Heterotropic Modulation of Selectin Affinity by Allosteric Antibodies Affects Leukocyte Rolling

Sebastian B. Riese, Christian Kuehne, Thomas F. Tedder, Rupert Hallmann, Erhard Hohenester and Konrad Buscher

*J Immunol* published online 15 January 2014
http://www.jimmunol.org/content/early/2014/01/16/jimmunol.1302147

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/01/16/jimmunol.1302147.DCSupplemental

Subscription
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Heterotropic Modulation of Selectin Affinity by Allosteric Antibodies Affects Leukocyte Rolling

Sebastian B. Riese,* Christian Kuehne,* Thomas F. Tedder,† Rupert Hallmann,‡ Erhard Hohenester,§ and Konrad Buscher‡,*

Selectins are a family of adhesion receptors designed for efficient leukocyte tethering to the endothelium under shear. As a key property to resist premature bond disruption, selectin adhesiveness is enhanced by tensile forces that promote the conversion of a bent into an extended conformation of the N-terminal lectin and epidermal growth factor–like domains. Conformation-specific Abs have been invaluable in deciphering the activation mechanism of integrins, but similar reagents are not available for selectins. In this study, we show that the anti-human L-selectin mAbs DREG-55 and LAM1-5 but not DREG-56, DREG-200, or LAM1-1 heterotropically modulate adhesion presumably by stabilizing the extended receptor conformation. Force-free affinity assays, flow chamber, and microkinetic studies reveal a ligand-specific modulation of L-selectin affinity by DREG-55 mAb, resulting in a dramatic decrease of rolling velocity under flow. Furthermore, secondary tethering of polymorphonuclear cells was blocked by DREG-200 but significantly boosted by DREG-55 mAb. The results emphasize the need for a new classification for selectin Abs and introduce the new concept of heterotropic modulation of receptor function. The Journal of Immunology, 2014, 192: 000–000.

By nature, the bonds that bind selectin to endothelial- or leukocyte-expressed ligands are subjected to high tensile forces imposed by hydrodynamic flow. Cell flattening (6), microvillus receptor presentation (7, 8), the formation of upstream membrane tethers, and downstream slings (9) describe cell adaptions to rolling under high shear. Importantly, also intrinsic receptor binding properties effectively modulate bond stability. A threshold of shear force is required for L-selectin–mediated binding, which was the first indication of the striking role of blood flow on selectin mechanics (10). Leukocyte rolling on immobilized ligands requires selectins to engage in fast but transient ligand interactions with high association (K_a) and dissociation rates (K_d) (11). Surprisingly, it was demonstrated that tensile forces enhance selectin-mediated adhesion and stabilize cell rolling by decreasing K_d in low shear conditions (12, 13), promoting the formation of so-called “catch bonds”.

The first study on altered L-selectin receptor function detected affinity changes upon leukocyte activation, but the precise mechanism remained unresolved (14). Domain-swapping experiments suggested a role for the EGF-like domain in ligand binding (15, 16), and crystal structure analysis subsequently revealed a flexible hinge between the N-terminal lectin and EGF-like domain of selectins (17, 18). Whereas sLe^a is bound by a bent conformation of P-selectin, cocystalization with PSGL-1 glycoprotein revealed an extended conformation (17). The transition from the bent to the extended state involves several subdomain movements in the lectin domain (19). One major component of this allosteric pathway is the tetrasaccharide sialyl Lewis x (sLex) with sulfated 1,4-(Fuc-Gal) internal repeat. A common minimal ligand determinant identified was the tetrasaccharide sialyl Lewis x with terminal α2,3-linked sialic acid and α1,3-linked fucose units that decorate a variety of O-glycans, for example, the leukocyte-expressed P-selectin glycoprotein ligand I (PSGL-1). In most inflammatory conditions, E- and P-selectin are major counterreceptors for PSGL-1, but also trans-interactions with L-selectin (CD62L) on passing leukocytes were found to be relevant for mediating secondary capture (3, 4). In lymphoid tissue, particularly in high endothelial venules (HEVs), the predominant ligand entity for L-selectin–mediated rolling is peripheral lymph node addressin (PNAd), a molecular complex of different sialomucins (5). Importantly, only sLe^a with sulfated N-acetylglucosamine on PNAd shows L-selectin binding activity (5). The great variety of different ligands, selectin expression patterns, and relevant posttranslational modifications reflects the precise tissue- and cell type–specific manner of leukocyte recruitment.

Received for publication August 14, 2013. Accepted for publication December 18, 2013.

This work was funded by Interdisziplina¨res Zentrum fu¨r Klinische Forschung Grant SEED 04/12 of the University of Muenster (to K.B.). E.H. is a Wellcome Trust Senior Research Fellow in Basic Biomedical Science (Grant 083942/Z/07/Z).

Experiments were performed by S.B.R., K.B., and C.K.; T.F.T. provided LAM Abs; and K.B. designed the study and analyzed data; and K.B. and R.H. wrote the manuscript.

Address correspondence and reprint requests to Dr. Konrad Buscher, Institute of Physiological Chemistry and Pathobiology, University of Muenster, 48149 Muenster, Germany; or to Dr. Erhard Hohenester, Charité–University of Medicine Berlin, 10117 Berlin, Germany; or to Dr. Thomas F. Tedder, Department of Physiology, Imperial College London, London SW7 2AZ, United Kingdom; or to Department of Anaesthesiology, Intensive Care, and Pain Medicine, University of Muenster, 48149 Muenster, Germany.

Acknowledgments

The online version of this article contains supplemental material.

