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The α(1,3)fucosyltransferases FucT-IV and FucT-VII Exert Collaborative Control over Selectin-Dependent Leukocyte Recruitment and Lymphocyte Homing

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Summary

E-, P-, and L-selectin counterreceptor activities, leukocyte trafficking, and lymphocyte homing are controlled prominently but incompletely by α(1,3)fucosyltransferase FucT-VII-dependent fucosylation. Molecular determinants for FucT-VII-independent leukocyte trafficking are not defined, and evidence for contributions by or requirements for other FucTs in leukocyte recruitment is contradictory and incomplete. We show here that inflammation-dependent leukocyte recruitment retained in FucT-VII deficiency is extinguished in *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ mice. Double deficiency yields an extreme leukocytosis characterized by decreased neutrophil turnover and increased neutrophil production. FucT-IV also contributes to HEV-born L-selectin ligands, since lymphocyte homing retained in *FucT-VII*$^{-/-}$ mice is revoked in *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ mice. These observations reveal essential FucT-IV-dependent contributions to E-, P-, and L-selectin ligand synthesis and to the control of leukocyte recruitment and lymphocyte homing.

Introduction

Blood leukocytes emigrate to extravascular compartments via a process requiring several molecules acting in carefully orchestrated temporal sequence (Vestweber and Blanks, 1999). Leukocytes are captured, decelerate, and engage in shear flow-dependent rolling on the endothelial surface. Rolling authorizes activation of the leukocyte, fosters its firm adhesion to the endothelium, and thus enables transendothelial leukocyte migration. Adhesive interactions that mediate leukocyte rolling are negotiated by the selectins and their counterreceptors (Vestweber and Blanks, 1999).

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Leukocyte E- and P-selectin counterreceptors PSGL-1, ESL-1, CD24, and L-selectin maintain optimal counterreceptor function only when properly modified by glycans (Lowe, 1997). L-selectin-dependent recognition of PSGL-1 and other ligands expressed by neutrophils previously recruited to the endothelial surface may also be glycan dependent (Vestweber and Blanks, 1999). L-selectin ligands expressed by the endothelial cell proper also affect leukocyte recruitment, though these ligands and their glycosylation requirements are not defined (Vestweber and Blanks, 1999). L-selectin is also an essential participant in homing of lymphocytes to lymph nodes and Peyer's patches. Lymphocyte homing requires adhesion to properly glycosylated HEV-born L-selectin counterreceptors like GlyCAM-1 and CD34 (Hemmerich and Rosen, 2000).

Glycans that contribute to E-, P-, and L-selectin counterreceptor activity are constructed by glycosylation reactions in which terminal steps are catalyzed by α(1,3)fucosyltransferases (FucTs). Of six known FucTs, two (FucT-IV and FucT-VII) are expressed in leukocytes and represent candidates for controlling leukocyte selectin ligand activity (Lowe, 1997), with the caveats that other unknown FucTs may be expressed in leukocytes and could contribute to selectin ligand activity and that fucose-independent selectin ligands have been described (Lowe, 1997). FucT-VII has been assigned a major role in this process, as FucT-VII null mice exhibit profound defects in leukocyte E- and P-selectin counterreceptor activity and HEV-born L-selectin ligand activity (Malý et al., 1996). Prominent selectin ligand activities remain in the absence of FucT-VII; however, a requirement for fucosylation in such activities has not been defined, and the identities and contributions of other genes that determine this additional level of control have been unknown.

FucT-IV has had an uncertain role in the control of selectin ligand activity (Lowe, 1997). It is not known if FucT-IV can contribute to the synthesis of HEV-derived L-selectin ligands or even if it is expressed by HEV, and the ability of FucT-IV to direct expression of E- and P-selectin ligands in cultured cells is an unpredictable function of the host's glycosylation phenotype (Goelz et al., 1990, 1994; Li et al., 1996; Wagers et al., 1997; Snapp et al., 1997). A biochemical explanation for these observations is not available. Though the leukocytes in *FucT-IV*$^{-/-}$ mice exhibit a subtle increase in rolling velocity in noninflamed dermal microvasculature (Weninger et al., 2000), it is not clear if the glycosylation phenotype of leukocytes, in vivo, will authorize FucT-IV-dependent synthesis of selectin ligands relevant to leukocyte trafficking.

To define the physiological participation of FucT-IV in selectin ligand synthesis and in the control of leukocyte trafficking, we have constructed and characterized mice deficient in FucT-IV and in both FucT-IV and FucT-VII. These studies expose a FucT-VII-independent role for FucT-IV in selectin-dependent leukocyte recruitment, since neutrophils in the doubly null mice, unlike FucT-VII null leukocytes, are virtually devoid of E- and P-selec-

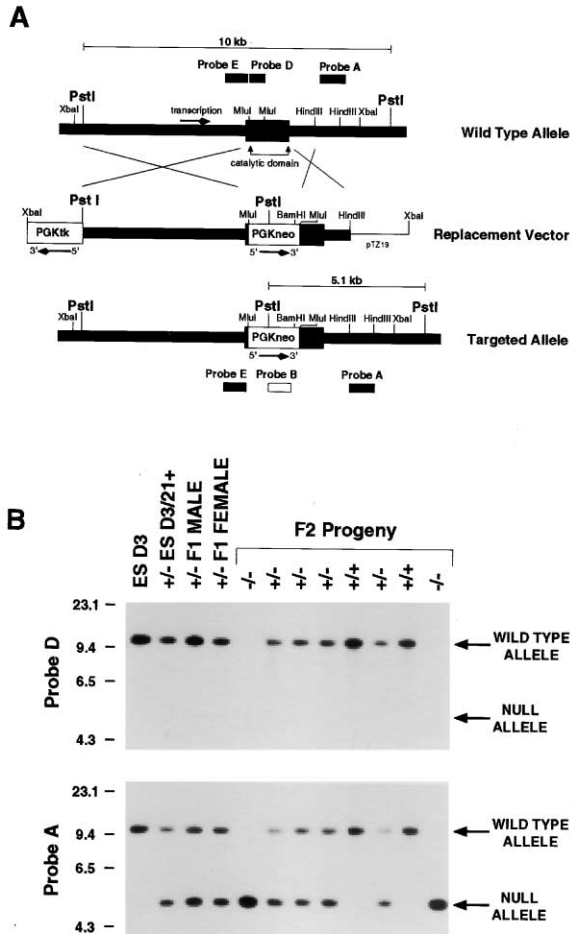

Figure 1. Targeted Disruption of the *FucT-IV* Locus

(A) Structure of WT and mutant *FucT-IV* loci. A G418 resistance segment (PGKneo) replaces an MluI segment corresponding to an essential part of FucT-IV's catalytic domain (a thick solid line denotes the single coding exon; Gersten et al., 1995). A thymidine kinase resistance cassette (PGKtk) is proximal to the 5′ PstI site in a SalI site in the targeting vector plasmid pTZ18 (data not shown).

(B) Southern blot analysis of WT and mutant *FucT-IV* loci. Blots were prepared using PstI-digested DNA from WT ES cells (lane ES D3) from a *FucT-IV*$^{+/-}$-targeted ES cell (lane ES D3/21+), from *FucT-IV*$^{+/-}$ progeny of a chimeric male derived from ES clone D3/21+ (lanes F1 MALE and F1 FEMALE), and from progeny of these two animals (F2 Progeny). Blots were hybridized with probe D (top; null allele is invisible to probe D), stripped, and rehybridized with probe A (bottom). Arrows denote fragments from WT or null alleles. Fragment sizes in kb are at left.

