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Leukocyte-endothelial cell interaction is enhanced in podocalyxin-deficient mice

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Abbreviations: EC, endothelial cell; MLEC, mouse lung endothelial cell; Podxl, podocalyxin; HEV, high endothelial venule; Tie2, tunica intima endothelial kinase 2; LPS, lipopolysaccharide; ICAM-2, intercellular adhesion molecule 2; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor

Abstract

The highly sialoglycosylated extracellular domain of podocalyxin (Podxl) is a constituent of the endothelial glycocalyx of most blood vessels but it is unknown if Podxl plays a prominent role in the function of the glycocalyx as a regulator of leukocyte-endothelial adhesion. We have recently found that mice lacking Podxl in the vascular endothelium develop histological lesions compatible with severe vasculitis resulting in organ failure and premature death. In this work, we show that these mice have an increased quantity of resident leukocytes within the peritoneal cavity in both basal and inflammatory conditions. Adhesion of macrophagic cells to lung endothelial cells from Podxl-deficient mice was increased under inflammatory stimuli. Both, chemokine binding and chemokine-mediated adhesion of immune cells were significantly higher in Podxl-deficient endothelial cells. Moreover, glycocalyx function assessed by measuring the anticoagulant capacity of endothelial cell monolayers to inactivate thrombin was significantly altered in the absence of Podxl. Overall, the results suggest that Podxl is an essential component of the glycocalyx and has an important so far unknown role in preventing leukocyte-endothelial cell adhesion under resting and inflammatory conditions.

Key words: podocalyxin; endothelial cell; glycocalyx; leukocyte-endothelial cell adhesion; inflammation

1. Introduction

Podocalyxin (Podxl) is an extensive N- and O-glycosylated sialoglycoprotein thought to be the major constituent of the glycocalyx of podocytes (Kerjaschki et al., 1984), playing an essential role in the development and function of the kidney glomerular filtration barrier (Doyonnas et al., 2001). Podxl is also normally expressed on vascular endothelial cells (ECs) in blood vessels of all calibers in a variety of tissues (lung, heart, small intestine, pancreas, kidney), but it is not present in the sinusoidal capillaries of liver and spleen (Horvat et al., 1986; Miettinen et al., 1990). Podxl was identified as one of the ligands for L-selectin in high endothelial venules (HEV) but this function has not been recognized in other types of vessels (Sassetti et al., 1998).

Recently, we and other authors have attempted to gain insights into the role of Podxl by generating conditional KO mice with selective deletion of *podxl* in vascular endothelial cells (Debruin et al., 2014; Horrillo et al., 2016). Both studies showed evidence of the role of Podxl in maintaining endothelial barrier integrity. Vascular permeability, assessed by *in vivo* assays as well as by transwell assays with monolayers of lung endothelial cells, was found to be increased in Podxl-deficient mice, being attributed to defective interactions with extracellular matrix components (Debruin et al., 2014) and to delayed recovery of adherence junctions after injury (Horrillo et al., 2016).

The endothelial glycocalyx, a negatively charged carbohydrate-rich mesh covering the luminal surface of vessels, is a key regulator of vascular permeability, cell adhesion, and inflammation (Reitsma et al., 2007; Yang and Schmidt, 2013). In recent years, research on glycocalyx has undergone a new impetus as it has been shown that its deterioration underlies a number of clinical conditions, such as ischemia, sepsis and inflammation, atherosclerosis, and diabetes (Becker et al., 2015; Tarbell and Cancel, 2016). The glycocalyx is composed of a wide variety of cell-bound proteoglycans and glycoproteins (Weinbaum et al., 2007). Proteoglycans are proteins attached to negatively charged glycosaminoglycans (GAGs), which are of several types: heparan sulfate, chondroitin sulfate, keratan sulfate, dermatan sulfate, and hyaluronic acid. The most abundant glycosaminoglycans are heparan sulfates, which account for more than 50% of the GAG pool. The main heparan sulfate proteoglycans found in endothelial cells are the transmembrane syndecans, that interact through their cytosolic domain with the cytoskeleton, and the glypicans anchored to the membrane and enriched in caveolae. Glycoproteins that are part of the glycocalyx include selectins, integrins, immunoglobulin receptors, and other membrane proteins such as CD44 and Podxl. Sialic acids located at the distal ends of the carbohydrate chains attached to these proteins are believed to contribute significantly to the overall negative charge on the cell surface, which is important for anti-adhesive repulsion between circulating cells and the vascular wall (Kelm et al., 1997).