Abbreviations used in this article: EGF, epidermal growth factor; FOV, field of view; HEV, high endothelial venule; PBL, primary blood lymphocyte; PMN, polymorphonuclear leukocyte; PNAd, peripheral lymph node addressin; PPEM, polyphosphoinositol; PSGL-1, P-selectin glycoprotein ligand 1; SCR, short consensus repeat; sLe^a, sialyl Lewis x (NeuAc–α2,3-Gal–β1,4–(Fuc–α1,3)GalNAc–β1,3); and K_a and K_d (ref) (11). Surprisingly, it was demonstrated that tensile forces enhance selectin-mediated adhesion and stabilize cell rolling by decreasing K_d in low shear conditions (12, 13), promoting the formation of so-called “catch bonds”.

The first study on altered L-selectin receptor function detected affinity changes upon leukocyte activation, but the precise mechanism remained unresolved (14). Domain-swapping experiments suggested a role for the EGF-like domain in ligand binding (15, 16), and crystal structure analysis subsequently revealed a flexible hinge between the N-terminal lectin and EGF-like domain of selectins (17, 18). Whereas sLe^a is bound by a bent conformation of P-selectin, cocystalization with PSGL-1 glycoprotein revealed an extended conformation (17). The transition from the bent to the extended state involves several subdomain movements in the lectin domain (19). One major component of this allosteric pathway is the 83–89 loop that relocates in close vicinity to the ligand-binding interface. Thereby new noncovalent interactions are formed, in-
cluding Glu\textsuperscript{88} ligation to the calcium ion and the PSGL-1 fucose unit, and Arg\textsuperscript{88} binding to a sulfated tyrosine of the PSGL-1 polypeptide. A second sulfate tyrosine is bound by His\textsuperscript{115} in P-selectin. The corresponding residue in L-selectin is alanine, a substitution that partially explains the lower affinity of L-selectin for PSGL-1 (20). To date, L-selectin crystal data are available only for the unbound state (PDB 3CFW), but the high phylogenetic conservation and molecular dynamic simulations suggest fundamentally similar ligand-binding modes for all selectins (21).

Tensile forces acting on a selectin/ligand complex favor the extended conformation, aligning the long axis of receptor with the direction of the force applied (21, 22). It is thought that this property gives rise to catch bonds; however, there is no clear consensus about the underlying mechanism. In the “allosteric model,” pivoting about the EGF–lectin interdomain hinge causes a restructuring of the distal ligand binding interface to a high-affinity conformation (19, 22). In contrast, the “sliding-rebinding” model is based on alignment of the lectin domain with the acting force, thereby enabling repetitive contacts to several carbohydrate epitopes on the same ligand (21, 23).

In all studies on selectin mechanochemistry, only genetically modified receptors have been studied (21, 22, 24). This is due to the fact that, in contrast to, for example, integrins, no reagents are known that modulate specific conformational states of selectins. We discovered that the anti–L-selectin mAbs DREG-55 and LAM1-5 dramatically reduce L-selectin–mediated lymphocyte rolling velocity under shear, consistent with the selective binding to a high-affinity conformation (21). This is due to the need for a new classification of anti-selectin mAbs according on the structure–function relationship. Moreover, our data highlight the concept of heterotropic modulation of selectin affinity and demonstrate how such anti-selectin mAbs can provide further insights on the structure–function relationship. Moreover, our data highlight the need for a new classification of anti-selectin mAbs according to their distinct stimulatory or inhibitory activities.

Materials and Methods

Reagents and Abs

HBSS containing Ca\textsuperscript{2+} and Mg\textsuperscript{2+} was obtained from Invitrogen. DREG-55 and DREG-200 mAb hybridomas were a gift from E. Butcher (Stanford University). DREG-56 (sc-18851) was purchased from Santa Cruz Biotechnology, and the secondary Ab goat anti-human IgG (Fc specific)–FITC (P9512) was from Sigma-Aldrich. Mouse anti-human L-selectin LAM1-1 and LAM1-5 mAbs were collected from ascites fluid (16). Purified human L-selectin–coated PSGL-1–Fc, and E-selectin–Fc chimeras were obtained from R&D Systems. PNA extracts from human tonsils were provided by S. Rosen (University of California, San Francisco). Streptavidin was from Calbiochem and biotinylated sLex-polyacrylamide was from Leuctin (Moscow, Russia). DREG-55 Fab fragments were generated with an IgG Fab purification kit according to the manufacturer’s instructions (Thermo Scientific). SDS-PAGE under reducing and nonreducing conditions confirmed the generation of monovalent Fab and the exclusion of Fc.

Cell isolation and culture

The Jurkat cell line clone E6-1 (American Type Culture Collection) was cultured in RPMI 1640 medium supplemented with 10% FCS and penicillin/streptomycin at 37°C and 5% CO\textsubscript{2}. EDTA-anticoagulated blood was obtained from healthy volunteers and separated by a Ficoll 1.077 and 1.119 g/ml gradient. The isolation yielded >90% viable human primary blood lymphocytes (PBLs) and polymorphonuclear leukocytes (PMNs) as verified by flow cytometry. Isolated cells were kept on ice for a maximum of 4 h.

L-selectin–coated microspheres

Carboxylated polystyrol microspheres (beads, 6 μm diameter) were coupled to protein G using a carbodiimide coupling kit (Polyscience). Human L-selectin–Fc (complete extracellular domain of L-selectin) was incubated with the beads for 1 h at room temperature, subsequently blocked by 2 mg/ml BSA in PBS, and successful coating was verified by flow cytometry. Protein G–coupled beads without addition of L-selectin–Fc did not show any interaction in PSGL-1–coated flow chambers under shear.

