During inflammation, circulating polymorphonuclear neutrophils (PMNs) receive signals to cross the endothelial barrier and migrate through the extracellular matrix (ECM) to reach the injured site. Migration requires complex and poorly understood interactions of chemokines, chemokine receptors, ECM molecules, integrins, and other receptors. Here we show that the ECM protein lumican regulates PMN migration through interactions with specific integrin receptors. Lumican-deficient (Lum−/−) mice manifest connective tissue defects, impaired innate immune response, and poor wound healing with reduced PMN infiltration. Lum−/− PMNs exhibit poor chemotactic migration that is restored with exogenous recombinant lumican and inhibited by anti-lumican antibody, confirming a role for lumican in PMN migration. Treatment of PMNs with antibodies that block β2, β1, and α5 integrin subunits inhibits lumican-mediated migration. Furthermore, immunohistochemical and biochemical approaches indicate binding of lumican to β2, α5, and α6 integrin subunits. Thus, lumican may regulate PMN migration mediated by MAC-1 (α5β2) and LFA-1 (α5β2), the two major PMN surface integrins. We detected lumican on the surface of peritoneal PMNs and not bone marrow or peripheral blood PMNs. This suggests that PMNs must acquire lumican during or after crossing the endothelial barrier as they exit circulation. We also found that peritoneal PMNs do not express lumican, whereas endothelial cells do. Taken together these observations suggest a novel endothelial lumican-mediated paracrine regulation of neutrophils early on in their migration path.

Polymorphonuclear neutrophils (PMNs) play a major role in the development of inflammatory responses to host injury and infection. Their functions include destruction of invading bacteria and recruitment of macrophages and lymphocytes to the affected site (1). Circulating PMNs sense injury and pathogen signals, cross the vascular endothelium, and migrate to the target tissue; two series of events control this process. The first leads to the slowing down and adherence of circulating PMNs on the vascular endothelium followed by their transendothelial migration or extravasation and activation (2). The second controls the directional migration of PMNs to the injured site through the endothelial basement membrane, a specialized type of ECM, and subsequently the deeper interstitial ECM, along chemokine and cytokine gradients. Leukocyte-to-leukocyte and leukocyte-to-endothelium interactions are important before extravasation. These are mediated by interactions between selectins and their ligands and by β2 (MAC-1 and LFA-1) and β1 (VLA-4–6) integrin interactions with cell adhesion proteins ICAM and PECAM (3). The directional migration of PMNs through the ECM is a complex, multistep process that involves several α and β integrin interactions with ECM proteins. Thus far, a few basement membrane proteins, laminins, entactin, and fibronectin have been identified as specific ligands in regulating migration of PMNs after extravasation (4–6). Additional interstitial ECM proteins and their receptors that modulate PMN migration have yet to be identified. Here we show that the ECM protein lumican is a novel regulator of PMN migration.

Lumican is a secreted collagen-binding ECM protein of the cornea, dermal, and tendon stroma, arterial wall, and the intestinal submucosa (7–9). It is a member of the small leucine-rich repeat proteoglycans (10); these were initially investigated in the context of binding collagen and regulating tissue structure and biomechanics (11, 12). A body of literature is beginning to indicate that these proteoglycans interact with cytokines, growth factors, and cell surface receptors to modulate cell adhesion, proliferation, and migration (13–16). Lumican and biglycan, another member of this family of proteoglycans, have been recently shown to regulate host response to pathogen-associated molecular patterns (17, 18). Thus, lumican-deficient (Lum−/−) mice are hypersensitive to bacterial lipopolysaccharide (LPS) endotoxins, and Lum−/− macrophages in culture produce lower levels of pro-inflammatory cytokines in response to LPS (18). Lumican facilitates innate immune response by binding LPS and CD14, the glycolipid phosphatidylinositol-linked cell surface adapter protein that transfers the LPS signal to toll-like receptor 4 (18). In a corneal injury model neutrophil influx is delayed in the Lum−/− mice (19, 20). Although this may be partly due to impaired innate immune...
response, it raises the possibility that lumican may have an additional role in neutrophil migration. Here we elucidate a role for lumican in PMN migration. We show that poor chemotactic migration of Lum−/− PMNs can be rescued by exogenous recombinant lumican (rLum) and blocked specifically with antibodies against lumican, β2, β1, and α4 integrins. Our results also show that lumican localizes on the surface of extravasated PMNs through its interactions with β2 integrins. The likely source of lumican on neutrophils is the vascular endothelium.