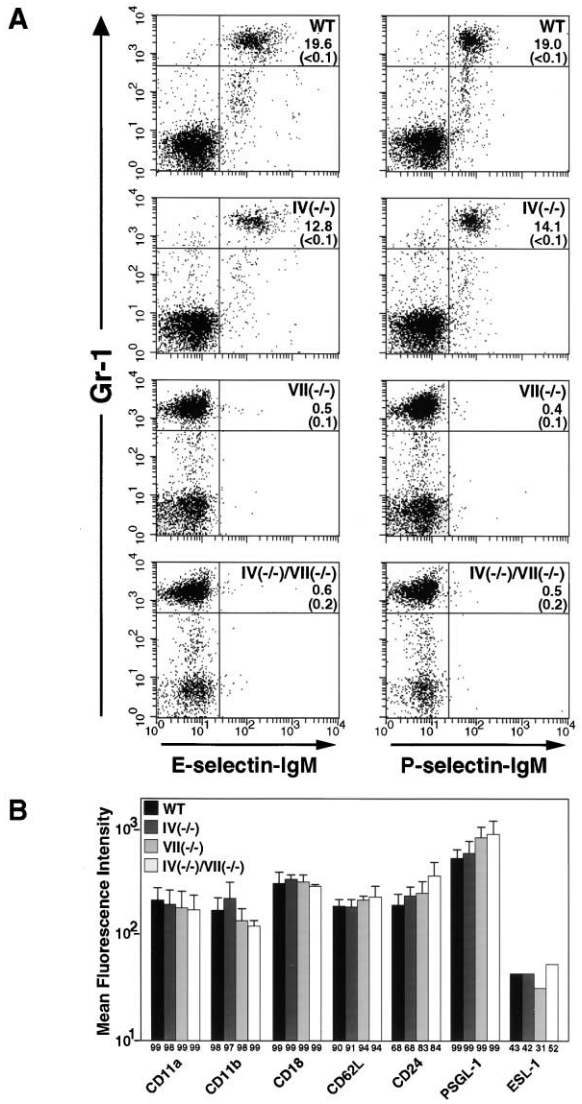

Figure 2. Flow Cytometry Analysis of Neutrophil Selectin Ligand Expression

(A) Selectin-IgM chimera binding. Blood leukocytes stained with the granulocyte-selective anti-Gr-1 antibody were incubated with an E- or P-selectin-IgM chimera and then with fluoresceinated anti-IgM and subjected to flow cytometry analysis as described (Malý et al., 1996). Upper right-hand quadrant numbers denote the fraction of all leukocytes that are positive for both Gr-1 and for E- or P-selectin ligands in the absence or presence (in parentheses) of 1 mM EDTA. Gr-1^{lo} cells are F4/80^{+} and Ly6G^{-}, identifying them as monocytes (data not shown).

(B) Expression of leukocyte adhesion molecules by Gr-1-positive cells. Blood leukocytes stained with Gr-1 were incubated with antibodies specific for the molecules indicated below the panel. Fluoresceinated reagents and flow cytometry analysis were used to detect the anti-leukocyte antibodies. Numbers below the histograms denote the fraction of Gr-1-positive cells that stain above thresholds set with negative control antibodies. Histogram bar height corresponds to the mean fluorescent intensities of these positive cells (±SEM; n ≥ 3 experiments, except for ESL-1, where n = 1).

tin ligand activities and the ability to emigrate in acute inflammation. FucT-IV also contributes to HEV-derived L-selectin ligands, since such ligands from doubly null mice, unlike FucT-VII null ligands, do not support lymphocyte homing. These observations assign an in vivo role to FucT-IV in leukocyte recruitment and lymphocyte homing.

Results

Disruption of the Mouse FucT-IV Locus

Male chimeras derived from targeted ES cell clones (Figure 1) generated *FucT-IV*$^{+/-}$ progeny. Intercrosses of heterozygous progeny yielded litters of normal size with Mendelian transmission of the null allele. *FucT-IV*$^{-/-}$ mice are fertile, yield normal litter sizes, are healthy, exhibit grossly normal behavior, maintain a normal life span, and are not susceptible to endogenous microbes

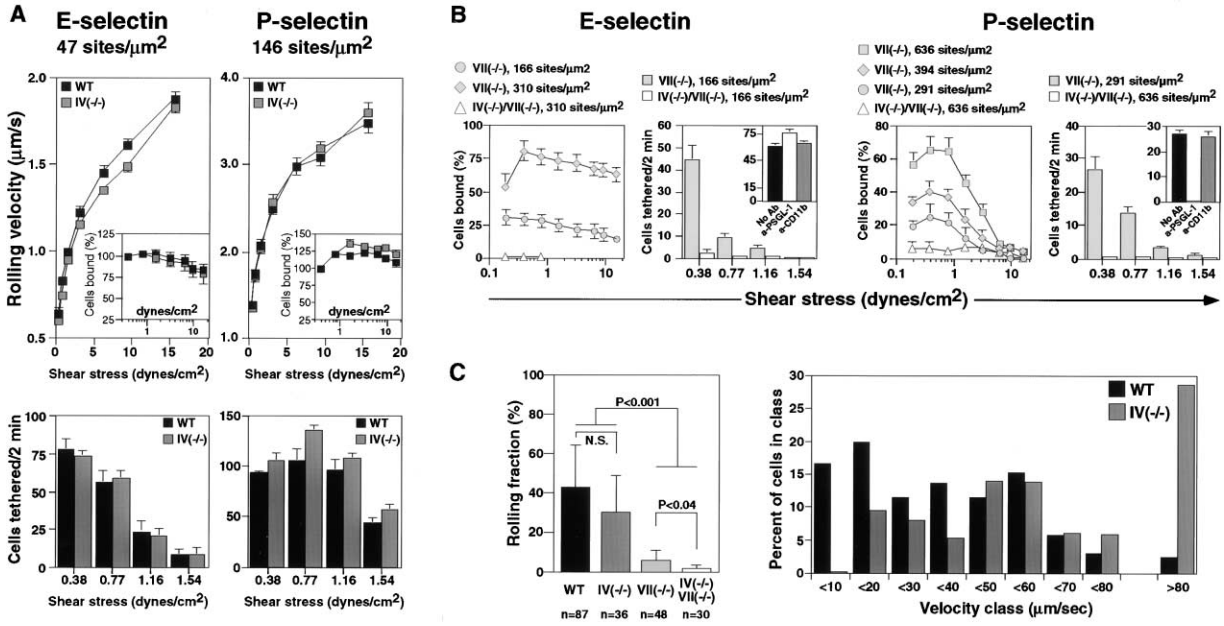

Figure 3. Selectin-Dependent Neutrophil Rolling In Vitro

(A) Rolling adhesion of blood neutrophils from WT and *FucT-IV*$^{-/-}$ mice. Rolling velocity data (upper graphs) are derived from two to four runs of each genotype cells and are representative of data obtained in at least three separate experiments (different cells and different plates). The number of cells (from multiple experiments) analyzed at any given shear stress ranged from 137 to 508. The percent cells bound data (upper graph inset) is inclusive of two to four runs from two (P-selectin) or three (E-selectin) separate experiments. Tethering data (lower graphs) are inclusive of two to four runs from three separate experiments. Statistical analyses disclosed no significant difference in any parameter between WT and *FucT-IV*$^{-/-}$ cells at any shear stress. At the substrate densities used, tethering and rolling of *FucT-VII*$^{-/-}$ and *FucT-IV*$^{-/-}$*/FucT-VII*$^{-/-}$ cells did not occur at any shear stress.

(B) Rolling adhesion of blood neutrophils from *FucT-VII*$^{-/-}$ and *FucT-IV*$^{-/-}$*/FucT-VII*$^{-/-}$ mice. Percent cells bound (left) and tethering efficiencies (right) data are inclusive of two to three runs of each genotype, through the range of shear stresses, from two separate experiments. Insets depict the results of tethering experiments conducted in the presence of anti-mouse PGSL-1 (4RA10) or anti-mouse CD11b monoclonal antibodies.

(C) Leukocyte rolling in venules of the exteriorized cremaster muscle. Leukocyte rolling fractions (left) and the frequency distribution of leukocyte rolling velocities in vivo (right) was determined in postcapillary venules using intravital microsopy. Total leukocyte flux was determined during an interval of ≥60 s. Hemodynamics were similar in all groups (data not shown). At right, the velocities of individual rolling leukocytes were measured, and the fraction of rolling cells with a velocity corresponding to a velocity class (bottom of the figure) was determined.

in a specific pathogen-free vivarium. Gross and histological examinations of *FucT-IV*$^{-/-}$ mouse organs disclose no abnormalities, including epithelia where the *FucT-IV* locus is transcribed (Gersten et al., 1995). Standard blood chemistry values in these animals are normal.