To date, the individual contribution of the glycocalyx components in maintaining its protective functions remains unclear. Given its high sialic acid and sulfate content, Podxl has been proposed to largely provide the anionic charge found within the glycocalyx of endothelial cells (Dekan et al., 1991). In addition, Podxl has been recently identified as a novel keratan sulfate proteoglycan (Toyoda et al., 2017). In this work we show that mice lacking endothelial Podxl exhibit increased recruitment of inflammatory cells in the peritoneum and that the endothelial cells of these mice have greater adhesive capacity under inflammatory conditions *in vitro*. On the basis of these findings, we have explored the possibility that Podxl may play a relevant role on the functional properties of glycocalyx, by analyzing leukocyte adhesion dependent on the chemokine-GAG interaction, and the capacity of endothelial cell monolayers to inactivate procoagulant enzymes.

2. Materials and methods

2.1. Animals

All procedures were performed in accordance with the guidelines of the European Community Council Directive 86/609 EEC and were approved by the Institutional Animal Care and Use Committee. Generation of mice with a restricted deletion of Podxl in the vascular endothelium (Podxl^{f/f};Tie2-Cre mice) has been previously reported (Horrillo et al., 2016). Mice used in all the experiments were maintained under controlled conditions of light and temperature, and all experiments were conducted using age (10-12-week old) and sex-matched control and Podxl-deficient mice that are siblings from the same litters.

2.2. Collection and flow cytometry analysis of peritoneal cavity (PerC) leukocytes

Mice untreated or treated with intraperitoneal injection of 1 mL 4% thioglycolate (Sigma-Aldrich) for 4, 24, or 48 h were killed using CO₂, and peritoneal leukocytes were collected by lavage of the cavity with PBS containing 5 mM EDTA. After centrifugation, cells were incubated 2 min in 1 mL of RBC buffer (170 mM NH₄Cl, 12 mM KHCO₃, 0.9 mM EDTA, pH 7.6) to lyse residual red cells and, then, centrifuged and resuspended in DMEM and counted using a hemocytometer.

For flow cytometry analysis, PerC cells were incubated for 30 min at 4°C with labeled antibodies against CD19 (clone 1D3, eBioscience), F4/80 (clone Cl:A3-1, AbD Serotec), Ly6-G (clone RB6-8C5, eBioscience), CD5 (clone 53-7.3, Biolegend), CD11b (clone M1/70, BD Pharmingen), and CD3ε (clone 145-2C11, BD Pharmingen), using isotype-matched antibodies as negative control and color compensation as appropriate for dual labeling. Samples were analyzed in a Coulter flow cytometer model FC500. A more detailed analysis of B-cell population was performed by simultaneous labeling with anti-CD19, anti-CD5 and anti-CD11b antibodies. According to the expression of these markers, B cells were considered as belonging to B1a (CD19+/CD5+/CD11b+), B1b (CD19+/CD5-/CD11b+), B1c (CD19+/CD5+/CD11b-) or B2 (CD19+/CD5-/CD11b-) subsets (Tung and Herzenberg, 2007; Rosado et al., 2014; Hastings et al., 2006).

