PNAS RESEARCH ARTICLE | IMMUNOLOGY AND INFLAMMATION

CCR3-dependent eosinophil recruitment is regulated by sialyltransferase ST3Gal-IV

Roland Immler[a], Katrin Nussbaumer[a], Axel Doerner[b], Omar El Bounkari[c], Silke Huber[d], Janine Abisch[a], Matteo Napoli[a], Sarah Schmidt[a], Andreas Margraf[a], Monika Pruenster[a], Ina Rohwedder[a], Baerbel Lange-Sperandio[e], Marcus A. Mall[f,g,h], Renske de Jong[i], Caspar Ohnmacht[i], Juergen Bernhagen[c,j,k], David Voehringer[d,l], Jamey D. Marth[m], David Frommhold[a,n,1], and Markus Sperandio[a,1,2]

Edited by Nives Zimmermann, Cincinnati Children's Hospital Medical Center, Cincinnati, OH; received October 31, 2023; accepted April 3, 2024 by Editorial Board Member Carl F. Nathan

Eosinophil recruitment is a pathological hallmark of many allergic and helminthic diseases. Here, we investigated chemokine receptor CCR3-induced eosinophil recruitment in sialyltransferase *St3gal4*$^{-/-}$ mice. We found a marked decrease in eosinophil extravasation into CCL11-stimulated cremaster muscles and into the inflamed peritoneal cavity of *St3gal4*$^{-/-}$ mice. Ex vivo flow chamber assays uncovered reduced adhesion of *St3gal4*$^{-/-}$ compared to wild type eosinophils. Using flow cytometry, we show reduced binding of CCL11 to *St3gal4*$^{-/-}$ eosinophils. Further, we noted reduced binding of CCL11 to its chemokine receptor CCR3 isolated from *St3gal4*$^{-/-}$ eosinophils. This was accompanied by almost absent CCR3 internalization of CCL11-stimulated *St3gal4*$^{-/-}$ eosinophils. Applying an ovalbumin-induced allergic airway disease model, we found a dramatic reduction in eosinophil numbers in bronchoalveolar lavage fluid following intratracheal challenge with ovalbumin in *St3gal4*-deficient mice. Finally, we also investigated tissue-resident eosinophils under homeostatic conditions and found reduced resident eosinophil numbers in the thymus and adipose tissue in the absence of ST3Gal-IV. Taken together, our results demonstrate an important role of ST3Gal-IV in CCR3-induced eosinophil recruitment in vivo rendering this enzyme an attractive target in reducing unwanted eosinophil infiltration in various disorders including allergic diseases.

eosinophil | sialylation | asthma | inflammation | chemokines

Significance

Eosinophils play a major role in allergic and helminthic diseases. Recruitment of eosinophils into the inflamed tissue is regulated by eosinophil chemokine receptor CCR3 interacting with the chemokine CCL11. Our work provides evidence that sialylation of CCR3 by the sialyltransferase ST3Gal-IV is crucial for CCR3-binding to CCL11 and hence critical for eosinophil trafficking under baseline and inflammatory conditions including eosinophil recruitment into the lung during allergic airway disease.

Eosinophils are granulocytes developing in the bone marrow from myeloid progenitors. Similar to neutrophils, eosinophils are released from the bone marrow into the blood circulation with a rather short residence time of around 18 h (1). Eosinophils have mainly been implicated in allergic airway diseases (e.g., asthma) and in helminthic diseases with tissue-destructive functions through release of its granular load including major basic protein, and eosinophil peroxidase (2). Interestingly, recent evidence sheds light on eosinophil function as an immunemodulatory cell regulating a whole variety of biological processes. These range from recruitment of allergen-specific T cells to local inflammatory sites, T cell selection in the thymus, plasma cell survival, adipose tissue homeostasis, cardiac inflammation, ovulatory cycle, and mammary gland development to reports that it may act as antigen-presenting cell (1–5). In addition, accumulating evidence also implies a beneficial role of eosinophils in tumor immunity through cross talk of eosinophils with other leukocyte subsets including T cells and innate lymphoid cells (ILC2) (6). Understanding the exact molecular mechanisms of eosinophil trafficking is therefore instrumental for potentially interfering with specific eosinophil functions in various diseases (5, 7, 8).

Eosinophil trafficking has been studied under in vivo and under static and dynamic in vitro conditions (7, 9). Similar to other leukocyte subsets, the recruitment of eosinophils follows a cascade of adhesion and activation events beginning with the capture of eosinophils to the endothelium followed by eosinophil rolling along the endothelium (9). Several reports have addressed the role of selectins and selectin ligands in eosinophil rolling. In vitro flow chamber assays using immobilized P-selectin and E-selectin revealed that eosinophil rolling is mostly mediated by P-selectin binding to PSGL-1 (10, 11). In fact, P-selectin-dependent eosinophil rolling turned out to be more robust than P-selectin-dependent rolling of neutrophils (12). In contrast, the ability of eosinophils to roll on E-selectin was less pronounced than for neutrophils demonstrating the predominant role of P-selectin in eosinophil rolling (10, 13). During rolling, eosinophils screen the endothelial surface for additional activation signals such as eosinophil-specific chemokines immobilized on the endothelium. Chemokine–chemokine receptor interactions trigger the activation of eosinophil-expressed integrins with subsequent firm eosinophil arrest on the

Author contributions: R.I., K.N., J.D.M., D.F., and M.S. designed research; R.I., K.N., A.D., O.E.B., S.H., J.A., M.N., S.S., A.M., M.P., I.R., R.d.J., C.O., D.F., and M.S. performed research; B.L.-S. and M.A.M. contributed new reagents/analytic tools; R.I., K.N., A.D., O.E.B., S.H., J.A., M.N., S.S., A.M., M.P., I.R., B.L.-S., M.A.M., R.d.J., C.O., J.B., D.V., D.F., and M.S. analyzed data; and R.I., J.D.M., D.F., and M.S. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission. N.Z. is a guest editor invited by the Editorial Board.

[1]D.F. and M.S. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: markus.sperandio@lmu.de.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2319057121/-/DCSupplemental.

Published April 30, 2024.

endothelium. Several eosinophil targeting chemokines including CCL11 (eotaxin-1) have been identified to bind to the eosinophil-expressed chemokine receptor CCR3 (14). CCL11 binding to CCR3 triggers the activation of α4β1 integrin (VLA-4) on eosinophils and mediates firm eosinophil arrest on inflamed endothelium via interacting with VCAM-1 (15).

Recently, posttranslational sialylation has been identified to influence binding of several chemokines to their specific chemokine receptors (16–22). Our group could identify α2,3 sialyltransferase ST3Gal-IV to be essential for CXCR2-triggered firm neutrophil arrest in inflamed cremaster muscle venules in vivo (18). In addition, Döring and colleagues showed that CCL5-dependent recruitment of monocytes into atherosclerotic plaques was significantly impaired in the absence of ST3Gal-IV (20). These findings suggest a fundamental role of ST3Gal-IV-dependent posttranslational α2,3-sialylation for chemokine-induced leukocyte recruitment affecting various but not all leukocyte subsets and chemokine-chemokine receptor systems (18).

In this study, we identified ST3Gal-IV to be critical for CCR3-mediated eosinophil recruitment. We found that recruitment of *St3gal4*-deficient eosinophils was significantly reduced in cremaster muscle tissue stimulated with CCL11. In addition, we show that *St3gal4*-deficient eosinophils adhered less efficiently than wild type (WT) eosinophils in flow chambers coated with P-selectin, VCAM-1, and CCL11. Most strikingly, ovalbumin-induced eosinophil recruitment into bronchoalveolar lavage (BAL) fluid was almost completely abolished in the absence of ST3Gal-IV. Finally, binding of CCL11 to CCR3 on *St3gal4*-deficient eosinophils was significantly reduced. This also affected internalization of CCR3, which was completely absent in CCL11-stimulated *St3gal4*-deficient eosinophils suggesting a crucial role of the sialyltransferase ST3Gal-IV for eosinophil recruitment in vivo.

Results

Eosinophil Extravasation into the Inflamed Peritoneal Cavity Following Intraperitoneal Thioglycollate (TG) Injection. As reported previously, intraperitoneal injection of TG leads to extravasation of eosinophils into the inflamed peritoneal cavity over a course of 24 to 50 h after injection (23). Using the TG-induced peritonitis model, we investigated eosinophil influx into the peritoneum of WT and *St3gal4*$^{-/-}$ mice 24 h after injection. Eosinophil recruitment to the peritoneum was significantly reduced to 40% in *St3gal4*$^{-/-}$ compared to WT animals (Fig. 1*A*), suggesting that eosinophil recruitment is impaired in *St3gal4*-deficient mice. Interestingly, intraperitoneal injection of TG led to a marked decrease of systemic blood eosinophil counts 24 h after stimulation in WT mice (Fig. 1*B*), but not in *St3gal4*$^{-/-}$ mice (Fig. 1*C*). Notably, baseline systemic counts for eosinophils were not significantly different between WT and *St3gal4*$^{-/-}$ mice (Fig. 1*D*).

The chemokine CCL11 has been shown to be instrumental for eosinophil recruitment during an inflammatory response through interacting with chemokine receptor CCR3 on eosinophils (14, 24). To investigate a potential contribution of ST3Gal-IV in CCL11-CCR3 dependent eosinophil transmigration, we performed transwell assays using in vitro differentiated bone marrow eosinophils from WT and *St3gal4*$^{-/-}$ mice (*SI Appendix*, Fig. S1*A*) and CCL11 as chemoattractant. As expected, CCL11 resulted in marked transmigration of WT eosinophils compared to unstimulated controls (Fig. 1*D* and *SI Appendix*, Fig. S1*B*). In contrast, *St3gal4*$^{-/-}$ eosinophils did not respond to CCL11, indicating that ST3Gal-IV is involved in CCL-11-triggered eosinophil transmigration. Next, we tested whether ST3Gal-IV also has a role in leukotriene LTB_4-stimulated eosinophil transmigration. A contribution of LTB_4 in mediating eosinophil extravasation had been demonstrated earlier (25). We found a modest increase in transmigration of LTB_4 treated WT eosinophils compared to controls but only a mild increase in LTB_4-stimulated *St3gal4*$^{-/-}$ eosinophils (Fig. 1*E*). As we noted that transmigration of unstimulated eosinophils was already markedly higher in WT than in *St3gal4*$^{-/-}$ eosinophils, we went on and analyzed the relative increase in transmigration between unstimulated and stimulated WT and *St3gal4*$^{-/-}$ eosinophils. While WT and *St3gal4*$^{-/-}$ eosinophils showed a significant difference in their ratios between the number of unstimulated and CCL11-stimulated eosinophils in the transwell assay (Fig. 1*F*), this ratio was similar following stimulation with LTB_4 suggesting that LTB_4 stimulated transmigration of eosinophils is not directly dependent on ST3Gal-IV. In addition, these results also imply that other yet unknown factors impair eosinophil transmigration in the absence of ST3Gal-IV, without stimulation.