Flow cytometry–based affinity assay

Jurkat cells (3 × 10\textsuperscript{6}) were washed in calcium-free PBS and resuspended in HBSS buffer. Then, 10 μg/ml DREG-55 mAb, DREG-200 mAb or PBS (untreated), and 10 μg/ml PSGL-1–Fc or E-selectin–Fc were added and incubated for 10 min at room temperature. After washing, cells were resuspended in ice-cold PBS/1% FCS buffer followed by a 30-min incubation with 1:1000 anti-human IgG-FITC on ice. Flow cytometry was performed and the mean fluorescence intensity was calculated relative to the sample with DREG-200 Ab (negative control).

Laminar flow assays

Bidi μ-slit VI\textsuperscript{0.1} (0.1 mm high) were coated for 2 h at room temperature with 30 μg/ml PSGL-1–Fc or 5 μg/ml E-selectin–Fc, unless otherwise stated, followed by 1 h blocking with 2 mg/ml BSA in PBS. Alternately, PNA was applied overnight at 4°C. For flow chambers on sLex\textsuperscript{0.1}, 10 μg/ml streptavidin coating overnight at 4°C was followed by 20 μg/ml biotinylated sLex-polyacrylamide coating for 2 h at room temperature. Cells (0.3 × 10\textsuperscript{6}/ml) in HBSS were preincubated for 5 min with the indicated Ab at room temperature. For representative video recordings, the Ab was directly added to the distal cell suspension reservoir of the running flow chamber system. Digital videos were recorded with an inverted phase contrast microscope (Axiovert M200; Zeiss) equipped with a CCD camera (ORCA; Hamamatsu) at 37°C and ×10 magnification. Cells were introduced at a high flow rate (>20 dynes/cm\textsuperscript{2}) for 1 min, and the flow rate was subsequently adjusted to the desired shear stress level. After an equilibration of 2 min, at least three different fields of view (FOV) were recorded. Rolling velocities were determined by offline analysis using Fiji (25). For detachment assays, cells were allowed to settle and the flow rate was increased stepwise to 3, 6, and 10 dynes/cm\textsuperscript{2}. After 1 min equilibration, a snapshot of three different FOVs were taken and the number of interacting cells was counted and expressed relative to 1 dynecm\textsuperscript{-2}. PMN string formation was analyzed using 20 μg/ml E-selectin–coated chambers after 3 min equilibration of flow and six adjacent FOVs (∼1300 × 1000 μm\textsuperscript{2}) were recorded. More than three cells aligned in the direction of the flow were considered. Most five cell diameters distance between two cells were considered a string.

Microkinetic velocity measurement

For motion analysis in high-temporal resolution, digital videos were acquired at ×20 or ×40 magnification with 100 frames/s (Neo 5.5 CMOS camera; Andor) at room temperature. The recorded rolling motion was then subjected to an automated frame-by-frame analysis using a snake model–based algorithm in MATLAB (MathWorks). The z-coordinates were translated into step distances and velocities by customized Excel macros. At 0.96 dynecm\textsuperscript{-2} a velocity threshold of 28 μm/s was found suitable for discriminating between load-bearing (deceleration) and breaking (acceleration) bonds in untreated and DREG-55 Ab-treated (10 μg/ml) samples. Measurements were repeated twice in triplicates with 13–16 analyzed cells per group, totaling 68–116 tether events. The logarithmic expression of tethered cells allowed the determination of −K\textsubscript{off} from the slope of the linear regression. Importantly, under the experimental conditions used this value is only an apparent K\textsubscript{d} owing to multibond tethers; however, it can be used for comparison of dissociation kinetics in the same cellular system.

Statistical analysis

The significance of results was determined using the Student t test. A p value <0.05 was considered to be statistically significant.

Results

DREG-55 and LAM1-5 Abs induce L-selectin–mediated slow lymphocyte rolling

L-selectin–dependent rolling was investigated in a parallel plate flow chamber on the immobilized ligand PSGL-1–Fc. Untreated PBLs showed fast and stable rolling at 2 dynes/cm\textsuperscript{2} (Fig. 1A). Anti-human L-selectin mAb DREG-200 blocked any interactions, proving the specificity of rolling (not shown). DREG-55 mAb but not isotype anti-human IgG1 preincubation at saturating concentrations triggered a massive decrease in mean rolling velocity from 113 to 13 μm/s. Fast rolling was directly converted into slow homogeneous rolling within a subsecond period, and hardly any detachment of cells was observed (Supplemental Video 1). Minimal
and maximal velocities measured were 48 and 136 m/s for untreated and 9 and 28 m/s for treated PBLs, respectively, showing a more uniform slow rolling phenotype. To investigate whether this effect includes a cellular response, the assay was repeated using L-selectin–coated microspheres yielding comparable results (Fig. 1A).