2.3. *In vitro* vascular permeability assay

Human umbilical vein endothelial cells (HUVECs) cells were grown in EBM-2 medium supplemented with EGM-2 Single Quots (Lonza). 10⁶ cells at passage 3 were transfected using the Amaxa nucleofactor system with 30 pmol of Podxl-specific or scrambled siRNAs (Ambion) and, then, 0.25 x 10³ cells were seeded on collagen-coated transwell inserts and grown for a total period of 48 h. Cells were treated with 100 ng/mL rhTNFα (R&D) for 16 h, and with 250 ng/mL rhVEGF (Immunotools) or 100 ng/mL LPS (Sigma) for 2 h. Then, FITC-dextran (1:20) was added and, after 1 h, fluorescence was determined in the low chamber using a Varioskan plate reader. For western blot analysis of Podxl expression, 20 µg of protein lysate from HUVEC cells nucleofected with Podxl-specific or scrambled siRNAs were analyzed with anti-human Podxl (Novusbio) and anti-VE-cadherin (ThermoScientific).

2.4. Mouse lung endothelial cell (MLEC) isolation and culture

Mouse lung endothelial cell (MLEC) cultures were prepared from two control and two KO mice as previously described (Horrillo et al., 2016). Briefly, after digestion with collagenase A, the tissue/cell suspension was resuspended in complete DMEM and, 24 h later, the medium was changed to endothelial cell growth medium consisting of 50% DMEM-50% Hams F-12 medium supplemented with penicillin and streptomycin, 20% fetal bovine serum, 20 µg/mL endothelial cell growth supplement from bovine neural tissue (ECGS, Sigma) and 0.1 mg/mL heparin sodium salt (Sigma) and the cells cultured for 2 days. Cells were then sorted using anti-ICAM-2 antibody (553326, BD Pharmingen) coupled to sheep anti-rat IgG Dynabeads (Invitrogen), plated and cultured during 3 days. The endothelial identity and

purity of the cells was confirmed by flow cytometry using FITC-labeled anti-ICAM-2 antibody (557444, BD Pharmingen), and only cultures with percentages of positive cells greater than 95% were used for experiments.

2.5. Adhesion of macrophagic PU5-1.8 cells to MLECs

MLECs from control and Podxl-deficient mice were grown to confluence on 96-well plates and treated with 50 ng/mL TNF α plus 100 ng/mL IL-1 β for 6 h. ICAM-2 was blocked by incubation with 5 μ g/mL anti-ICAM-2 for 30 min. PU5-1.8 cells were stained with 2.5 μ M calcein-AM for 30 min and, after washing, L-selectin was blocked by incubation with 5 μ g/mL anti-L-selectin (MEL-14, eBioscience) for 30 min. Then, 5x10⁴ PU5-1.8 cells were plated onto monolayers of MLECs in triplicate and incubated for 30 min. Non-adherent cells were removed by washes with PBS and adherent cells were quantified using a Varioskan plate reader.

2.6. Adhesion of PerC leukocytes to MLECs

Control mice were treated with intraperitoneal injection of 1 mL 4% thioglycolate and, after 4 h, PerC leukocytes were collected as before and labeled with 2.5 μ M calcein-AM for 30 min. MLECs from control and Podxl-deficient mice were grown to confluence on 96-well plates and treated with chemokines CCL20 (20 ng/mL, R&D Systems 760-M3), CCL21 (20 nM, Peprotech 250-13), CXCL2 (10 ng/mL, Peprotech 250-15), CXCL12 (10 ng/mL, Peprotech 250-20A), or CXCL13 (50 nM, Peprotech 250-24) for 1 h. Following chemokine incubation, medium was removed and 5x10⁴ calcein-labeled PerC cells were added to each well and incubated for 20 min. Non-adherent cells were removed by washes with PBS and adherent cells were quantified using a Varioskan plate reader.

2.7. Analysis of chemokine binding to MLECs

MLECs from control and Podxl-deficient mice were grown to confluence on 12-well plates and incubated with CXCL13 (50 nM) for 3 h, followed by thorough washing with PBS to remove unbound chemokine. Then, 100 μ L of Triton lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 0.05% Tween-20) was dispensed directly onto the cell monolayer; thus, the bound CXCL13 was removed and solubilized together with triton-soluble cell proteins. After 30 min on ice, the lysate was subjected to centrifugation to remove non-solubilized membrane residues and 20 μ g of protein lysate were analyzed by western blot with polyclonal goat anti-CXCL13 (AF470) and anti-ICAM-2 (AF774) antibodies (R&D Systems).