**EXPERIMENTAL PROCEDURES**

**Mice—**C57BL/6 (Lum+/+ and Lum−/−) were housed in a specific pathogen-free mouse facility according to The Johns Hopkins University Institutional Animal Care and Use Committee. Gender-matched 6–8-week-old mice were used.

**Antibodies—**Antibodies against mouse and human lumican were rabbit polyclonal (Covance Research Products). Monoclonal antibodies to mouse integrins were obtained from eBioscience, BD Biosciences, and BioLegend. For flow cytometry, the following fluorescence-labeled antibodies were purchased from eBioscience: anti-CD11a-phycocerythrin/cyanin7 (PE/Cy7), anti-CD11b-PE/Cy7, anti-CD18-PE, anti-CD49d-PE, anti-CD49e-PE, fluorescein isothiocyanate-labeled donkey anti-rabbit IgG, and anti-granulocyte-differentiation antigen (Gr1)-allophycocyanin. For confocal microscopy, biotinylated antibodies (anti-CD11a, anti-CD11b, anti-CD18, anti-CD49d, and anti-CD49e) were purchased from BD Biosciences.

**PMN Isolation and Purification—**PMNs from peritoneal lavage were purified by Histopaque gradient centrifugation (21) and used for migration assay and confocal microscopy. Bone marrow and peripheral blood were collected, cleared of red blood cells (ACK lysing buffer, Invitrogen), and resuspended in 10% fetal bovine serum in PBS for flow cytometry.

**Transwell Migration Assay—**Bottom chambers were filled with 100 μl of RPMI 1640 medium with or without 0.1 ng/ml Cxcl1 (BIOSOURCE) or 100 nM bacterial tripeptide (Sigma). PMNs (1 × 10^5) in 100 μl of RPMI were placed on the insert (6.5 mm, 3 μm pore size) with or without rLum (3 μg/ml) or antibodies (5 μg/ml) against integrin or lumican. After 2 h of incubation at 37 °C, 5% CO₂, PMNs that migrated to the bottom chambers were counted manually under a microscope.

**Immunoprecipitation—**Peritoneal lavage cells were extracted with 20 ml Tris-HCl, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, pH 8.0, and precleared with goat IgG and protein G beads. Specific integrins were immunoprecipitated using anti-integrin-conjugated protein G beads. Co-immunoprecipitated lumican was detected by anti-lumican immunoblotting. Densitometric analysis of triplicate data were performed using the NIS-Elements AR 3.0 software (Nikon Instrument Inc.).

**Under-agarose Directional Migration Assay—**Agarose plates were prepared as described (22). A central well received purified PMNs (1.0 × 10^5), whereas 10 μl of 1 ng/ml Cxcl1 or BSA solution were added to wells on either side. Dishes were incubated at 37 °C for 2 h and photographed using a Zeiss LSM 410 confocal microscope with ×20/0.8 objective.

Confocal Microscopy—PMNs (2 × 10^5) on coverslips were fixed in 4% paraformaldehyde, blocked with Image-iT™FX signal enhancer (Invitrogen), followed by 3% BSA in PBS for 30 min, and treated with an endogenous biotin-blocking kit (Molecular Probes). The coverslips were incubated with primary antibodies against lumican and integrins and fluorescently labeled secondary antibodies (Alexa Fluor 488 for lumican and streptavidin-Alexa Fluor 594 for the integrin antibodies). Imaging was performed on a Zeiss LSM 510 single photon Meta Confocal microscope using oil ×40/1.3 or oil ×100/1.4 objectives. Z-stacks for three-dimensional reconstructions were collected at 0.15-μm z axis interval and 3-μm total height with a ×100 objective. Three-dimensional structures of PMNs stained with lumican and integrins were constructed from Z-stacked data (Volocity software, version 4.2.1).