Eosinophil Extravasation in Whole Mounts of CCL11-Treated Cremaster Muscles. To explore the contribution of ST3Gal-IV in CCL11-triggered eosinophil extravasation in vivo, we intrascrotally injected CCL11 and performed Giemsa-staining of whole mount cremaster muscles obtained 4 h later. In WT mice, CCL11 induced pronounced recruitment of eosinophils into cremaster muscle tissue compared to intrascrotal injection of normal saline (Fig. 2 *A* and *B*). Of note, CCL11-induced eosinophil extravasation was significantly reduced in WT mice pretreated with the $G_{\alpha i}$-blocker pertussis toxin (PTx), demonstrating that CCL11/CCR3-induced signaling in eosinophils is dependent on $G_{\alpha i}$-signaling as shown previously (26). In contrast, the number of extravascular eosinophils following CCL11 stimulation was strongly reduced in *St3gal4*$^{-/-}$ compared to CCL11-treated WT mice. In addition, the $G_{\alpha i}$-blocker PTx was unable to further reduce eosinophil extravasation in *St3gal4*$^{-/-}$ mice suggesting that ST3Gal-IV is critical for CCL11-dependent eosinophil recruitment in vivo.

CCL11-Triggered Eosinophil Adhesion in an Ex Vivo Flow Chamber Assay. Using an autoperfused ex vivo flow chamber system (27, 28), we studied the contribution of ST3Gal-IV on eosinophil adhesion under flow conditions. Eosinophil adhesion was significantly higher in WT compared to *St3gal4*$^{-/-}$ mice in flow chambers coated with P-selectin, VCAM-1 and CCL11 (Fig. 2*C*). In contrast, we noted only a few adherent eosinophils with no difference in the number of adherent cells between WT and *St3gal4*-deficient mice in flow chambers coated with P-selectin and VCAM-1, P-selectin alone, CCL11 alone or VCAM-1 alone. This suggests that i) CCL11 is crucial for induction of eosinophil adhesion and ii) sialylation by ST3Gal-IV is required for CCL11-triggered eosinophil adhesion under flow. To exclude lower expression of adhesion relevant molecules on eosinophils being responsible for the observed reduction in eosinophil adhesion under flow, we performed FACS analysis on the surface expression of $\alpha_4\beta_1$ integrin, the key integrin on eosinophils mediating firm eosinophil adhesion in vivo and in vitro (15). We found no differences in surface expression of α_4 and β_1 integrin subunits between *St3gal4*$^{-/-}$ and WT eosinophils (Fig. 2 *D* and *E*), indicating that impaired eosinophil recruitment in *St3gal4*$^{-/-}$ mice is not a consequence of altered $\alpha_4\beta_1$ integrin availability.

ST3Gal-IV Regulates Baseline Numbers of Tissue-Resident Eosinophils in Some Organs. Both, CCR3 and CCL11 have been shown to be critical for eosinophil recruitment to various organs under homeostatic conditions (29–32). We therefore

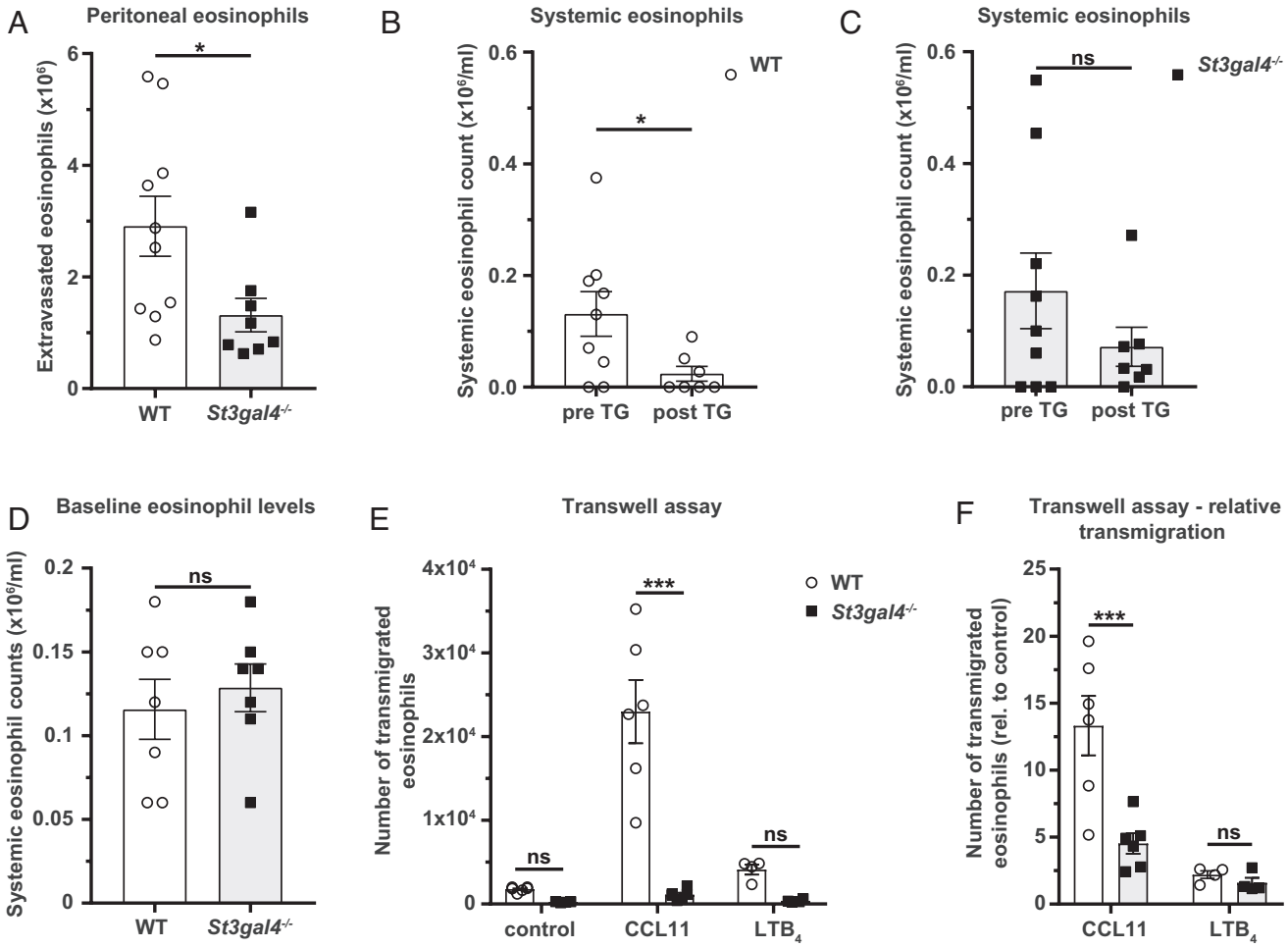

Fig. 1. Eosinophil recruitment during inflammation in vivo and in vitro. (*A*) Peritoneal eosinophil influx was assessed 24 h after injection of 4% TG into WT and *St3gal4*$^{-/-}$ mice. Number of eosinophils (n ≥ 8 mice per group, Mann–Whitney *U* test) was assessed from Haema quick stained peritoneal lavage samples. (*B* and *C*) Systemic eosinophil counts of (*B*) WT and (*C*) *St3gal4*$^{-/-}$ mice (n ≥ 7 per group; Mann–Whitney *U* tests) are shown before TG injection (pre TG) and after peritoneal lavage (post TG). (*D*) Systemic eosinophil counts of WT and *St3gal4*$^{-/-}$ mice (n = 7 mice per group, Mann–Whitney *U* test). (*E* and *F*) Differentiated bone marrow eosinophils from WT and *St3gal4*$^{-/-}$ mice were allowed to transmigrate toward a CCL11 and LTB_4 gradient (both 10 nM) for 45 min in a transwell assay. (*E*) Numbers of transmigrated eosinophils were analyzed by flow cytometry and (*F*) transmigration relative to unstimulated control was calculated (n = 4 to 6 mice per group, two-way ANOVA, Sidak's multiple comparisons). Data are presented as mean ± SEM, **P* < 0.05, ****P* < 0.001, ns: not significant.

speculated that baseline eosinophil levels in the thymus and the small intestine are affected by genetic deletion of ST3Gal-IV as well. To address this, we analyzed baseline eosinophil numbers within the thymus, the adipose tissue and the small intestine by flow cytometry (*SI Appendix*, Fig. S2). Interestingly, numbers of eosinophils were significantly decreased in the thymus as well and in adipose tissue of *St3gal4*$^{-/-}$ compared to WT mice (Fig. 2 *F* and *G*), indicating that the CCR3/CLL11 axis depends on ST3Gal-IV activity under inflammatory, but also under homeostatic conditions. Of note, we did not observe any difference in the number of resident eosinophils in small intestine (Fig. 2*H*) suggesting alternative and yet unknown mechanisms in regulating the number of resident eosinophils in the small intestine.

Endothelial Expression of VCAM-1 in CCL11 Stimulated Cremaster Muscle Venules. To elucidate underlying mechanisms of impaired eosinophil recruitment in *St3gal4*$^{-/-}$ mice, we assessed endothelial expression of eosinophil-specific adhesion molecule VCAM-1 in CCL11-stimulated and control cremaster muscle venules in *St3gal4*$^{-/-}$ mice and WT mice (Fig. 3 *A*–*D*). In both, WT and *St3gal4*$^{-/-}$ mice, there was no endothelial VCAM-1 expression detectable under baseline conditions (Fig. 3*E*). However, intrascrotal injection of CCL11 induced VCAM-1 expression in WT cremaster muscle venules as well as in *St3gal4*$^{-/-}$ mice, although this was not as pronounced as in WT mice.