2.8. Functional analysis of MLEC monolayer's capacity to inactivate FXa and thrombin

To evaluate the functionality of live surface glycocalyx, we assessed its anticoagulant capacity to inactivate FXa and thrombin (Dimitrievska et al., 2016). Briefly, for FXa colorimetric assay 1.5x10⁴ MLECs from control and Podxl-deficient mice were seeded on gelatin-coated 96-well plates and grown for 24 h. Cells were incubated with 75 μ L of increasing concentrations (0.25 to 2 μ g/mL) of antithrombin (Innovative Research) in 50 mM Tris buffer pH 8.4 for 5 min at 37°C. Thereafter, 75 μ L of 50 mU/mL FXa (Sigma-Aldrich, F9302) in PBS containing 1 mg/mL BSA was added and incubation continued for 2 min. 100 μ L of the supernatants were then transferred into a new 96-well plate and preincubated at 37°C in a Varioskan plate reader. 25 μ L of 1.5 mM chromogenic substrate S-2222 (Chromogenix) was added, mixed, and the increase of absorbance at 405 nm was recorded during 20 min.

For thrombin colorimetric assay, cells were incubated with 75 μ L of increasing concentrations (0.1 to 1 μ g/mL) of antithrombin for 5 min at 37°C. Then, 75 μ L of 2 U/mL thrombin (Sigma-Aldrich, T6884) in PBS was added and incubation continued for 2 min. 100 μ L of the supernatants were then transferred into a new 96-well plate and preincubated at 37°C in a Varioskan plate reader. 12.5 μ L of 1 mM chromogenic thrombin substrate S-2238 (Eurogentec) was added, mixed, and the increase of absorbance at 405 nm was recorded

during 20 min.

2.9. Statistical analysis

The values are expressed as mean $\pm$ SD and comparisons between two groups were made by 2-tailed *t* test. Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Downregulation of Podxl in HUVEC cells increases cell permeability

We have previously shown (Horrillo et al., 2016) that confluent monolayers of lung endothelial cells from mice (MLECs) lacking endothelial Podxl exhibit increased permeability to dextran as well as increased macrophage transmigration in transwell assays. In the present study, to further confirm the effect of the absence of Podxl on permeability, Podxl expression was downregulated in HUVEC cells by transfection of Podxl-specific siRNAs. As shown in Fig. 1, Podxl-depleted HUVEC cells were more permeable to FITC-dextran in response to various barrier-disruptive stimuli.

3.2. Increased recruitment of immune cells in the peritoneal cavity (PerC) of Podxl-deficient mice

In an attempt to confirm the permeability failure of either mouse or human Podxl-deficient endothelial cells detected in the *in vitro* assays, we performed *in vivo* experiments of leukocyte recruitment in control and Podxl-deficient mice (KO mice). We used flow cytometry to analyze resident leukocytes in resting peritoneal cavity (PerC) as well as recruitment of blood cells during the inflammatory response induced by thioglycollate administration (Leijh et al., 1984). The number of total immune cells within the unstimulated PerC is significantly higher in KO mice than in control mice and this difference is also observed in animals treated with thioglycollate for 4, 24 or 48 h (Fig. 2A). The increased cellularity in KO mice is mainly due to an increase in B-cells, as indicated by anti-CD19 labeling (Fig. 2B). However, macrophage and granulocyte populations detected with anti-F4/80 and anti-Ly-6G antibodies, respectively, are similar in unstimulated control and KO mice. Both, the peak value achieved by the neutrophil population 4 h after thioglycollate administration and the progressive increase in the macrophage population, are also similar (Fig. 2B). The number of T lymphocytes, identified with CD3 ϵ labeling, was very low and similar in control and KO mice (not shown). The results of a more detailed analysis of the B-cell population by simultaneous labeling with anti-CD19, anti-CD5 and anti-CD11b antibodies indicate that all subsets of B-cell analyzed are increased in KO mice (Fig. 3).