Flow Cytometry—Wild type and Lum−/− PMNs were labeled with Gr1-allophycocyanin as a PMN marker and anti-mouse lumican antibody followed by fluorescein isothiocyanate-labeled secondary antibody. The control sample was stained with fluorescein isothiocyanate-labeled anti-rabbit IgG or PE or PE/Cy7-labeled rat IgG. Samples were analyzed using the FACSCalibur flow cytometer (BD Biosciences) and CellQuest software. PMNs were identified by forward scatter and side scatter, gated by Gr1-allophycocyanin, and analyzed for lumican.

**Quantitative Real Time PCR—**qRT-PCR was performed on cDNA from peritoneal PMNs and mouse embryonic fibroblasts (MEFs) with SYBR Green real time PCR mixture (Applied Biosystems) as described before (23). Pro-inflammatory factors, including tumor necrosis factor-α (2 ng/ml), interleukin-1β (2 ng/ml), and LPS (1 ng/ml), were incubated with peritoneal PMNs for 2 h before isolating total mRNA.

**Induction of Keratitis and Immunohistochemistry—**Mice were anesthetized, and the central epithelium of the cornea was injured and exposed to 10 μg of LPS as before (20). After 6 h the eyes were enucleated and frozen for immunohistochemistry (24). After cryoprotection, the eyes were infused for 30 min with an embedding media consisting of 2 parts of 20% sucrose in phosphate buffer to 1 part of OCT (Tissue-Tek). The molds containing sample were submerged in isopentane cooled by dry ice for 15 min and then stored at −80 °C. Parasagittal sections (20 μm thick) of the eyes were cut.

**Cell Adhesion Assay—**Cell adhesion assays were performed on the 96-well plate as described previously (5). Each well was coated with 200 μl of 0.2 mg/ml type I collagen, fibronectin, Matrigel®, or BSA overnight at 4 °C. The plates were blocked with 1% BSA at room temperature for 1 h and rinsed three times with PBS. After adding purified peritoneal Lum+/+ or Lum−/− PMNs (2 × 10^5) to the wells, the plates were incubated at 37 °C, 5% CO₂ for 1 h. Unattached cells were rinsed off with PBS, and 20 μl of CellTiter 96 AQUOS solution (Promega) was added to each well and incubated at 37 °C, 5% CO₂ for 1.5 h. Absorbance at 490 nm, which is proportional to the number of the cells in the well, was measured.

**RESULTS**

**Migration Deficiencies in Lum−/− PMNs—**Mouse CXC motif chemokine ligand 1 (Cxcl1), also known as KC, Gro1, and Scy1, is a functional homolog of human interleukin-8 and a
strong mediator of PMN migration through interactions with its receptor Cxcr2 (25, 26). We used Cxcl1 as a chemoattractant in an under-agarose migration assay to compare PMN migration in wild type and Lum−/− mice. Purified peritoneal PMNs were placed in a central well cored out of a layer of agarose in a 35-mm dish. Wells on either side were filled with Cxcl1 or BSA as a control. Within 2 h, wild type PMNs had migrated across the edge toward the Cxcl1- and not the BSA-containing wells. In contrast Lum−/− PMNs showed very little preferential migration toward Cxcl1, suggesting a migration deficiency in Lum−/− PMNs (Fig. 1A).