Selectin Ligand Expression by *FucT-IV*$^{-/-}$ Neutrophils

E- and P-selectin ligand expression on *FucT-IV*$^{-/-}$ leukocytes was initially characterized using E- and P-selectin/IgM chimeric flow immunocytometry reagents (Malý et al., 1996). Both chimeras bind equivalently to wild-type (WT) and FucT-IV-deficient neutrophils (Gr-1^{hi}) or monocytes (Gr-1^{lo}) at saturating (Figure 2) or subsaturating (data not shown) concentrations. Defects in selectin ligand activity were also sought, using in vitro analyses of shear flow- and E- and P-selectin-dependent cell adhesion (Figure 3). Neutrophil rolling velocities were assessed to approximate the effective bond lifetime of the set of selectin/counterreceptor couples established between the cells and the adhesive substrata (Puri et al., 1997). On E- or P-selectin substrates at densities (47 and 146 sites per square micometer, respectively) at which *FucT-VII*$^{-/-}$ neutrophils neither tether nor roll (data not shown), WT and *FucT-IV*$^{-/-}$ neutrophils roll at equivalent velocities (Figure 3A). Neutrophil tethering efficiencies were determined to assess the rate of formation of initial adhesive interactions between cellular selectin counterreceptors and the selectin-substituted surface (van der Merwe, 1999). These assays disclose that WT and *FucT-IV*$^{-/-}$ neutrophils exhibit virtually indistinguishable tethering efficiencies on E- and P-selectin (Figure 3A). Controlled detachment assays quantitated resistance of neutrophils to detachment from selectin-coated substrata to gauge the effective instantaneous number of selectin-counterreceptor bonds and their reactive compliance (Puri et al., 1997). In this assay, WT and *FucT-IV*$^{-/-}$ neutrophils exhibit identical resistance to detachment from both selectins. Considered together, these results imply that any contributions provided by FucT-IV to the intrinsic or cellular k_{on} or k_{off} of E- or P-selectin counterreceptor interactions or to the density availability or reactive compliance of the molecules that contribute to such interactions (van der Merwe, 1999) are too modest to be measured in these in vitro assays or are masked by a predominant contribution by FucT-VII.

FucT-IV-Dependent E- and P-Selectin Ligand Activities Unveiled by the Absence of FucT-VII

FucT-VII$^{-/-}$ neutrophils tether to P-selectin at a P-selectin density of 291 sites/μm^2 (Figure 3B) and resist de-

tachment from P-selectin (Figure 3B). *FucT-VII*$^{-/-}$ neutrophils also engage in shear-dependent tethering to and resist detachment from E-selectin (Figure 3B). These observations identify FucT-VII-independent E- and P-selectin ligand activities. To determine if FucT-IV contributes to these activities and to further define the role of each enzyme in leukocyte trafficking, mice deficient in both enzymes were generated by crossing *FucT-IV*$^{-/-}$ and *FucT-VII*$^{-/-}$ mice. Doubly deficient mice are vigorous and fertile and are not susceptible to environmental microbes in a specific pathogen-free vivarium. The major organs of the doubly deficient mice are grossly and histologically normal (except as addressed below), as are standard blood chemistry values.

Selectin ligand activities of *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ neutrophils were characterized in controlled detachment or tethering assays under conditions where *FucT-VII*$^{-/-}$ neutrophils adhere to E- and P-selectin (Figure 3B). In these experiments, *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ neutrophils do not bind to E- or P-selectin (Figure 3B) (nor in a flow cytometry assay; Figure 2A). Neutrophils deficient in both FucTs are thus devoid of E- and P-selectin ligand activity, demonstrating that FucT-IV directs expression of the E- and P-selectin ligand activities retained in the absence of FucT-VII. FucT-IV-dependent P-selectin counterreceptor activity localizes exclusively to PSGL-1, since P-selectin-dependent tethering by *FucT-VII*$^{-/-}$ neutrophils is blocked by an anti-PSGL-1 monoclonal antibody (Figure 3B). E-selectin-dependent tethering of *FucT-VII*$^{-/-}$ neutrophils is not blocked by anti-PSGL-1 (Figure 3B), however, which assigns FucT-IV-dependent E-selectin counterreceptor activity to molecules distinct from PSGL-1. Defective selectin ligand activity in FucT-IV and/or FucT-VII deficiency derives from deficits of specific α(1,3) linked fucose residues, since deficiency of FucT-IV alone or in combination with FucT-VII deficiency does not diminish expression of four glycoproteins (Figure 2) implicated in the display of E- and/or P-selectin ligand activities in mice (PSGL-1, ESL-1, CD24) or humans (L-selectin).

FucT-IV Directs FucT-VII-Independent Leukocyte Selectin Ligand Expression In Vivo

Intravital microscopy was used to determine if FucT-IV contributes to selectin ligand activities in the inflamed cremasteric microvasculature, where trauma induces leukocyte rolling that occurs ~1–2 hr postsurgery (Ley et al., 1995). The diameters of the microvessels studied did not differ significantly between the strains, nor did the shear rate or shear stress within these vessels (data not shown). In this model, the mean leukocyte rolling fraction is decreased in *FucT-IV*$^{-/-}$ mice (Figure 3C), although this reduction does not achieve statistical significance. In contrast to either singly deficient strain, the doubly deficient mice are devoid of any rolling activity (Figure 3C). FucT-IV-dependent selectin ligand activity thus accounts for residual leukocyte rolling observed in *FucT-VII*$^{-/-}$ mice (Figure 3C).

The mean rolling velocity of leukocytes in *FucT-IV*$^{-/-}$ mice (73.9 $\pm$ 42.4 μm/sec) exceeds that in WT mice (33.1 $\pm$ 16.0 μm/sec); no leukocytes roll at very slow velocities ($<$10 μM/sec) (Figure 3C), and the fraction of leukocytes rolling at $<$40 μM/sec is markedly reduced. This deficit is accompanied by a proportional increase in the fraction of leukocytes rolling at velocities $>$80 μM/sec (Figure 3C). While slow rolling in normal skin is primarily determined by E-selectin (Weninger et al., 2000), trauma-induced rolling in cremaster muscle venules is largely determined by P-selectin (Ley et al., 1995). Only after treatment with inflammatory cytokines such as TNFα do E-selectin and the β2 integrins determine rolling velocity in this tissue (Jung et al., 1998). However, neither of these molecules contributes to leukocyte rolling in cremasteric venules in the absence of cytokine treatment (K. Ley, personal communication). Thus, the lack of slow rolling cells here suggested a role for FucT-IV-dependent P-selectin ligands in vivo, although such contributions are not evident when selectin ligand activity is assessed in the flow chamber (Figure 3).

FucT-IV and FucT-VII Control Blood Leukocyte Number through Contributions to Neutrophil Production and Intravascular Half-Life

In *FucT-IV*$^{-/-}$ mice, modest increments are seen in blood neutrophils, monocytes, and eosinophils (increases of 20%, 6%, and 10%, respectively; Figure 4A). By contrast, there is a 2.9-fold increase in circulating leukocytes in doubly deficient mice, accounted for by an 18.4-fold increase in neutrophils, and significant increases in monocytes, eosinophils, and lymphocytes (Figure 4A). The leukocytosis in *FucT-VII*$^{-/-}$ mice (Malý et al., 1996) is intermediate between those in *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ mice and in WT or *FucT-IV*$^{-/-}$ mice (Figure 4A).

To determine if selectin ligand defects in FucT-deficient mice contribute to leukocytosis through altered neutrophil production, neutrophils newly released from the marrow were quantitated (Lord et al., 1991). In WT mice, neutrophil precursors yield [^{3}H]thymidine-labeled neutrophils appearing in the circulation ~72 hr after [^{3}H]thymidine injection, where they come to represent more than 50% of circulating neutrophils (Lord et al., 1991). This fraction does not vary between WT mice and FucT-deficient strains (Figure 4B). However, the total number of newly synthesized neutrophils is elevated to an intermediate and substantial degree, respectively, in mice deficient in FucT-VII or both FucT-IV and FucT-VII (Figure 4B). These observations imply that neutrophil production in these mice is increased in a genotype-specific manner that aligns with their respective graded deficits in selectin ligand activity.

To determine if alterations in leukocyte circulation time also contribute to the leukocytosis in these mice, we assessed the relative rates of disappearance of WT or FucT mutant neutrophils from the circulation. WT and *FucT-IV*$^{-/-}$ neutrophils leave at similar rates (Figure 4C), whereas *FucT-VII*$^{-/-}$ neutrophils circulate significantly longer than WT or *FucT-IV*$^{-/-}$ cells (Figure 4C), and *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ neutrophils circulate longer than *FucT-VII*$^{-/-}$ cells (Figure 4C). The leukocyte integrins CD11a, CD11b, and CD18 are expressed equivalently by neutrophils (Figure 2B) in all three strains. It is therefore unlikely that alterations in the integrin receptor/counterreceptor systems perturb circulating leukocyte half-times in FucT-deficient mice. These observations thus imply that the leukocyte selectin ligand defects in the FucT-deficient mice contribute to a prolongation in leukocyte circulatory times and that such prolongation contributes to the leukocytosis in these animals.