To further explore CCL11-triggered increase in endothelial expression of VCAM-1 (33, 34) we investigated direct and indirect effects of CCL11 on VCAM-1 expression using cultured endothelial cells. To this end, we performed flow cytometric analysis of murine aortic endothelial cells (MAECs) treated either directly with CCL11 or with supernatant of CCL11 stimulated leukocytes. Direct endothelial stimulation with CCL11 did not up-regulate VCAM-1 compared to control treatment (Fig. 3*F*). However, endothelial cells treated with supernatant of CCL11-incubated murine leukocytes showed a significant increase in VCAM-1 expression compared to unstimulated and CCL11-stimulated endothelial cells suggesting that CCL11 does not directly affect endothelial VCAM-1 expression, but contributes indirectly to the upregulation of endothelial VCAM-1 by yet unknown mechanisms.

CCL11 Binding to *St3gal4*$^{-/-}$ Eosinophils and to CCR3. Next, we investigated CCL11 binding to the surface of WT and *St3gal4*$^{-/-}$ eosinophils by flow cytometry. Using FITC-labeled CCL11, we found a significant reduction in binding of CCL11 to *St3gal4*-deficient compared to WT eosinophils (Fig. 4*A*) suggesting that ST3Gal-IV-dependent α2,3 sialylation is critical for CCL11 binding to eosinophils. Because CCR3 is the cognate receptor

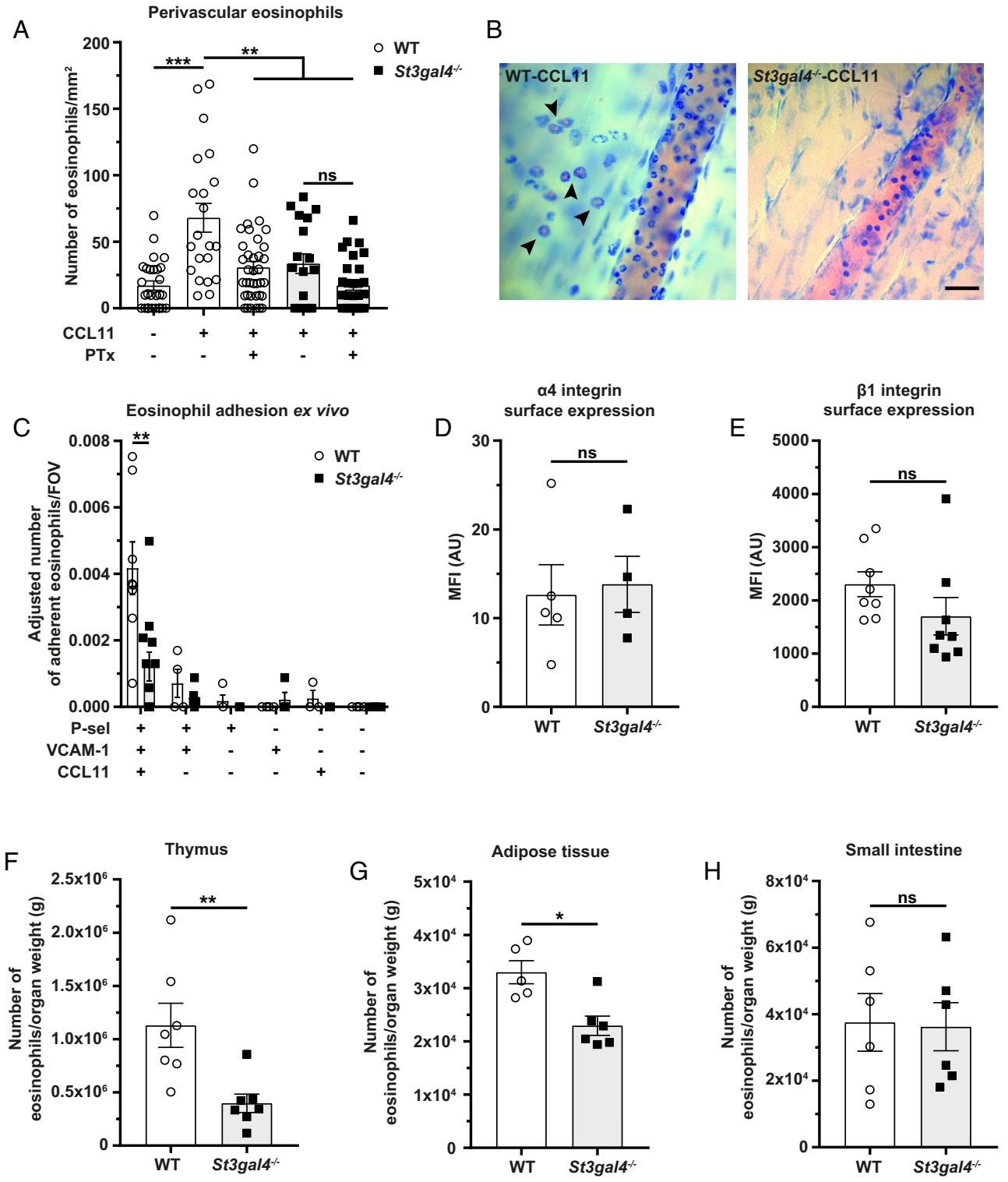

Fig. 2. Eosinophil trafficking during inflammation and under baseline conditions. (*A*) Giemsa stained whole mounts of CCL11-treated cremaster muscles of WT and *St3gal4*$^{-/-}$ mice were analyzed for the number of perivascular eosinophils per mm^2 perivascular surface area. WT mice receiving an intrascrotal injection of normal saline served as negative controls. In certain experiments, the $G\alpha_i$-blocker Pertussis toxin (PTx, 4 μg/mice) was intravenously administered 15 min before CCL11 injection to inhibit $G_{\alpha i}$-dependent signaling (n > 18 vessels from three mice per group, one-way ANOVA, Tukey's multiple comparisons). (*B*) Two representative micrographs illustrating eosinophil extravasation in CCL11-treated cremaster muscle whole mounts of WT and *St3gal4*$^{-/-}$ mice (Scale bar: 50 μm). (*C*) Number of adherent eosinophils from WT and *St3gal4*$^{-/-}$ mice were counted in an ex vivo microflow chamber system. Chambers were coated with various combinations of adhesion-relevant molecules including P-selectin, VCAM-1, and CCL11 and number of adherent eosinophils was assessed in flow chambers (n ≥ 3 chambers per group from ≥3 mice per group; two-way ANOVA, Sidak's multiple comparisons). (*D* and *E*) Surface expression of (*D*) α_4 integrin and (*E*) β_1 integrin was assessed on WT and *St3gal4*$^{-/-}$ eosinophils by flow cytometry (n ≥ 4 mice per group, Mann–Whitney *U* tests). (*F–H*) Baseline eosinophil levels were assessed in (*F*) thymus, (*G*) adipose tissue, and (*H*) small intestine of WT and *St3gal4*$^{-/-}$ mice (n ≥ 5 mice per group, Mann–Whitney *U* tests). Data are presented as mean ± SEM (*A* and *C–H*) and as representative micrographs (*B*) *P < 0.05, **P < 0.01, ***P < 0.001, ns: not significant.

