the innate immunity yields to adaptive immunity, the associated migration of lymphocytes occurs within days (53–55), with $CD4^+$ T-cell infiltration preceding $CD8^+$ T-cells (13, 14, 56). Indeed, the presence of $CD4^+$ T-cells seems to be critical for the subsequent recruitment of activated $CD8^+$ T-cells (56, 57). On the other hand, B-cells are not typically recruited to sites of acute inflammation (14, 15, 58, 59).

Our studies here highlight critical differences in the capacity of human PBMCs to bind to vascular E-selectin, the first step in leukocyte extravasation at inflammatory sites (3). The data indicate that the ability of human monocytes, B-cells and $CD4^+$ and $CD8^+$ T-cells to engage inflamed endothelium under hemodynamic shear conditions is integrally related to the content and breadth of glycoproteins that display sLe^X on these cells. In particular, monocytes possess the highest ability to bind to E-selectin under hemodynamic shear stress and consistently show the highest expression of sLe^X–bearing glycoproteins among PBMC subsets. Among circulating human monocytes, the classical subset exhibits the highest sLe^X expression, whereas non-classical monocytes show dramatically lower expression. Importantly, our results are in agreement with studies in mice, in which classical monocytes ($Ly\text{-}6C^{hi}$) demonstrated greater binding to E-selectin under flow conditions and were recruited preferentially to atherosclerotic lesions compared with non-classical monocytes ($Ly\text{-}6C^{lo}$) (60, 61). Moreover, murine classical monocytes migrate to inflamed tissues, whereas non-classical monocytes migrate to non-inflamed endothelium (62) and patrol healthy tissues (63). Regarding the sLe^X-bearing scaffolds, in addition to the E-selectin-reactive glycoform of PSGL-1 (CLA), our studies identify three E-selectin-binding glycoproteins not previously known to be expressed on human monocytes, including a currently-unidentified E-selectin ligand of ~65–70 kDa that is unrelated to L-selectin or to the structure known as MPO-EL (30). Notably, while HCELL has previously been described to function as an E-selectin ligand on human HSPCs (10, 29), leukemic blasts (35) and certain solid malignancies (64), and CD43E expression has been reported on both human (28) and murine T-cells (65–68), HSPCs (10) and on murine neutrophils (69), our dataset is the first to describe expression of these two E-selectin ligands on human monocytes. This striking plethora of E-selectin ligands on human blood monocytes indicates that these cells are distinctly specialized to engage E-selectin-bearing endothelial beds.

Among circulating human lymphocytes, $CD4^+$ T-cells exhibit the greatest ability to bind to E-selectin under shear stress conditions and, accordingly, express higher levels of E-selectin-binding determinants than do $CD8^+$ T-cells or B-cells. As previously reported, $CD4^+$ T-cells express the glycoprotein E-selectin ligands CLA and CD43E (28), however our studies reveal that these cells can also express HCELL. $CD8^+$ T-cells exhibit lower amounts of CLA and CD43E compared to $CD4^+$ T-cells and do not express HCELL, whereas B-cells completely lack expression of glycoproteins with E-selectin ligand activity. HCELL is the most potent E-selectin ligand expressed on human cells (3, 10), and expression of HCELL on human monocytes and $CD4^+$ T-cells would contribute to the observed greater capacity of these cells to undertake tethering/rolling adhesive interactions on TNF-α-stimulated endothelium under hemodynamic shear stress conditions (Figure 1a and 1b). Importantly, our data show that, similarly to HCELL expression on human HSPCs, HCELL on $CD4^+$ T-cells displays its E-selectin-reactive sialofucosylated determinants on *N*-glycans; however,

the observed PNGase-F-resistance of monocyte HCELL suggests that it displays its E-selectin binding determinants on *O*-glycans. In any case, CD44 immunoprecipitated from monocytes is markedly more reactive with E-Ig than that of $CD4^+$ T cells, indicating that, proportionally, monocyte CD44 is far more highly decorated with sLe^X compared to that of T-cells.

