

Sialyltransferase ST3Gal-IV controls CXCR2-mediated firm leukocyte arrest during inflammation

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Recent *in vitro* studies have suggested a role for sialylation in chemokine receptor binding to its ligand (Bannert, N., S. Craig, M. Farzan, D. Sogah, N.V. Santo, H. Choe, and J. Sodroski. 2001. *J. Exp. Med.* 194:1661–1673). This prompted us to investigate chemokine-induced leukocyte adhesion in inflamed cremaster muscle venules of $\alpha 2,3$ sialyltransferase (ST3Gal-IV)-deficient mice. We found a marked reduction in leukocyte adhesion to inflamed microvessels upon injection of the CXCR2 ligands CXCL1 (keratinocyte-derived chemokine) or CXCL8 (interleukin 8). In addition, extravasation of ST3Gal-IV^{-/-} neutrophils into thioglycollate-pretreated peritoneal cavities was significantly decreased. *In vitro* assays revealed that CXCL8 binding to isolated ST3Gal-IV^{-/-} neutrophils was markedly impaired. Furthermore, CXCL1-mediated adhesion of ST3Gal-IV^{-/-} leukocytes at physiological flow conditions, as well as transendothelial migration of ST3Gal-IV^{-/-} leukocytes in response to CXCL1, was significantly reduced. In human neutrophils, enzymatic desialylation decreased binding of CXCR2 ligands to the neutrophil surface and diminished neutrophil degranulation in response to these chemokines. In addition, binding of $\alpha 2,3$ -linked sialic acid-specific *Maackia amurensis* lectin II to purified CXCR2 from neuraminidase-treated CXCR2-transfected HEK293 cells was markedly impaired. Collectively, we provide substantial evidence that sialylation by ST3Gal-IV significantly contributes to CXCR2-mediated leukocyte adhesion during inflammation *in vivo*.

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Abbreviations used: CF-CXCL8, human carboxyfluorescein-labeled CXCL8; fMLP, *N*-formyl-Met-Leu-Phe; GPCR, G α protein-coupled receptor; ICAM-1, intercellular adhesion molecule 1; MAL-II, *Maackia amurensis* lectin II; PTx, pertussis toxin; ST3Gal-IV, $\alpha 2,3$ sialyltransferase; WBC, white blood cell.

The recruitment of neutrophils into inflamed tissue is an important immunological process that proceeds along a well-defined cascade of events beginning with the capture of neutrophils to the inflamed endothelium and followed by neutrophil rolling along the endothelium (1).

Both capture and rolling are mediated by selectins binding to selectin ligands (2). During rolling, neutrophils are in intimate contact with the inflamed endothelium, enabling endothelial-bound chemokines to interact with specific chemokine receptors expressed on the surface of neutrophils. This in turn triggers the activation of β_2 integrins, which eventually leads to firm neutrophil arrest (3). The CXC chemokine

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CXCL8 has been demonstrated to induce human neutrophil arrest from neutrophil rolling in a dynamic *in vitro* assay (4). Using intravital microscopy, a subsequent report demonstrated that triggering of neutrophil arrest in unstimulated mouse cremaster muscle venules was almost completely dependent on CXCR2-mediated events (5). In contrast, stimulating the cremaster muscle by intrascrotal injection of TNF- α led to neutrophil arrest, which was not strictly dependent on CXCR2 but occurred in an overlapping fashion with an E-selectin-mediated mechanism (5).

During leukocyte recruitment, an essential role of posttranslational glycosylation has been identified for selectin ligand function. This became particularly apparent in mice with a genetic deletion in glycosyltransferases such as fucosyltransferase VII or core 2 GlcNAcT-I (6, 7). Both enzymes are critically involved in the synthesis of core 2-decorated O-glycans carrying the selectin binding motif sialyl Lewis X as a capping group. Deletion of these enzymes led to a profound impairment of selectin-dependent leukocyte rolling *in vivo* (6, 8). Recently, it has been demonstrated that posttranslational glycosylation also influences the function of chemokine receptors (9, 10). Ludwig et al. demonstrated that N-glycosylation of human CXCR2 protects this receptor from proteolytic degradation and removal from the surface of neutrophils, whereas it does not interfere with its trafficking function (9). Bannert et al. used a canine thymocyte cell line to generate cells stably expressing WT CCR5 and CCR5 mutants. In CCR5 mutants, serine or threonine residues were changed to alanine at putative O-glycosylation sites at the N terminus of CCR5 (10). Functional analysis of the cell lines carrying the different CCR5 mutants revealed that CCR5 binding to the CC chemokines macrophage inflammatory proteins 1 α and 1 β was strongly dependent on a sialic acid carrying O-glycan linked to serine 6 at the N terminus of CCR5 (10). Although

these findings point toward an important role of glycosylation and sialylation in the function of some chemokine receptors, the contribution of distinct glycosyltransferases in the proper synthesis of carbohydrate structures crucial for chemokine receptor function has not been investigated. Recently, mice deficient in the α 2,3 sialyltransferase (ST3Gal-IV) have been generated (11). *In vivo* studies on P-, E-, and L-selectin-mediated leukocyte rolling in inflamed cremaster muscle venules of ST3Gal-IV^{-/-} mice revealed a complete absence of L-selectin ligand function and a partial impairment in E-selectin-dependent leukocyte rolling, whereas P-selectin-dependent rolling was not affected (12, 13).

In this study, we will present substantial evidence that leukocyte adhesion and extravasation are impaired in ST3Gal-IV^{-/-} mice. Because of the rather mild defect at the level of leukocyte rolling, the impairment in leukocyte adhesion and extravasation in ST3Gal-IV^{-/-} mice cannot be explained by the observed rolling defect. We therefore hypothesized that sialylation by ST3Gal-IV contributes to chemokine-triggered neutrophil arrest *in vivo*. To this end, we investigated leukocyte arrest mechanisms in unstimulated and TNF- α -stimulated cremaster muscle venules of ST3Gal-IV^{-/-} mice *in vivo* and in various static and dynamic assays *in vitro*.

RESULTS

Leukocyte adhesion in trauma-induced inflammation

Leukocyte adhesion was assessed in 58 cremaster muscle venules of 13 ST3Gal-IV^{-/-} mice and compared with leukocyte adhesion in 37 venules of 13 WT control mice. Hemodynamic parameters between the groups showed no significant difference in vascular diameter, blood flow velocity, wall shear rate, and systemic white blood cell (WBC) count (Table I). Leukocyte adhesion in postcapillary venules of the cremaster muscle during the first hour after exteriorization

Table I. Hemodynamic parameters (mean \pm SEM of diameter, centerline velocity, shear rate, and WBC) of cremaster muscle venules in ST3Gal-IV^{-/-}, CXCR2^{bm-/-}, and WT control mice

		Mice (n)	Venules (n)	Diameter (μ m)	Blood flow velocity (μ m/s)	Shear rate (1/s)	WBC (/ μ l)
Cremaster venules in trauma-induced inflammation	ST3Gal-IV ^{-/-}	13	58	30 \pm 1	2,800 \pm 200	2,400 \pm 200	5,500 \pm 600
	WT	13	37	32 \pm 1 n.s.	3,100 \pm 200 n.s.	2,500 \pm 200 n.s.	5,600 \pm 800 n.s.
Chemokine-treated cremaster venules in trauma-induced inflammation	ST3Gal-IV ^{-/-}	10	19	31 \pm 1	2,000 \pm 200	1,700 \pm 100	5,700 \pm 700
	CXCR2 ^{bm-/-}	5	5	31 \pm 3	1,600 \pm 100	1,500 \pm 300	4,800 \pm 100
	WT	19	30	30 \pm 1 n.s.	2,300 \pm 200 n.s.	1,900 \pm 200 n.s.	6,300 \pm 500 n.s.
Cremaster venules in TNF- α -induced inflammation	ST3Gal-IV ^{-/-}	16	90	30 \pm 1	2,300 \pm 100	1,900 \pm 100	3,400 \pm 500
	CXCR2 ^{bm-/-}	8	76	31 \pm 1	1,800 \pm 110	1,500 \pm 120	2,600 \pm 200
	WT	16	67	32 \pm 1 n.s.	2,100 \pm 100 n.s.	1,700 \pm 100 n.s.	2,800 \pm 400 n.s.