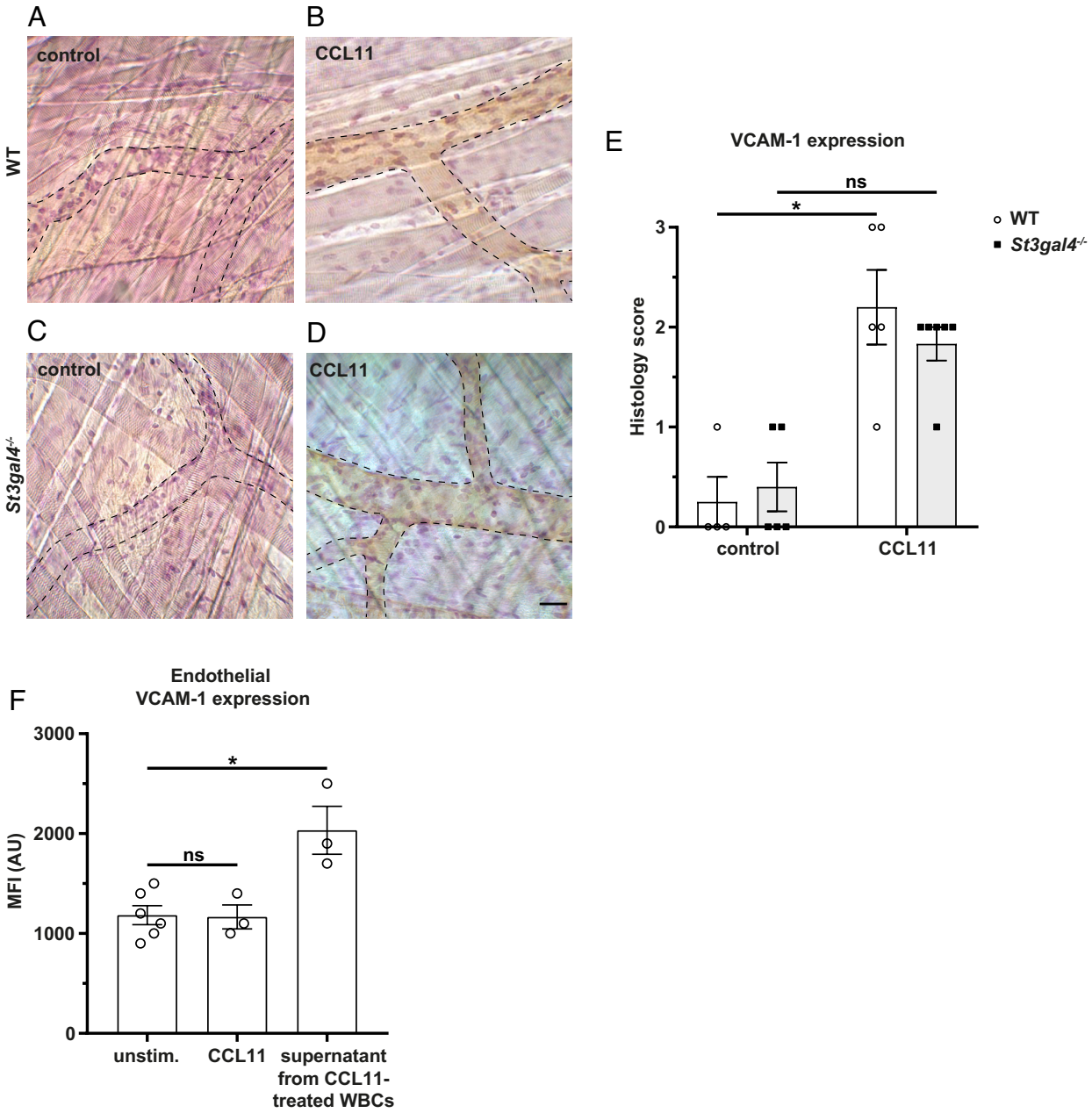

Fig. 3. Expression of VCAM-1 in CCL11-stimulated cremaster muscles venules. (*A–D*) Immunohistochemistry was performed to assess endothelial expression of VCAM-1 in CCL11-stimulated cremaster muscle venules of WT and *St3gal4*$^{-/-}$ mice. The primary anti-VCAM-1 antibody was injected systemically and incubated for 10 min. Cremaster muscle whole mounts were then fixed and permeabilized. Biotinylated secondary antibody, peroxidase-conjugated streptavidin, and diaminobenzidine (DAB) were used to detect endothelial expression (brown signal) on HE counter-stained background. Representative micrographs for VCAM-1 staining are depicted (three mice per group; Scale bar: 50 μm) (*E*). Micrographs of Giemsa-stained cremaster muscles from WT and *St3gal4*$^{-/-}$ mice were histologically scored based on their VCAM-1 staining (n ≥ 4 micrographs of three mice per condition; Kruskal–Wallis test, Dunn's multiple comparisons). (*F*) VCAM-1 expression was assessed in unstimulated and CCL11-stimulated immortalized MAECs by flow cytometry. In addition, VCAM-1 surface expression was assessed in MAECs incubated with supernatant from CCL11-stimulated leukocytes (n ≥ 3 individual experiments; Kruskal–Wallis test, Dunn's multiple comparisons). Data are presented as mean ± SEM (*E* and *F*) and as representative micrographs (*A–D*), *$P < 0.05$, ns: not significant.

of CCL11 and prominently expressed on eosinophils, we next explored whether binding of CCL11 to eosinophil-expressed CCR3 is affected in the absence of ST3Gal-IV. To do this, we performed pull-down experiments using cell lysates from bone marrow differentiated WT and *St3gal4*$^{-/-}$ eosinophils preincubated with CCL11, followed by addition of biotinylated anti-mCCL11 antibody and ensuing capture by streptavidin beads. CCR3/CCL11 complexes in the lysates were revealed by Western blot using anti-CCR3 antibody. We found a significant reduction of CCR3/CCL11 complexes retrieved from lysates of *St3gal4*$^{-/-}$ compared to WT eosinophils (Fig. 4*B*). Since reduced binding of CCL11 to *St3gal4*$^{-/-}$ eosinophils might be caused by diminished expression of CCR3 on unstimulated *St3gal4*$^{-/-}$ eosinophils, we conducted flow cytometric analysis of peripheral blood eosinophils which revealed similar expression of CCR3 on *St3gal4*$^{-/-}$ and WT eosinophils (Fig. 4*C*). Similarly, CCR3 expression levels of tissue eosinophils from thymus, adipose tissue, and small intestine did not differ between the genotypes (*SI Appendix*, Fig. S3 *A–C*). Of note, CCR3 surface expression of eosinophils cultivated from primary bone marrow cells was slightly but significantly higher in *St3gal4*$^{-/-}$ compared to WT eosinophils (*SI Appendix*, Fig. S3*D*).

Engagement of CCR3 on eosinophils by CCL11 has been reported to induce rapid downregulation and internalization of CCR3, which is also required for some CCL11-dependent effector functions

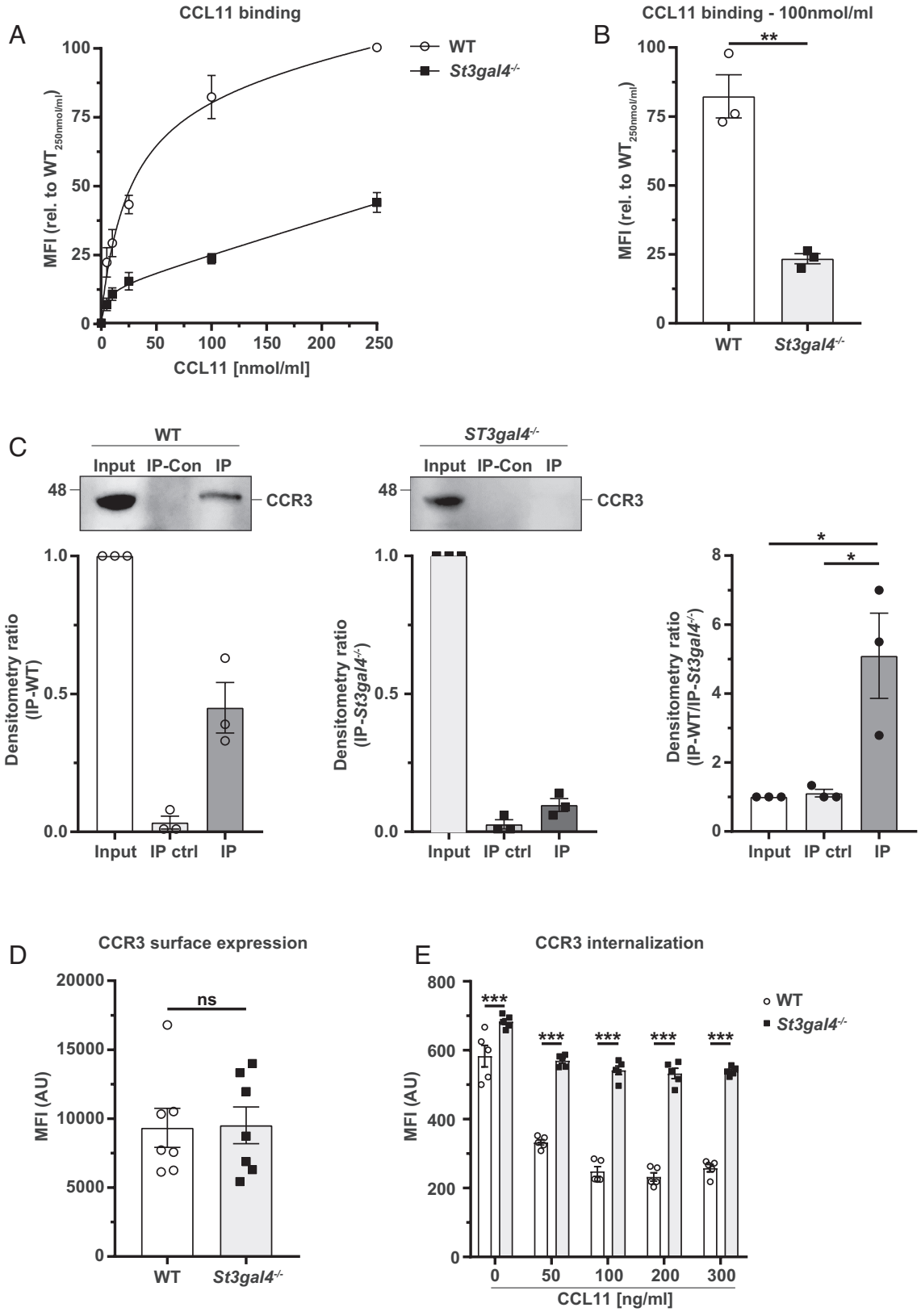

Fig. 4. CCL11 binding to CCR3. (*A*) CCL11 binding to isolated eosinophils from WT and *St3gal4*$^{-/-}$ mice was investigated for different CCL11 concentrations and analyzed by flow cytometry. (*B*) CCL11 binding to isolated eosinophils from WT and *St3gal4*$^{-/-}$ mice at 100 nmol/mL (n = 3 mice per group, unpaired Student's *t* test). (*C*) CCR3 binding to CCL11 was assessed in a pulldown and immunoprecipitation assay. Data are presented as mean ± SEM of densitometry ratio measurements of CCR3 (n = 3 mice per group; one-way ANOVA with Tukey's multiple comparisons). (*D*) Baseline expression of CCR3 on peripheral blood eosinophils from WT and *St3gal4*$^{-/-}$ mice obtained by flow cytometry (n = 7 mice per group, Mann–Whitney *U* test). (*E*) Internalization of surface-expressed CCR3 in eosinophils from WT and *St3gal4*$^{-/-}$ mice after stimulation with increasing concentrations of CCL11 (n = 5 mice per group; two-way ANOVA, Sidak's multiple comparisons). Data are presented as mean ± SEM and as representative Western blots (*C*), **P* < 0.05, ***P* < 0.01, ****P* < 0.001, ns: not significant.

including actin polymerization and shape change (35–37). Therefore, we set out to investigate internalization of CCR3 upon stimulation of bone marrow cultivated eosinophils with CCL11. We found a rapid and pronounced internalization of CCR3 in WT eosinophils as described earlier (37) (Fig. 4*D*). In contrast, internalization was completely absent in *St3gal4*$^{-/-}$ eosinophils. In conjunction, these findings demonstrate that CCL11 binding to CCR3 and internalization of CCR3 is strongly reduced in *St3gal4*$^{-/-}$ eosinophils.