3.3. Lack of Podxl increases the adhesive capacity of endothelial cells

As a possible cause of the previously reported increase of macrophage transmigration through monolayers of Podxl-deficient mouse lung endothelial cells (MLECs) (Horrillo et al., 2016) and the increased recruitment of leukocytes in KO mice (Fig. 2), we explored the adhesive capacity of endothelial cells *in vitro*. We first analyzed the adhesion of macrophagic PU5-1.8 cells to primary cultures of MLECs from control and KO mice. As shown in Fig. 4A, the increase in adhesion under inflammatory stimuli is considerably higher in cells lacking Podxl. In both cell types, this effect was partially reversed when cells were incubated with an anti-L-selectin antibody, this blocking being significantly greater in cells from the KO animals. The confluent monolayers of MLECs have the cobblestone-like appearance typical of primary cultures of endothelial cells, with nuclei protruding above flattened cytoplasm. As shown in Fig. 4B, no appreciable differences were observed in the morphology of the monolayers after the inflammatory stimulus and adhesion of the macrophage cells.

We have previously reported a significant 10-20% increase in ICAM-2 surface expression in MLECs from KO mice (Horrillo et al., 2016). This increase does not correspond to an increase in the total ICAM-2 content assessed by western blot (Fig. 5B). Moreover, ICAM-2 blockade does not have a significant effect on the adhesion of macrophagic cells (Fig. 4).

3.4. Podxl-deficient endothelial cells exhibit increased chemokine-mediated adhesion of PerC cells

In the *in vivo* recruitment experiments we found that resident leukocytes in KO animals increase basically at the expense of B cells. It is well known that chemokines and their interactions with the glycocalyx' glycosaminoglycans (GAG) widely regulate the entry of B cells into the peritoneal cavity (Baggiolini, 1998; Salanga and Handel, 2011). Since Podxl is one of the glycoproteins that, together with proteoglycans, make up the glycocalyx that covers the endothelial surface (Weinbaum et al., 2007), we found of interest to analyze the ability of several chemokines to mediate adhesion of immune cells to control and Podxl-deficient endothelial cells. The results show that chemokines from both the CXC and CC families induced significantly higher adhesion of leukocytes to MLECs lacking Podxl (Fig. 5A). In agreement with these findings, Podxl-deficient cells showed a greater ability to retain the chemokine CXCL13, a potent chemoattractant for both B1 and B2 subsets of B lymphocytes (Fig. 5B).

3.5. MLECs lacking Podxl have a greater ability to inactivate thrombin

Another approach to explore whether the absence of Podxl has an impact on glycocalyx functionality was through *in vitro* assays to quantify the antithrombogenic ability of control and Podxl-deficient MLECs (Dimitrievska et al., 2016). In these assays we measured the potential of ECs' glycocalyx to inactivate 50 mU FXa and 2 U thrombin in the absence or in the presence of increasing concentrations of antithrombin (AT). AT binds glycosaminoglycans in the endothelial glycocalyx and becomes active; subsequently, activated AT inhibits the coagulation proteases by forming equimolar complexes, and the activity of unbound proteases is recorded using specific chromogenic substrates. Thus, the absorbance values generated by the residual amounts of active FXa or thrombin are indicative of the functional capacity of the glycocalyx in MLEC monolayers. In our experimental conditions, cells lacking Podxl exhibited a similar ability than control cells to inhibit FXa activity. No differences were found either in the slopes of the curves or in the maximum value recorded (Fig. 6A). However, compared with control cells, Podxl-deficient cells have a greater capacity to inactivate thrombin. As shown in Fig. 6B, the slopes of curves corresponding to AT concentrations of 0.1, 0.25, and 0.5 $\mu\text{g/mL}$ were significantly reduced in cells lacking Podxl.