n.s., nonsignificant (P > 0.05).

of the cremaster muscle was significantly lower in ST3Gal-IV^{-/-} than in WT control mice, suggesting a defect in firm leukocyte arrest in ST3Gal-IV^{-/-} mice (Fig. 1 A). As recently demonstrated, leukocyte adhesion in untreated cremaster muscle venules is mediated via interactions between chemokine receptor CXCR2 expressed on the surface of rolling neutrophils and endothelial-bound chemokines, including keratinocyte-derived chemokine (CXCL1) (5). We analyzed leukocyte adhesion in untreated mice before and 3 min after systemic injection of 600 ng CXCL1 or the human prototype CXC chemokine CXCL8 (600 ng/mouse). Microvascular parameters during those experiments were similar between the groups (Table I). In WT mice, the number of adherent leukocytes increased by 457 ± 83 cells/mm² after CXCL1 injection and by 422 ± 87 cells/mm² after CXCL8 (Fig. 1 B; and Video 1, available at <http://www.jem.org/cgi/content/full/jem.20070846/DC1>). In contrast, leukocyte adhesion in ST3Gal-IV^{-/-} mice increased only by 73 ± 17 cells/mm² after CXCL1 injection and by 73 ± 34 cells/mm² after CXCL8 injection (Fig. 1 B; and Video 2), suggesting that CXCL1- and CXCL8-induced firm adhesion are dependent on ST3Gal-IV. Next, leukocyte adhesion was investigated in mice pretreated with 4 μ g pertussis

toxin (PTx), an inhibitor of G α_i protein-coupled receptors, 3 h before cremaster muscle exteriorization. Similar to a previous report (5), PTx pretreatment of WT control mice led to a significant attenuation in the increase of adherent leukocytes after CXCL1 injection (83 ± 21 cells/mm²) when compared with WT control mice without PTx (Fig. 1 B). Similar results were obtained in PTx-pretreated control mice after injection of CXCL8 (88 ± 33 cells/mm²). In ST3Gal-IV^{-/-} mice, no significant influence of PTx pretreatment was noted on the number of accumulated leukocytes after CXCL1 or CXCL8 injection (45 ± 27 and 88 ± 40 cells/mm², respectively; Fig. 1 B), suggesting that leukocyte adhesion triggered by CXCR2 is significantly impaired in ST3Gal-IV^{-/-} mice. Finally, we investigated the increase in the number of adherent leukocytes in cremaster muscle venules of CXCR2^{bm-/-} mice after systemic injection of CXCL1 or CXCL8. Similar to ST3Gal-IV^{-/-} mice, leukocyte adhesion after CXCL1 increased only by 80 ± 16 and 88 ± 41 cells/mm² after CXCL8 (Fig. 1 B). This was significantly different to the results observed in WT mice, demonstrating that CXCR2 is crucial in mediating CXCL1/CXCL8-dependent leukocyte adhesion in this setting.

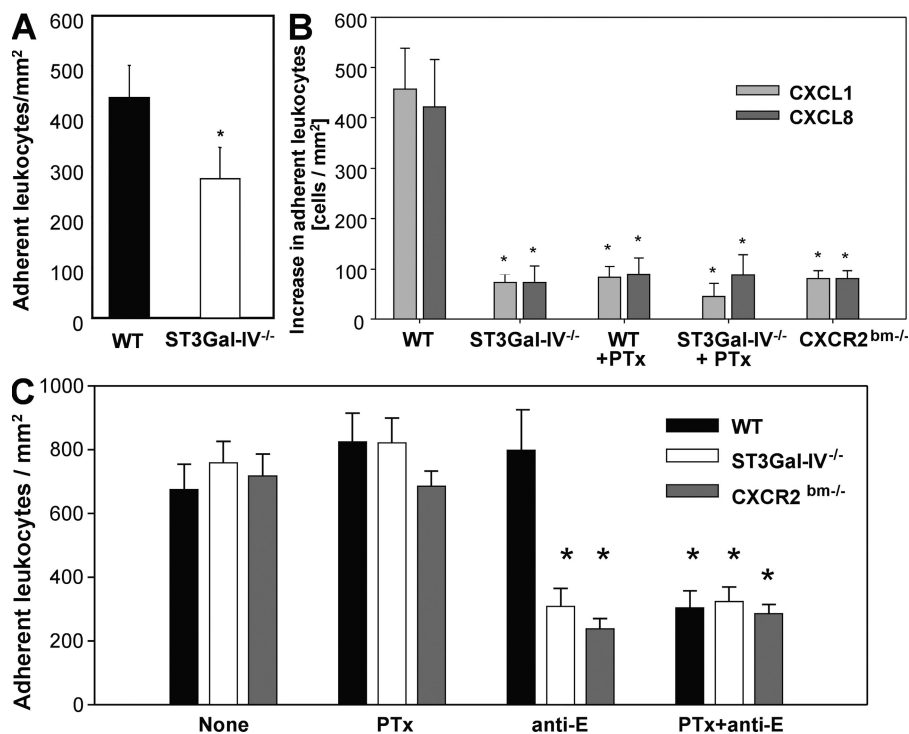


Figure 1. Leukocyte adhesion during inflammation in vivo. (A) Number of adherent leukocytes (mean \pm SEM per mm² of surface area) in unstimulated mouse cremaster muscle venules of WT control and ST3Gal-IV^{-/-} mice. (B) Increase in the number of adherent leukocytes (mean \pm SEM per mm² of surface area) 3 min after systemic injection of 600 ng CXCL1 or CXCL8 in WT control, ST3Gal-IV^{-/-}, WT control (plus 4 μ g PTx), ST3Gal-IV^{-/-} (plus 4 μ g PTx), and CXCR2^{bm-/-} mice. PTx was injected 3 h before cremaster muscle exteriorization. (C) Leukocyte adhesion (mean \pm SEM) was observed in 3-h TNF- α -treated cremaster muscle venules of WT control, ST3Gal-IV^{-/-}, and CXCR2^{bm-/-} mice. Additionally, pretreatment of TNF- α -treated WT control, ST3Gal-IV^{-/-}, and CXCR2^{bm-/-} mice was conducted with 4 μ g PTx and/or 100 μ g E-selectin mAb 9A9 5 min before TNF- α injection. Data in A–C were obtained from at least three independent experiments per group. *, $P < 0.05$ versus WT control mice (A and C); *, $P < 0.05$ versus WT control mice treated with CXCL1 or CXCL8, respectively (B). Videos 1 and 2 are available at <http://www.jem.org/cgi/content/full/jem.20070846/DC1>.

To exclude the possibility that a reduction in leukocyte rolling is responsible for the impaired firm leukocyte arrest observed in ST3Gal-IV^{-/-} and CXCR2^{bm-/-} mice, we assessed leukocyte rolling in unstimulated cremaster muscle venules of WT control, ST3Gal-IV^{-/-}, and CXCR2^{bm-/-} mice. We found a significant increase in the leukocyte rolling flux fraction in ST3Gal-IV^{-/-} (46 ± 5%) and CXCR2^{bm-/-} (51 ± 7%) mice compared with control mice (32 ± 4%; P < 0.05; Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20070846/DC1>), reflecting the inability of ST3Gal-IV^{-/-} and CXCR2^{bm-/-} leukocytes to transit from established rolling into firm arrest.