FIGURE 1. Anti-human L-selectin Abs of the DREG and LAM family differentially regulate L-selectin–dependent rolling on PSGL-1. (A) The rolling velocity of human PBLs (dashed line) and L-selectin (CD62L)–coated beads (solid line) was investigated in a parallel plate flow chamber on immobilized PSGL-1–Fc. PBLs rolled on 30 μg/ml PSGL-1 at 2 dynes/cm² and beads on 20 μg/ml PSGL-1 at 3 dynes/cm². Anti–L-selectin mAb DREG-55 or isotype anti-human IgG1 (10 μg/ml) was added prior to the experiment. Mean velocities were 112.8 versus 13.3 m/s for PBLs and 36.4 versus 4.8 m/s for beads (untreated versus DREG-55 mAb). Means ± SD are shown of n = 3–4. (B) Jurkat T cells rolling on PSGL-1–Fc at 2 dynes/cm². No interaction indicates full inhibitory activity with cell displacement equal to the hydrodynamic flow. Means ± SD of four experiments. (C) Cumulative frequency histogram of the rolling velocity showing concentration-dependent effects of DREG-55 mAb. Mean velocities are 13.1, 19.5, 39.5, and 111.5 m/s at 10, 1, 0.1, and 0 μg/ml DREG-55 mAb concentration, respectively. There were 32–79 analyzed cells per condition, representative of two independent experiments. (D) Jurkat cells rolling on 30 μg/ml PSGL-1 were subjected to increasing shear forces in a flow chamber. Cells were either left untreated (○) or incubated with 10 μg/ml DREG-55 mAb (●) prior to the experiment. The number of interacting cells was determined for each shear level at 1, 1.5, 3, 6, and 10 dynes/cm² and expressed relatively to 1 dyne/cm². Means ± SD of three experiments. (E) Rolling velocity of Jurkat cells on 20 μg/ml immobilized PSGL-1 at 2 dynes/cm² with or without addition of LAM1-1 or LAM1-5. Means ± SD of three to four experiments, >100 cells analyzed per condition. **p < 0.01, ***p < 0.001.

FIGURE 2. Microkinetic analysis of DREG-55 mAb-induced slow rolling. (A) The impact of the PSGL-1 ligand density on Jurkat cell rolling was determined with and without a saturating amount of DREG-55 mAb. Mean rolling velocities are 117.7 ± 5.8 (30 μg/ml coated ligand), 161.5 ± 17.2 (10 μg/ml), and 225.6 ± 16.8 μm/s (6 μg/ml) and decrease to 25.3 ± 3.7 (30 μg/ml), 26.7 ± 3.7 (10 μg/ml), and 29.9 ± 2.7 μm/s (6 μg/ml) upon 10 μg/ml DREG-55 mAb treatment. Data show mean ± SD of n = 3 with at least 30 cells analyzed per experiment. (B and D) High-resolution velocity profile of Jurkat cells rolling on low-density PSGL-1 recorded at 100 frames/s. A representative 2-s time course of an individual untreated (B) and DREG-55 Ab-treated (D) Jurkat cell rolling at 0.96 dyne/cm² on 6 μg/ml immobilized PSGL-1 is shown. (C) Comparative k_{off} values were calculated from (B) and (D) as described in Materials and Methods. **p < 0.01, ***p < 0.001.
A similar phenotype was observed using human primary PMNs (not shown) and the human T lymphocyte cell line Jurkat (Fig. 1B). Importantly, monovalent DREG-55 mAb Fab fragment was sufficient to reduce rolling velocity, excluding Fc receptor participation and dimerization effects. Additional DREG-200 mAb, EDTA, or KPL-1 mAb (blocking anti-human PSGL-1 mAb) treatment blocked the slow rolling interaction and induced detachment showing the L-selectin–dependent nature of this phenomenon (Fig. 1B). DREG-55 Ab titration revealed a concentration-dependent effect, with 10 μg/ml yielding the lowest velocity (Fig. 1C). A further increase up to 100 μg/ml did not significantly alter rolling (not shown).

To investigate DREG-55 Ab implications on shear resistance, Jurkat cells were allowed to settle onto a PSGL-1–Fc-coated flow chamber in the presence or absence of DREG-55, followed by a stepwise increase of shear, and the number of rolling cells at each shear level was measured. The proportion of slow rolling Jurkat cells in the presence of DREG-55 mAb was reduced with increasing shear compared with untreated cells (Fig. 1D). In a range from 1 to 6 dynes/cm² no significant change in the rolling flux of untreated cells was detected, whereas DREG-55 Ab treatment promoted detachment already at 1.5 dynes/cm², with complete abolishment of rolling at 6 dynes/cm². Similarly, the capture rate was significantly decreased by DREG-55 mAb (not shown).

It was reported previously that monoclonal anti-human L-selectin Abs of the LAM family showed stimulatory L-selectin binding activity in a soluble binding assay using polyphosphomonoester (PPME) polysaccharide or fucoidan (16, 26). Indeed, LAM1-5 Ab significantly reduced the rolling velocity of Jurkat cells on PSGL-1 by 36% whereas another member of this family, LAM1-1, exerted full inhibitory function (Fig. 1E).

**Bond dissociation rates are decreased by DREG-55 mAb**

Because ligand density is a crucial parameter affecting rolling mechanics, we investigated the impact of increased ligand spacing. Dilution of immobilized PSGL-1–Fc revealed 6 μg/ml to be the minimal coating concentration required to support rolling at 2 dynes/cm². A significant increase in rolling velocity was already observed at 10 μg/ml but more accentuated at 6 μg/ml PSGL-1–Fc using untreated Jurkat cells. However, DREG-55 Ab treatment triggered similar slow rolling velocities at all densities of PSGL-1–Fc tested (Fig. 2A), suggesting decreased bond dissociation.