For a quantitative analysis of migration, we used transwell assays, where PMNs were placed in the insert and chemoattractant-containing medium in the bottom chamber of transwells. Compared with the significant migration seen in Lum+/+ peritoneal PMNs, Lum−/− PMNs migrated minimally in response to the bacterial tripeptide fMLP or Cxcl1 (Fig. 1, B and C). LPS alone did not promote chemotactic migration in vitro. However, in vivo LPS induces cytokines and chemokines through TLR4 signaling and the consequent migration of PMNs (19, 20). Incubation of PMNs with rLum or its presence alone in the bottom chamber did not promote migration, indicating that lumican itself is not a chemoattractant (Fig. 1C). However, preincubation of Lum−/− PMNs with exogenous rLum restored their chemotactic migration to Cxcl1, to a level comparable with that seen in Lum+/+ PMNs. Moreover, anti-lumican antibody treatment blocked migration of Lum+/+ or rLum-treated Lum−/− PMNs toward Cxcl1. These results clearly define a role for lumican in PMN migration.

The observed poor migration of Lum−/− PMNs toward Cxcl1 and fMLP could be due to reduced cell surface integrins or the Cxcl1 receptor, Cxcr2. We ruled out this possibility based on FACS analyses of Lum+/+ and Lum−/− peritoneal PMNs that show comparable surface levels of CD11a (αI), CD11b (αII), CD18 (β2), CD29 (β1), CD49d (αL), CD49e (αS) integrins, and Cxcr2 (Fig. 2). Reduced migration of Lum−/− PMNs could also be due to altered cell adhesion to the ECM. We compared adhesion of Lum+/+ and Lum−/− peritoneal PMNs on the ECM substrates Matrigel® (27), fibronectin, and collagen type I (Fig. 3). The assayed adhesion properties were comparable in Lum−/− versus Lum+/+ PMNs, indicating that the migration deficiency in the former was not due to initial differences in adhesion.
Blocking Specific Integrins Abrogates Lumican-mediated PMN Migration—To identify specific integrins regulating lumican-mediated PMN migration, we assayed migration of Lum\(^{-/-}\) PMNs that were preincubated with rLum in the presence or absence of integrin blocking antibodies (Fig. 4). PMN migration was inhibited by 56 and 55% in the presence of CD18 (\(\beta_2\)) and CD29 (\(\beta_1\)) blocking antibodies, respectively. Blocking CD11b (\(\alpha_M\)) inhibited migration by 37%, whereas anti-CD11a (\(\alpha_4\)), anti-CD49d (\(\alpha_2\)), and anti-CD49e (\(\alpha_5\)) had no inhibitory effects on chemotactic migration (Fig. 4). Therefore, lumican seems to affect PMN migration that is mediated by \(\beta_2\) and \(\beta_1\) integrins specifically. Because blocking CD11b (\(\alpha_M\)) also reduced migration, MAC-1 (CD11b/CD18) is clearly implicated in lumican-mediated migration.

Lumican Found on Peritoneal and Not Bone Marrow or Peripheral Blood PMNs—Lumican is normally found in association with collagen fibrils in interstitial ECMs. We questioned whether lumican is present on the surface of PMNs to promote migration. Purified peritoneal PMNs were immunostained with anti-lumican and viewed by confocal microscopy (Fig. 5A). The presence of lumican was evident on the surface of wild type and not Lum\(^{-/-}\) mouse PMNs. To determine whether localization of lumican on PMN surfaces was universal for bone marrow-derived cells, peripheral blood, and peritoneal lavage, we analyzed these populations by flow cytometry. Total bone marrow, peripheral blood, and peritoneal lavage cells were stained for Gr1 (PMN marker) and lumican. A significant portion (\(\sim\)13%) of the peritoneal lavage Gr-1-positive cells was positive for lumican (Fig. 5B). In contrast, Gr1-positive bone marrow and peripheral blood cells were negative for lumican (Fig. 5C).

**FIGURE 3.** Peritoneal Lum\(^{+/+}\) and Lum\(^{-/-}\) PMNs show similar adhesion to ECM. Peritoneal PMNs were allowed to adhere to 96-well plates coated with type I collagen, fibronectin, Matrigel, and BSA. There was no significant difference in adhesion between Lum\(^{+/+}\) and Lum\(^{-/-}\) PMNs (n = 3).