Leukocyte adhesion in TNF-α-induced inflammation

Next, we observed leukocyte adhesion in 90 TNF-α-treated (3 h) cremaster muscle venules of 16 ST3Gal-IV^{-/-} mice and in 76 venules of 8 CXCR2^{bm-/-} mice, and compared it with leukocyte adhesion in 67 venules of 16 WT animals. In this model, transition from leukocyte rolling to firm adhesion after TNF-α pretreatment is mediated in an overlapping fashion involving CXCR2 and E-selectin (5). Microvascular parameters for the groups are presented in Table I and show similar vessel diameters, centerline velocities, wall shear rates, and WBC counts. Leukocyte adhesion 3 h after TNF-α treatment

was similar between WT (674 ± 80 cells/mm²), ST3Gal-IV^{-/-} (759 ± 66 cells/mm²), and CXCR2^{bm-/-} (717 ± 68 cells/mm²) mice (Fig. 1 C). In agreement with a previous study, pretreatment with the E-selectin blocking mAb 9A9 or PTx did not affect neutrophil arrest in WT control mice (799 ± 128 and 823 ± 91 cells/mm², respectively; Fig. 1 C) (5). Similarly, ST3Gal-IV^{-/-} or CXCR2^{bm-/-} mice pretreated with PTx revealed no changes in neutrophil arrest (821 ± 78 and 686 ± 47 cells/mm², respectively) compared with WT control mice. In contrast, ST3Gal-IV^{-/-} or CXCR2^{bm-/-} mice pretreated with E-selectin blocking mAb 9A9 showed significantly reduced leukocyte adhesion (308 ± 56 and 239 ± 31 cells/mm², respectively; P < 0.05 vs. WT control; Fig. 1 C) when compared with WT control mice, suggesting that leukocyte arrest triggered by CXCR2 is considerably affected in ST3Gal-IV^{-/-} mice. This was also supported by experiments in ST3Gal-IV^{-/-} and CXCR2^{bm-/-} mice pretreated with both PTx and E-selectin blocking mAb 9A9, where leukocyte adhesion was not further reduced (324 ± 45 and 295 ± 30 cells/mm², respectively; Fig. 1 C). In addition, in WT control mice pretreated with PTx and E-selectin blocking mAb 9A9, leukocyte adhesion was also significantly decreased (304 ± 54 cells/mm²; Fig. 1 C), confirming the crucial role of ST3Gal-IV for firm leukocyte adhesion in vivo.

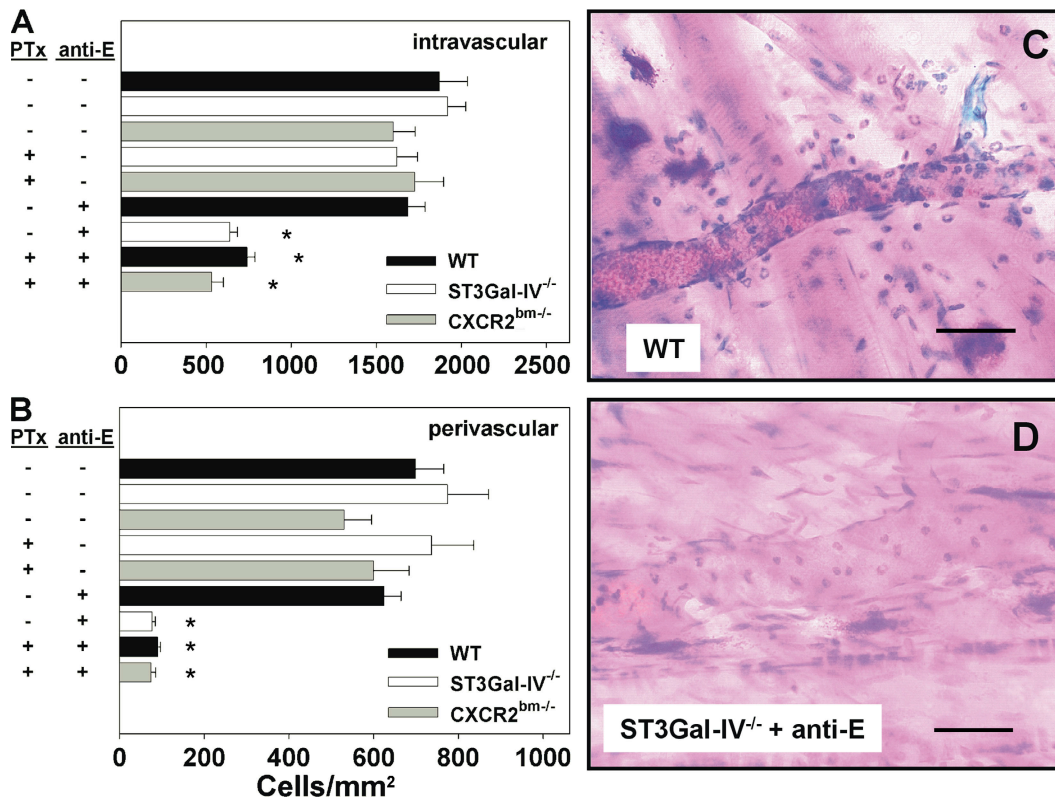


Figure 2. Cremaster muscle whole mount. Giemsa-stained whole-mounts of TNF-α-treated cremaster muscles of WT, ST3Gal-IV^{-/-}, and CXCR2^{bm-/-} mice were analyzed for the number of intravascular (A) and perivascular (B) leukocytes (mean ± SEM per mm² surface area). In addition, two typical micrographs are presented illustrating intra- and perivascular leukocyte distribution in TNF-α-treated cremaster muscle whole mounts of WT control mice (C) and ST3Gal-IV^{-/-} mice pretreated with E-selectin blocking mAb 9A9 (D). Data in A and B were obtained from at least three independent experiments per group. *, P < 0.05 versus WT mice. Bars, 50 μm.

Next, we assessed leukocyte rolling in WT, ST3Gal-IV^{-/-}, and CXCR2^{bm-/-} mice. We found no difference in the leukocyte rolling flux fraction in TNF- α -stimulated cremaster muscle venules of ST3Gal-IV^{-/-} ($21 \pm 4\%$) and CXCR2^{bm-/-} ($15 \pm 2\%$) mice compared with WT control mice ($20 \pm 3\%$; Fig. S1 B).

Leukocyte adhesion and extravasation in whole mounts of TNF- α -treated cremaster muscles

To investigate the contribution of ST3Gal-IV on the composition and number of extravasated neutrophils, we performed Giemsa staining of whole-mount cremaster muscles pretreated with TNF- α and classified leukocytes into neutrophils, eosinophils, and mononuclear cells. In ST3Gal-IV^{-/-} and CXCR2^{bm-/-} mice, the number of intravascular and extravasated neutrophils was similar to that observed in WT mice (Fig. 2, A–C). Relative frequencies of intravascular and extravasated neutrophils, mononuclear cells, and eosinophils were comparable between ST3Gal-IV^{-/-} and WT mice, suggesting that leukocyte recruitment in ST3Gal-IV^{-/-} mice is not impaired after stimulation with TNF- α (unpublished data). Pretreatment of ST3Gal-IV^{-/-} or CXCR2^{bm-/-} mice with the G α_1 blocker PTx did not significantly influence the number of intra- and extravascular leukocytes nor the relative leukocyte frequencies (Fig. 2; unpublished data).