To dissect the mechanistic underpinning(s) of cell type-specific differences in E-selectin binding, we performed qRT-PCR studies of relevant glycosyltransferases and glycosidases that regulate sLe^X synthesis on B-cells and monocytes. Based on these results, we then performed α(1,3)-exofucosylation of these cells to probe the relative expression level of (natively) unfucosylated sialylated type 2 lactosaminyl glycan acceptors. Human blood B-cells and monocytes each have abundant transcript levels of relevant α(2,3)-sialyltransferases that create sialylated lactosamines. In contrast, compared to monocytes, B-cells have relatively low gene expression of the α(1,3)-fucosyltransferases involved in the biosynthesis of sLe^X, but, notably, express FTVII transcript levels equivalent to that of monocytes. FTVII is generally considered to be the principal fucosyltransferase that creates sLe^X (3), and, as such, the pattern of transcript levels of FTVII and of α(2,3)-sialyltransferases that modify terminal lactosamines would predict that B-cells would readily display sLe^X. Indeed, we had predicted that transcript levels for mRNA encoding all α(1,3)-fucosyltransferases would be low among B-cells, particularly for FTVII transcripts, given the abundance of information supporting a key role for FTVII in regulation of sLe^X synthesis among lymphocytes (70–74). However, B-cells also have high transcript levels of glycosyltransferases that compete against sLe^X display, such as ST6GalI and ST3GalI (see Figure 5). Thus, overall, B-cells exhibit a “transcript signature” of glycosyltransferases that favors a lower expression of sLe^X synthesis compared to monocytes. Notably, among both monocytes and B-cells, α(1,3)-exofucosylation increased E-selectin-binding activity (see Figure 6d), but much more dramatically on monocytes than on B-cells; these findings indicate that, compared to monocytes, there is much lower synthesis of acceptor terminal sialylated type-2 lactosamine units on B-cells for subsequent shaping by α(1,3)-fucosyltransferases to create the sLe^X determinant. Moreover, though α(1,3)-exofucosylation of monocytes increased E-selectin binding for all glycoprotein ligands (CLA, CD43E, HCELL and the ~65 kDa molecule), sLe^X expression was most prominently enforced on CD43E, indicating that this monocyte glycoprotein natively displays the greatest content of underfucosylated terminal sialylated type-2 lactosamine units. Interestingly, this “glycan signature” of monocyte CD43 is recapitulated among B-cells, as α(1,3)-fucosylation of the cell surface only generates CD43E (see Figure 6c). Thus, although human circulating B-cells uniformly display CD44 and yet express CD43 on only a small percentage of the cells (see Figure 3b) (75–77), of all membrane glycoproteins on blood B-cells, CD43 is uniquely decorated with sialylated type-2 lactosaminyl glycans. Altogether, the results of qRT-PCR studies of blood B-cells, coupled with the fact that these cells have lower expression of the pertinent proteins that serve as scaffolds for display of E-selectin-binding carbohydrate motifs (see Figure 3b) (75, 78–80), account for the absence of E-selectin binding activity by these cells.

The elaboration of host defense critically depends on the coordinated escape of circulating immune cells from the vasculature into sites of inflammation. Though chemoattractants such

as chemokines provide directional cues for entry of leukocytes into inflammatory foci (81–83), Step 1 effectors drive the kinetics of leukocyte recruitment as chemokine signaling cannot proceed without prerequisite adhesive interactions of circulating leukocytes at target endothelial beds (84). Accordingly, the observed differences in the structural biology and expression levels of E-selectin ligands among human blood monocytes and lymphocytes provide mechanistic insights into the well-known temporal pattern of PBMC accumulation in the evolution of host immunity. In particular, the abundance of E-selectin ligands on monocytes, and absence of such structures on B-cells, is a reflection of a distinct repertoire of glycosyltransferases that program display of sLe^X on a variety of specialized glycoproteins. Though prominent E-selectin ligand expression mediates monocyte tropism to inflamed endothelial beds, thereby licensing their role as key effectors of antigen recognition and immunoregulation, their capacity to engage E-selectin contributes to the pathogenesis of vascular inflammatory diseases such as atherosclerosis and giant cell arteritis (52, 85). As such, our increasing knowledge of the structural biology of E-selectin ligands and of the tapestry of glycosyltransferases and glycosidases that shape cell-specific variations in E-selectin ligand expression among human PBMC subsets could yield novel therapeutic approaches to potentiate tissue infiltrates to bolster host defense, and, conversely, to ameliorate and/or prevent pathologic consequences of such infiltrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper include

CHO-E	Chinese hamster ovary cells transfected with full-length human E-selectin cDNA
CHO-M	mock-transfected CHO cells
CLA	Cutaneous Lymphocyte Antigen
FT	α(1,3/4)-fucosyltransferase
HCELL	Hematopoietic Cell E-selectin/L-selectin Ligand
HSPCs	Hematopoietic Stem and Progenitor Cells
MPO	Myeloperoxidase
PNGase-F	Peptide-*N*-Glycosidase

PSGL-1	P-selectin Glycoprotein Ligand-1
qRT-PCR	Quantitative real-time PCR
SNA	*Sambucus nigra* lectin
ST3	α(2,3)-sialyltransferase
ST6	α(2,6)-sialyltransferase