**FIGURE 4.** Antibodies against CD18, CD11b, and CD29 inhibit lumican-mediated PMN migration. Migration toward Cxcl1 was measured in peritoneal Lum\(^{-/-}\) PMNs incubated with rLum and with or without specific anti-integrin antibodies. Anti-CD11b (\(\alpha_M\)), -CD18 (\(\beta_2\)), and -CD29 (\(\beta_1\)) inhibited PMN migration. *, p \(<\) 0.05 between migration with or without integrin-blocking antibodies considered as significantly different. The results shown are means \(\pm\) S.D. (n = 3) and are one of three similar experiments.

**FIGURE 5.** Lumican is present on peritoneal PMNs. A, immunostaining and confocal microscopy shows lumican (green) on the surface of Lum\(^{+/+}\) peritoneal PMNs. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue). Lum\(^{-/-}\) PMNs are negative for lumican as expected. Magnification = \(\times\)40; scale bar, 20 \(\mu\)m. B and C, analysis of lumican associated with PMNs by flow cytometry. Cells were stained with Gr1 (PMN marker) and lumican-specific antibodies and analyzed by FACS. Dot plots show expression of lumican versus Gr1. The peritoneal lavage (B) shows a significant percentage of Gr1\(^+\) cells positive for lumican, indicating the presence of lumican-positive PMNs. Lumican expression was at a basal level in bone marrow cells (BMC) and peripheral blood (C), indicating the absence of lumican-positive PMNs in these two groups.
Lumican-mediated Neutrophil Migration

The lumican detected on peritoneal PMNs may arise from two potential sources; lumican is either synthesized by peritoneal PMNs, or it is deposited on the surface, sometime during or after trans-endothelial migration of circulating PMNs. qRT-PCR measurements could not detect lumican transcripts in peritoneal PMNs, even after induction by pro-inflammatory factors. MEFs used as a positive control, on the other hand, expressed lumican, with increased levels after treatment with LPS (Fig. 6A). Thus, peritoneal PMNs do not express lumican. We next considered the vascular endothelium as a likely source of lumican. Indeed, human umbilical vein endothelial cell (HUVEC) total protein extracts, immunoblotted for lumican, showed a band similar in size to the lumican core protein (Fig. 6B lane 3). These results suggest that endothelial lumican may regulate peritoneal PMN migration after it is deposited on extravasating PMNs.

Lumican Integrin Interactions—Blocking specific integrins inhibited rLum-mediated chemotactic migration. This suggests that lumican may serve as a ligand for these receptors. We examined this possibility in two ways as follows: first by assaying for co-localization of lumican and integrins by immunostaining and confocal microscopy, and second by testing co-immunoprecipitation of lumican with specific integrins. The strongest immunostaining of PMNs was seen with anti-CD18 and anti-CD11b and to a lesser extent with anti-CD11a (Fig. 7A). The merged data for lumican (green) and integrin (red) showed significant overlap (yellow) between lumican and CD18 (Fig. 7, A and B, and supplemental movie S1) and between lumican and CD11b immunostaining (Fig. 7A). A quantitative estimate of immunostain overlaps was obtained from three independent PMN images as follows: lumican immunostaining showed the highest overlap with CD18, then CD11b, and CD11a (Fig. 7C).

We sought biochemical evidence for lumican-integrin interactions by immunoprecipitating integrins from peritoneal lavage extracts and subsequent immunoblotting with anti-lumican. The results showed co-precipitation of lumican with CD18, CD11b, and CD11a (Fig. 7D), suggesting that lumican is a ligand for the MAC-1 (CD11b/CD18) and possibly LFA-1 (CD11a/CD18) receptor complexes. In a previous study we showed binding between lumican and CD14. To determine whether binding of lumican to CD18 is dependent on lumican-CD14 interactions, we also immunoprecipitated CD18 from CD14-deficient (CD14−/−) mouse peritoneal PMNs; lumican still co-precipitated with CD18 in this lysate (Fig. 7E). Therefore, lumican-CD18 interactions appear to be independent of its interactions with CD14. Lum−/− mouse peritoneal lavage, used as a negative control, does not co-precipitate lumican with CD18 as expected (Fig. 7E).

