Supplementary Information

Materials and Methods

Selectin ligands and selectin-binding FACS. Cell surface sLeX, sLeA and PSGL-1 presentation as well as static E-/P-selectin-binding were determined on all three PCa cell lines and appropriate positive control cell lines (Table S1) as described before (17).

Flow adhesion assays on HPMEC and immobilized rhP-Selectin. E-selectin, P-selectin, ICAM-1 and VCAM-1 expression was determined by incubating non-stimulated and TNFα-stimulated (10 ng/mL rhTNFα from Peprotech, London, UK for 4h) HPMEC with FITC-conjugated mAbs for human E- or P-selectin (clone BBIG-E5 and Cat.#BBA34, both from R&D systems, Wiesbaden, Germany), PE-conjugated anti-human ICAM-1 (clone HA58, eBioscience) and a mAb for human VCAM-1 (clone 1.4C3, Dako) fluorescence labelled with goat-anti-mouse Ig-APC secondary antibody (BD Biosciences). Corresponding isotype controls were used for each antibody and propidium iodide staining was performed prior to flow cytometry.

Next, flow adhesion assays on HPMEC monolayers and an immobilized rhP-selectin/Fc chimera (R&D systems) were performed and analyzed as described before (26). Additional TNFα-stimulated HPMEC were blocked with a mAb for human E-selectin (clone HAE-1f, BioLegend, San Diego, USA) 30 min before running the flow adhesion assay. Each experiment was recorded in three viewing fields per flow chamber and was repeated at least twice.

Immunohistochemical analysis of spontaneous lung metastases. In addition to morphological analyses of spontaneous lung metastases, co-immunostainings for S1P1 (polyclonal rabbit, SantaCruz#25489) and human vimentin (monoclonal mouse-
anti-human, Dako#M7020) were performed on consecutive lung tissue slides to ascertain the presence of intrastromal PC-3 cells in selectin deficient mice and to confirm the human origin of these cellular deposits. Briefly, lung sections from E-/P-selectin/-/- mice were de-paraffinized, pre-treated with Dako Retrieval Solution (S1699, pH 6), blocked with 3% H₂O₂ in methanol (1:10), incubated with anti-vimentin (1:150) for 1 h, washed, incubated with biotinylated rabbit-anti-mouse secondary antibody for 30 min, washed, complexed with a peroxidase-conjugated avidin-biotin-complex (Vector) and developed with DAB (Sigma). Afterwards, the sections were incubated with anti-S1P₁ (1:25) overnight and a biotinylated swine-anti-rabbit secondary antibody, complexed with an alkaline phosphatase-conjugated avidin-biotin-complex (Vector) and developed with permanent red (Dako).

Statistical analyses. Glycosylation array data were analyzed based on the ΔCt method using an Excel-based template (SA Bioscience) and were represented as x-fold up- or down-regulation in comparison to PPEC. Statistical evaluations of the in vivo-experiments were performed with GraphPad Prism software version 5. The HPA- and E-selectin-binding patterns on TMAs were statistically evaluated using SPSS 19 software package (SPSS Inc., Chicago, USA). Kaplan-Meier curves were plotted for biochemical recurrence analyses and compared using log-rank tests in patients with completed follow-up (≥ 1 month). Student’s t-, Fisher exact, Kruskal-Wallis, and Mann-Whitney-U-tests were performed as appropriate to assess associations between variables. Univariate linear regression model was used to compare HPA-binding status with mean PSA-values. Multivariate cox regression analysis was applied to test HPA-binding as an independent predictive marker. Statistical significance was assigned at two-tailed p-values <0.05.
Results

*Sialylated Lewis antigens and E-/P-selectin-mediated dynamic flow adhesion are not detectable in PCa.* All tested PCa cells do not present detectable levels of sLe$^A$ or sLe$^X$ on their surface, whereas PC-3 and DU-145 (but not VCaP) express low levels of PSGL-1. Consequently, they are not able to bind rhE-selectin under static conditions and show only a weak binding of rhP-selectin (Fig. S1 A, B).

E-selectin, ICAM-1 and VCAM-1 are remarkably up-regulated at the HPMEC surface following activation by TNF$\alpha$. Of these, only ICAM-1 is constitutively expressed on HPMEC (Fig. S1 C). P-selectin is not detectable on normal or activated cells (not shown). Under physiological blood flow conditions that reflect endothelial shear stress found in post-capillary venules in the lungs (<1 dyn/cm²; (48)), all tested PCa cell lines do not adhere to non-stimulated or stimulated HPMEC and not to immobilized rhP-selectin (Fig. S1 D). HT29, PaCa5061 and EOL-1 were used as internal positive controls as appropriate (Fig. S1 A, B, D; Table S1). Flow adhesion of HT29 and PaCa5061 is abolished by E-selectin blockade (Fig. S1 D).

**Immunohistochemical analysis of spontaneous lung metastases.** S1P$_1$ was found to be a suitable marker to label vascular endothelium in different vessel sections of murine lungs. Lung capillaries in alveolar septae were stained as well (Fig. 4, Fig. S2). Spontaneous PC-3 lung metastases were not labelled by a human-specific pan-cytokeratin antibody (Dako M3515, not shown), but by the human-specific vimentin antibody suggesting a particular relevance of epithelial-to-mesenchymal transition during metastatic spread of PC-3 in our s.c. xenograft model (Suppl. Fig. S2). Moreover, double-stainings for S1P$_1$ and vimentin of lungs of E-/P-selectin$^{-/-}$ mice illustrate the intravascular or intrastromal presence of PC-3 cells.
Hence, selectins are apparently not essential for extravasation of circulating PC-3-cells.

**Legend to Supplementary Figures**

**Fig. S1. Selectin ligands, selectin binding and dynamic adhesion of PCa cells.**

A-B, All tested PCa cells do not present sLe\(^A\) or sLe\(^X\) at their surface, whereas PC-3 and DU-145 express low levels of PSGL-1. Consequently, they are not able to bind rhE-selectin under static conditions and show only a weak binding of rhP-selectin. C, E-selectin, ICAM-1 and VCAM-1 are up-regulated on HPMEC following incubation with rhTNF\(\alpha\). D, PCa cells do not adhere to untreated or TNF\(\alpha\)-activated HPMEC or immobilized rhP-selectin under shear stress conditions found in post-capillary venules (<1 dyn/cm\(^2\); (48)). Flow adhesion on HPMEC is abolished by blocking E-selectin. HT29, PaCa5061 and EOL-1 were used as positive controls as appropriate. *\(p<0.05\) and **\(p<0.001\) vs. -TNF\(\alpha\); #\(p<0.05\) and ##\(p<0.001\) vs. +TNF\(\alpha\); ++\(p<0.001\).

Bars represent mean ± SD of three independent experiments.

**Fig. S2. Intrastromal PC-3 lung metastatic cells in E-/P-selectin\(^{-}\) mice.**

Combined immunostainings for murine S1P1 (vascular endothelium, red) and human vimentin (PC-3, brown) illustrate the intravascular or intrastromal location of metastatic PC-3 cells in lungs of E-/P-selectin\(^{-}\) mice and confirm the human origin of such deposits.