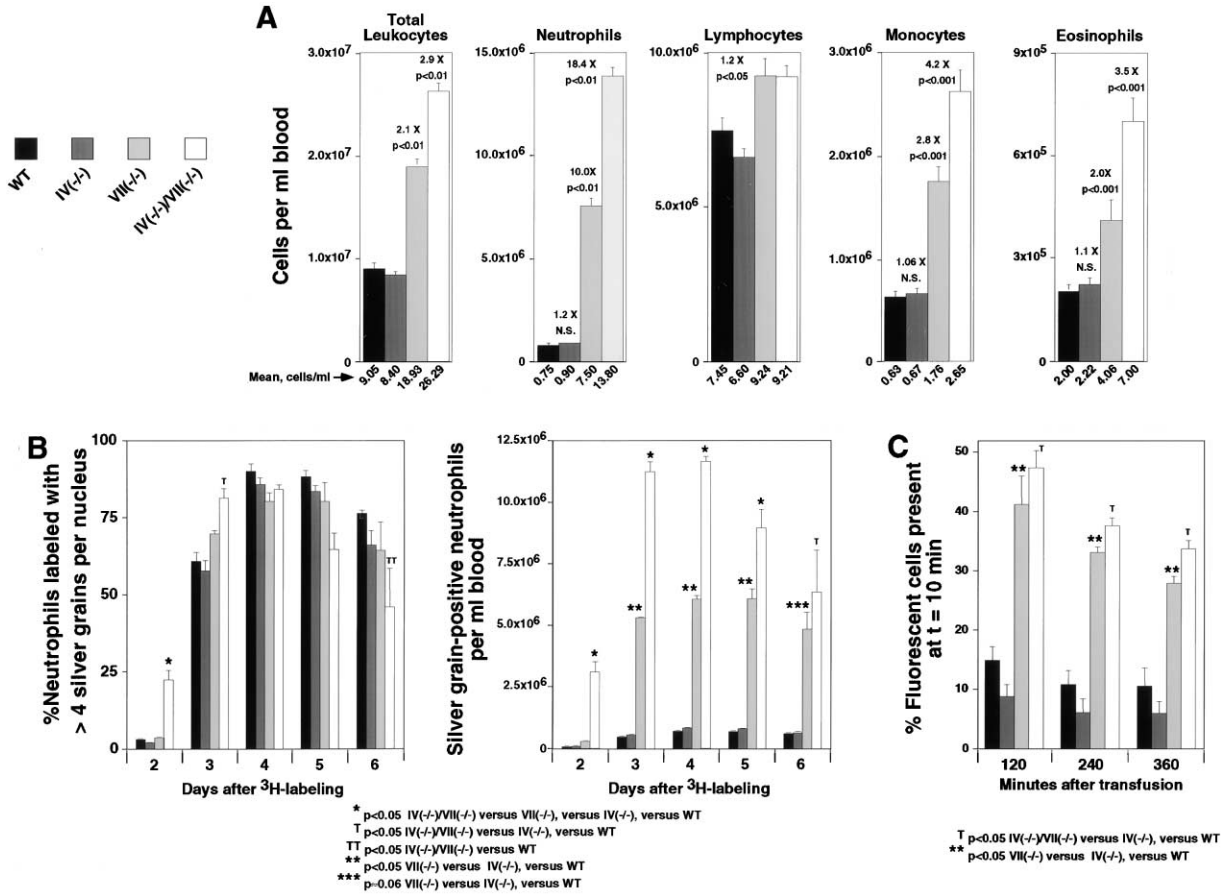

Figure 4. Blood Leukocyte Counts and Granulocyte Kinetics

(A) Blood leukocyte counts. Data are mean values (below each bar) ± SEM determined on 20 mice of each genotype. Pairwise comparisons between WT and *FucT-IV*$^{-/-}$ leukocyte types disclosed a significant difference only for neutrophils ($p = 0.023$).

(B) Neutrophil production. The fraction of ^{3}H-labeled peripheral blood neutrophils in mice of each genotype was determined every 24 hr after intravenous administration of [^{3}H]thymidine (left panel; $n = 4$ per genotype). The right panel displays the absolute number of ^{3}H-labeled peripheral blood neutrophils in each strain, determined from the fraction of such cells and the blood neutrophil count.

(C) Neutrophil turnover. CMFDA-labeled peripheral blood neutrophils were administered intravenously to a recipient of the same genotype. Blood was taken at 10 min and at the indicated intervals after neutrophil transfusion, and the fraction of CMFDA-positive neutrophils was determined by flow cytometry. Data ($n = 4$ per genotype) represent the fraction of the number of CMFDA-positive neutrophils present at each time point relative to the number at 10 min after transfusion.

FucT-IV Contributes to Leukocyte Trafficking in Inflammation

In vivo models of selectin-dependent neutrophil recruitment were used to define contributions by FucT-IV to this process in inflammation. In irritant-induced cutaneous inflammation, where neutrophil recruitment is E- and P-selectin dependent yet L-selectin independent (Catalina et al., 1999), WT and *FucT-IV*$^{-/-}$ neutrophils are recruited to the dermis to an identical extent (Figure 5A). Recruitment is reduced substantially in *FucT-VII*$^{-/-}$ mice, however, and residual recruitment in FucT-VII-deficiency is virtually absent in *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ mice (Figure 5A). In a zymosan-induced, P- and E-selectin-dependent model of dermal neutrophil recruitment (Homeister et al., 1998), WT and *FucT-IV*$^{-/-}$ neutrophils emigrate equivalently, and FucT-VII deficiency yields a significant yet partial decrement in recruitment that is fully compromised in *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ mice (Figure 5B). Finally, in selectin-dependent thioglycollate-elicited neutrophil recruitment (Mayadas et al., 1993), WT and *FucT-IV*$^{-/-}$ neutrophils accumulate equivalently. The severe but partial deficit in recruitment in FucT-VII deficiency (Malý et al., 1996) (Figure 5C) is further and significantly reduced in *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ mice. The neutrophilia in *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ mice persists throughout inflammation in each model (data not shown), indicating that the severe deficit in neutrophil recruitment in these mice is accounted for by the virtual absence of neutrophil E- and P-selectin ligand activities. FucT-IV thus provides a contribution to selectin-dependent neutrophil recruitment in inflammation that is masked by FucT-VII-dependent ligand expression but unveiled in mice also deficient in FucT-VII.

FucT-IV-Dependent HEV-Derived L-Selectin Ligands

Lymphocyte homing requires adhesive interactions between L-selectin on lymphocytes and sulfated, glycosylated counterreceptors on HEV (Hemmerich and Rosen, 2000). Normal HEV-born L-selectin counterreceptor activity is FucT-VII dependent, but *FucT-VII*$^{-/-}$ peripheral