Next, microkinetic analysis on low-density ligand was performed at high temporal resolution (100 frames/s, Supplemental Video 2). Fig. 2B depicts a representative velocity profile of an individual untreated Jurkat cell during 2 s at 0.96 dyne/cm². Regular cell decelerations with intermitting accelerations from 6 to 300 μm/s were observed. Notably, at the applied shear stress and frame rate, no complete cell arrest was detected. The addition of DREG-55 mAb dramatically altered the microkinetics of L-selectin–dependent rolling. Enduring slow motions ranging from complete stop to 15 μm/s were interrupted by short forward movements at medium velocity (Fig. 2D). Although the velocity patterns most likely represent multibond interactions in this experimental setup, a comparative $K_{off}$ was determined on the basis of tether durations as described in Materials and Methods, revealing an almost 10-fold increase (Fig. 2C). Step distances were markedly reduced from 20.9 to 1.2 μm upon DREG-55 mAb treatment (not shown).

**FIGURE 3.** DREG-55 mAb-induced slow rolling is ligand specific. Flow chamber assays were performed with Jurkat cells at 2 dynes/cm² using different immobilized L-selectin ligands. Numbers on top of the bars indicate the mean rolling velocity. No interaction indicates full inhibitory activity of DREG-55 mAb. DREG-200 Ab blocked all interactions (not shown); $n$ = 3–4, means ± SD.

**FIGURE 4.** DREG-55 mAb increases force-free affinity of L-selectin. Jurkat cells were incubated with human PSGL-1–Fc or E-selectin–Fc in the presence of DREG-55 or DREG-200 mAb and the fluorescence of the secondary anti-human IgG-FITC Ab was measured by flow cytometry. In untreated samples, only soluble ligand and secondary Ab but no DREG Ab were added. Mean fluorescence intensity is expressed relative to DREG-200 mAb samples. Means ± SD of three to four experiments. *$p < 0.05$, ***$p < 0.001$.

**FIGURE 5.** PMN string formation via L-selectin–PSGL-1 interactions is enhanced by DREG-55 mAb. Flow chambers were coated with 20 μg/ml E-selectin, and human PMN string formation was analyzed at 1 dyne/cm² as described in Materials and Methods. (A) Typical readouts for untreated and DREG-55 and DREG-200 mAb-treated PMNs under flow are shown. The arrow indicates the direction of the flow. (B) Determination of the number of strings. Five FOVs were averaged per experiment. Mean ± SD, $n$ = 3–4. (C) Histogram showing the average lengths of all strings in the untreated ($n$ = 60) and DREG-55–treated ($n$ = 89) condition. *$p < 0.05$. 

**ALLOSTERIC SELECTIN Abs**

To investigate DREG-55 Ab implications on shear resistance, Jurkat cells were allowed to settle onto a PSGL-1–Fc-coated flow chamber in the presence or absence of DREG-55, followed by a stepwise increase of shear, and the number of rolling cells at each shear level was measured. The proportion of slow rolling Jurkat cells in the presence of DREG-55 mAb was reduced with increasing shear compared with untreated cells (Fig. 1D). In a range from 1 to 6 dynes/cm² no significant change in the rolling flux of untreated cells was detected, whereas DREG-55 Ab treatment promoted detachment already at 1.5 dynes/cm², with complete abolishment of rolling at 6 dynes/cm². Similarly, the capture rate was significantly decreased by DREG-55 mAb (not shown).

It was reported previously that monoclonal anti-human L-selectin Abs of the LAM family showed stimulatory L-selectin binding activity in a soluble binding assay using polyphosphomonoester (PPME) polysaccharide or fucoidan (16, 26). Indeed, LAM1-5 Ab significantly reduced the rolling velocity of Jurkat cells on PSGL-1 by 36% whereas another member of this family, LAM1-1, exerted full inhibitory function (Fig. 1E).

**Bond dissociation rates are decreased by DREG-55 mAb**

Because ligand density is a crucial parameter affecting rolling mechanics, we investigated the impact of increased ligand spacing. Dilution of immobilized PSGL-1–Fc revealed 6 μg/ml to be the minimal coating concentration required to support rolling at 2 dynes/cm². A significant increase in rolling velocity was already observed at 10 μg/ml but more accentuated at 6 μg/ml PSGL-1–Fc using untreated Jurkat cells. However, DREG-55 Ab treatment triggered similar slow rolling velocities at all densities of PSGL-1–Fc tested (Fig. 2A), suggesting decreased bond dissociation.

Next, microkinetic analysis on low-density ligand was performed at high temporal resolution (100 frames/s, Supplemental Video 2). Fig. 2B depicts a representative velocity profile of an individual untreated Jurkat cell during 2 s at 0.96 dyne/cm². Regular cell decelerations with intermitting accelerations from 6 to 300 μm/s were observed. Notably, at the applied shear stress and frame rate, no complete cell arrest was detected. The addition of DREG-55 mAb dramatically altered the microkinetics of L-selectin–dependent rolling. Enduring slow motions ranging from complete stop to 15 μm/s were interrupted by short forward movements at medium velocity (Fig. 2D). Although the velocity patterns most likely represent multibond interactions in this experimental setup, a comparative $K_{off}$ was determined on the basis of tether durations as described in Materials and Methods, revealing an almost 10-fold increase (Fig. 2C). Step distances were markedly reduced from 20.9 to 1.2 μm upon DREG-55 mAb treatment (not shown).
DREG-55 mAb–induced slow rolling is ligand specific

Physiological L-selectin ligand epitopes vary considerably. Whereas a crucial binding motif in PSGL-1 includes the sulfated tyrosine residues of the protein scaffold in addition to an adjacent core 2–based O-glycan capped with sLeα (27), binding to HEV-expressed PNAd depends on carbohydrates from sLeα with sulfated N-acetylgalcosamine (5).