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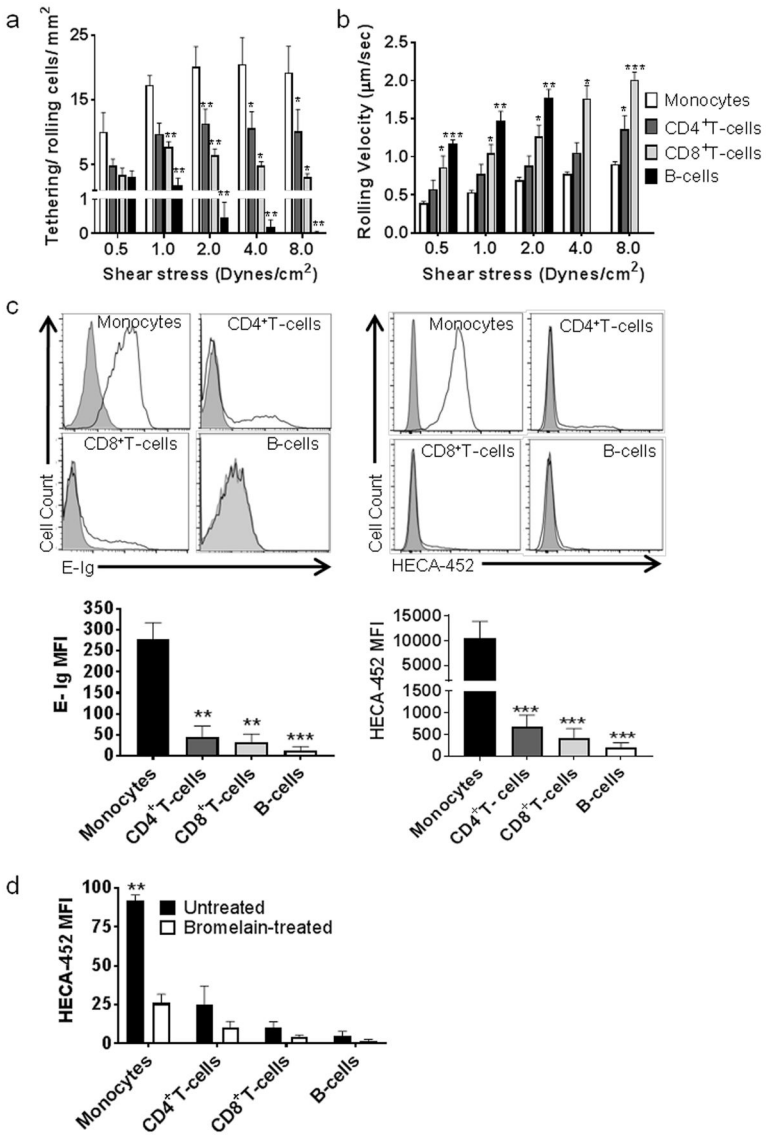

Figure 1. Native human blood monocytes exhibit greater E-selectin binding activity compared to native human blood lymphocytes

(a and b) *Parallel-plate flow chamber analysis of tethering and rolling interactions of PBMCs on E-selectin-expressing endothelial cells*. Freshly isolated human blood monocytes, $CD4^+$ and $CD8^+$ T-cells and B-cells were perfused over TNF-α-stimulated HUVECs in flow with shear stress ranging from 0.5 to 8 dynes/cm^2. **(a)** The number of cells rolling on the HUVEC monolayer at shear stress levels of 0.5, 1, 2, 4 and 8 dynes/cm^2 was quantified, normalized to 1×10^6 cells/mL of infusate, and corrected by subtracting the values obtained using a function-blocking mAb to E-selectin. Monocytes and $CD4^+$ T-cells engaged in rolling and adhesive interactions at shear stress levels of up to 8 dynes/cm^2, whereas $CD8^+$ showed rolling interactions most efficiently at 1–2 dynes/cm^2, and B-cells engaged endothelium only at 0.5–1.0 dynes/cm^2 shear stress. The number of rolling cells for monocytes was ~2-fold greater than $CD4^+$ T-cells, >3-fold greater than $CD8^+$ T-cells, and >10-fold greater than B-cells. **(b)** Rolling velocities of PBMCs on TNF-α activated HUVEC