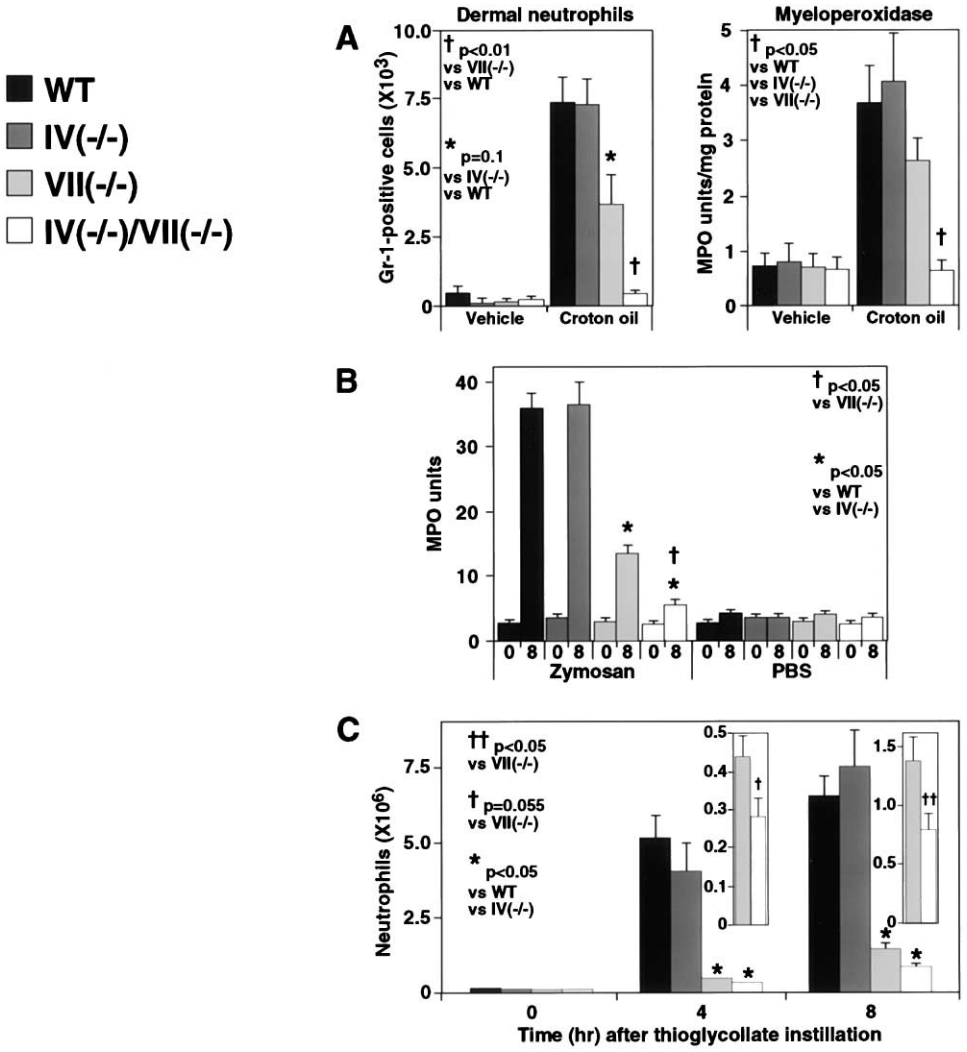

Figure 5. Neutrophil Trafficking in Acute Inflammation

(A) Irritant dermatitis. Ears were treated with croton oil or vehicle for 24 hr. A single-cell suspension of the dermis was then prepared, and the $CD45^+Gr\text{-}1^+Thy\ 1.2^-$ cells per ear were quantitated by flow cytometry (left; n = 9 or 10 per group). Alternatively, myeloperoxidase (MPO) activity was quantitated in the homogenized ear (right; n = 5 per group).

(B) Zymosan-induced dermatitis. Ears were injected subcutaneously with vehicle (PBS) or zymosan and were assayed for myeloperoxidase activity (n = 6 per group) immediately (0 hr) or at 8 hr.

(C) Thioglycollate-induced peritonitis. Peritoneal lavage fluid was collected immediately (0 hr) or 4 or 8 hr after instillation of thioglycollate into the peritoneal space, and peritoneal neutrophils were quantitated (n = 6 per group; data from *FucT-VII*$^{-/-}$ and doubly deficient mice expanded in the inset).

lymph nodes (PLN) are not completely barren of lymphocytes, support residual lymphocyte homing activity, and are of normal size (Malý et al., 1996). These observations infer the existence of active L-selectin ligands in the absence of FucT-VII. However, residual L-selectin counterreceptor activity on *FucT-VII*$^{-/-}$ HEV is inconsistently detected with an L-selectin-IgM immunohistochemical probe (Malÿ et al., 1996, and data not shown).

In an effort to identify such residual L-selectin counterreceptor activity and to more precisely define the contributions of FucT-IV and FucT-VII to HEV-derived selectin ligands, GlyCAM-1 was isolated from the mice and probed for its ability to support shear-dependent lymphocyte tethering in vitro. Tethering is the same on WT and *FucT-IV*$^{-/-}$ GlyCAM-1 but is reduced substantially on *FucT-VII*$^{-/-}$ GlyCAM-1 (Figure 6A). FucT-VII-deficient GlyCAM-1 nonetheless supports residual L-selectin-dependent lymphocyte tethering at a higher GlyCAM-1 substitution density. This tethering is clearly FucT-IV dependent, since GlyCAM-1 from *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ mice does not support lymphocyte tethering. FucT-IV therefore contributes to HEV-born L-selectin ligand activity. FucT-VII dominates in its contribution to selectin ligand activity on HEV, however, as decrements in ligand activity on *FucT-IV*$^{-/-}$ HEV are not observed with an L-selectin-IgM probe (data not shown) and since WT and *FucT-IV*$^{-/-}$ GlyCAM-1 exhibit identical tethering efficiencies.

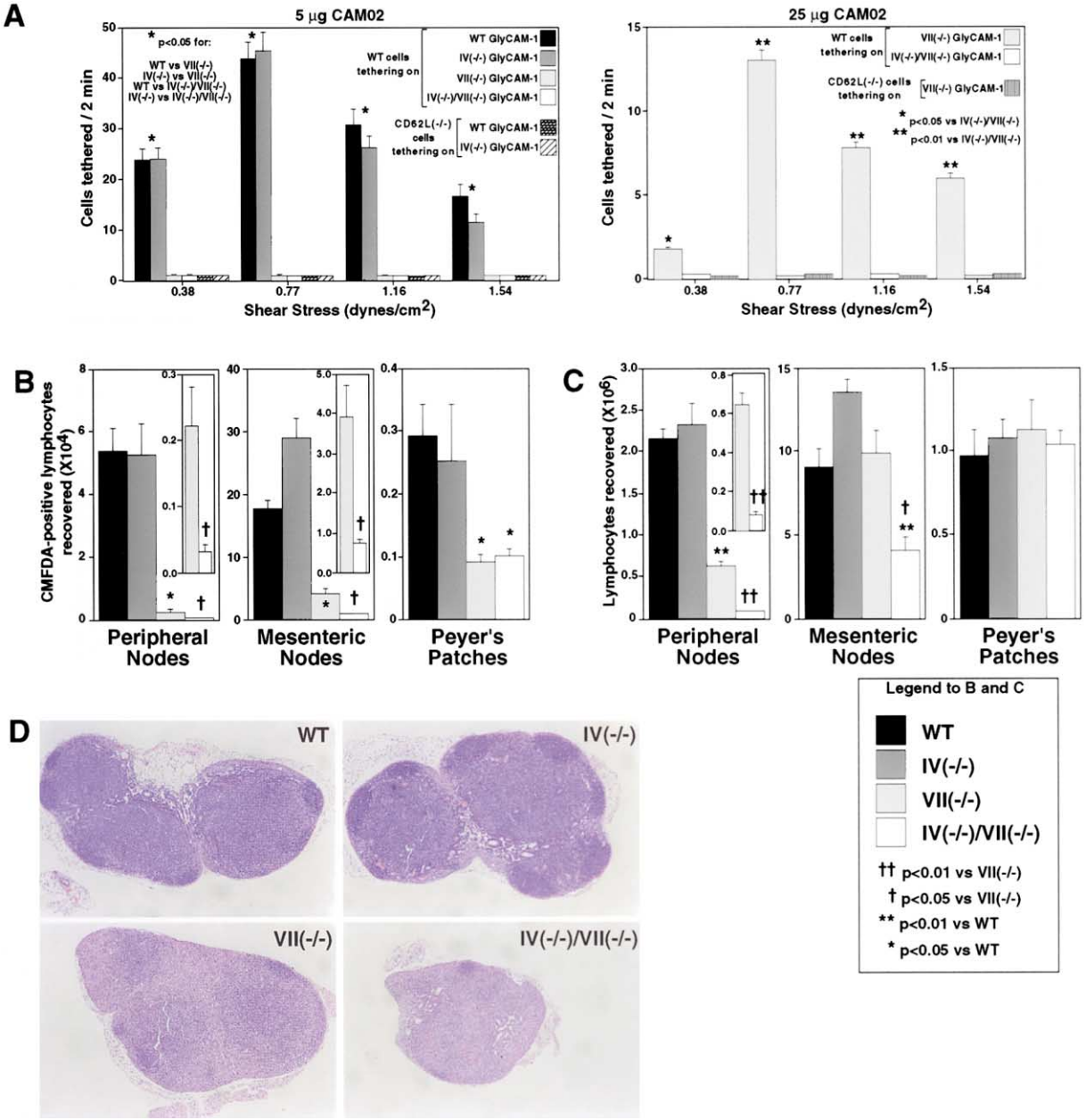

Figure 6. Selectin Ligand Expression and Lymphocyte Homing

(A) Lymphocyte tethering on GlyCAM-1. WT or *L-selectin*$^{-/-}$ lymphocyte tethering was determined using a flow chamber coated with GlyCAM-1 isolated from each mouse strain. Low or high GlyCAM-1 substitution densities were achieved using plates coated with low (5 μg/ml; left) or high (25 μg/ml; right) concentrations of the anti-GlyCAM-1 antibody CAM02. Data are derived from three to five independently prepared adhesion surfaces (3 to 17 observations).