In Vivo Lumican Co-localizes with CD18 on PMNs en Route to the Cornea—To gain insight into the physiological relevance of lumican binding to integrins, we investigated if lumican localized with CD18 (β2) integrins on the surface of PMNs migrating to the injured cornea. Earlier studies have shown that significant influx of circulating PMNs into the injured cornea takes about 6 h (19, 20). Therefore, whole eyes were harvested 6 h after injuring the cornea with a circular wound. Adjacent frozen sections were immunostained for Gr1 (PMN marker), CD18, and lumican (Fig. 8). The Lum+/- eye sections showed overlapping immunostaining for CD18 and lumican on infiltrating PMNs (Fig. 8, A and B). Importantly, whereas lumican is a major component of the cornea, the PMNs transmigrating from the limbal vasculature into the anterior chamber already had lumican on their surface before entering the cornea. As expected, the Lum−/- PMNs and cornea were negative for lumican (Fig. 8C).

DISCUSSION

The extracellular matrix is an important regulator of inflammation and an integral component of the complex process involved in neutrophil recruitment and guidance to the site of inflammation. However, contributions of different ECM proteins to this process remain elusive for the most part. The endothelial basement membrane is the first ECM type encountered by an extravasating PMN. The basement membrane is often viewed as providing ligands for specific interactions leading to the adherence and activation of circulating PMNs, or as a substrate undergoing specific degradation as PMNs cross this barrier (28). The interstitial ECM is thought to provide signals later on in the path of migrating PMNs (5). We began exploring lumican as an interstitial ECM protein driving the later stages of
PMN migration to the site of inflammation. Instead, our results identify lumican as a versatile ECM protein, possibly regulating early events, in PMN migration. Lumican seems to promote PMN migration through its interactions with β₂ integrins. The source of this lumican is not the abundant form present in the interstitial ECM, but it is one that is likely derived from the endothelium and deposited on the surface of extravasating PMNs.

In *in vitro* chemotaxis assays, *Lum*<sup>−/−</sup> PMNs migrated poorly in response to bacterial fMLP or the chemokine Cxcl1, a migration deficiency restored by supplementing exogenous rLum. This suggests a form of “outside-in” signaling (29) by lumican. Migration could be specifically inhibited by antibodies that block β₂ (CD18) and β₁ (CD29) integrins, the two major subfamilies of leukocyte integrin receptors (3). In general, the β₂ integrins, LFA-1 and MAC-1, regulate adhesion of neutrophils to the endothelium, their activation, and extravasation (2, 3, 30, 31). The β₁ subunit carrying receptors are known to regulate PMN interactions with the ECM (32). Using immunohistology and confocal microscopy, we found robust expression of CD18, CD11b, and to a lesser extent CD11a on peritoneal PMNs. On immunostaining peritoneal PMNs for lumican, we noted significant overlaps in their staining pattern with that of CD18, CD11b, and CD11a, implying co-localization of lumican with these integrins. Association between lumican and each of these integrin subunits was confirmed by co-immunoprecipitation of lumican with CD18, CD11b, and CD11a from peritoneal PMN preparations. We further found that the β₁ integrin (CD29) immunoprecipitate did not have lumican, indicating a lack of binding between lumican and this integrin subunit. Therefore, the effects of lumican on β₁-mediated PMN migration is likely indirect. An earlier study suggested lumican interactions with β₁ integrin subunit in melanoma cells (33). Lumican-mediated Neutrophil Migration