Next, we investigated the number of intra- and perivascular neutrophils in TNF- α -stimulated cremaster muscle venules of ST3Gal-IV^{-/-} and WT control mice pretreated with the E-selectin blocking mAb 9A9. Blocking E-selectin in WT control mice did not change the number of intravascular neutrophils nor neutrophil extravasation when compared with untreated WT control mice (Fig. 2, A and B). In addition, the composition of perivascular leukocytes was similar between E-selectin mAb 9A9-pretreated WT control mice and untreated WT control mice (unpublished data). In ST3Gal-IV^{-/-} mice pretreated with the E-selectin blocking mAb 9A9, intravascular as well as perivascular neutrophils were significantly decreased compared with WT control mice (Fig. 2, A–D). However, the composition of extravasated leukocytes in E-selectin blocking mAb 9A9-pretreated ST3Gal-IV^{-/-} mice did not significantly change compared with WT mice, suggesting that the emigration of mononuclear cells and eosinophils was also impaired (unpublished data). Next, we investigated intra- and perivascular neutrophils in WT control and CXCR2^{bm-/-} mice pretreated with the E-selectin blocking mAb 9A9 and PTx (Fig. 2, A and B). We found a similar reduction in the number of perivascular neutrophils as in E-selectin blocking mAb 9A9-pretreated ST3Gal-IV^{-/-} mice, confirming that ST3Gal-IV is involved in CXCR2-dependent extravasation of neutrophils.

Thioglycollate-induced peritonitis

As reported previously, neutrophil arrest inhibition through blockade of G α_1 protein-coupled receptor (GPCR)-dependent signals leads to impaired extravasation of neutrophils into the inflamed peritoneal cavity after intraperitoneal injection

of thioglycollate (5). We investigated neutrophil influx into the peritoneum of WT, ST3Gal4^{-/-}, and CXCR2^{bm-/-} mice 4 h after thioglycollate injection. Neutrophil recruitment to the peritoneum was significantly reduced to 60% in ST3Gal-IV^{-/-} mice (4.5×10^6 cells; $n = 9$; Fig. 3), to 57% in WT mice pretreated with PTx (4.3×10^6 cells; $n = 6$), and to 35% in CXCR2^{bm-/-} mice (2.5×10^6 cells; $n = 3$) when compared with WT mice (7.5×10^6 cells; $n = 7$; $P < 0.05$). These results suggest that chemokine-triggered signaling is impaired in ST3Gal-IV^{-/-} mice, which was also confirmed by the observation that pretreatment of ST3Gal-IV^{-/-} mice with PTx did not cause a further significant reduction in neutrophil extravasation after thioglycollate injection (2.7×10^6 cells; $n = 5$; Fig. 3).

Ligand binding and functional activity of CXCR2 on ST3Gal-IV^{-/-} neutrophils

Because CXCR2 was found to carry several N-glycosylation sites with potential terminal sialylation (9), we tested whether deficiency of ST3Gal-IV would directly affect the biology of this receptor. To address the possibility that a lower expression of CXCR2 on ST3Gal-IV^{-/-} leukocytes contributes to the decrease in the number of adherent leukocytes, we initially performed flow cytometric analysis of CXCR2 expression on GR-1-positive leukocytes of ST3Gal-IV^{-/-} and WT control mice (Fig. 4 A). CXCR2 expression was slightly higher in ST3Gal-IV^{-/-} compared with WT control mice, excluding a lower expression of CXCR2 in ST3Gal-IV^{-/-} mice to be responsible for the observed defect in leukocyte adhesion in these mice. We then questioned whether ST3Gal-IV deficiency would affect ligand binding to CXCR2. For the ligand binding assays, we chose to use human carboxyfluorescein-labeled CXCL8 (CF-CXCL8), because bound CF-CXCL8 can be directly detected by flow cytometric analysis of leukocyte populations within mouse blood. Human CXCL8

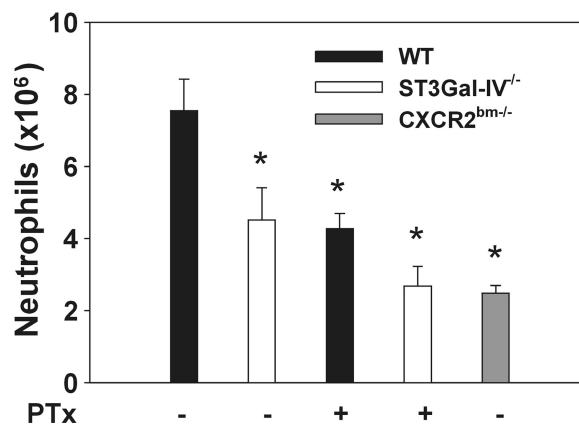


Figure 3. Thioglycollate-induced peritonitis. Peritoneal neutrophil influx was assessed 4 h after injection of 4% thioglycollate into WT ($n = 7$), ST3Gal-IV^{-/-} ($n = 9$), WT (plus 4 μ g PTx; $n = 6$), ST3Gal-IV^{-/-} (plus 4 μ g PTx; $n = 5$), and CXCR2^{bm-/-} ($n = 3$) mice. The number of neutrophils ($\times 10^6$; mean \pm SEM) was assessed from Turks-stained peritoneal lavage samples. *, $P < 0.05$ versus WT control mice.

binds with high affinity to mouse CXCR2 (14) and exerts the same *in vivo* effect as CXCL1 when injected into WT cremaster muscle venules (Fig. 1 B and Video 1). Human CXCL8 can therefore be used for binding studies in mouse neutrophils. The specificity of CF-CXCL8 binding was demonstrated by its decreased binding to neutrophils from CXCR2^{bm-/-} mice (Fig. 4 B) When compared with WT leukocytes, leukocytes from ST3Gal-IV^{-/-} mice showed a considerably reduced ability to bind CF-CXCL8. A reduction in CF-CXCL8 binding was also observed when WT neutrophils were treated with neuraminidase, which removes terminal sialic acids from surface glycoproteins. These data point toward a role of α 2,3-linked sialic acid residues for efficient binding of CXCR2 ligands to mouse neutrophils.

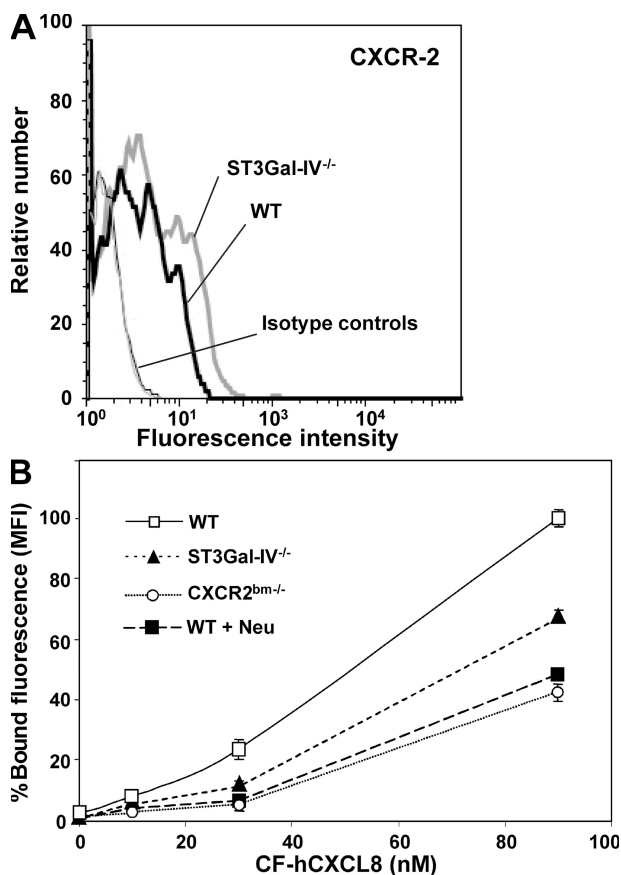


Figure 4. CXCR2 expression and ligand binding of ST3Gal-IV^{-/-} leukocytes. (A) Surface expression of CXCR2 on GR-1-positive leukocytes of ST3Gal-IV^{-/-} and WT mice was analyzed by flow cytometry. Leukocytes were incubated with an allophycocyanin-labeled GR-1 mAb, a PE-labeled anti-CXCR2 mAb, and a PE-labeled isotype control. (B) For ligand binding studies, leukocytes from the peripheral blood of WT ($n = 4$), ST3Gal-IV^{-/-} ($n = 3$), and CXCR2^{bm-/-} ($n = 3$) mice were incubated with different dilutions of CF-CXCL8 and subsequently analyzed for bound fluorescence by flow cytometry. Peripheral blood leukocytes from WT mice were treated with 100 mU/ml neuraminidase for 60 min and investigated for CF-CXCL8 binding. Data are given as mean \pm SD. $P < 0.05$ for WT versus ST3Gal-IV^{-/-}, CXCR2^{bm-/-}, and WT + Neu for 30 and 90 nM CF-CXCL8, respectively. MFI, mean fluorescence intensity.