cells were calculated as the distance traveled divided by the time period of observation. Blood monocytes exhibited marked slower rolling velocities than $CD4^+$ T-cells, $CD8^+$ T-cells and B-cells. Statistical significance (*p<0.05; **p<0.01; ***p<0.001) was determined using paired *t*-test and refers to the difference in E-selectin-dependent rolling interactions between monocytes and the other PBMC subsets (either $CD4^+$ T, $CD8^+$ T or B-cells). Values are mean ± SEM of 5 independent experiments. **(c)** *Representative flow cytometry histograms of E-Ig (left) and HECA-452 (right) staining of monocytes, $CD4^+$ and $CD8^+$ T-cells and B-cells*. As shown, human monocytes express the highest levels of E-selectin ligands, followed by $CD4^+$ and $CD8^+$ T-cells, whereas B-cells lack E-Ig and HECA-452 reactivity. Filled histograms represent incubation with E-selectin-Ig in the absence of Ca^{2+} (EDTA treatment) or with isotype control, and open histograms represent incubation with E-Ig (in presence of 2 mM Ca^{2+}) or HECA-452. Statistical significance (**p<0.01; ***p<0.001) was determined using paired *t*-test and refers to the difference between monocytes and the other PBMC subsets (either $CD4^+$ T, $CD8^+$ T or B-cells). Graph values are mean ± SEM of 4 (E-Ig) and 7 (HECA-452) independent experiments. **(d)** *Effects of protease treatment on sLe^X expression of native human PBMCs*. Untreated (black bars) or bromelain-treated (white bars) cells were stained with HECA-452 mAb and analyzed by flow cytometry. Bromelain digestion decreased HECA-452 reactivity in all PBMC subsets tested. Statistical significance (**p<0.01) was determined using paired *t*-test and refers to the difference in HECA-452 reactivity between bromelain-treated and untreated cells. Data are mean ± SEM of 3 independent experiments.

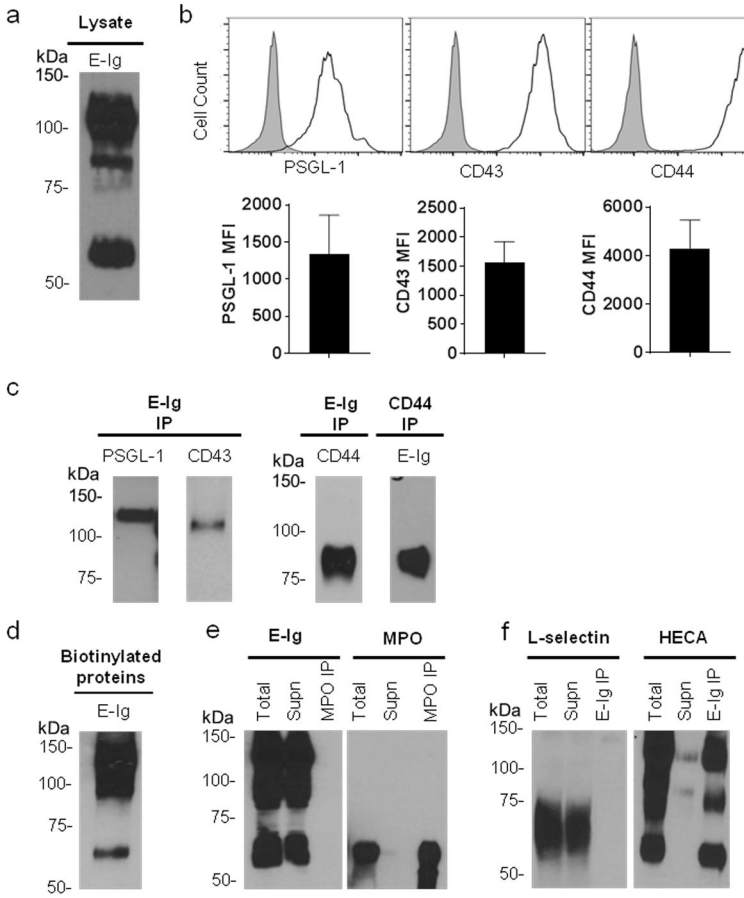

Figure 2. Identification of glycoprotein E-selectin ligands on native human blood monocytes

(a) *Western blot analysis of whole cell lysate of human monocytes stained with E-Ig.* E-Ig staining of monocyte lysates resolved under reduced SDS-PAGE conditions revealed three principal bands at ~120–130 kDa, ~80–90 kDa and ~70 kDa. (**b)** *Representative flow cytometry histograms of PSGL-1, CD43 and CD44 expression in human blood monocytes*. Filled histograms represent incubation with isotype control and open histograms represent incubation with specific antibodies. As shown, human monocytes uniformly express PSGL-1, CD43 and CD44. Graph values are mean ± SEM of 6 independent experiments. **(c)** *Western blot analysis of glycoprotein E-selectin ligands in human blood monocytes.* E-Ig-immunoprecipitated (E-Ig IP) proteins from cell lysates of human blood monocytes were resolved by SDS-PAGE, blotted, and stained with anti-PSGL-1 and anti-CD43 antibodies (Left panel) or with anti-CD44 antibody (Right panel). In addition, CD44 was immunoprecipitated (CD44 IP) from cell lysates of human monocytes, resolved by SDS-PAGE, blotted, and stained with E-Ig (Right panel). Human monocytes express PSGL-1, CD43 and CD44 scaffolds that display E-selectin-reactive epitopes. **(d)** *E-selectin-reactive 65–70 kDa unidentified glycoprotein in monocytes is a cell surface protein.* Monocyte surface proteins were labeled with biotin derivative products and lysed. Biotinylated proteins were collected on streptavidin beads, resolved by SDS/PAGE, blotted and stained with E-Ig. Three E-Ig-reactive bands were observed, including the 65–70 kDa unidentified glycoprotein, confirming its expression on the cell surface. **(e)** *Western blot analysis of E-Ig*