Eosinophil Recruitment into the Lung During Ovalbumin Induced Airway Disease. To study the contribution of ST3Gal-V on eosinophil recruitment in allergic disease, we used a murine model of ovalbumin (OVA)-induced airway disease (38). After sensitization and challenge with OVA or placebo (tracer aluminum served as control), we performed BAL and assessed the number of eosinophils extravasated into the bronchoalveolar space. In WT mice, sensitization and challenge with OVA evoked profound extravasation of eosinophils into the bronchoalveolar space when compared to the control group where eosinophils were almost absent in BAL-fluid (BALF) (Fig. 5 *A*–*C*). In contrast, in *St3gal4*$^{-/-}$ mice OVA did not induce accumulation of eosinophils in the bronchoalveolar space. Thus, in *St3gal4*$^{-/-}$ mice, OVA and placebo treatment resulted in similarly low BALF eosinophils, suggesting that ST3Gal-IV is critically involved in eosinophil recruitment during allergic airway disease.

Finally, we analyzed levels of CCL11 in BAL fluid during OVA-induced airway disease by ELISA. CCL11 concentration in BALF was low under control conditions in both WT and *St3gal4*$^{-/-}$ mice (Fig. 5*D*). While bronchoalveolar CCL11 levels in response to OVA were only marginally increased over controls in WT mice, they were significantly elevated in *St3gal4*$^{-/-}$ mice when compared with respective controls, most likely reflecting the inability of CCL11 to recruit St3gal4-deficient eosinophils into the bronchoalveolar space. In addition, it inversely mirrors the impairment of ST3Gal-IV-controlled and CCR3-mediated CCL11 uptake/internalization by eosinophils in the absence of ST3Gal-IV.

Discussion

Eosinophil recruitment into inflamed tissue as observed in a whole variety of allergic and helminthic disorders follows a cascade of adhesion and activation events comparable to those reported for other innate immune cells (9). Eosinophils roll on P-selectin, interact with eosinophil-specific chemokines including CCL11 (eotaxin-1), CCL24 (eotaxin-2), and CCL26 (eotaxin-3) but also with less specific chemokines such as CCL5 (RANTES) and CCL7 (MCP-3),

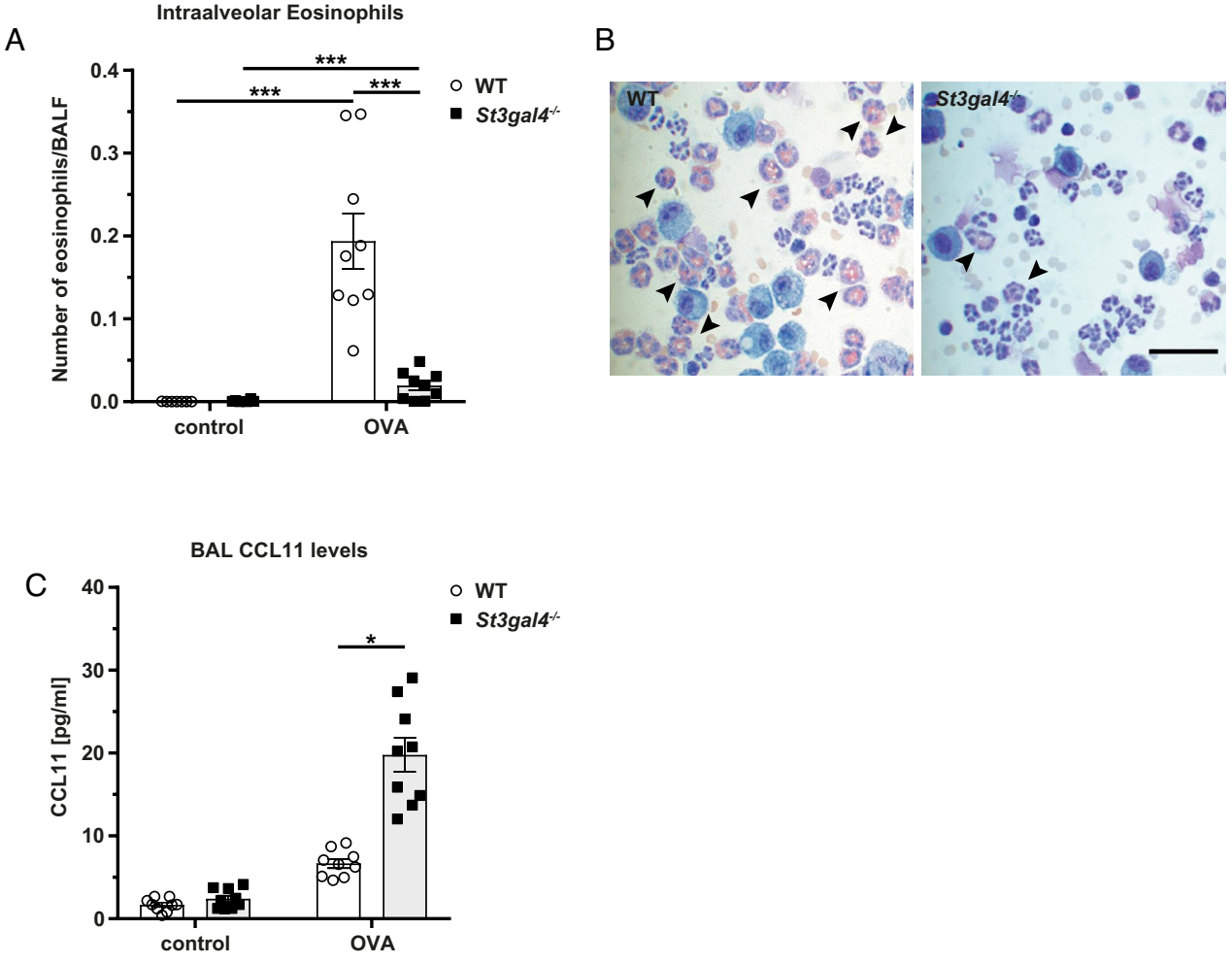

Fig. 5. Eosinophil recruitment into the lung in ovalbumin-induced airway disease. (*A*) In a model of ovalbumin-induced acute airway disease (OVA), the number of intra-alveolar eosinophils (n = 9 mice per group; two-way ANOVA, Sidak's multiple comparisons) obtained by BAL at day 28 was analyzed in WT and *St3gal4*$^{-/-}$ mice. Negative controls were sensitized and challenged with the tracer aluminum hydroxide only (control). (*B*) Two representative micrographs of cytospin preparations of BAL fluid illustrate bronchoalveolar eosinophil accumulation during ovalbumin-induced airway disease in WT and *St3gal4*$^{-/-}$ mice (arrows: eosinophils; Scale bar: 20 μm). (*C*) CCL11 concentrations in the BAL fluid supernatant for the respective groups were assessed using ELISA (n = 9 mice per group; two-way ANOVA, Tukey's multiple comparisons). Data are presented as mean ± SEM (*A* and *C*) and as representative micrographs (*B*), **P* < 0.05, ****P* < 0.001.

which trigger activation of α4β1 integrin (VLA-4) leading to firm eosinophil arrest on the inflamed endothelium (39). In this study, we identify the sialyltransferase ST3Gal-IV to be critically involved in CCL11-induced eosinophil recruitment. Intrascrotal injection of CCL11 leads to eosinophil extravasation into cremaster muscle tissue of WT, but not of *St3gal4*-deficient mice. In the TG-induced peritonitis model, eosinophil recruitment 24 h after TG injection was significantly reduced in the absence of ST3Gal-IV. In addition, ex vivo flow chamber assays demonstrated a severe eosinophil adhesion defect in *St3gal4*$^{-/-}$ mice in chambers coated with P-selectin, VCAM-1 and CCL11 suggesting defective eosinophil recruitment already within the intravascular compartment. ST3Gal-IV has been reported to contribute to selectin ligand formation and leukocyte rolling during inflammation in vivo (40, 41). However, these studies show that P-selectin dependent leukocyte rolling is affected only mildly in the absence of ST3Gal-IV. This is an important finding as rolling of eosinophils is predominantly P-selectin mediated and less dependent on E-selectin (13, 42). Therefore, potential changes in eosinophil rolling are unlikely to contribute to the marked eosinophil recruitment defect observed in the absence of ST3Gal-IV. This points toward a critical role of chemokine–chemokine receptor-dependent mechanisms as the main cause of defective eosinophil recruitment in *St3gal4*$^{-/-}$ mice. Posttranslational sialyation of chemokine receptors by ST3Gal-IV has already been described to be functionally relevant for several chemokine receptor systems including CXCR2 and CCR5 (18, 20). Our work adds CCR3, the main chemokine receptor expressed on eosinophils, to the list of chemokine receptors regulated by ST3Gal-IV. We show that binding of CCL11 to CCR3 expressed on eosinophils was significantly reduced in the absence of ST3Gal-IV. Interestingly, reduced binding of CCL11 to CCR3 also affected rapid internalization of CCR3 on *St3gal4*$^{-/-}$ eosinophils. As internalization of CCR3 is required for some of the effector functions of eosinophils (including shape change and actin polymerization) during chemokine-induced eosinophil activation (35, 36), loss of ST3Gal-IV leads to impaired eosinophil effector functions at two levels: i) direct signaling effects triggered by CCL11 binding to surface-expressed CCR3 leading to eosinophil arrest and ii) CCR3-internalization dependent effector functions which are necessary for efficient eosinophil extravasation. Interestingly, the absence of ST3Gal-IV did not only affect eosinophil recruitment in respective inflammation models but also the number of resident eosinophils in the thymus and adipose tissue, while baseline eosinophil numbers were not altered in the small intestine. In contrast to the thymus and adipose tissue, the small intestine is considered a tissue with high numbers of eosinophils and recruitment of eosinophils already occurs during fetal development (32, 43). Why the number of small intestine resident eosinophils is not affected in the absence of ST3Gal-IV is currently unknown, but the findings might indicate that recruitment mechanisms exist that are distinct to those in other organs.