(B) Lymphocyte homing. WT MLN lymphocytes labeled with CMFDA were injected into the tail vein of WT or FucT-deficient mice. Mice were sacrificed 1 hr later, a suspension of lymphocytes was prepared from each lymphoid organ, and CMFDA-positive lymphocytes in each were quantitated by flow cytometry (Malý et al., 1996). At least 100,000 lymphocytes per lymphoid organ were analyzed (n = 9 recipient animals).

(C) Lymph node cellularity. Lymphocytes recovered from each lymphoid organ were quantitated (Malý et al., 1996) (n = 4 to 9 mice of each strain).

(D) Peripheral lymph node histology. PLN were fixed, embedded, sectioned, and stained with H&E. 50× magnification.

Defective Lymphocyte Homing in *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ Mice

Homing of wild-type lymphocytes to PLN and MLN is dramatically reduced in *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ recipients (reductions of 86% and 81%, respectively) relative to *FucT-VII*$^{-/-}$ recipients (Figure 6B), indicating that homing to PLN and MLN in *FucT-VII*$^{-/-}$ mice is primarily FucT-IV dependent. Reduced homing efficiencies in *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ mice are accompanied by reductions of 88% and 59% in the lymphocyte content of their PLN and MLN, respectively, relative to *FucT-VII*$^{-/-}$ mice (Figure 6C). These reductions correlate quantitatively

with the relative contributions assigned to L-selectin-dependent homing to these lymphoid organs (Bargatze et al., 1995).

In contrast, lymphocyte homing to PP is the same in *FucT-VII*$^{-/-}$ and *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ mice (Figure 6B). This implies a subtle role, if any, for FucT-IV in the generation of selectin ligands by PP HEV. Recovery of resident lymphocytes from the PP of all four strains is equivalent (Figure 6C), suggesting that $\alpha 4\beta 7$-dependent homing to PP compensates for L-selectin ligand deficiency in FucT-deficient PP (Bargatze et al., 1995). The modest lymphocytosis observed in the absence of FucT-VII (Figure 4A) may also contribute to compensated lymphocyte homing to PP. Lymphocyte homing to the spleen does not vary as a function of FucT genotype (data not shown), implying that neither FucT is required for this process.

PLN are small in *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ mice, as in L-selectin null mice (Arbones et al., 1994), in contrast to a normal size in WT, *FucT-IV*$^{-/-}$, and *FucT-VII*$^{-/-}$ mice (Figure 6D) and are notably hypocellular. Primary follicles are small and infrequent, but the overall nodal architecture is normal. L-selectin expression on *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ splenic lymphocytes is normal, and CMFDA-labeled WT lymphocytes coinjected with CMTMR-labeled *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ lymphocytes home with equal efficiencies to PLNs in WT recipients (*FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$:WT $= 1.05 \pm 0.15$; n $= 5$). The severe reductions in lymphocyte content in PLN and MLN of doubly deficient mice relative to *FucT-VII*$^{-/-}$ mice thus derive from a FucT-IV-dependent defect in HEV-born L-selectin counterreceptor activities.

FucT-IV-Dependent Synthesis of Sulfated L-Selectin Ligand Capping Groups In Vitro

A biochemical basis for the FucT-IV-dependent contribution to HEV-born selectin ligand activity was sought using in vitro FucT assays and precursors to sulfated glycan capping groups implicated in L-selectin ligand activity (Figure 7) (Hemmerich and Rosen, 2000). Fucosylation likely represents the terminal synthetic step for each capping group, since 6-sulfation of *N*-acetylglucosamine precedes galactosylation (Figure 7), α(1,3) fucosylation blocks 6-sulfation of galactose by known galactose 6-sulfotransferase activities, and α(1,3)fucosylation blocks α(2,3)sialylation of terminal galactose moieties (Torii et al., 2000; Hemmerich and Rosen, 2000).

FucT-IV can utilize a 6-sulfated α(2,3)sialyl N-acetyllactosamine substrate to form the 6-sulfo sialyl Lewis x structure implicated in L-selectin ligand activity (Figure 7) and can use its nonsulfated isomer to form the sialyl Lewis x structure but forms only trace amounts of 6′-sulfo sialyl Lewis x with the precursor to this structure (Figure 7). FucT-VII also utilizes the 6-sulfated and nonsulfated precursors, with a rank order preference similar to FucT-IV, but does not utilize the 6′-sulfated precursor (Figure 7). FucT-IV, like FucT-VII, can thus catalyze terminal fucosylation events in the biosynthesis of the glycans implicated as essential components of HEV-born L-selectin ligands.

Nonsialylated glycans may occur proximally within the proposed L-selectin ligand biosynthetic scheme (Figure 7). Fucosylation of such substrates will prevent α(2,3)sialylation required for HEV-born L-selectin ligand activity (Lowe, 1997), possibly leading to nonproductive pathways. However, FucT-VII does not utilize any nonsialylated substrate (Figure 7) and is thus not likely to participate in such pathways. In contrast, FucT-IV utilizes each nonsialylated precursor, although the sulfated isomers are used at substantially lower relative rates (Figure 7). FucT-IV thus might divert some substrate away from synthesis of glycans that contribute to L-selectin ligand activity, and its deletion could thus lead to paradoxically enhanced FucT-VII-dependent ligand expression. Although homing efficiencies and lymphocyte recoveries increase in *FucT-IV*$^{-/-}$ mesenteric nodes (Figure 6), these parameters are identical in *FucT-IV*$^{-/-}$ and WT PLNs (Figure 6), and we find no differences between these two strains in L-selectin ligand activities in PLNs or MLNs (data not shown).

Discussion

Although FucT-VII clearly provides a major contribution to the control of E-, P-, and L-selectin ligand activities in vivo (Malý et al., 1996), molecules responsible for selectin ligand activities retained in *FucT-VII*$^{-/-}$ mice have not been defined. Although FucT-IV and its fucosylated glycans have been candidates in this context, it has been unclear if FucT-IV contributes to leukocyte E- and P-selectin ligand expression or to selectin-dependent leukocyte recruitment, since prior studies have involved transfected cell lines yielding inconsistent conclusions (Lowe, 1997) or are of uncertain physiological relevance for leukocyte recruitment (Weninger et al., 2000). Moreover, a role for FucT-IV in controlling HEV-specific L-selectin ligand expression has not been suggested or examined. Our studies demonstrate that leukocyte E- and P-selectin ligand activities relevant to leukocyte recruitment are the sum of unequal contributions provided by FucT-IV and FucT-VII. These studies further disclose that FucT-IV collaborates with FucT-VII in elaborating L-selectin ligands on HEV, with the conclusion that the two enzymes work in concert to control leukocyte recruitment and lymphocyte homing.