reactivity of myeloperoxidase (MPO) expressed on native human blood monocytes. MPO was immunoprecipitated from monocyte lysates, resolved by SDS-PAGE, blotted, and stained with anti-MPO mAb (Right panel) or E-Ig (Left panel). “Total” represents whole lysate and “Supn” corresponds to the cleared lysate, i.e., following MPO immunoprecipitation (“MPO IP”). As shown, MPO is not an E-selectin ligand in native human blood monocytes. **(f)** *Western blot analysis of L-selectin staining of E-selectin ligands immunoprecipitated from circulating human monocytes.* E-selectin ligands were immunoprecipitated from monocyte lysates, resolved by SDS-PAGE under non-reducing conditions, blotted, and stained with HECA-452 mAb (Right panel) or anti-L-selectin mAb (Left panel). “Total” represents whole lysate and “Supn” corresponds to the cleared lysate, i.e., following E-selectin ligand immunoprecipitation (“E-Ig IP”). L-selectin is not an E-selectin ligand on circulating monocytes, as indicated by lack of reactivity with anti-L-selectin mAb on the E-Ig IP product.

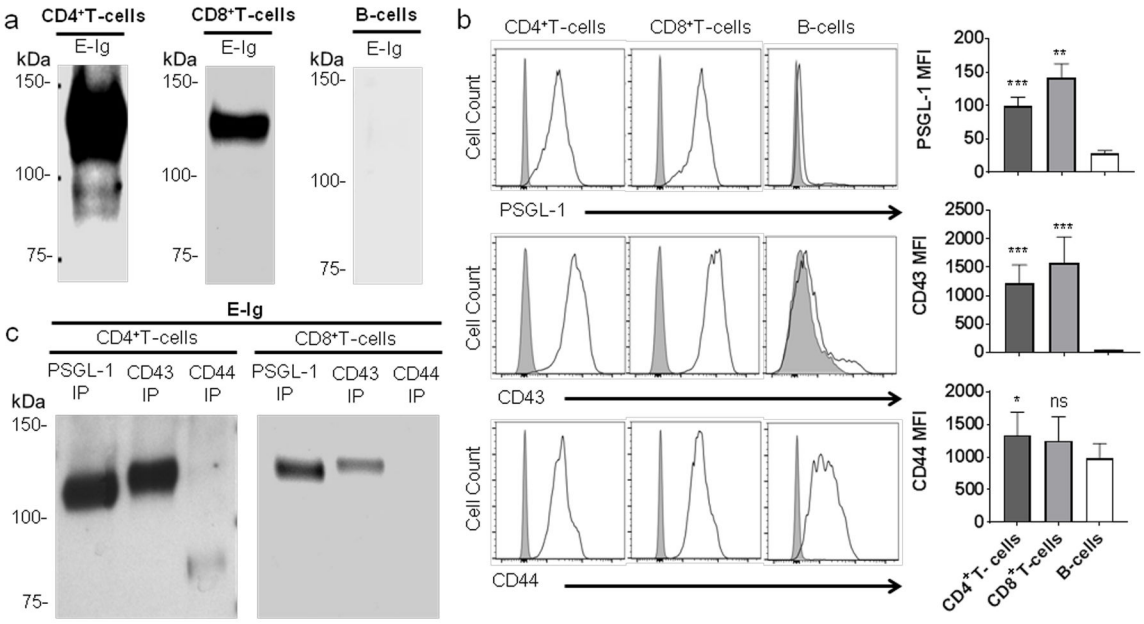

Figure 3. Identification of E-selectin ligands expressed on different subsets of lymphocytes