While posttranslational sialylation by ST3Gal-IV as shown here enhances eosinophil recruitment, sialylation by sialyltransferases ST3Gal-III and ST6Gal-I has been reported to exert negative regulatory functions on eosinophil recruitment (44–46). These contrasting effects between ST3Gal-IV vs. ST3Gal-III and ST6Gal-I can be attributed to differences in carbohydrate substrate specificity. In fact, although ST3Gal-III utilizes similar carbohydrate substrates (Galβ1-3GlcNAc and Galβ1-4GlcNAc) on glycoproteins as ST3Gal-IV, subtle differences in preferred substrate carbohydrate structures lead to distinct modifications of target glycoproteins by the two sialyltransferases. ST3Gal-III has been described to contribute to the generation of 6′-sulfo-sialyl Lewis X, but not to 6-sulfo-sialyl Lewis X or sialyl Lexis X, two important carbohydrate determinants on selectin ligands, which are generated by the enzymatic activity of ST3Gal-IV (and ST3Gal-VI) (41). 6′-sulfo-sialyl Lewis X in contrast is a critical ST3Gal-III-dependent carbohydrate moiety and found on ligands of murine Siglec-F and human Siglec-8 (the closest functional paralog of Siglec-F) (47). Siglec-F exhibits a negative regulatory role on eosinophil recruitment during allergen-induced airway disease and induces eosinophil apoptosis as shown in *St3gal3*-deficient mice (48, 49). ST6Gal-I, the other sialyltransferase with a negative regulatory role on eosinophil function, has been postulated to exert its influence on eosinophil trafficking mostly through exosialylation by liver-secreted (and potentially B cell-secreted) ST6Gal-I of yet unknown surface carbohydrate substrates on hematopoietic cells (46, 50). These findings emphasize the complex regulation of sialylation on eosinophil trafficking and uncover also challenges and risks of therapeutically targeting sialylation.

To demonstrate that impaired CCR3-dependent eosinophil recruitment as observed in the absence of ST3Gal-IV is also relevant in eosinophil-dependent pathologies, we investigated eosinophil extravasation in an ovalbumin-induced airway disease model. Eosinophil extravasation into the bronchoalveolar space was dramatically reduced following OVA-stimulation. This indicates that post-translational sialylation by ST3Gal-IV is critical for successful eosinophil recruitment to the lung. Similar impairment in eosinophil recruitment has been observed in CCR3-deficient mice (14, 31), indicating that chemokine-receptor interaction of other CCR3 binding chemokines (i.e., RANTES) might also be affected. Considering the fact that RANTES binding to CCR5 is also reduced in the absence of ST3Gal-IV (20), it is likely that CCR3-binding chemokines including RANTES interact with CCR3 in a sialylation-dependent fashion.

Taken together, we identify ST3Gal-IV as a critical regulator of eosinophil recruitment on the level of CCR3-dependent eosinophil effector functions. Loss of ST3Gal-IV does not only reduce binding of CCR3 to CCL11, it also leads to a complete absence of CCR3 internalization. This has significant consequences on eosinophil recruitment in allergen-induced airway disease. Therefore, blocking ST3Gal-IV in eosinophils might be an interesting therapeutic strategy to reduce eosinophil-dependent tissue damage in disorders with unwanted eosinophil recruitment including allergic airway diseases.

Material and Methods

Animals. *St3gal4*$^{-/-}$ mice were generated as described earlier (51), backcrossed over at least seven generations into the C57BL/6NCrl background, and housed in barrier facilities under SPF conditions. Mouse experiments were approved by the Regierungspräsidium Karlsruhe, Baden-Württemberg, Germany, AZ 35-9185.81/G-67/03 and by the Regierung von Oberbayern AZ 55.2-1-54-2531-175/09.

TG-Induced Peritonitis Model. Peritoneal recruitment of eosinophils was induced by i.p. injection of 1 mL freshly prepared TG (Sigma) as reported earlier (18). Twenty-four hours later, mice were killed and the peritoneum flushed with 5 mL chilled PBS and massaged for 10 s. Then, eosinophil counts of the peritoneal lavage fluid were assessed using Haema quick (Niepoetter) staining. Systemic eosinophil counts were assessed before TG injection and after peritoneal lavage using Haema quick staining of tail vein blood samples.

Transwell Assays. Eosinophils were isolated and differentiated for 14 d from bone marrow of *St3gal4*$^{-/-}$ and WT mice as described elsewhere (52). Transwell assays were conducted as reported previously (53). Briefly, 3×10^5 bone marrow-derived eosinophils in HBSS buffer [containing 0.1% of glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.25% BSA, and 10 mM HEPES (Sigma-Aldrich), pH 7.4] were added to the upper compartment of the transwell filters in 24

wells plates (5 μm pore size, Corning) and allowed to migrate toward a CCL11 (10 nM, R&D Systems) or LTB_4 (10 nM, Sigma-Aldrich) gradient for 45 min at 37 °C. HBSS alone was used as negative control. Migrated cells were collected and counted by flow cytometry (CytoFlex S, Beckman Coulter) using Flow-Count Fluorspheres (Beckman Coulter) and FlowJo software for later analysis. Eosinophils were defined as Siglec-F^+ population (Siglec-F-Alexa647, clone 83103, BD Pharmingen, 5 μg/mL). Differentiation resulted in 95 ± 1% (WT) and 94 ± 1% (*St3gal4*$^{-/-}$) Siglec-F^+ cells, respectively (*SI Appendix*, Fig. S1*A*).

Preparation and Histology of Cremaster Muscle Whole Mounts. To induce an eosinophil inflammation, rmCCL11 (1 μg, R&D Systems) was injected intrascrotally. Mice injected with isovolumetric normal saline served as controls. After 4 h, anesthesia was induced by i.p. injection of ketamine (125 mg/kg body weight; Pfizer) and xylazine (12.5 mg/kg body weight; Alverta) and thereafter, surgical preparation of the cremaster muscle was conducted as described previously (54). To count extravascular eosinophils, cremaster muscle whole mounts were prepared as reported using a Zeiss microscope with a 100x/1.4NA oil immersion objective (Zeiss) (55). In some experiments, the $G_{\alpha i}$-blocker PTx (4 μg/mice; from *Bordetella pertussis*, Sigma-Aldrich) was intravenously administered 15 min before CCL11 to inhibit $G_{\alpha i}$-dependent signaling via CCR3.

Ex Vivo Microflow Chamber. Microflow chambers (20 × 200 μm cross-section, VitroCom) were used as previously described (28) and coated with different combinations of rmP-selectin (20 μg/mL), rmVCAM-1 (15 μg/mL), and rmCCL11 (15 μg/mL) (all R&D Systems) for 2 h at room temperature as indicated. Blocking of chambers was performed for 1 h using 5% casein (Pierce Chemicals). Chambers were then connected to PE 10 tubing (B&D) inserted into the mouse carotid artery, as described (27). Observation of eosinophil adhesion in the flow chamber was conducted using an upright microscope (Olympus BX51, Olympus) with a saline immersion objective (SW 20×/0.5NA, Olympus). Flow chambers were perfused with whole blood via the carotid catheter for 10 min and recorded using a CCD color video camera (model DXC-390, SONY Corporation). Flow chambers were subsequently fixed and stained with an eosinophil staining solution (containing 0.5% eosin, 0.5% phenol, and 0.5% formaldehyde). Number of adherent eosinophils (within a distance of 20 mm) was counted using a Leica DM750 microscope (equipped with a 40×/0.65NA HI plan objective) and normalized to the eosinophil flux (number of systemic eosinophils that pass the flow chamber at the given flow rate). Systemic eosinophil counts were assessed using Haema quick® staining of carotid artery blood samples.

Eosinophil Baseline Levels in Tissues. To investigate eosinophil baseline levels in small intestine, adipose tissue, and thymus of *St3gal4*$^{-/-}$ and WT mice, organs were harvested and single cell suspensions were generated as previously described (56). Briefly, small intestine was flushed with PBS, cut longitudinally, and washed twice with PBS (containing 2% BSA, 5 mM EDTA, 1%P/S) for 25 min at 37 °C. The samples were weighed out and subsequently digested using collagenase type IV and VIII [both 250 U/mL, Sigma-Aldrich, in HBSS buffer, containing 0.1% of glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.25% BSA, and 10 mM HEPES (Sigma-Aldrich), pH7.4] for 50 min at 37 °C. Perigonadal adipose tissue was collected, weighed out, and digested using liberase (0.2 mg/mL Sigma-Aldrich, in HBSS) for 50 min at 37 °C. Cell suspensions as well as collected and weighed thymus were passed through a cell strainer, centrifuged and Fc-block was added (anti CD16/CD32, clone 93, BioLegend) for 10 min. Cells were subsequently stained with anti-CD45-PB (clone 104, BioLegend), anti-CCR3-FITC (clone J073E5, BioLegend), or FITC-isotype control (clone RTK2758, BioLegend), respectively, and anti-Siglec-F-Alexa647 (clone 83103, BD Pharmingen, all 5 μg/mL). Cells were fixed and eosinophils (CD45$^+$/Siglec-F^+) population was counted using a CytoFlex S flow cytometer, Flow-Count Fluorospheres (both Beckman Coulter) and FlowJo software, including CCR3 expression levels of tissue eosinophils. Therefore, mean fluorescence intensities of isotype controls were subtracted from CCR3 mean fluorescence intensities. Numbers of eosinophils in peripheral blood were assessed using a hematocytometer (ProCyte, IDEXX Laboratories).

Immunohistochemistry. To investigate ST3-Gal-IV-dependent expression of endothelial VCAM-1 in response to CCL11 stimulation, we performed immunohistochemical analysis of whole mounts of CCL11 and normal saline-treated cremaster muscles of *St3gal4*$^{-/-}$ and WT mice. As described previously, primary antibody against VCAM-1 (MVCAMA 429; 30 μg/mouse; Bio-Rad) was systemically injected and incubated for 10 min (57) before the mouse was killed to target antibody binding to surface-expressed antigens within the vasculature. Secondary antibody application and staining were then performed on whole mounts obtained postmortem. Analysis of stained slides was conducted on a Leica DMRB upright microscope and a 25×/0.75NA oil immersion objective (both Leica). Photographs of the samples were taken using a color CCD camera (KAPPA). Images were histologically scored in a blinded fashion with 0 (no VCAM-1 staining), 1 (weak VCAM-1 staining), 2 (intermediate VCAM-1 staining), and 3 (strong VCAM-1 staining).