4. Discussion

In this work we present data supporting that Podxl has an important role in the functionality of the endothelial glycocalyx. This cell surface layer is currently the focus of much investigation to determine its detailed composition and pathophysiological relevance in processes including inflammation and vascular permeability (Yang and Schmidt, 2013; Becker et al., 2015; Tarbell and Cancel, 2016). To date, the individual contribution of the glycocalyx components to its functionality and its overall negative charge on the cell surface, important for attenuating firm adhesion of blood leukocytes and platelets, remains unclear. In this sense, mice lacking syndecan-1, one of the more abundant components of endothelial glycocalyx, synthesize and maintain a hemodynamically functional glycocalyx layer (Savery et al., 2013). In another study, endomucin, a O-sialoglycoprotein present in the glycocalyx of venules and capillaries, but not of arteries, in adult tissues, has been shown to prevent leukocyte contact with adhesion molecules both under resting and inflammatory conditions

(Zahr et al., 2016). Unlike endomucin, Podxl is expressed in major and minor arterial and venous vessels of most tissues. Previously, we had reported that mice lacking Podxl in the vascular endothelium die prematurely around three months of age, exhibiting a phenotype characterized by severe vasculitis and degenerative changes in vital organs (Horrillo et al., 2016). In the present study, we corroborate our proposal on the role of Podxl in the maintenance of endothelial integrity by showing enhanced permeability of Podxl-depleted human endothelial cells and, also, a significantly increased recruitment of inflammatory cells in the PerC of Podxl-deficient mice (KO mice). We also show data suggesting that these effects are explained, at least partially, by improved leukocyte-endothelial cell interaction probably due to functional defect of the Podxl-devoid glycocalyx.

Normally, unstimulated PerC in mice is populated by CD3⁺ T cells, B lymphocytes, macrophages, and by a low number of monocytes and neutrophils. We found that the total population of resident leukocytes is significantly increased in the KO animals, the number of cells being even greater than that collected in control mice treated with thioglycollate. Cell phenotype analysis revealed that resident leukocytes in KO animals increase basically at the expense of B cells, identified by CD19 labeling. The peritoneal cavity normally harbors B1 and B2 cell populations and, unlike B2 cells that arise from the bone marrow throughout life, B1 cells appear to emerge early from fetal liver precursors and become a population of self-renewing cells in the peritoneal cavity of adult mice (Kantor and Herzenberg, 1993; Tung and Herzenberg, 2007). The B1 cell population is further divided into B1a and B1b subsets, according to the differential expression of CD5. A third subset of B1 peritoneal cells has been phenotyped as CD5⁺/Mac-1⁻ and termed B1c (Hastings et al., 2006). Virtually, all subsets of B cells are increased in Podxl-deficient mice. Although the mechanisms regulating the entry and accumulation of B cell populations in the peritoneal cavity are not well understood, it is known that these processes are largely governed by the activity of chemokines (Baggiolini, 1998). In addition to activating G protein-coupled signaling receptors, chemokines mediate their biological effects by interacting with the glycosaminoglycans (GAG) of the endothelial glycocalyx via electrostatic interactions between the GAG's negatively charged residues and the chemokine's positively charged arginine and lysine residues (Salanga and Handel, 2011; Kufareva et al., 2015). Thus, it is possible that the absence of Podxl on the endothelial glycocalyx somehow affects the GAG-chemokine interactions and results in a greater accumulation of B lymphocytes in the peritoneal cavity of KO mice.

Podxl is one of the ligands of L-selectin mediating leukocyte extravasation in specialized postcapillary HEV (Sasseti et al., 1998), but this pro-adhesive function has not been recognized in other vascular endothelia. Conversely, the highly sialylated and sulfated extracellular domain of Podxl contributing to the negative charge on the surface glycocalyx may have a major role in the maintenance of an anti-adhesive and hence anti-inflammatory endothelial cell surface (Dekan et al., 1991; Kelm et al., 1997). Supporting this assumption, we found that, under inflammatory stimuli, adhesion of macrophages mediated by L-selectin is significantly enhanced in MLEC monolayers lacking Podxl, which might indicate that Podxl acts constraining the adhesive function of the endothelial surface layer. Although ICAM-2 has been reported to facilitate the neutrophil-endothelial cell interaction (Halai et al., 2014), the increased ICAM-2 surface exposure displayed by Podxl-deficient MLECs (Horrillo et al., 2016) does not appear to play an adhesive role in these experimental conditions as indicated by the lack of effect of ICAM-2 blockade.