(a) *Western blot analysis of E-Ig-reactive proteins in native human blood CD4$^+$ T-cells, CD8$^+$ T-cells and B-cells.* Lysates of lymphocytes were resolved by SDS-PAGE electrophoresis, and immunoblotted with E-Ig chimera. CD4$^+$ and CD8$^+$ T-cells show two and one E-Ig reactive band, respectively, whereas B-cells lack E-Ig staining. **(b)** *Flow cytometry analysis of PSGL-1, CD43 and CD44 expression on native human blood lymphocytes.* Circulating CD4$^+$ and CD8$^+$ T-cells express high levels of PSGL-1, CD43 and CD44, whereas circulating B-cells only express CD44 and a small amount of CD43. Filled histograms represent incubation with isotype control and open histograms represent incubation with specific antibodies. Statistical significance (*p<0.05; **p<0.01, ***p<0.001) was determined using paired *t*-test and refers to the difference between B-cells and the other lymphocyte subsets (either CD4$^+$ T or CD8$^+$ T-cells). Graph values are mean ± SEM of 6 independent experiments. **(c)** *Identification of glycoprotein E-selectin ligands expressed on native human blood T lymphocytes*. PSGL-1, CD43 and CD44 were immunoprecipitated from whole cell lysates of circulating human CD4$^+$ and CD8$^+$ T-cells. Immunoprecipitates were then resolved by SDS-PAGE, blotted and stained with E-selectin-Ig chimera (E-Ig). Western blots reveal expression of E-selectin ligands CLA, CD43E and HCELL on human CD4$^+$ T-cells, and CLA and CD43E on human CD8$^+$ T-cells.

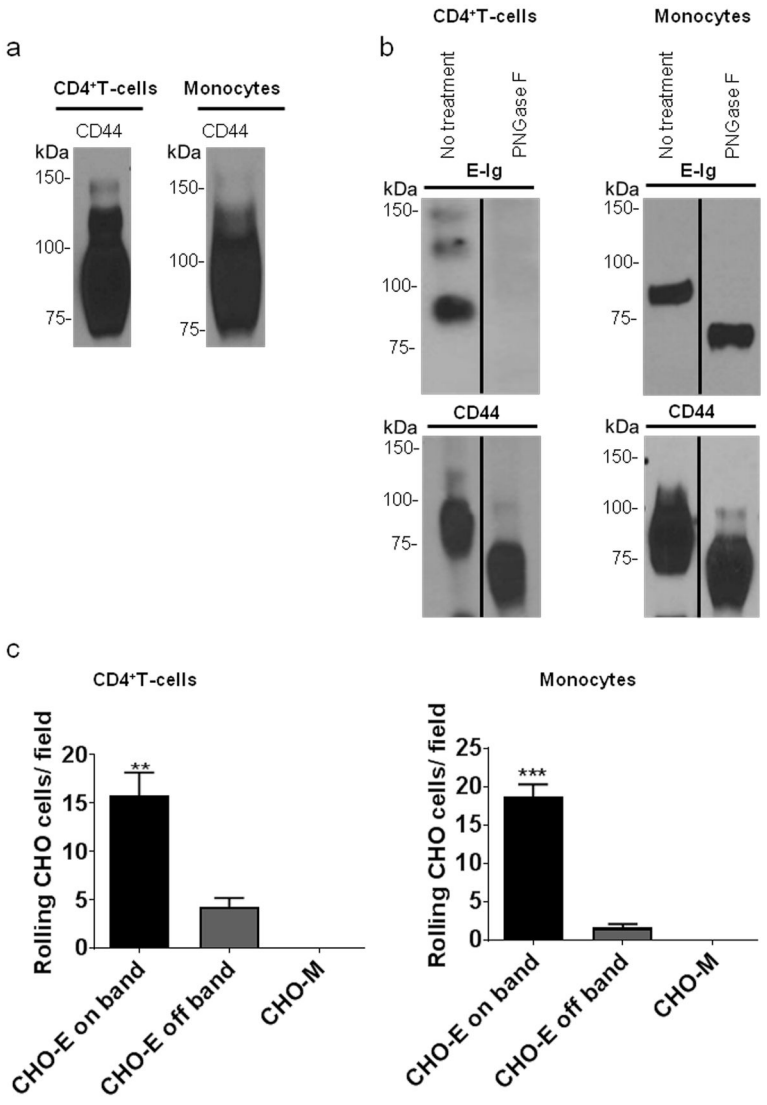

Figure 4. HCELL is a functional E-selectin ligand on circulating human monocytes and CD4$^+$ T-cells

(a) *Circulating human CD4$^+$ T-cells and monocytes express two variant isoforms of CD44.* Whole cell lysates of CD4$^+$ T-cells (Left panel) and monocytes (Right panel) were resolved by SDS-PAGE, blotted and stained with CD44 mAb. In addition to the major expression of the standard ("CD44s") isoform (~90kDa), western blots reveal the expression of two CD44 variant isoforms (~120 and 150 kDa) on native human blood CD4$^+$ T-cells and monocytes. **(b)** *CD44 expressed by human blood monocytes displays E-selectin binding determinants on O-glycans while CD44 expressed by human CD4$^+$ T-cells displays E-selectin binding determinants on N-glycans.* CD44 was immunoprecipitated from whole cell lysates of CD4$^+$ T-cells (Left panel) and monocytes (Right panel). CD44 immunoprecipitates were buffer-treated or treated with PNGase-F. Samples were resolved by SDS-PAGE, blotted, and stained with either E-Ig (Top panel) or CD44 (Bottom panel). In contrast to results obtained from PNGase-F digestion of HCELL of circulating human CD4$^+$T-cells, the persistence of E-Ig staining after PNGase-F digestion of HCELL from monocytes shows that CD44