Leukocyte adhesion in ST3Gal-IV^{-/-} neutrophils in the microflow chamber assay

Next, we investigated the role of ST3Gal-IV on leukocyte arrest using a recently described autoperfused microflow chamber system coated with recombinant mouse P-selectin, intercellular adhesion molecule 1 (ICAM-1), and CXCL1 (15). This combination of proteins mediates rolling and firm arrest of WT neutrophils (15). Rolling of WT and ST3Gal-IV^{-/-} leukocytes was noted in all flow chambers in which P-selectin was immobilized (Fig. 5 A). However, a marked reduction in the rolling of control leukocytes was noted in the presence of ICAM-1 and CXCL1, which was most likely caused by the concomitant significant increase in the number of adherent leukocytes (Fig. 5 A). As depicted in Fig. 5 B, adhesion of WT leukocytes was only observed in flow chambers in which all three molecules (P-selectin, ICAM-1, and CXCL1) were immobilized. Leukocytes from ST3Gal-IV^{-/-} mice also firmly adhered only in those flow chambers in which P-selectin, ICAM-1, and CXCL1 were immobilized on the surface. However, the number of adherent leukocytes was significantly lower than observed with control leukocytes under the same conditions (Fig. 5 B). These results strongly suggest that ST3Gal-IV significantly contributes to the successful arrest of leukocytes in the presence of CXCL1, ICAM-1, and P-selectin.

Transendothelial migration of neutrophils

To study the contribution of neutrophil-expressed ST3Gal-IV on neutrophil transmigration, CXCL1-triggered transendothelial migration of ST3Gal-IV^{-/-} neutrophils through an endothelial cell line monolayer (b.End5) was investigated. As depicted in Fig. 5 C, transendothelial migration was significantly reduced for ST3Gal-IV^{-/-} compared with WT neutrophils. In addition, transendothelial migration of neuraminidase-pretreated control neutrophils was also markedly impaired (Fig. 5 C), suggesting that α 2,3 sialylation by ST3Gal-IV is important for CXCL1-induced neutrophil transmigration. Pretreatment of the endothelial cell line b. End5 with neuraminidase before addition of WT neutrophils did not reduce the number of migrated neutrophils, suggesting that the impairment in neutrophil recruitment seen in ST3Gal-IV^{-/-} mice is caused by the loss of crucial α 2,3-linked sialic acid residues on neutrophils, not on endothelial cells (Fig. 5 C). To rule out any significant effect of CXCL1-independent transmigration, we also performed transmigration experiments without CXCL1. Spontaneous neutrophil transmigration (without CXCL1) was similar for WT and ST3Gal-IV^{-/-} neutrophils ($0.07 \pm 0.04 \times 10^6$ vs. $0.06 \pm 0.01 \times 10^6$ cells, respectively) and accounted for 20 and 17% of migrated neutrophils, respectively, compared with the total number of migrated WT neutrophils observed during CXCL1-induced transmigration (unpublished data).

Effect of enzymatic desialylation on the binding and activity of CXCR2 on human neutrophils

Biochemical studies on the presence of terminal sialic acids on CXCR2 were performed with human CXCR2, which

could be precipitated from CXCR2-transfected HEK293 cells and subsequently probed for the binding of the *Maackia amurensis* lectin II (MAL-II), which preferentially recognizes carbohydrate chains containing sialic acid residues α 2,3 linked to penultimate galactose residues (16). Digestion of precipitates with neuraminidase clearly abolished MAL-II binding but not detection of CXCR2, indicating that CXCR2 carries terminal sialic acids (Fig. 6 A). In addition, binding of CF-CXCL8 to HEK293 cells was clearly dependent on the presence of CXCR2, because CF-CXCL8 did not bind to untransfected HEK293 cells (unpublished data). However, as seen for mouse neutrophils (compare with Fig. 4 B), binding was considerably reduced (by 46%; not depicted) when the cells were desialylated by treatment with neuraminidase.

To address whether terminal sialylation is also implicated in the ligand binding and function of CXCR2 on human neutrophils, isolated human neutrophils were treated with different dosages of neuraminidase. As demonstrated by binding assays with the radioactively labeled CXCR2 ligand 125 I-labeled neutrophil-activating peptide 2 (CXCL7), treatment with neuraminidase resulted in reduced binding of the

chemokine to neutrophils (Fig. 6 B). For these experiments, CXCL7 was chosen as a radioligand, because this chemokine displays high affinity binding to CXCR2 and low affinity binding to CXCR1, whereas CXCL8 binds to both CXCR1 and CXCR2 with high affinity (17). Scatchard plot analysis revealed that CXCL7 binding to high affinity sites was shifted from 6.6 to 20 nM after neuraminidase treatment, whereas the low affinity binding was not affected (Fig. 6 C). The total number of binding sites was not changed upon treatment with neuraminidase, which was consistent with the unaffected expression level of CXCR2 and CXCR1 on the surface of these cells, as determined by flow cytometry (Fig. S2 A, available at <http://www.jem.org/cgi/content/full/jem.20070846/DC1>). Binding of platelet factor 4 (CXCL4), which is not dependent on either of the two receptors (CXCR1 or CXCR2), was not affected by neuraminidase treatment (Fig. 6 B).

Functionally, CXCL7 and CXCL8 induce lysosomal degranulation of neutrophils. We investigated the release of elastase from primary granules of CXCL8- and CXCL7-treated neutrophils and found that elastase release was considerably