FucT-IV$^{-/-}$ granulocytes exhibit normal shear-dependent adhesion to E- and P-selectin in vitro (Figure 3A) yet maintain subtle elevations in blood leukocyte counts (Figure 4A) and shifts toward higher leukocyte rolling velocities in the cremaster muscle microvasculature (rolling induced by inflammation accompanying surgical trauma; Figure 3C) or in uninflamed dermal microvessels (constitutive rolling in a noninflamed vascular bed) (Weninger et al., 2000). FucT-IV thus exerts subtle control over selectin-dependent leukocyte adhesion in vivo but not in vitro when FucT-VII is also expressed. In *FucT-IV*$^{-/-}$ mice, the discordance between increased rolling velocity and normal neutrophil recruitment may be reconciled by the possibility that increased rolling velocities do not occur when inflammation and E- and P-selectin expression are robust. Alternatively, *FucT-IV*$^{-/-}$ neutrophils may roll faster than WT neutrophils in acutely inflamed vasculature, but this may not diminish their transit time in the inflamed microvessel to a degree that yields a deficit in leukocyte arrest and emigration (Jung et al., 1998). These observations align with those made in

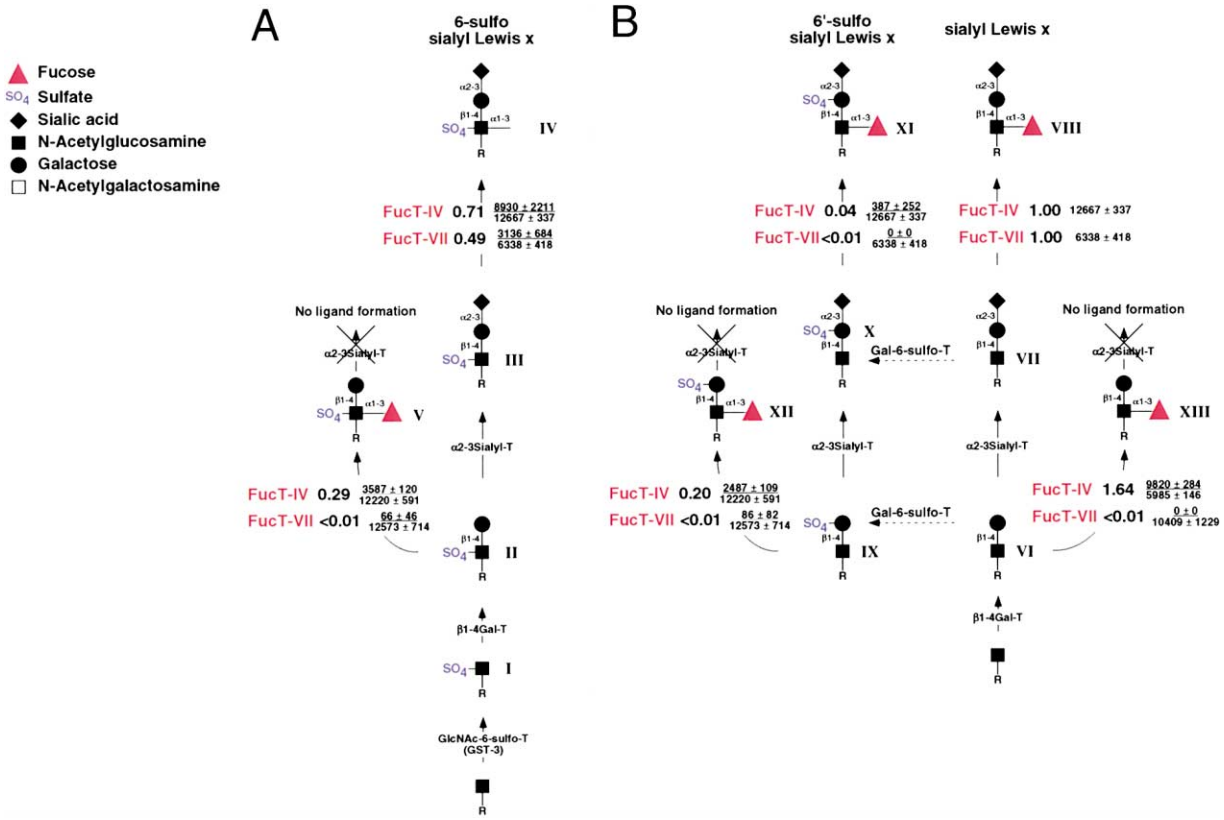

Figure 7. Fucosylation of Sulfated, Sialylated Glycans Implicated in L-Selectin Ligand Activity

Precursor product relationships proposed for 6-sulfo-sialyal Lewis x (compound IV) (A) and for sialyl Lewis x (compound VIII) and 6′-sulfo-sialyl Lewis x (compound XI) (B) have been reviewed (Hemmerich and Rosen, 2000; Lowe, 1997). Recombinant mouse FucT-IV or VII were assayed in vitro for their ability to use various precursors on this pathway. Rates of utilization of various acceptor substrates, relative to an α(2,3)sialyl LacNAc-based substrate (compound VII; [B]), were determined and are shown (as a percent and as absolute numbers) adjacent to arrows depicting the relevant precursor-product relationship. Enzyme activities are in mUnits observed with an acceptor (numerator), relative to the α(2,3)sialyl LacNAc acceptor (denominator). FucT-IV can also add fucose to internal GlcNAc residues (data not shown). R = glycoproteins, glycolipids, or alkyl groups.

E-selectin deficiency, where increased leukocyte rolling velocities (Kunkel et al., 1996; Ley et al., 1998) are generally not accompanied by decrements in granulocyte recruitment unless the functions of P-selectin (Labow et al., 1994; Frenette et al., 1996) or CD18 (Jung et al., 1998) are also abrogated.

Increased leukocyte rolling velocities in vivo in *FucT-IV*$^{-/-}$ mice contrasts with equivalent adhesion of WT and *FucT-IV*$^{-/-}$ granulocytes to E- and P-selectins in vitro (Figure 3). Strain-specific differences might be considered accountable since intravital microscopy studies used recombinant inbred (RI) strains, while in vitro adhesion assays (Figure 3) used cells from C57Bl/6J inbred strains. This is unlikely, however, since RI WT and *FucT-IV*$^{-/-}$ granulocytes are indistinguishable with respect to rolling velocity, detachment, and tethering, and strain-specific differences are not observed in blood leukocyte counts or inflammation assays (data not shown). Instead, subtle FucT-IV-dependent increments in rolling velocities may not be evident in a flow chamber because this assay does not recapitulate the complexity of leukocyte-endothelial cell interactions within a blood vessel. This may include leukocyte activation events that modulate leukocyte rolling velocities, perhaps involving integrin engagement, independent of or syngergistic with modulation of selectin ligand activities.

In vitro, PSGL-1 displays virtually all FucT-IV-dependent P-selectin counterreceptor activity expressed by *FucT-VII*$^{-/-}$ blood neutrophils. FucT-IV thus can fucosylate PSGL-1-associated glycans to construct a PSGL-1 glycoform with P-selectin counterreceptor activity. This apparently conflicts with the observation that a P-selectin-Ig chimera fails to precipitate PSGL-1 from *FucT-VII*$^{-/-}$ bone marrow granulocytes (Huang et al., 2000). PSGL-1 modified solely by FucT-IV may have a very high off rate, insufficient for biochemical purification, whereas the shear-dependent rolling assay may more sensitively detect PSGL-1-born, FucT-VII-independent selectin counterreceptor activity. It is also possible that marrow-derived granulocytes and peripheral blood neutrophils maintain structurally and functionally distinct PSGL-1 glycoforms.

E-selectin counterreceptor activity on *FucT-VII*$^{-/-}$ granulocytes resists inhibition by the adhesion blocking anti-PSGL-1 antibody. ESL-1 likely contributes most of this FucT-IV-dependent, PSGL-1-independent E-selectin counterreceptor activity, since an E-selectin-Ig chimera immunoprecipitates normal amounts of ESL-1

from *FucT-VII*$^{-/-}$ bone marrow neutrophils but none from *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ mice (Huang et al., 2000). A confirmation of this possibility will await the availability of adhesion blocking anti-ESL-1 antibodies.

FucT-VII and FucT-IV contribute to maintaining homeostasis in blood leukocyte number, in part, by influencing the circulating neutrophil half-life. Prolonged neutrophil circulatory times are also characteristic of and may contribute to leukocytosis in selectin deficiency (Johnson et al., 1995). Deletion of a constitutive, selectin-dependent leukocyte emigration and turnover process likely accounts for these observations in selectin-deficient mice and applies to the partial and complete deficiency of neutrophil selectin counterreceptor activities seen in *FucT-VII*$^{-/-}$ and *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ mice, respectively. Increased neutrophil production may also contribute to leukocytosis in FucT null mice. Interactions between P-selectin and PSGL-1 suppress marrow progenitor cell proliferation (Levesque et al., 1999). Since recognition of PSGL-1 by P-selectin is fucosylation dependent, "inactive" PSGL-1 characteristic of FucT deficiency may relieve this suppression, leading to progenitor cell expansion and increased leukocyte production. Exploration of FucT-dependent perturbations in marrow progenitor cell biology are the subject of another report (S. Kale, J.B.L., and M.W. Long, unpublished data).