**A**

**DAPI**

**Lumican**

**Integrins**

**Merge**

**CD11a**

**CD11b**

**CD18**

**CD49d**

**CD49e**

**B**

**C**

**Pearson Correlation**

**CD11a**

**CD11b**

**CD18**

**CD49d**

**CD49e**

**D**

**Control IgG**

**CD18**

**CD11b**

**CD11a**

**CD49d**

**CD49e**

**IP: Integrins**

**WB: anti-mLum**

**75 kDa**

**50 kDa**

**37 kDa**

**E**

**Lum**<sup>−/−</sup> **CD14**<sup>−/−</sup> **Lum**<sup>−/−</sup>

**CD18**

**Control IgG**

**Control IgG**

**Control IgG**

**Control IgG**

**IP: CD18**

**WB: anti-mLum**

**75 kDa**

**50 kDa**

**37 kDa**

**IgG H**

**Lum**

**IgG L**
Lumican-mediated Neutrophil Migration

FIGURE 7. Lumican interacts with specific integrins on the surface of Lum+/+ PMNs. A, confocal microscopy of purified peritoneal PMNs showing 4,6-diamidino-2-phenylindole (DAPI)-stained nucleus (blue), immunostained lumican (green), integrins (red), and a merged image of lumican and specific integrins. There were significant overlaps (yellow) in immunostaining of lumican with CD18 and CD11b. Scale bar, 3 μm. B, reconstructed three-dimensional structure of PMNs by Z-stack analysis of immunostained lumican (green) and CD18 (red). See also supplemental movie S1. C, extent of overlap between anti-Lum and anti-integrin immunostaining of three independent PMN images reconstructed by Z-stack analysis shown as mean Pearson correlation ± S.D. Lum−/− peritoneal PMN whole cell lysate was immunoprecipitated (IP) with anti-CD18, -CD11b, -CD11a, -CD49d, -CD49e, and -CD29 antibodies, resolved by SDS-PAGE, and immunoblotted with anti-lumican detecting a lumican glycoprotein band of ∼40 kDa (Lum). WB, Western blot. E, CD18 was immunoprecipitated from Lum+/+, CD14+/−, and Lum−/− peritoneal lavage and immunoblotted with anti-lumican. Lumican co-precipitates with CD18 from wild type and CD14−/− lysates and not Lum−/− lysate were used as a negative control.

FIGURE 8. Co-localization of lumican and CD18 on PMNs migrating to injured Lum+/+ mouse cornea. Six h after corneal wounding, whole eye parasagittal sections were stained with Gr1 (purple), lumican (green), CD18 (red), and nucleus (blue). A, section shows Gr1 and lumican-positive PMNs in the anterior chamber (ac) before entering the cornea (co). These PMNs were also positive for CD18. Note that lumican is a major ECM protein of the cornea, and thus the cornea is stained brightly by anti-lumican antibody. Magnification = ×40. Scale bar, 50 μm. B, high magnification (×100) of PMNs from the anterior chamber from composite A showing lumican (green) and CD18 (red) immunostaining and staining overlaps (yellow) in the merged image. Scale bar, 10 μm. C, Lum−/− cornea used as a negative control shows no staining of lumican and positive immunostaining for Gr1 and CD18. Scale bar, 50 μm. DAPI, 4,6-diamidino-2-phenylindole; DIC, differential interference contrast.

can-mediated adhesion of melanoma cells could be inhibited by anti-β3 antibodies, although no biochemical evidence was provided for binding between lumican and the β1 integrin subunit in that study.