Immunoprecipitation. Bone marrow-derived eosinophils of *St3gal4*$^{-/-}$ or WT mice were lysed on ice with IP-lysis buffer [1× cell lysis buffer, Cell Signaling; 100 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich), and 1× protease and phosphatase inhibitors (Roche)]. The cleared cell lysates were subsequently incubated with prewashed DYNAL™ Dynabeads™ M-280 Streptavidin (Thermo Fisher) for 2 h at 4 °C (preclearing step). After centrifugation, the supernatants were incubated with rmCCL11 and biotinylated anti-mouse CCL11 (clone 42285, R&D Systems) or IgG-isotype control at 4 °C with constant rotation overnight. The precipitated proteins were washed twice with IP-lysis Buffer and analyzed on SDS-PAGE followed by Western blotting using anti-CCR3 antibody (Abcam).

Flow Cytometry. Mouse blood was obtained from the retroorbital sinus and collected in 300 μL 20 U/mL Heparin in saline. Fc-receptors were blocked with anti-mouse CD16/CD32 (eBioscience). For analysis of surface expression of CCR3 and VLA-4 subunits α_4 and β_1, whole blood was either incubated with anti-CCR3-FITC (clone J073E5, BioLegend), anti-CD29-PB (β1 subunit; clone HMβ1-1, BioLegend), or anti-CD49d-FITC (α4 subunit; clone R1-2, BioLegend) antibodies, or respective isotype controls (all 5 μg/mL). Subsequently, red blood cells were lysed and leukocytes were fixed using FACS lysing solution (BD BioScience). Samples were analyzed by flow cytometry (Gallios or CytoFlex S, Beckman Coulter) and FlowJo software (including isotype subtraction). Eosinophils were defined as Siglec-F^+ population (clone E50-2440, BD Bioscience 5 μg/mL).

For binding analysis of CCL11 to CCR3, cells were incubated with CCL11-Alexa647 (Almac) at the indicated concentrations (in HBSS). After 2 h of incubation on 4 °C, cells were washed, fixed in 1% PFA, and analyzed using a Gallios flow cytometer and FlowJo software. Eosinophils were defined as Siglec-F^+-PE cells (clone E50-2440; BD Pharmingen; 5 μg/mL) and analyzed for CCL11 binding. CCL11 binding to WT cells was set to 100% at a CCL11 concentration of 250 nmol/L.

To investigate CCL11-stimulated endothelial VCAM-1 expression, we performed flow cytometric analysis of cultured MAECs treated either directly with CCL11 or with supernatant of CCL11 stimulated leukocytes. MAECs were isolated and cultured as described previously (55). MAECs were grown to near confluence in 6-well plates (Greiner) and incubated with saline or CCL11 at 2 μg/10^6 cells/mL for 4 h at 37 °C. In a second approach, bone marrow derived-leukocytes were stimulated with CCL11 at 2 μg/10^6 cells/ml for 4 h at 37 °C. Next, the supernatant was incubated with cultured MAECs for 4 h at 37 °C. In all experiments, cells were harvested with Accutase (PAA) and washed with PBS containing 1% BSA before they were incubated in the dark for 45 min on ice with a PE-conjugated anti-mouse VCAM-1 mAb (clone 429 MVCAM.A, BioLegend) or respective isotype control (eBioscience). Unstimulated cells served as controls. Gated endothelial cells were analyzed using a four-decade FACS Scan LSRII with DIVA software package (B&D).

Internalization Assay. The internalization assay was performed as previously described (58). Briefly, bone marrow–differentiated eosinophils from *St3gal4*$^{-/-}$ or WT mice were stimulated with different concentrations of rmCCL11 (50, 100, 200, and 300 ng/mL) for 25 min at 37 °C. The cells were subsequently washed three times with ice-cold PBS followed with flow cytometry buffer (PBS supplemented with 0.5% BSA) and incubated with PE-conjugated anti-CCR3 antibody (clone 83101, R&D Systems) or the corresponding isotype control (BD Pharmingen) for 1 h in the dark at 4 °C. After incubation, the cells were intensively washed and analyzed by BD FACSVerse™ (BD Biosciences). Quantification of the measurements was performed using FlowJo software.

Eosinophil Recruitment during Ovalbumin-Induced Acute Airway Disease. For induction of acute airway disease in mice, we used the established model of ovalbumin-induced acute airway disease (38). Briefly, mice were sensitized i.p. at day 0, 14 and 21 with 100 μg ovalbumin at 1 mg aluminum hydroxide hydrate (both Sigma-Aldrich) in 100 μL PBS. On days 26 and 27, we preformed intratracheal ovalbumin challenge with 50 μg Ovalbumin in 50 μL PBS during anesthetic inhalation of isoflurane (Baxter). At day 28, mice were anesthetized, the trachea was cannulated and a BAL of the left lung was performed using a rinse solution containing PBS and protease inhibitor solution (Protease Inhibitor Cocktail, Sigma) to harvest infiltrated cells. For leukocyte differentiation, stained cytospin preparations were analyzed on a Leica DMRB upright microscope and a 25x/0.75NA oil immersion objective (both Leica). Finally, CCL11 concentration in BALF was analyzed using Quantikine ELISA (R&D Systems) according to the manufacturer's instructions.

Statistics. Graph pad prism 8 and Adobe Illustrator were used for statistical analysis and to generate the figures. Group sizes were chosen on the basis of previous experiments. Statistical tests were carried out according to the groups being compared. For pairwise comparisons, either a Mann–Whitney *U* test or a Student's *t* test was carried out. For more than two groups, either a one-way ANOVA (including Tukey's post hoc test), a Kruskal–Wallis test (including Dunn's post hoc test) or a two-way ANOVA (including Sidak's or Tukey's post hoc test, respectively) was used. Statistical significance was set at $P < 0.05$.

Data, Materials, and Software Availability. All study data are included in the article and/or supporting information.

ACKNOWLEDGMENTS. We thank Dorothee Gössel, Melitta Weissinger, Inna Babushkina, and Susanne Bierschenk for their technical support. M.S. received generous support for this study from the Mizutani Foundation (Grant Ref. Nr. 090063). Support came also from the Deutsche Forschungsgemeinschaft (DFG) TRR359 project B02 (R.I. and M.S.) and B07 (M.S.) and TRR332 project C02 (M.S.). D.F. was supported by DFG (FR3068/4–1) and Bundesministerium für Bildung und Forschung (BMBF) (01GL1746E). J.B. received support from DFG (CRC1123-A3) and R.d.J. was supported by the Humboldt foundation (grant number 1200905–HFST-P). C.O. is supported by the European Research Council (ERC Starting grant project number 716718) and the DFG (project number 395357507–SFB1371 and grant number OH 282/1-1 within FOR2599). D.V. is supported by DFG FOR2599 project 4. J.D.M.'s contributions were supported by NIH grants HL131474, DK048247, AI151371, and HL158677.

Author affiliations: [a]Institute of Cardiovascular Physiology and Pathophysiology, Walter Brendel Center of Experimental Medicine, Ludwig-Maximilians-Universität München, PLanegg-Martinsried 82152, Germany; [b]Department of Neonatology, University of Heidelberg, Heidelberg 69120, Germany; [c]Division of Vascular Biology, Institute for Stroke and Dementia Research, Ludwig-Maximilians-Universität, München 81377, Germany; [d]Institute of Immunology, Ludwig-Maximilians-Universität München, München 80336, Germany; [e]von Haunersches Kinderspital, Klinikum der Universität München, Ludwig-Maximilians-Universität, München 80336, Germany; [f]Department of Pediatric Respiratory Medicine, Immunology and Critical Care Medicine, Charité–Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität Berlin, Berlin 13353, Germany; [g]Berlin Institute of Health at Charité–Universitätsmedizin Berlin, Berlin 10117, Germany; [h]German Centre for Lung Research, Associated Partner Site, Berlin 13353, Germany; [i]Center of Allergy and Environment (ZAUM), Technical University and Helmholtz Center Munich, München 80802, Germany; [j]Munich Cluster for Systems Neurology, München 81377, Germany; [k]Munich Heart Alliance, München 80336, Germany; [l]Department of Infection Biology, University of Erlangen, Erlangen 91054, Germany; [m]Sanford Burnham Prebys Medical Discovery Institute, Infectious and Inflammatory Diseases, San Diego, CA 92037; and [n]Children's Hospital Memmingen, Memmingen 87700, Germany