Im mobilized PSGL-1–Fc, E-selectin–Fc, PNAd, and sLeα supported specific L-selectin–mediated rolling of Jurkat cells (Fig. 3). However, only PSGL-1–Fc and E-selectin–Fc were sufficient to allow DREG-55–induced slow rolling, whereas on PNAd and sLeα all rolling cells detached. In contrast, DREG-200 Ab blocked rolling on all ligands (not shown). A similar selective modulation of rolling on PSGL-1–Fc and E-selectin–Fc was obtained using DREG-55 mAb Fab fragments (not shown). These results indicate that specific ligand requirements for DREG-55 mAb-induced L-selectin–mediated slow rolling are met by PSGL-1 and E-selectin but not by sLeα or PNAd.

Shear-free affinity of L-selectin is upregulated by DREG-55 Ab

To directly address the question of force-free (shear-independent) affinity modulation, a flow cytometry–based assay for ligand affinity was performed. Jurkat cells were incubated with or without mouse anti-human DREG-55 or DREG-200 Ab together with soluble human PSGL-1–Fc or E-selectin–Fc chimeric proteins, and the specific binding of a secondary anti-human IgG-FITC Ab was detected. Samples with the blocking DREG-200 Ab served as negative control. Because L-selectin bonds are characterized by low-affinity interactions (28), the binding of soluble E-selectin–Fc or PSGL-1–Fc to untreated Jurkat cells was absent under these experimental conditions (Fig. 4). DREG-200 mAb did not induce significant changes relative to untreated samples excluding possible cross-reactions of the anti-human IgG Ab to mouse DREG-Ig or of DREG Abs to cellular Fc receptors. In contrast, coincubation of Jurkat cells with DREG-55 mAb led to a 1.6- and 1.4-fold higher binding of soluble PSGL-1–Fc and E-selectin–Fc, respectively. This result suggests that the treatment of Jurkat cells with DREG-55 mAb exposes a higher affinity binding site on the L-selectin molecule in the absence of shear (Fig. 4).

Secondary capture of PMNs is amplified by DREG-55 mAb

Intercellular interactions between free-flowing and adherent neutrophils occur via transient PSGL-1–L-selectin bonds and serve as a mechanism to boost neutrophil recruitment at inflammatory sites (“secondary capturing”) (29). Using isolated PMNs under flow, the formation of cells lined up in the direction of the flow (strings) can be observed in vitro. Because L-selectin shows a higher affinity to PSGL-1 in the DREG-55 mAb modified state (Fig. 4), we investigated whether there is a functional relevance in secondary capture. Therefore, untreated, DREG-55 or DREG-200 mAb Fab fragment–treated human PMNs were perfused into an E-selectin–coated flow chamber at 1 dyne/cm². An average of approximately two strings per FOV was found in untreated conditions, and addition of DREG-200 mAb Fab abolished most of the secondary but not primary capture (Fig. 5A, 5B). In contrast, DREG-55 mAb Fab treatment intensified string formation and yielded longer strings (Fig. 5B, 5C).

**FIGURE 6.** Mapping of DREG Ab epitopes onto the N-terminal lectin domain. (A) Sequence alignment of the N-terminal portion of human selectins. The four switch regions in the lectin domain that undergo conformational changes upon selectin extension (19) are underlined and labeled S1–S4. Residues involved in PSGL-1 binding are indicated in orange (PSGL-1 polypeptide) and magenta (PSGL-1 glycan). Residues involved in calcium binding are marked by cyan circles. Residues whose mutation abolishes mAb DREG-55 and DREG-200 binding (30) are indicated in blue and green, respectively. (B) Drawings of P-selectin crystal structures in the bent (left panel) and extended (right panel) conformation (17). The lectin domain is at the top and the EGF-like domain is at the bottom. The pivot at the interdomain hinge is indicated by a filled black triangle and switch regions S2 and S3 are labeled. The calcium ion is shown as a cyan sphere. The PSGL-1 ligand is shown in orange (polypeptide) and magenta (glycan). The two sulfated tyrosines are shown in atomic detail. Arg85, which reorients dramatically upon PSGL-1 binding, is shown in red. Residues whose mutation abolishes DREG-55 and DREG-200 mAb binding are indicated by blue and green Ca spheres, respectively. The putative DREG-55 mAb epitope in the extended conformation is indicated by a blue oval.
**DREG-55 mAb targets switch regions of L-selectin**

Previous random mutagenesis of human L-selectin had identified the amino acid positions 11, 56, 89, 105, 107, and 111 as critical for DREG-55 mAb binding (30) (Fig. 6A). Because only the bent conformation of L-selectin has been crystallized to date, we mapped these positions onto the bent and extended conformations of P-selectin (Fig. 6B). Three of the critical residues (105, 107, 111) surround the calcium binding site and are unlikely to be involved directly in DREG-55 mAb binding. Residues 55, 87, and 89 (and possibly 11) form a plausible convex epitope. Interestingly, these residues are located in the two major switch regions that allosterically couple selectin extension to increased affinity for ligand (19). DREG-200 mAb interacts with a distant region unaffected by conformational changes at L-selectin residues 45, 46, and 47 (30), and the latter is known to interact with the sulfated Tyr7 (Tyr48 of the propeptide) of PSGL-1, thus explaining its blocking activity (Fig. 6B) (31).

**Discussion**

Selectin-mediated tethering and rolling under shear is dependent on multiple factors, for example, receptor-binding kinetics. However, the role of different selectin conformations in bond stabilization is not yet fully understood. To this end, we analyzed the allosteric impact of different Abs on selectin rolling with the conclusion that 1) selectin-mediated rolling can be regulated by exogenous soluble molecules without directly interfering with the binding site for the ligand PSGL-1; 2) the anti–L-selectin mAbs LAM1-5 and DREG-55 show such activity, the latter presumably by enforcing a loop reorientation in the lectin domain; and 3) a high-affinity conformation of L-selectin can be adopted in shear-free conditions, supporting the allosteric model of catch bonds.