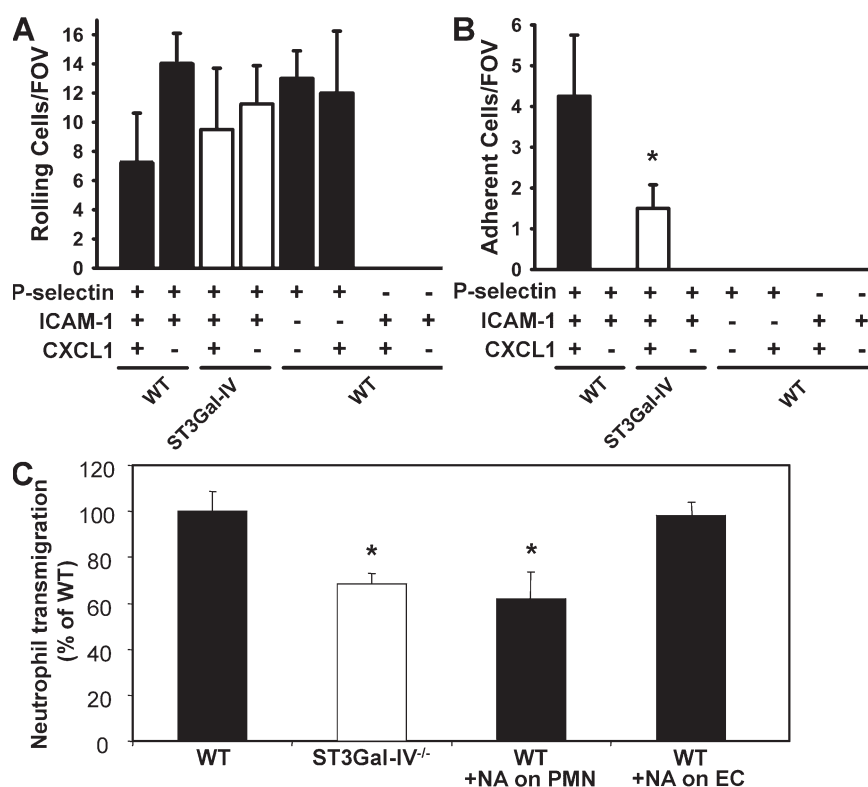


Figure 5. Adhesion in the flow chamber and transmigration of ST3Gal-IV^{-/-} neutrophils in vitro. The number of rolling and adherent leukocytes from ST3Gal-IV^{-/-} and WT mice was determined in blood-perfused microflow chambers. (A) Rolling and (B) adherent leukocytes in autoperfused flow chambers (5.94 dynes/cm²) coated with 20 μ g/ml P-selectin and 15 μ g/ml ICAM-1 with or without 15 μ g/ml CXCL1. Data in A and B are presented as mean \pm SEM from at least four mice and four flow chambers. *, $P < 0.05$ versus WT mice (B). (C) The percentage of transmigrated neutrophils from WT control mice, ST3Gal-IV^{-/-} mice, and WT control mice pretreated with neuraminidase was assessed in a transwell assay in which transwells were coated with an immortalized endothelial cell line, b.End5. In addition, transmigration of WT control neutrophils through a neuraminidase-pretreated b.End5 monolayer was analyzed. Data in C were determined from at least three independent experiments per group. *, $P < 0.05$ versus untreated WT control neutrophils (C). FOV, field of view.

reduced when human neutrophils were pretreated with neuraminidase (Fig. 6 D). Notably, elastase release in response to the preferential CXCR2 ligand CXCL7 was more profoundly reduced than that after CXCL8 stimulation, which is explained by the fact that CXCL8 binds and functions via both CXCR1 and CXCR2 (17). Importantly, enzymatic digestion did not lead to a decreased reactivity of neutrophils in general, as stimulation with the tripeptide *N*-formyl-Met-Leu-Phe (fMLP) induced similar responses in untreated and enzyme-treated neutrophils (Fig. 6 D). These findings were further corroborated by measuring CXCL7-induced lactoferrin release from secondary granules, which was significantly diminished in neuraminidase-treated neutrophils (Fig. S2 B). In contrast, lactoferrin release triggered by CXCL4 (which does not bind to CXCR2) was not influenced by the enzymatic treatment (Fig. S2 B). Thus, terminal sialic acids are found on CXCR2, and deficient sialylation in neutrophils decreases ligand binding and activity of the receptor.

DISCUSSION

Chemokine receptors and their ligands are involved in many immunological processes requiring specific and high affinity interactions between chemokine receptors and their respective ligands (18–21). Specificity is in part achieved by post-translational modification of chemokine receptors, as recently demonstrated for CXCR2 and CCR5 (9, 10).

The chemokine receptor CXCR2 (mouse homologue of IL-8 receptor B) is involved in chemokine-induced leukocyte adhesion and migration (22–24). Two studies identified CXCR2 as playing a critical role during the pathogenesis of ventilator- or LPS-induced inflammatory lung injury (25, 26). In addition, CXCR2 was also shown to participate in the recruitment of macrophages into established atherosclerotic lesions (27).

In contrast to the variety of publications illustrating CXCR2-mediated effects in a wide spectrum of diseases, little is known about how CXCR2 achieves specific binding to its ligands. We present for the first time in vivo evidence that

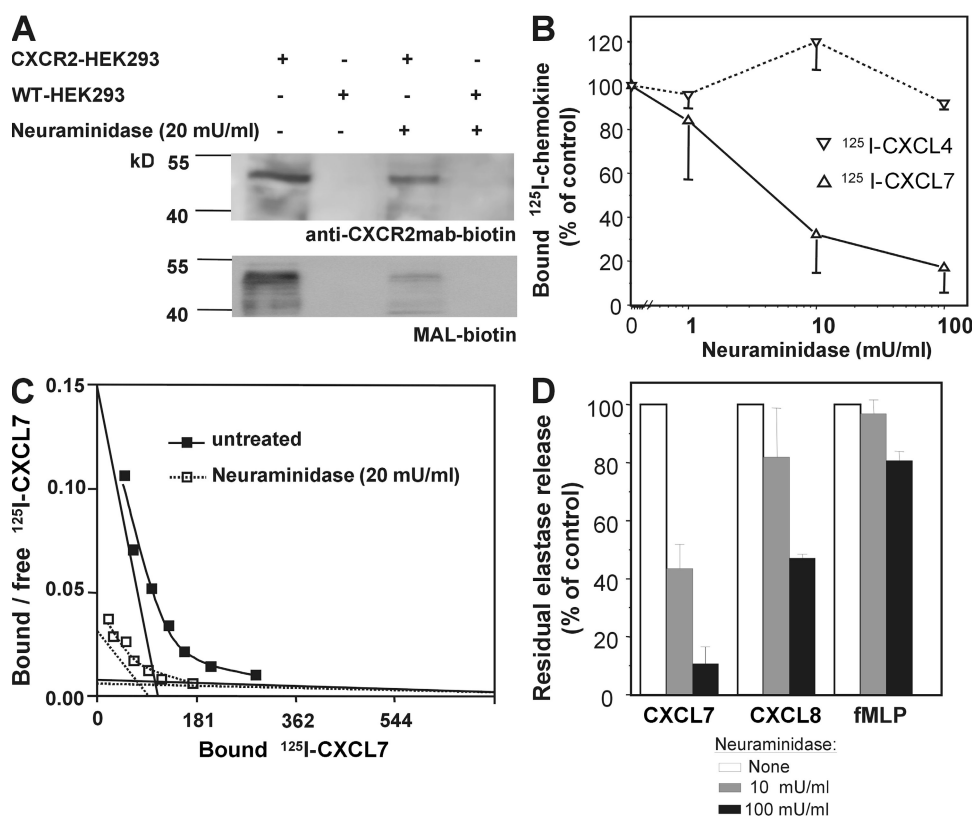


Figure 6. Effect of enzymatic desialylation on chemokine binding and function of human CXCR2. (A) Cell lysates of CXCR2-transfected and untransfected HEK293 cells were incubated with an mAb to CXCR2, followed by precipitation with protein A. Precipitates were treated with 20 U/ml neuraminidase for 60 min at 37°C, or were left untreated and analyzed by Western blotting for binding of biotinylated anti-CXCR2 antibody or biotinylated MAL-II, respectively. (B) Isolated human neutrophils were treated with the indicated dosages of neuraminidase for 30 min at 37°C, washed, and subsequently assayed for binding of ^{125}I -labeled CXCL7 and ^{125}I -labeled CXCL4. Specifically bound radioactivity was expressed as the percentage of WT control cells receiving no enzyme treatment. (C) Scatchard plot for CXCL7 binding to untreated and neuraminidase-treated (20 U/ml) human neutrophils. The binding data were curve fit to determine affinity constants for high and low affinity binding sites. (D) Neutrophils were treated with the indicated dosages of neuraminidase for 30 min at 37°C, washed, and subsequently assayed for elastase release in response to increasing concentrations of CXCL7, CXCL8, and fMLP. The residual responsiveness of neuraminidase-treated neutrophils was expressed as the percentage of that determined for untreated cells. Data in A–D represent means \pm SD determined in three different experiments, each with blood from a different donor.

