Lumican is required for neutrophil extravasation following corneal injury and wound healing

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Summary

An important aspect of wound healing is the recruitment of neutrophils to the site of infection or tissue injury. Lumican, an extracellular matrix component belonging to the small leucine rich proteoglycan (SLRP) family, is one of the major keratan sulfate proteoglycans (KSPGs) within the corneal stroma. Increasing evidence indicates that lumican can serve as a regulatory molecule for several cellular processes, including cell proliferation and migration. In the present study, we addressed the role of lumican in the process of extravasation of polymorphonuclear leukocytes (PMNs) during the early inflammatory phase present in the healing of the corneal epithelium following debridement. We used *Lum*$^{-/-}$ mice and a novel transgenic mouse, *Lum*$^{-/-}$*,Kera-Lum*, which expresses lumican only in the corneal stroma, to assess the role of lumican in PMN extravasation into injured corneas. Our results showed that PMNs did not readily invade injured corneas of *Lum*$^{-/-}$ mice and this defect was rescued by the expression of lumican in the corneas of *Lum*$^{-/-}$*,Kera-Lum* mice. The presence of lumican in situ facilitates PMN infiltration into the peritoneal cavity in casein-induced inflammation. Our findings are consistent with the notion that in addition to regulating the collagen fibril architecture, lumican acts to aid neutrophil recruitment and invasion following corneal damage and inflammation.

Key words: Lumican, Neutrophil, Knockout mouse, Transgenic mouse, Cornea, Wound healing

Introduction

Recruitment of neutrophils from the circulating blood to sites of infection and tissue injury represents one of the important elements of innate immunity. Cell adhesion molecules such as integrin(s) and galectin(s) have been strongly implicated in neutrophil extravasation and tissue infiltration (Sato et al., 2002; Werr et al., 1998); however, the role of extracellular matrix (ECM) components has not been extensively studied in this process. One such component of the ECM, lumican, belongs to the small leucine rich proteoglycan (SLRP) family and is one of the major keratan sulfate proteoglycans present in the corneal stroma. The non-glycanated lumican core protein is widely distributed in many interstitial connective tissues, e.g. sclera, aorta, cartilage, liver, skeletal muscle, kidney, pancreas, brain, placenta, bone and lung (Carlson et al., 2005; Ying et al., 1997; Chakravarti et al., 1998; Ezura et al., 2000; Funderburgh et al., 1991; Funderburgh et al., 1993; Krull and Gressner, 1992). In the cornea, lumican is in the glycanated form, meaning that keratan sulfate glycosaminoglycan chains (KS-GAGs) have been added to the core protein. It has been postulated that binding of lumican core protein to collagen molecules regulates the fibril diameter, whereas the extended KS-GAG side chain modulates fibril spacing and corneal hydration. The necessity of lumican in regulating collagen matrix assembly required for tissue integrity and function are best exemplified by corneal opacity and skin fragility observed in lumican-knockout (*Lum*$^{-/-}$) mice (Carlson et al., 2005; Saika et al., 2000). Abnormally large collagen fibrils and disorganized interfibrillar spacing are found in the stroma of *Lum*$^{-/-}$ mice. It has been suggested that a key role for lumican in the posterior stroma is in maintaining normal fibril architecture, probably by regulating fibril assembly and maintaining the optimal KS-GAG content a requirement for corneal transparency (Chakravarti et al., 1998).

Increasing evidence suggests that lumican also serves as a regulatory molecule for several cellular functions, such as promoting cell proliferation and migration, suppressing apoptosis in the injured corneal epithelium, and regulating expression of keratocan (*Kera*) and aldehyde dehydrogenase (*Aldh*) by keratocytes (Kao et al., 2006; Kao and Liu, 2002). One process in which lumican serves as a regulatory molecule is posterior capsular opacification (PCO), a major complication following cataract surgery. Following cataract surgery, lens epithelial cells undergo cell proliferation and epithelial–mesenchymal transition (EMT). During this process, lumican is transiently expressed by the transformed lens epithelial cells followed by expression of α-smooth muscle actin (α-SMA) and type I collagen. This process ultimately leads to the formation of opaque scar tissue. Interestingly, lens epithelial cells from *Lum*$^{-/-}$ mice show a decrease and delay in α-SMA expression and postponed EMT induction by TGFβ-2 in vitro, suggesting that lumican modulates EMT in mouse lens cells (Saika et al., 2004).