L-selectin is well known as a signaling molecule (32, 33). Hence, the increase in L-selectin–mediated ligand binding could result from outside–in signaling events leading to reorganization of adhesion receptors on the leukocyte surface (34). However, several lines of evidence argue against this in DREG-55 Ab-induced slow rolling. First, anti–L-selectin or anti–PSGL-1 blocking Abs abolish lymphocyte interaction in both DREG-55 mAb-treated and untreated samples completely (Fig. 1B) excluding the involvement of integrins or other adhesion molecules. Second, K. Buscher’s unpublished observations demonstrate that energy depletion by azide and 2-deoxy-glucose in Jurkat cells does not impair the observed phenotype. Third, neither the use of Fab fragments (Fig. 1B) nor confocal imaging (not shown) supported a role for receptor dimerization or clustering, respectively. Finally, slow rolling was also inducible using microspheres coated with the extracellular domain of human L-selectin (Fig. 1A). We therefore conclude that the observed findings are best explained by an effect of DREG-55 mAb on an intrinsic property of the L-selectin molecule and not by avidity or signaling-dependent effects.

Analysis of the DREG-55 mAb binding site and investigation of its functional impact provide some information on the underlying structural mechanisms governing the slow rolling phenotype. It is known that sLe^\* binds to P-selectin in the bent state whereas the ligand PSGL-1 triggers the extended conformation, thereby enabling additional bonds to sulfated tyrosines of the PSGL-1 backbone (27, 35). Because the 83–88 loop of the L-selectin lectin domain is targeted by DREG-55 mAb (Fig. 6) and this loop binds the Tyr7 residue of PSGL-1 only in the extended conformation (17), we hypothesized that this loop movement is important for the observed increase of L-selectin affinity to PSGL-1. Flow chamber assays using different ligands support this hypothesis. DREG-55 Ab decelerated L-selectin–mediated leukocyte rolling only on PSGL-1 and E-selectin, whereas on PNAd and sLe^\* it exerted blocking functions (Fig. 3). This differential support of DREG-55 mAb on rolling indicates that specific structural requirements of the ligand are necessary to stabilize the bonds to the DREG-55 mAb-enforced selectin conformation, for example, sulfated tyrosines that are available on PSGL-1 but not on sLe^\*. Despite the use

**Table I. Compilation of published and our own data on anti-human selectin mAbs binding the N-terminal lectin domain and the relation of target epitopes to relevant conformational switch regions as defined by Springer (19)**

<table>
<thead>
<tr>
<th>mAb Clone</th>
<th>Selectin</th>
<th>Epitope Residues</th>
<th>Switch</th>
<th>Action</th>
<th>Ligand</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP-5C7, 12B6, BBIG-E6</td>
<td>E/P</td>
<td>21, 22, 23, 119, 120</td>
<td>S3</td>
<td>X</td>
<td>sLe^*</td>
<td>(44, 45)</td>
</tr>
<tr>
<td>C215</td>
<td>P</td>
<td>76–83</td>
<td>S3</td>
<td>X</td>
<td>sLe^*</td>
<td>(46)</td>
</tr>
<tr>
<td>TQ-1</td>
<td>L/E</td>
<td>ND</td>
<td></td>
<td>X</td>
<td>PSGL-1</td>
<td>(16)</td>
</tr>
<tr>
<td>LAM1-1</td>
<td>L</td>
<td>ND</td>
<td></td>
<td>X</td>
<td>HEV, PPME</td>
<td>(16, 26), this study</td>
</tr>
<tr>
<td>LAM1-7, 8, 9, 10, 11, 12, 13</td>
<td>L</td>
<td>ND</td>
<td></td>
<td>X</td>
<td>PPME</td>
<td>(16, 26)</td>
</tr>
<tr>
<td>LAM1-5</td>
<td>L</td>
<td>ND</td>
<td></td>
<td>X</td>
<td>HEV, PPME, PSGL-1</td>
<td>(16, this study)</td>
</tr>
<tr>
<td>LAM1-6</td>
<td>L</td>
<td>ND</td>
<td></td>
<td>X</td>
<td>HEV</td>
<td>(16, 26)</td>
</tr>
<tr>
<td>LAM1-108, 110, 115, 116, 120</td>
<td>L</td>
<td>ND</td>
<td></td>
<td>X</td>
<td>HEV</td>
<td>(33)</td>
</tr>
<tr>
<td>DREG-55</td>
<td>L</td>
<td>11, 56, 87, 89, 105, 107, 111</td>
<td>S1, 3</td>
<td>X</td>
<td>Intercellular, PSGL-1, E-selectin, HEV, PNAd, sLe^*</td>
<td>(30, 41), this study</td>
</tr>
<tr>
<td>DREG-56</td>
<td>L</td>
<td>ND</td>
<td></td>
<td>X</td>
<td>PPME</td>
<td>(X)</td>
</tr>
<tr>
<td>DREG-200</td>
<td>L</td>
<td>45, 46, 47</td>
<td></td>
<td>X</td>
<td>PPME, HEV, PSGL-1, E-selectin, PNAd, sLe^*</td>
<td>(30, 41), this study</td>
</tr>
</tbody>
</table>

Known binding sites and differential ligand interactions are indicated as previously published. Depending on the respective column, X means either inhibitory (I), stimulatory (S), or nonfunctional (NF). (X) means the same effect but weak.
of Fab fragments, we cannot exclude that steric hindrance is responsible for the inability of Ab-occupied L-selectin to adhere to immobilized PNAd or sLeX\(^\text{a}\). Furthermore, note that selectin beads to sulfated tyrosines alone are not sufficient for high-affinity binding but rather require additional bonds to adjacent carbohydrate units (31).