CXCR2-triggered leukocyte arrest during inflammation is strongly dependent on posttranslational sialylation mediated by ST3Gal-IV. This can be deduced from results in ST3Gal-IV^{-/-} mice, where baseline as well as CXCL1- or CXCL8-triggered leukocyte adhesion in trauma-induced inflammation was significantly lower than in WT control mice. However, leukocyte adhesion was comparable to either WT control and ST3Gal-IV^{-/-} mice pretreated with PTx, an inhibitor of GPCRs, or CXCR2^{bm-/-} mice, suggesting an important role for α 2,3-sialylation in CXCR2 function in vivo. In a second approach, we found that leukocyte adhesion in TNF- α -stimulated cremaster muscle venules was similar between ST3Gal-IV^{-/-}, CXCR2^{bm-/-}, and WT control mice. In this model, leukocyte adhesion occurs in an overlapping fashion by E-selectin- and chemokine-triggered CXCR2 activation (5). Although blockade of GPCRs with PTx did not have any effect on leukocyte adhesion in ST3Gal-IV^{-/-}, CXCR2^{bm-/-}, or WT mice, pretreatment of ST3Gal-IV^{-/-} and CXCR2^{bm-/-} mice but not WT mice with E-selectin blocking mAb 9A9 led to a significant reduction of leukocyte adhesion, suggesting that CXCR2-triggered firm arrest is defective in ST3Gal-IV^{-/-} mice.

To challenge our results and directly investigate CXCR2-dependent leukocyte adhesion in a more reductionist system in which all “endothelial players” are known, we used a recently described ex vivo flow chamber system and coated the surface of the rectangular flow chamber with recombinant mouse P-selectin, ICAM-1, and CXCL1 (15). Although we observed some adhesion of ST3Gal-IV^{-/-} leukocytes in the flow chamber, the number of adherent cells was significantly lower than that seen in control mice. These data strongly support the hypothesis that CXCR2-mediated events leading to firm leukocyte arrest are impaired in ST3Gal-IV^{-/-} mice. In addition, the results suggest that the adhesion defect is located on neutrophils. This observation was confirmed and expanded into the human system in direct ligand binding assays using fluorescently labeled CXCL8 and radiolabeled CXCL7. We found a significant and dose-dependent reduction in the binding of CF-CXCL8 to ST3Gal-IV^{-/-} neutrophils when compared with control neutrophils. In addition, we observed a strong reduction in ¹²⁵I-labeled CXCL7 binding to neuraminidase-pretreated human neutrophils. Recently, Reutershan et al. identified a critical role for endothelial CXCR2 in the successful migration of neutrophils into LPS-stimulated lung tissue (26). We have not formally ruled out a role for endothelial CXCR2 in leukocyte adhesion and migration in stimulated cremaster muscle venules in vivo. However, we did not detect an effect of endothelial CXCR2 in mediating neutrophil transmigration in the chemotaxis assay using the brain endothelial cell line b.End5, suggesting that various endothelial compartments may differ in their ability to express functional CXCR2.

In a previous study, we reported that human CXCR2 is *N*-glycosylated at two distinct sites. This was shown by enzymatic deglycosylation and Western blotting of the immunoprecipitated receptor (9). In this study, we demonstrate

that enzymatic removal of terminal sialic acids results in reduced CXCR2 ligand binding to human neutrophils. When using ¹²⁵I-labeled CXCL7 as a high affinity CXCR2 ligand for binding studies with human neutrophils, we found a strong reduction in the affinity of CXCR2 when neutrophils were treated with neuraminidase, as shown by Scatchard analysis of the binding kinetics. In contrast, neuraminidase treatment of neutrophils neither affected the number of binding sites nor the expression level of CXCR2. These data suggest that sialic acids on neutrophils are required to maintain high ligand binding affinity rather than surface expression of the receptor. Although defective sialylation of CXCR2 in ST3Gal-IV^{-/-} mice could not be directly demonstrated at the molecular level, we were able to demonstrate that human CXCR2 carries terminal sialic acids, which were sensitive to neuraminidase treatment. As a consequence of reduced receptor interaction, we observed decreased adhesion, transmigration, and degranulation of mouse or human leukocytes in response to CXCR2 ligands. To confirm that glycosylation defects would not affect the responsiveness of neutrophils in general, we stimulated neutrophils independent of CXCR2 using fMLP and CXCL4. Treatment with those proinflammatory mediators did not alter neutrophil responsiveness after desialylation. This does not exclude a requirement for terminal sialic acids for ligand binding by other chemokine receptors such as CCR5, for which an important role of glycosylation has also been demonstrated (10). However, CCR7-dependent migration of dendritic cells was not affected in ST3Gal-IV^{-/-} mice (unpublished data), suggesting that sialylation cannot be considered a general requirement for optimal chemokine receptor-mediated events. Accordingly, our results suggest that posttranslational sialylation may be a new regulatory mechanism by which chemokine-triggered recruitment of some leukocyte subpopulations may occur. However, at present it remains unclear how cell type-specific expression of the different sialyltransferases contributes to chemokine receptor-mediated leukocyte adhesion. For the ST3Gal family of sialyltransferases, six different isoenzymes have been described (28). Previous studies of ST3Gal sialyltransferases have revealed different substrate specificity and tissue expression of the various isoenzymes (29). ST3Gal-IV shares substrate specificity mostly with ST3Gal-VI and, to a minor degree, with ST3Gal-III. This may indicate that ST3Gal-VI (besides ST3Gal-IV) could also be involved in the regulation of chemokine receptor-triggered leukocyte arrest. Currently, ST3Gal-VI^{-/-} mice are not available. Future studies using ST3Gal-VI^{-/-} mice and ST3Gal-IV^{-/-}/ST3Gal-VI^{-/-} mice will help to further characterize the role of sialylation on chemokine receptor function.

Collectively, our findings provide novel evidence for a crucial role of the sialyltransferase ST3Gal-IV in CXCR2-mediated neutrophil adhesion and recruitment under inflammatory conditions. Besides initiating the development of new therapeutic strategies in the treatment of CXCR2-mediated diseases, these results should also serve in stimulating additional

studies in elucidating the role of posttranslational sialylation/glycosylation on chemokine receptor function.

MATERIALS AND METHODS

Animals. ST3Gal-IV^{-/-} mice and CXCR2^{-/-} bone marrow chimeric mice were generated as previously described (12, 30) and housed in barrier facilities under specific pathogen-free conditions. Animal experiments were approved by the Regierungspräsidium Karlsruhe (AZ 35–9185.81/G–67/03), the Bezirksregierung Köln (AZ 50.203.2 AC37, 19/04), and the University of Virginia Animal Care and Use Committee.

Reagents. E-selectin blocking mAb 9A9 (rat IgG1; 30 µg per mouse) was a gift from B. Wolitzky (MitoKor, San Diego, CA). Mouse keratinocyte-derived chemokine (CXCL1) and human CXCL8 were obtained from Pepro-Tech and injected at 600 ng per mouse. *Bordetella pertussis* PTx was purchased from Sigma-Aldrich (4 µg per mouse), and rmP-selectin and mMICAM-1 were purchased from R&D Systems. In certain experiments, rmTNF-α (R&D Systems) was injected intrascrotally at 500 ng per mouse 3 h before intravital microscopy was begun. Biotinylated mAb to CXCR2 (clone RII115; reference 9) was provided by E. Brandt (Research Center Borstel, Borstel, Germany). Biotinylated MAL-II was obtained from Vector Laboratories.

Intravital microscopy. Intravital microscopy of the cremaster muscle was conducted as reported previously with an upright microscope (model 512815/20; Leica) with a saline immersion objective (SW 40/0.75NA) (8). Experiments were recorded via a charge-coupled device camera system (CF8/1; Kappa) on an S-VHS recorder (Panasonic). Supplemental videos were generated by digitizing and compressing the appropriate video sequences from recorded videotapes using Final Cut 5.1 (Apple) and Premiere Pro 1.5 (Adobe).

Data analysis of intravital experiments. Microvascular parameters were measured using a digital image processing system (31). The rolling leukocyte flux fraction was defined as previously described (32) by dividing leukocyte rolling flux by total leukocyte flux, estimated as (WBC) $v_b \pi (d/2)^2$. (WBC) is the actual systemic leukocyte count, v_b is the blood flow velocity, and d is the venular diameter.