Our proposal of the so far unrecognized relevance of Podxl in glycocalyx functionality is also supported by the results of two types of *in vitro* assays. First, adhesion of PerC cells mediated by chemokines is increased in endothelial cell monolayers lacking Podxl. Since these cells bind more CXCL13 than control cells and, as mentioned above, leukocyte transmigration depends in part on the GAG-chemokine interaction, it is reasonable to conclude that the absence of Podxl in some way facilitates GAG-chemokine interaction. Noteworthy, recruitment of B cells, but not of macrophages, is mainly directed by the chemokine CXCL13 (Ansel et al., 2002; Hopken et al., 2004). Second, in addition to regulation of leukocyte

adhesion, another important function of the glycocalyx is vascular protection through inhibition of coagulation (van Hinsbergh 2012). Among GAGs, heparan sulfate is known to bind and, consequently, activate antithrombin (Chappell et al., 2009; Becker et al., 2010). Recently, a new *in vitro* assay has been developed to directly determine the anticoagulant capacity of glycocalyx to inactivate FXa and thrombin dependent on antithrombin binding and activation (Dimitrievska et al., 2016). We performed these assays as an approach to explore the functionality of the Podxl-deficient glycocalyx, and found that the capacity to inactivate thrombin is increased in MLECs from KO mice compared with that of cells derived from control animals. Again, these findings suggest that depletion of Podxl alters the adherent properties of the endothelial surface. The lack of Podxl and/or sialic acid could alter the electrostatic environment and, consequently, certain heparan sulfate chains that are normally buried may be more accessible to antithrombin binding. However, the apparently higher binding of antithrombin to the endothelium lacking Podxl does not result in greater inactivation of FXa. One possibility is that the effect on FXa inactivation was overlapped with the direct inhibition produced by the presence of tissue factor pathway inhibitor, as described in HUVEC monolayers (Kato, 2002). Alternatively, since inactivation of thrombin by heparin requires that heparin bind to both antithrombin and thrombin (Hirsh et al., 2001), it is possible that the greater inactivation of thrombin in the absence of Podxl was due to a greater binding of thrombin to the glycocalyx lacking Podxl.

In summary, we provide data suggesting that the absence of Podxl on the endothelial surface leads to an alteration in the physical organization of the glycocalyx that favors the adhesion and transmigration of leukocytes. Podxl could be necessary to maintain the stability of electrostatic interactions that operate in the functionally normal glycocalyx. The effect of the lack of Podxl was evidenced by leukocyte recruitment *in vivo* as well as by *in vitro* adhesion assays with MLECs stimulated by inflammatory cytokines or homeostatic chemokines. Overall, our observations suggest that Podxl plays a key role, hitherto unknown, in preventing leukocyte-endothelial cell adhesion under resting and inflammatory conditions, thus revealing Podxl as a therapeutic molecule to be considered in the reemergent glycotherapy field (Hudak and Bertozzi, 2014).

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Conflict of interest statement

The authors declare no conflict of interest.

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Figures

Figure 1

Figure 1. Increased permeability in Podxl-deficient HUVEC cells. (A) Downregulation of Podxl expression in HUVEC cells nucleofected with Podxl-specific siRNAs. (B) HUVEC cells transfected with scrambled or Podxl-specific siRNAs were seeded on collagen coated transwell inserts and grown for a total period of 48 h. Cells were treated with 100 ng/mL TNF α for 16 h, or with 250 ng/mL VEGF or 100 ng/mL LPS for 2 h, and permeability to

FITC-dextran was measured as described under Methods. Values are means of three independent experiments. By t-test * $p < 0.02$.