In humans, E-selectin is an L-selectin ligand of unknown physiological relevance (36, 37). Because E-selectin also supports DREG-55 Ab-induced slow rolling, with a similar reduction in velocity of rolling (74%) as observed with cells rolling on PSGL-1 (Fig. 3), it is tempting to speculate on a common ligand motif in both E-selectin and PSGL-1. However, the absence of detailed functional and structural data from E-selectin–L-selectin bonds prevents investigation of this possibility.

An important question is how the increased selectin affinity under static conditions induced by DREG-55 mAb (Fig. 4) translates to lower shear resistance (Fig. 1D). A similar counterintuitive behavior was described for the A28H mutant of P-selectin (24). This mutation filled a structural cleft within the lectin domain favoring the extended state with high-affinity binding to PSGL-1. Similar to our assumptions on DREG-55 mAb, a predicted allosteric change was the movement of the 83–89 loop. Yeast expressing this mutant also showed a decreased rolling velocity on immobilized PSGL-1–Fc compared with wild-type P-selectin but detached faster at higher shear forces. It was hypothesized that this behavior might result from a slower interconversion between bent and extended states in the mutant. These data are very similar to those obtained in the present study, supporting the allosteric model of selectin mechanochemistry with a two-state conformational equilibrium (24). We postulate that DREG-55 mAb binds specifically to the extended conformation of L-selectin and thereby increases the equilibrium constant in a concentration-dependent manner (Fig. 2C), resulting in enhanced ligand binding in shear-free conditions. An analogous mechanism is well documented for activating anti-integrin Abs (38–40).

The DREG family of Abs was raised by Kishimoto et al. (41) against a downregulated (DREG) adhesion molecule on PMA-activated human lymphocytes that blocked L-selectin binding in frozen section assays of lymphoid tissue. DREG-55, DREG-56, and DREG-200 Abs have been widely employed as blocking Abs ever since. The fact that DREG-55 mAb indeed blocks L-selectin binding to HEV-expressed PNAd and that mAb-induced slow rolling on PSGL-1 becomes unstable with increasing shear could explain why the allosteric activity has been overlooked for many years. Although, to our knowledge, our data provide the first detailed description on activating anti-selectin mAbs under flow, evidence for other such mAbs has been reported previously, but without recognition of their role in mechanochemistry. Shear-free affinity assays showed higher binding signals for the artificial L-selectin ligands PPME and fucoidin in the presence of LAM-1 or LAM-1-5 (16, 26). Our data indicate that LAM-1-5 but not LAM-1-1 mAb modulates L-selectin–mediated rolling on PSGL-1 under physiological shear (Fig. 1E). Published data and our own findings on anti-human selectin mAbs targeting the lectin domain are summarized in Table I.

The investigation of the complex affinity regulation of heterodimeric integrins was greatly facilitated by the use of conformation-sensitive Abs. Extensive work on functional residue mapping provided valuable insights on intramolecular remodeling and served as useful reagents to analyze mechanisms of action (42, 43). Nonfunctional, stimulatory/activation-specific and inhibitory categories classify integrin Abs according to their molecular activity. Precise knowledge about Ab effects is of high relevance, as it can shed light on the structure–function relationship of selectins and the wrong choice might adversely affect the interpretation of experimental data. In light of the results presented in this study, we propose a similar classification system for selectin Abs.

Our data indicate that L-selectin–mediated rolling can be regulated by exogenous molecules that do not block ligand recognition. This may be explained by the “allosteric pathway,” whereby alterations at sites distant to the ligand trigger changes in the binding interface through “transmission of allostery” (19, 24) and implies important functional consequences. We show that DREG-55 mAb renders more effective secondary tethering of PMNs (Fig. 5), a mechanism highly important for inflammatory neutrophil recruitment in vivo (29). It is therefore conceivable to develop small-molecule probes that induce or inhibit specific selectin conformations as a new therapeutic approach to modulate inflammation or lymphocyte homeostasis.

Acknowledgments
We are grateful to Klaus Ley for general advice, Steven Rosen for providing PNAd, Yaroslav Tsytsyura for technical support with microkinetic measurements, and Lydia Sorokin for critical reading of the manuscript.

Disclosures
The authors have no financial conflicts of interest.

References


SUPPLEMENTAL DATA

Suppl. Video 1

Induction of slow L-selectin mediated rolling by DREG-55 mAb.

Jurkat cells show L-selectin mediated rolling at steady state on immobilized PSGL-1 in a parallel plate flow chamber at 1.5 dyn/cm². 10 µg/ml anti-L-selectin antibody DREG-55 was added to the flow buffer without interruption of the flow. Magnification 10×. Detailed analysis of the rolling velocity is shown in figures 1 and 2 and in suppl. video 2.
Suppl. Video 2

L-selectin-mediated rolling in high temporal resolution

Untreated (top) and 10 µg/ml DREG-55 mAb treated (bottom) Jurkat cells rolling on 6 µg/ml immobilized PSGL-1 at 0.96 dyn/cm². Time-lapse video with a total duration of 1000 ms recorded at 100 fps and at 40× magnification. Scale bar shows 20 µm. Automatic cell tracking was performed as indicated in materials and methods and the velocity analysis is shown in figure 2.