Whole-mount histology. To differentially count intravascular and extravascular leukocytes, cremaster muscle whole mounts were prepared as previously described (33). Leukocyte differential counts were assessed using a microscope with a 100× 1.4NA oil immersion objective (Carl Zeiss, Inc.).

Thioglycollate-induced peritonitis model. Peritoneal recruitment of leukocytes was induced by intraperitoneal injection of 1 ml of freshly prepared thioglycollate (Sigma-Aldrich), as previously described (33). Some mice received 4 µg PTx 3 h before thioglycollate injection. 4 h after thioglycollate injection, mice were killed, and the peritoneum was rinsed with 5 ml of chilled PBS and massaged for 10 s. The total neutrophil count of the peritoneal lavage was assessed using Turks-stained samples.

Ex vivo microflow chamber. Microflow chambers (20 × 200-µm cross section; VitroCom) were used as previously described (15, 34). 20 µg/ml rmP-selectin, 15 µg/ml mMICAM-1, and 15 µg/ml rmCXCL1 were immobilized in the chamber for 2 h at room temperature. Chambers were blocked for 1 h with 10% casein (Thermo Fisher Scientific) and connected to tubing (PE 10; Becton Dickinson), which was inserted into the carotid artery. On the other chamber end, a tube (PE 50; Becton Dickinson) was connected and used to control wall shear stress. Microscopy was conducted using a microscope (Axioskop; Carl Zeiss, Inc.) with a saline immersion objective (SW 20/0.5NA). Images were recorded over 6 min for each chamber with a charge-coupled device color video camera (DXC-390; Sony) connected to an S-VHS recorder.

Flow cytometry. Expression of CXCR2 was analyzed on freshly isolated peripheral leukocytes from control and ST3Gal-IV^{-/-} mice. After centrifuga-

tion and red blood cell lysis with PharM-Lyse 10× solution (BD Biosciences), cells were suspended in PBS/1% BSA solution and incubated in the dark with a PE-conjugated mAb against CXCR2, an allophycocyanin-conjugated mAb against GR-1, and appropriate rat isotype controls (0.5 µg per 10⁶ cells; Becton Dickinson) for 30 min on ice. To study binding of CXCL8, blood leukocytes were incubated with different concentrations of CF-CXCL8 in the absence or presence of a 10-fold excess of unlabeled CXCL8 in PBS/1% BSA for 30 min on ice. Gated neutrophil populations were analyzed using a FACSCalibur or LSR II (both from BD Biosciences) with the FACSDiva software package (BD Biosciences).

Transendothelial migration assay. Transendothelial migration of neutrophils was analyzed as previously described (35). In brief, 5 × 10⁵ bone marrow-isolated neutrophils were added on top of a bEnd.5 cell monolayer that had been grown for 2 d in transwells (5 × 10⁴ bEnd.5 cells per well; Costar) with a 5-µm pore size, coated with 50 µg/ml laminin (Boehringer Mannheim). 16 h before adding neutrophils, bEnd.5 cells were stimulated with 5 nM TNF-α. Assays were run at 37°C and 10% CO₂ for 30 min in presence of 40 ng/ml CXCL1 in the bottom chamber. Migrated neutrophils were collected for cell counting (CASY; Schärfe-System). In some experiments, WT control neutrophils were incubated with 0.1 U neuraminidase from *Arthrobacter ureafaciens* (Roche) per 10⁶ cells at 37°C for 20 min.

Enzymatic deglycosylation. Isolated human neutrophils of CXCR2-transfected HEK293 cells were suspended at 10⁷ cells/ml in PBS containing 0.1% BSA and treated with the indicated dosages of recombinant N-glycosidase F or purified neuraminidase from *Vibrio cholerae* (both from Roche) for the indicated times. Subsequently, washed cells were investigated for radioligand binding and activation by chemoattractants, as described in iodination of chemokines and binding assays.

Immunoprecipitation and Western blotting. HEK293 cells were transfected to express CXCR2 using lipofectamine (Invitrogen) and were used for immunoprecipitation of CXCR2, as previously described (9). In brief, 0.5 × 10⁷ cells were suspended in 1 ml of ice-cold lysis buffer (PBS containing 1% Triton X-100 and a protease inhibitor mix; Roche) and incubated for 10 min. Cell lysates were cleared by centrifugation at 10,000 *g* for 10 min, and CXCR2 was precipitated with 5 µg/ml anti-CXCR2 antibody followed by the addition of 30 µl of protein A agarose (GE Healthcare). Washed precipitates were treated for 60 min with neuraminidase from *V. cholerae* at 37°C in PBS or left untreated. Subsequently, precipitates were analyzed by Western blotting for binding of biotinylated lectin MAL-II (20 µg/ml) or anti-CXCR2 antibody (2 µg/ml), which were detected by peroxidase-conjugated streptavidin (1:50,000; Roche) and addition of enhanced chemoluminescence substrate (GE Healthcare).

Iodination of chemokines and binding assays. Chemokines were radiolabeled by iodination at tyrosine residues using the chloramine T method (17). Before iodination, CXCL7 was chemically modified by the introduction of tyrosine residues, as previously described (17). PMN were suspended at 10⁷ cells/ml in Dulbecco's PBS (D-PBS)/2%BSA, and duplicate samples of 10⁶ cells were incubated on ice for 2 h with 10 nmol/liter of ¹²⁵I-labeled CXCL7 or 1 µmol/liter of ¹²⁵I-labeled CXCL4 in the presence or absence of a 10-fold excess of unlabeled CXCL7 or CXCL4, respectively. After twofold washing and sedimentation through 10% sucrose in D-PBS, bound radioactivity was determined in a γ counter, and unspecifically bound radioactivity determined in the presence of the unlabeled chemokines was subtracted.

Degranulation assays. Human polymorphonuclear cells from peripheral blood were generated as previously described (36) and suspended at 10⁷ cells/ml in D-PBS/0.1% BSA (low endotoxin BSA; Serva). Before the addition of secondary stimuli, cells were incubated at 37°C under agitation with 5 µg/ml cytochalasin B (Sigma-Aldrich) for the measurement of elastase release or with 9 ng/ml TNF-α for priming of lactoferrin release. Cells were then challenged with the secondary stimuli CXCL8 (1, 3, and 10 nM),

CXCL7 (1, 3, 10, and 30 nM), CXCL4 (1, 3, and 10 μ M), or fMLP (1, 3, and 10 nM) in D-PBS/0.1% BSA with 0.9 mM CaCl₂ and 0.5 mM MgCl₂. After 30 min of incubation at 37°C, the cells were sedimented, and supernatants were monitored for elastase enzymatic activity or lactoferrin release as previously described (37). Release rates for elastase and lactoferrin were expressed as the percentages of total content in detergent-treated PMN lysates prepared in 0.1% hexadecyltrimethylammonium bromide.

Statistics. The Sigma Stat 2.0 software package (SPSS Inc.) was used for statistical analysis. Microvascular parameters and leukocyte differentials between groups and treatments were compared with the one-way analysis of variance on ranks (Kruskal-Wallis) with a multiple pairwise comparison test (the Dunn's or Tukey test, as appropriate) or with the Student's *t* test, as appropriate. Statistical significance was set at $P < 0.05$, indicated by *.

Online supplemental material. Intravital microscopy was applied in Videos 1 and 2 to visualize leukocyte arrest in unstimulated cremaster muscle venules of WT (Video 1) and ST3Gal-IV^{-/-} (Video 2) mice before and after systemic injection of 600 ng CXCL8. Fig. S1 illustrates leukocyte rolling in untreated and TNF- α -treated cremaster muscle venules of WT, ST3Gal-IV^{-/-}, and CXCR2^{tm-/-} mice. Fig. S2 demonstrates the effect of enzymatic desialylation on expression and function of CXCR2 on human neutrophils. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20070846/DC1>.

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