Lumican has also been implicated in cell proliferation and metastasis of several cancers, such as breast, colorectal, pancreatic, lung, and benign prostatic hyperplasia (Leygue et al., 1998; Lu et al., 2002; Matsuda et al., 2008). Although the expression and form of lumican often correlates with the severity of cancer, reports

have also shown that overexpression of lumican can suppress transformation by Src and K-Ras. Despite these contradictory reports, and the role of lumican in cancer, the evidence strongly supports the notion that lumican can modulate several cellular functions in addition to serving as a component of the ECM.

Recent reports have shown that *Lum*$^{-/-}$ mice have immunological problems attributed to the Fas-Fas ligand and Toll-like receptor 4 pathways in lipopolysacchride (LPS)-induced inflammation (Vij et al., 2005); however, it is still unclear how lumican modulates the inflammatory response and in particular, neutrophil extravasation during wound healing. In addition, we recently reported an impaired ability of neutrophils to infiltrate the corneas of keratocan- and lumican-knockout mice, which also suggests an impaired inflammatory response (Carlson et al., 2007). In the present study, we used *Lum*$^{-/-}$ mice and *Lum*$^{-/-}$*,Kera-Lum* bi-transgenic mice, which express lumican only in the cornea, to examine the role of lumican on neutrophil extravasation into injured corneas. Our results demonstrate that lumican is required for efficient extravasation of polymorphonuclear leukocytes (PMNs) out of the blood vessels to sites of injury.

Results

PMN extravasation into injured corneas of *Lum*$^{+/+}$, *Lum*$^{-/-}$ and *Lum*$^{-/-}$*,Kera-Lum* mice

Twelve hours after a 2-mm-diameter corneal epithelial debridement, histological examination indicated that PMNs were present in the stroma of injured corneas of wild-type (*Lum*$^{+/+}$) mice, whereas few PMNs were found in corneas of *Lum*$^{-/-}$ and bitransgenic *Lum*$^{-/-}$*,Kera-Lum* mice. This trend was maintained 24 hours after wounding. In comparison with *Lum*$^{-/-}$ mice, significantly more PMNs were seen in injured corneas of *Lum*$^{-/-}$*,Kera-Lum* mice, although the number was significantly less than that of wild-type mice (Fig. 1A). These results were confirmed by immunofluorescent staining with a monoclonal anti-CD11b antibody 12 hours and 18 hours after corneal injury (Fig. 1B). Measurement of myeloperoxidase (MPO) activity in injured corneas also showed that there was a significant increase in enzyme activity in wild-type, *Lum*$^{-/-}$*,Kera-Lum* and *Lum*$^{-/-}$ mice 12 hours and 24 hours after wounding as compared with those of uninjured corneas of respective genotypes (Fig. 1C). Interestingly, the presence of the lumican transgene (*Kera-Lum*) significantly enhanced the invasion of PMNs into the cornea of *Lum*$^{-/-}$*,Kera-Lum* mice 12 hours and 24 hours after injury, as determined by MPO enzyme activity. At 48 hours after debridement, the MPO activity returned to a much lower level in all mice despite their genotypes, because epithelium debridement healed at this time point.

The impaired PMN invasion into the injured corneas in the absence of lumican might be due to alteration of PMN maturation during hematopoiesis and/or the requirement of lumican for PMN extravasation and invasion. The following series of experiments examined these possibilities. To further elucidate the role of lumican on PMN extravasation during inflammation, we analyzed the distribution of white blood cells isolated from bone marrow, circulating blood, peripheral blood, and the peritoneal cavity of experimental *Lum*$^{-/-}$ and *Lum*$^{+/-}$ mice that were intraperitoneally injected with casein.