displays sLe^X on *O*-glycans. Black lines represent lanes from the same blot that have been cropped. **(c)** *Blot rolling assay of HCELL isolated from circulating human CD4$^+$ T-cells and monocytes.* CD44 was immunoprecipitated from cell lysates of $CD4^+$ T-cells and monocytes. Immunoprecipitates were resolved by SDS-PAGE, blotted and stained with HECA-452 mAb. Functional E-selectin ligand activity was assessed by perfusion of E-selectin-transfected CHO cells (CHO-E) over blots at 0.17 dynes/cm^2. Non-specific adhesion was determined by assessing binding of CHO-E cells outside the HECA-452–reactive band (CHO-E off-band) and by perfusing mock-transfected CHO cells (CHO-M) over the blot. Statistical significance (**$p<0.01$; ***$p<0.001$) refers to the difference between specific E-selectin-dependent rolling interactions (CHO-E on band) and non-specific binding of CHO-E cells (CHO-E off band). Data are mean ± SEM of 3 independent experiments.

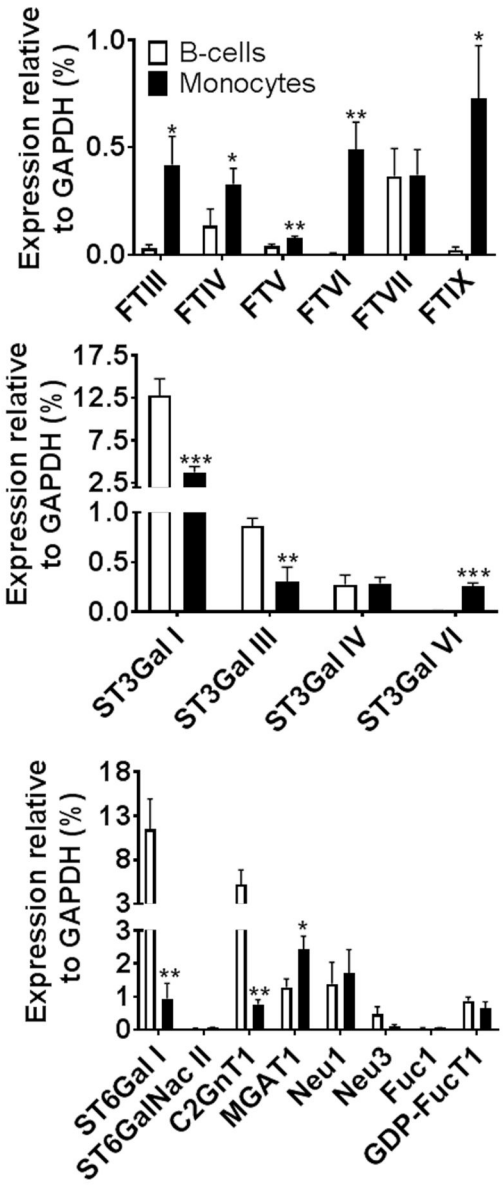

Figure 5. Analysis of glycosyltransferase gene expression on native human blood B-cells and monocytes

Real-time PCR analysis of glycosyltransferases involved in sLe^X biosynthesis in circulating human B-cells (white bars) and monocytes (black bars). Compared to monocytes, B-cells express lower levels of α(1,3)-fucosyltransferase-related transcripts that direct sLe^X synthesis, and higher levels of glycosyltransferases that compete against creation of sLe^X. Experiments were performed with a minimum of five healthy donors. The mRNA expression of each glycosyltransferase was normalized to GAPDH. Statistical significance (*$p<0.05$; **$p<0.01$; ***$p<0.001$) refers to the difference in gene expression between human B-cells and monocytes, as determined using unpaired *t*-test.