1. H. F. Rosenberg, K. D. Dyer, P. S. Foster, Eosinophils: Changing perspectives in health and disease. *Nat. Rev. Immunol.* **13**, 9–22 (2013).
2. L. Gigon, T. Fettrelet, S. Yousefi, D. Simon, H. U. Simon, Eosinophils from A to Z. *Allergy* **78**, 1810–1846 (2023).
3. E. A. Jacobsen, R. A. Helmers, J. J. Lee, N. A. Lee, The expanding role(s) of eosinophils in health and disease. *Blood* **120**, 3882–3890 (2012).
4. P. F. Weller, L. A. Spencer, Functions of tissue-resident eosinophils. *Nat. Rev. Immunol.* **17**, 746–760 (2017).
5. E. A. Jacobsen *et al.*, Eosinophil knockout humans: Uncovering the role of eosinophils through eosinophil-directed biological therapies. *Annu. Rev. Immunol.* **39**, 719–757 (2021).
6. S. Grisaru-Tal, M. E. Rothenberg, A. Munitz, Eosinophil-lymphocyte interactions in the tumor microenvironment and cancer immunotherapy. *Nat. Immunol.* **23**, 1309–1316 (2022).
7. P. C. Fulkerson, M. E. Rothenberg, Targeting eosinophils in allergy, inflammation and beyond. *Nat. Rev. Drug Discov.* **12**, 117–129 (2013).
8. H. J. Kim, Y. Jung, The emerging role of eosinophils as multifunctional leukocytes in health and disease. *Immune Netw.* **20**, e24 (2020).
9. D. Broide, P. Sriramarao, Eosinophil trafficking to sites of allergic inflammation. *Immunol. Rev.* **179**, 163–172 (2001).
10. P. Sriramarao *et al.*, E-selectin preferentially supports neutrophil but not eosinophil rolling under conditions of flow in vitro and in vivo. *J. Immunol.* **157**, 4672–4680 (1996).
11. D. H. Broide, D. Humber, P. Sriramarao, Inhibition of eosinophil rolling and recruitment in p-selectin– and intracellular adhesion molecule-1-deficient mice. *Blood* **91**, 2847–2856 (1998).
12. B. S. Edwards *et al.*, Expression of P-selectin at low site density promotes selective attachment of eosinophils over neutrophils. *J. Immunol.* **165**, 404–410 (2001).
13. K. D. Patel, R. P. McEver, Comparison of tethering and rolling of eosinophils and neutrophils through selectins and p-selectin glycoprotein ligand-1. *J. Immunol.* **159**, 4555–4565 (1997).
14. P. C. Fulkerson *et al.*, A central regulatory role for eosinophils and the eotaxin/CCR3 axis in chronic experimental allergic airway inflammation. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 16418–16423 (2006).
15. P. Sriramarao *et al.*, VCAM-1 is more effective than MAdCAM-1 in supporting eosinophil rolling under conditions of shear flow. *Blood* **95**, 592–601 (2000).
16. A. Ludwig, J. E. Ehlert, H. D. Flad, E. Brandt, Identification of distinct surface-expressed and intracellular CXC-chemokine receptor 2 glycoforms in neutrophils: N-glycosylation is essential for maintenance of receptor surface expression. *J. Immunol.* **165**, 1044–1052 (2000).
17. N. Bannert *et al.*, Sialylated O-glycans and sulfated tyrosines in the NH2-terminal domain of CC chemokine receptor 5 contribute to high affinity binding of chemokines. *J. Exp. Med.* **194**, 1661–1673 (2001).
18. D. Frommhold *et al.*, Sialyltransferase ST3Gal-IV controls CXCR2-mediated firm leukocyte arrest during inflammation. *J. Exp. Med.* **205**, 1435–1446 (2008).
19. E. Kiermaier *et al.*, Polysialylation controls dendritic cell trafficking by regulating chemokine recognition. *Science* **351**, 186–190 (2016).
20. Y. Doring *et al.*, Deficiency of the sialyltransferase St3Gal4 reduces Ccl5-mediated myeloid cell recruitment and arrest: Short communication. *Circ. Res.* **114**, 976–981 (2014).
21. M. Sperandio, The expanding role of alpha2-3 sialylation for leukocyte trafficking in vivo. *Ann. N. Y. Acad. Sci.* **1253**, 201–205 (2012).
22. S. Sitte, D. Doehler, M. Sperandio, J. D. Marth, D. Voehringer, The sialyltransferase ST3Gal-IV guides murine T-cell progenitors to the thymus. *Blood Adv.* **4**, 1930–1941 (2020).
23. D. A. Steeber *et al.*, Leukocyte entry into sites of inflammation requires overlapping interactions between the l-selectin and icam-1 pathways. *J. Immunol.* **163**, 2176–2186 (1999).
24. M. E. Rothenberg, J. A. MacLean, E. Pearlman, A. D. Luster, P. Leder, Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. *J. Exp. Med.* **185**, 785–790 (1997).
25. A. M. Tager *et al.*, BLTR mediates leukotriene B(4)-induced chemotaxis and adhesion and plays a dominant role in eosinophil accumulation in a murine model of peritonitis. *J. Exp. Med.* **192**, 439–446 (2000).
26. S. L. Cuvelier, K. D. Patel, Shear-dependent eosinophil transmigration on interleukin 4-stimulated endothelial cells: A role for endothelium-associated eotaxin-3. *J. Exp. Med.* **194**, 1699–1709 (2001).
27. M. L. Smith, M. Sperandio, E. V. Galkina, K. Ley, Autoperfused mouse flow chamber reveals synergistic neutrophil accumulation through P-selectin and E-selectin. *J. Leukoc. Biol.* **76**, 985–993 (2004).
28. M. Sperandio *et al.*, Ontogenetic regulation of leukocyte recruitment in mouse yolk sac vessels. *Blood* **121**, e118–28 (2013).
29. A. Mishra, S. P. Hogan, J. J. Lee, P. S. Foster, M. E. Rothenberg, Fundamental signals that regulate eosinophil homing to the gastrointestinal tract. *J. Clin. Invest.* **103**, 1719–1727 (1999).
30. A. N. Matthews *et al.*, Eotaxin is required for the baseline level of tissue eosinophils. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6273–6278 (1998).
31. A. A. Humbles *et al.*, The murine CCR3 receptor regulates both the role of eosinophils and mast cells in allergen-induced airway inflammation and hyperresponsiveness. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 1479–1484 (2002).
32. A. Gurtner, D. Crepaz, I. C. Arnold, Emerging functions of tissue-resident eosinophils. *J. Exp. Med.* **220**, e20221435 (2023).
33. G. Huang, L. Tao, S. Shen, L. Chen, Hypoxia induced CCL28 promotes angiogenesis in lung adenocarcinoma by targeting CCR3 on endothelial cells. *Sci. Rep.* **6**, 27152 (2016).
34. R. Salcedo *et al.*, Eotaxin (CCL11) induces in vivo angiogenic responses by human CCR3+ endothelial cells. *J. Immunol.* **166**, 7571–7578 (2001).
35. N. Zimmermann, J. J. Conkright, M. E. Rothenberg, CC chemokine receptor-3 undergoes prolonged ligand-induced internalization. *J. Biol. Chem.* **274**, 12611–12618 (1999).
36. N. Zimmermann, M. E. Rothenberg, Receptor internalization is required for eotaxin-induced responses in human eosinophils. *J. Allergy Clin. Immunol.* **111**, 97–105 (2003).
37. I. Sabroe *et al.*, The carboxyl terminus of the chemokine receptor CCR3 contains distinct domains which regulate chemotactic signaling and receptor down-regulation in a ligand-dependent manner. *Eur. J. Immunol.* **35**, 1301–1310 (2005).
38. M. L. Conrad *et al.*, Comparison of adjuvant and adjuvant-free murine experimental asthma models. *Clin. Exp. Allergy* **39**, 1246–1254 (2009).
39. S. R. Barthel, M. W. Johansson, D. M. McNamee, D. F. Mosher, Roles of integrin activation in eosinophil function and the eosinophilic inflammation of asthma. *J. Leukoc. Biol.* **83**, 1–12 (2008).
40. L. G. Ellies *et al.*, Sialyltransferase specifity in selectin ligand formation. *Blood* **100**, 3618–3625 (2002).
41. W. H. Yang, C. Nussbaum, P. K. Grewal, J. D. Marth, M. Sperandio, Coordinated roles of ST3Gal-VI and ST3Gal-IV sialyltransferases in the synthesis of selectin ligands. *Blood* **120**, 1015–1026 (2012).
42. J. Kitayama, R. C. Fuhlbrigge, K. D. Puri, T. A. Springer, P-selectin, L-selectin, and α_4 integrin have distinct roles in eosinophil tethering and arrest on vascular endothelial cells under physiological flow conditions. *J. Immunol.* **159**, 3929–3939 (1997).
43. J. C. Nussbaum *et al.*, Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature* **502**, 245–248 (2013).

44. M. Suzukawa *et al.*, Sialyltransferase ST3Gal-III regulates Siglec-F ligand formation and eosinophilic lung inflammation in mice. *J. Immunol.* **190**, 5939–5948 (2013).
45. T. Kiwamoto *et al.*, Mice deficient in the St3gal3 gene product Î±2,3 sialyltransferase (ST3Gal-III) exhibit enhanced allergic eosinophilic airway inflammation. *J. Allergy Clin. Immunol.* **133**, 240–247 (2014).
46. M. Nasirikenari *et al.*, Altered eosinophil profile in mice with ST6Gal-1 deficiency: An additional role for ST6Gal-1 generated by the P1 promoter in regulating allergic inflammation. *J. Leukoc. Biol.* **87**, 457–466 (2010).
47. T. Kiwamoto, T. Katoh, M. Tiemeyer, B. S. Bochner, The role of lung epithelial ligands for Siglec-8 and Siglec-F in eosinophilic inflammation. *Curr. Opin. Allergy Clin. Immunol.* **13**, 106–111 (2013).
48. M. Zhang *et al.*, Defining the in vivo function of Siglec-F, a CD33-related Siglec expressed on mouse eosinophils. *Blood* **109**, 4280–4287 (2007).
49. S. J. McMillan, H. E. Richards, P. R. Crocker, Siglec-F-dependent negative regulation of allergen-induced eosinophilia depends critically on the experimental model. *Immunol. Lett.* **160**, 11–16 (2014).
50. E. E. Irons *et al.*, B cells suppress medullary granulopoiesis by an extracellular glycosylation-dependent mechanism. *Elife* **8**, e47328 (2019).
51. L. G. Ellies *et al.*, Sialyltransferase ST3Gal-IV operates as a dominant modifier of hemostasis by concealing asialoglycoprotein receptor ligands. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 10042–10047 (2002).
52. K. D. Dyer *et al.*, Functionally competent eosinophils differentiated ex vivo in high purity from normal mouse bone marrow. *J. Immunol.* **181**, 4004–4009 (2008).
53. R. Immler *et al.*, Extratubular polymerized uromodulin induces leukocyte recruitment and inflammation in vivo. *Front. Immunol.* **11**, 588245 (2020).
54. R. Immler *et al.*, The voltage-gated potassium channel KV1.3 regulates neutrophil recruitment during inflammation. *Cardiovasc. Res.* **118**, 1289–1302 (2022).
55. D. Frommhold *et al.*, RAGE and ICAM-1 differentially control leukocyte recruitment during acute inflammation in a stimulus-dependent manner. *BMC Immunol.* **12**, 56 (2011).
56. A. Gurtner *et al.*, Active eosinophils regulate host defence and immune responses in colitis. *Nature* **615**, 151–157 (2023).
57. D. Frommhold *et al.*, RAGE and ICAM-1 cooperate in mediating leukocyte recruitment during acute inflammation in vivo. *Blood* **116**, 841–849 (2010).
58. S. Alampour-Rajabi *et al.*, MIF interacts with CXCR7 to promote receptor internalization, ERK1/2 and ZAP-70 signaling, and lymphocyte chemotaxis. *FASEB J.* **29**, 4497–4511 (2015).