Figure 2

Figure 2. Podxl-deficient mice exhibit increased recruitment of B-cells in the peritoneal cavity (PerC). Mice were treated with intraperitoneal injection of 1 mL 4% thioglycollate for different time periods and peritoneal cells were collected and labelled with antibodies against CD19 (clone 1D3, eBioscience), F4/80 (clone CI:A3-1, AbD Serotec), or Ly6-G (clone RB6-

8C5, eBioscience) for flow cytometry analysis, as described in Methods. **(A)** Total number of cells in the PerC of control and KO mice. Cell counting was performed manually using a Neubauer chamber. Numbers of analyzed animals are given in parentheses. **(B)** Number of cells labeled with antibodies against CD19, F4/80, and Ly6-G in the control and KO mice analyzed in panel A. The number of each cell type was calculated based on the percentage of positive cells in the flow cytometry analysis. Data represent the mean $\pm$ SD of analysis of four mice of each type.

Figure 3

Figure 3. All B-cell populations are increased in Podxl-deficient mice. Number of B1a, B1b, B1c, and B2 lymphocytes in the PerC of control and KO mice treated with intraperitoneal injection of 1 mL 4% thioglycollate for 48 h. Cells were simultaneously labeled with anti-CD19 (clone 1D3, eBioscience), anti-CD5 (clone 53-7.3, Biolegend) and anti-CD11b (clone M1/70, BD Pharmingen), and assigned to each B-cell subset as described in methods. Data represent the mean $\pm$ SD of analysis of four mice of each type.

Figure 4

Figure 4. Endothelial cells lacking Podxl have increased adhesive capacity. (A) Static adhesion assays of macrophagic PU5-1.8 cells to MLECs were performed as described in Methods. Each experiment was performed with MLEC cultures from two control or KO mice grown to confluence on 96-well plates and treated with 50 ng/mL TNFα plus 100 ng/mL IL-1β for 6 h. ICAM-2 was blocked by incubation with 5 μg/mL anti-ICAM-2 (553326, BD Pharmingen) for 30 min. Calcein-stained PU5-1.8 cells were incubated with 5 μg/mL anti-L-selectin (MEL-14, eBioscience) for 30 min and, then, 5x10⁴ cells were plated onto MLEC monolayers and incubated for 30 min. Values are means of four independent experiments performed in triplicate. By t-test * $p < 0.05$. **(B)** Representative images showing the morphology of MLEC monolayers from control and KO mice before and after incubation with TNFα/ IL-1β and PU5-1.8 cells. Photos were taken using a 20x objective. An area from each field is shown with greater magnification in the lower part of the panel.

Figure 5

Figure 5. Increased chemokine-mediated adhesion of PerC cells to Podxl-deficient endothelial cells. (A) PerC cells were collected from two control mice treated with intraperitoneal injection of 1 mL 4% thioglycollate for 4 h and labeled with calcein-AM. MLEC cultures from two control or KO mice were incubated in the absence or presence of chemokines CCL20 (20 ng/mL), CCL21 (20 nM), CXCL2 (10 ng/mL), CXCL12 (10 ng/mL), or CXCL13 (50 nM) for 1 h and, then, the medium was removed and 5×10^4 calcein-labeled PerC cells were added to each well and incubated for 20 min, as described in Methods. The results are mean values $\pm$ SD of four independent experiments performed in triplicate. (B) Representative experiment and quantification of western blot analysis of CXCL13 bound to MLECs. Cells grown to confluence were incubated with 50 nM CXCL13 and, after exhaustive washing to remove unbound chemokine, Triton lysis buffer was added and the cell lysate containing bound CXCL13 was analyzed by western blot as described in Methods. ns, not significant; *, $p < 0.05$.

Figure 6

Figure 6. MLECs lacking Podxl have a greater ability to inactivate thrombin. Confluent cells from control (CT) and KO mice in 96 well-plates were incubated with increasing concentrations of antithrombin (AT) and, then, with an equal volume of FXa (50 mU/mL) or thrombin (2 U/mL) was added, and residual amounts of unbound coagulation factors were detected using chromogenic substrates, as described in Methods. The graphs show the absorbance readings of residual FXa (**A**) and thrombin (**B**) generated from control and Podxl-deficient MLEC monolayers. The results are mean $\pm$ SD of four independent assays using four different MLEC cultures. (**C**) Values of the slopes of curves of panel B within the 2-6 min interval. ns, not significant.