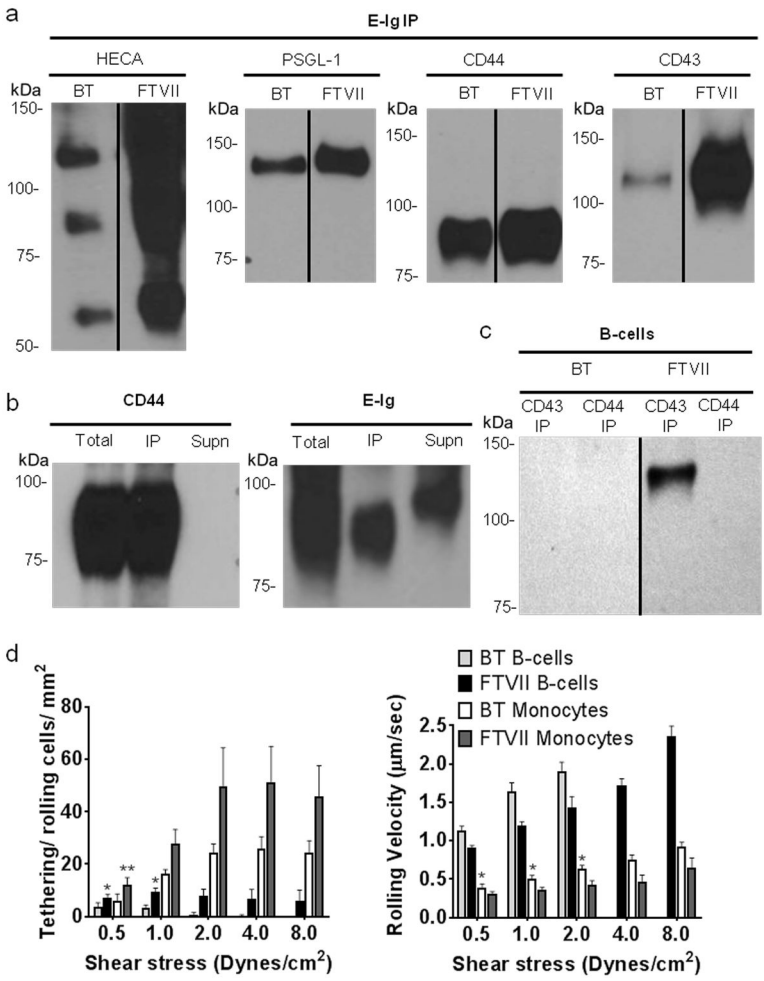

Figure 6. E-selectin ligand expression on monocytes and B-cells is increased by cell surface α(1,3)-fucosylation

(a) *FTVII-mediated exofucosylation of monocytes creates E-selectin ligands on multiple cell surface glycoproteins.* Human monocytes were treated with fucosyltransferase VII (FTVII) or buffer alone (BT). Cell lysate was immunoprecipitated with E-selectin-Ig (E-Ig IP), resolved by SDS-PAGE, and blotted with HECA-452, anti-PSGL-1, anti-CD44 or anti-CD43 antibodies. A marked increase in E-Ig reactivity on the CD43 scaffold was observed, with smaller increases of E-Ig reactivity on PSGL-1 and CD44. Black lines represent different lanes from the same blot that have been cropped. (**b)** *FTVII-mediated exofucosylation of monocytes creates a novel ~90-kDa E-selectin ligand.* CD44 immunoprecipitation was performed on cell lysate from FTVII-treated monocytes. Total cell lysate (Total), CD44 immunoprecipitate (IP), and cleared lysate (Supn) were resolved by SDS-PAGE and blotted with anti-CD44 mAb (Left panel) or E-Ig (Right panel). Following complete clearance of CD44, E-Ig-reactivity persists at ~90 kDa, indicating enforced expression of a presently unknown E-selectin ligand. (**c)** *FTVII-mediated exofucosylation of B-cells converts soley CD43 to an E-selectin ligand.* Human B-cells were treated with fucosyltransferase VII (FTVII) or buffer alone (BT), and lysates were immunoprecipitated with CD43 mAb (CD43 IP) or CD44 mAb (CD44 IP). Immunoprecipitated products were

resolved by SDS-PAGE and blotted with E-Ig. As shown in the blot, exofucosylation of B-cells creates E-selectin-binding determinants exclusively on the CD43 protein scaffold. Black line represents different parts from the same blot that have been cropped. (**d)** *FTVII treatment creates functional E-selectin ligands on monocytes and B-cells.* Human monocytes and B-cells were treated with fucosyltransferase VII (FTVII) or buffer alone (BT), and were subsequently perfused into a parallel-plate flow chamber seeded with TNF-α-stimulated HUVEC at shear stresses of 0.5, 1, 2, 4 and 8 dynes/cm^2. Both monocytes and B-cells exhibited increased tethering/rolling interactions and slower rolling velocities after FTVII treatment. To assure specificity of E-selectin-dependent rolling, we counted the number of cells rolling on the HUVEC monolayer and subtracted the number of rolling cells when the HUVEC monolayer was pre-incubated with a function blocking mAb to E-selectin. Statistical significance (*$p<0.05$; **$p<0.01$) refers to the difference in E-selectin-dependent tethering/rolling interactions between FTVII-treated and buffer-treated cell subsets, as determined using paired *t*-test. Values are mean ± SEM of 3 independent experiments.(86–89)