We focused on lumican-β3 integrin interactions because of its novelty and the possible implications of this interaction in early migration of neutrophils after endothelial extravasation. By flow cytometry we found Gr-1 (PMN marker)-positive cells from bone marrow or peripheral blood to be negative for surface lumican. On the other hand, a significant subset of peritoneal lavage Gr-1-positive cells is positive for lumican, suggesting surface acquisition of lumican some time during or after neutrophils exit the vasculature. qRT-PCR measurements of lumican mRNA clearly indicated that the peritoneal PMNs do not make the lumican deposited on their surfaces. The likely source of this lumican is the vascular endothelium. Indeed, we found cultured HUVECs to express lumican. Taken together these observations suggest a novel endothelial lumican-mediated paracrine regulation of neutrophils. This idea gains further support from our observations that β3 integrins, known to modulate PMN extravasation and early stages of migration (3), interact with PMN-surface lumican. Furthermore, confocal microscopy of corneal wounds showed that the infiltrating PMNs already have lumican on their surface, before entering the lumican-rich connective tissue of the cornea. Circulating neutrophils entering the cornea are derived from the microvasculature of the limbal network (34). Therefore, it is likely that lumican produced by the limbal microvascular endothelial cells interact with the PMN cell surface MAC-1 (αMβ2) and LFA-1 (αLβ2), as the PMNs extravasate and migrate to the injured cornea. The ECM-associated lumican in the collagenous stroma of the cornea may have a different role in binding Cxcl1 and providing an optimal chemotactic gradient for final migration of PMNs within the interstitial ECM (19).

Lumican exists as a glycoprotein in most tissues, and as a glycosaminoglycanated proteoglycan in certain tissues such as the cornea (7, 9, 35). Therefore, lumican functions can be impacted by the core protein and/or its keratan sulfate side chains. Two lines of evidence suggest that the lumican-integrin interaction relevant for PMN migration, as demonstrated here, involves the lumican core protein and not its glycosaminoglycanated side chains. First, lumican detected in the endothelial cell extract was a sharp band in the immunoblot, rather than a smeared band characteristic of the heterogeneously glycosaminoglycan-modified proteoglycan form. The endothelial lumican band was also similar in size to the lumican core protein. Second, recombinant lumican, which is a simple glycoprotein without glycosaminoglycan side chains, was sufficient for restoring migration deficiencies of Lum−/− PMNs. Decorin, another small leucine-rich repeat proteoglycan, presents a different scenario. Its role in endothelial cell adhesion and migration through interactions with α5β1 integrin requires the intact proteoglycan form (14). Integrins often interact with protein ligands at an Arg-Gly-Asp (RGD) sequence, in addition to other non-RGD regions (36–38). Lumican does not have an RGD sequence. However, the tandem leucine-rich repeat motifs, a feature of these proteoglycans, are involved in multiple interactions (16, 39, 40). Sites within these motifs may be involved in integrin binding.

Cell migration requires establishment of polarity and a series of adhesive and de-adhesive processes (41). Our in vitro cell adhesion assay indicates that adhesion of peritoneal PMNs to
Matrigel®, fibronectin, or collagen type I is not affected by lumican deficiency. On the other hand, in under-agarose assays, where migration was tested in the absence of any added ECM, Lum−/− PMNs performed poorly, consistently with the idea that lumican may be needed even before PMNs reach ECM-rich tissues in vivo. We speculate that lumican may be involved in the activation of β1 integrins; binding between lumican and β2 integrin may initiate an outside-in signaling that promotes cell mobility. Ligand binding by the extracellular domains of integrins and additional processes involving chemokine-receptor interactions is considered to alter plasma membrane lateral mobility, leading to transient high affinity states in integrin heterodimers, downstream signaling, and altered gene expression affecting migration outcomes (30). Lumican binding with β2 integrin may be modulating its affinity for chemokines and subsequent activation, or its affinity for other ECM ligands. Another study reported lumican and Cxcl1 interactions in corneal extracts (19). Thus, lumican binding to the β2 integrin subunit, as our results indicate, and Cxcl1 (19) may facilitate chemokine-mediated activation of β1 integrins. Activated β2 integrins are known to induce surface expression of β1 integrins on human PMNs, and this may facilitate PMN migration on ECM (42). This raises the question that the function of lumican on human PMNs, and this may facilitate PMN migration on tissues.

In summary, lumican possibly produced by the vascular endothelium binds to the surface of extravasating PMNs through its interactions with β2 integrins and promotes migration during inflammation. These findings question our perception of ECM proteins as mere adhesive and migration substrates. Instead, they may be recruited to closely interact with cell surface molecules and modulate their behavior early during inflammation.

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