Mucocutaneous infections suffered by selectin-deficient mice may contribute to their leukocytosis via cytokine-dependent events that stimulate hematopoietic progenitor cell proliferation (Frenette et al., 1996; Robinson et al., 1999). This is probably not operative in FucT-deficient mice since they do not develop such infectious complications. Infection in selectin-deficient mice and its absence in FucT-deficient mice is not clearly accounted for by variance between housing conditions since such differences persist when the mice are housed in the same vivarium (P. Frenette, personal communication). Mechanisms to account for these differences remain to be defined.

These studies disclose that FucT-VII-independent lymphocyte homing (Malý et al., 1996) is primarily fucosylation dependent and controlled by FucT-IV. FucT-IV and FucT-VII exhibit the same rank order preference, in vitro, for precursors to known L-selectin counterreceptor capping groups (sLex and 6-O-sulfosialyl Lewis x), implying that both enzymes may construct the same structures. In vitro, neither forms the 6′-O-sulfosialyl Lewis x structure, a capping group for which there is conflicting evidence for a role in L-selectin counterreceptor activity (Hemmerich and Rosen, 2000). These and previous observations (Malý et al., 1996) suggest that alternative pathways (like 6′-O-sulfation of sialyl Lewis x-type structures; Figure 7) may predominate in its synthesis. Which of these glycans contributes to L-selectin ligand in FucT-VII deficiency will require a characterization of the GlyCAM-1-associated glycan structures retained in *FucT-VII*$^{-/-}$ mice.

FucT-IV or FucT-VII can construct multiply and internally fucosylated structures (Niemela et al., 1998) associated with GlyCAM-1 (Hemmerich and Rosen, 2000) and PSGL-1 (Wilkins et al., 1996). The relevance of such multiply fucosylated structures to selectin counterreceptor activities is uncertain, however, since crystallographic studies disclose that the single external fucose of representative P- and E-selectin ligands seems sufficient for ligand-selectin interactions of high affinity (Somers et al., 2000). Glycan structural information derived from the FucT null mice will be required to more precisely define the molecular basis for how each enzyme contributes to E- and P-selectin counterreceptor activities.

Experimental Procedures

Generation of Targeted ES Cells

A 436 bp MluI segment of the mouse FucT-IV locus, encoding an essential portion of FucT-IV, was replaced with a 1.4 kb fragment carrying a G418 resistance selection cassette (PGKneo). Targeting events in D3 ES cell were identified by Southern blotting using PstI digestion and probe A (Figure 1A; 10 kb WT, 5.1 kb mutant; 3′ end of the *FucT-IV* locus external to the targeting construct). Nonhomologous integration events were excluded with PstI digestion and probe B (Figure 1A; 5.1 kb mutant allele; PGKneo cassette; data not shown). Homologous integration was confirmed with an XbaI-BamHI double digest and probe E (Figure 1A; 11 kb WT allele; 5.7 kb mutant allele; data not shown). Absence of the WT allele was confirmed with PstI digests and probe D, which is absent from the targeting construct.

Generation of *FucT-IV*$^{-/-}$ Mice and *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ Mice

Chimeric males derived from *FucT-IV*$^{+/-}$ ES cells were crossed with (C57BL/6J×DBA/2J)F1 females. *FucT-IV*$^{+/-}$ progeny were backcrossed onto C57Bl/6J WT mice for 14 generations. Heterozygotes were intercrossed to generate a *FucT-IV*$^{-/-}$ strain otherwise congenic with C57BL/6J mice. *FucT-IV*$^{+/-}$ progeny were also intercrossed to generate an RI strain of *FucT-IV*$^{-/-}$ mice. Backcrossing through nine generations generated a C57BL/6J *FucT-VII*$^{-/-}$ strain from RI *FucT-VII*$^{-/-}$ mice (Malý et al., 1996). C57BL/6J *FucT-VII*$^{-/-}$ and *FucT-IV*$^{-/-}$ mice were bred to generate a C57BL/6J *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ strain. RI *FucT-IV*$^{-/-}$/*FucT-VII*$^{-/-}$ and WT strains were also generated. C57BL/6J strains were used except for Figures 3C, 4B, 4C, and 5B.

Leukocyte Rolling In Vitro

Neutrophil-selectin adhesive interactions were analyzed using a parallel plate flow chamber whose bottom plate was coated with selectin-human IgG chimera at densities quantitated by radioimmunoassay (Puri et al., 1997). Neutrophils were purified from blood leukocytes obtained after hypotonic lysis using antibodies (CD4, CD8, B220, and Ter 119) and a magnetic column (Miltenyi Biotec) to remove T, B, and red cells. Data analysis was performed using an Inovision Corp. image processing system.

For cell velocity determination, neutrophils (1×10^6/ml) were drawn into the chamber and allowed to settle onto the selectin-chimera substrate. Flow was restarted and increased at set intervals to double the wall shear stress without interrupting cell flow. Stationary cells (≤10 μm movement) were excluded from analysis. The fraction of cells adherent at the end of each shear stress was calculated using the number of adherent cells at the end of the period of shear stress = 0.38 dyne/cm^2 as denominator.

For neutrophil tethering, a delineated region of the plate was coated with selectin-chimera, and the remainder of the plate was blocked with bovine serum albumin. Neutrophils were infused at a given shear stress, and tethering efficiency was determined, after two minutes, at the selectin-chimera/BSA interface by enumerating adherent rolling cells. Cells adherent via secondary tethers were excluded. Antibody blocking experiments used neutrophils that had been preincubated with anti-mouse PSGL-1 (mAb 4RA10, IgG1, 10 μg/ml, gift of D. Vestweber) or anti-mouse CD11b (mAb M1/70, IgG2b, 10 μg/ml, PharMingen).

For lymphocyte tethering, serum-derived GlyCAM-1 was displayed on the bottom plate of the flow chamber via capture with the anti-GlyCAM-1 antibody CAM02 (Singer and Rosen, 1996). A delineated region of the bottom plate was coated with CAM02 (in Tris-HCl [pH 9.2], 16 hr, 4°C) and the remainder blocked with bovine serum albumin. The antibody-coated portion was then incubated

with GlyCAM-1 (5 hr at 4°C and again for 16 hr at 4°C) isolated (Singer and Rosen, 1996) from the serum of WT or FucT-deficient mice to achieve equivalent GlyCAM-1 substitution densities. Tethering was determined as above. Lymphocytes were isolated from PLN of WT C57Bl/6J mice or from MLN of *L-selectin*$^{-/-}$ mice.

Intravital Microscopy

Intravital fluorescence microscopic observations and calculations were made as described (Ley and Gaehtgens, 1991; Weninger et al., 2000).

Neutrophil Kinetics

Neutrophil production was assessed by pulse labeling of neutrophil precursors with [^{3}H]thymidine (Lord et al., 1991). Mice were bled daily, cytospin preparations of the blood were processed for detection of [^{3}H]nuclei, and were Wright's stained to determine the fraction of labeled neutrophils ($\geq$4 silver grains per nucleus). Neutrophil turnover studies used CMFDA-labeled blood neutrophils (1.6×10^7) injected into the tail vein of recipients. Blood samples were taken at set intervals, and the fraction of circulating neutrophils derived from the transfused neutrophils was determined by flow cytometry using anti-Ly6G antibody as a neutrophil marker and CMFDA fluorescence to identify transfused cells.

Fucosyltransferase Assays

FucT assay conditions were chosen to ensure linear rates of product formation (Malý et al., 1996). Acceptor molecules consisted of the octyl glycosides of α(2,3)sialylated N-acetyllactosamine (sialyl-LacNAc-octyl), its 6-O- and 6′-O-sulfate esters (Misra et al., 2000), the nonsialylated isomers of these molecules (synthetic details to be reported elsewhere), or molecules previously described (Malý et al., 1996). One unit of enzyme activity forms 1 pmol of product in 1 hr, using sialyl-LacNAc-type acceptors.

Statistical Analysis

Two group comparisons were performed using the Student's t test (parametric; $n \geq 10$ per group) or the Mann-Whitney U test (nonparametric). Parametric comparisons among more than two groups were performed using Analysis of Variance with a Tukey-Kramer post-hoc test for all possible pairwise comparisons. Nonparametric comparisons among more than two groups were performed using the Kruskal-Wallis test and a subsequent Mann-Whitney U test with the Bonferroni correction for all possible pairwise comparisons. Data are presented as mean $\pm$ SEM unless noted. Intergroup differences were considered significant at $p \leq 0.05$.

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