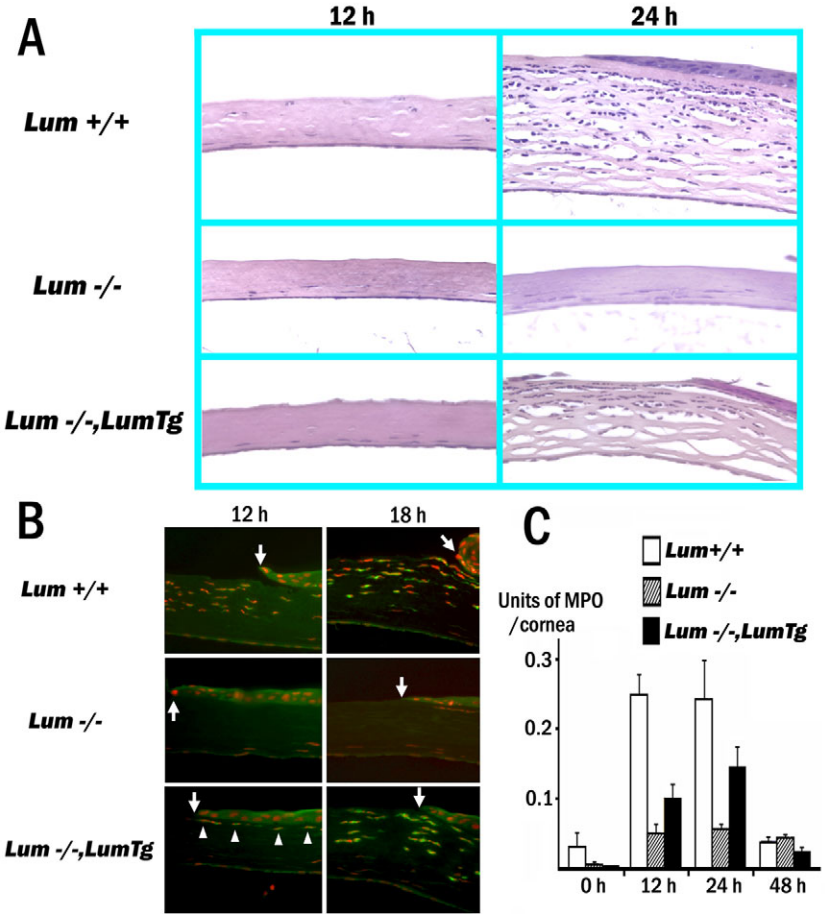

Fig. 1. Invasion of PMNs into injured corneas of *Lum*$^{+/-}$, *Lum*$^{-/-}$ and *Lum*$^{-/-}$*,Kera-Lum* mice. (**A**) Paraffin sections prepared from specimens 12 and 24 hours after cornea epithelial debridement stained with hematoxylin and eosin. At 12 hours, PMNs are found only in the cornea of wild-type mice. 24 hours after wounding, PMNs are found in the corneal stroma of wild-type mice. No PMNs are found in the wound area at 12 hours in *Lum*$^{-/-}$*,Kera-Lum*; however, by 24 hours the number of PMNs increased. Few PMNs, if any, are observed in *Lum*$^{-/-}$ mouse corneas. (**B**) Anti-neutrophil (CD-11b) antibody staining (green) with propidium iodide (PI) nuclear counter staining (red). The left-hand side of each panel shows the wound area whereas the right-hand side shows the peripheral cornea. Many CD11b^{+} cells are observed in the corneal stroma of wild-type mice 12 hours after injury. In the cornea of *Lum*$^{-/-}$*,Kera-Lum*, only the superficial stroma which is covered by epithelium has sporadic CD11b^{+} cells (arrowheads) 12 hours after wounding. 18 hours after wounding, corneal sections of wild-type and *Lum*$^{-/-}$*,Kera-Lum* mice show an increase of CD11b^{+} cells, whereas no CD11b^{+} cells are observed in *Lum*$^{-/-}$ mice. The corneal epithelium and endothelium show non-specific staining of anti-CD11b antibody in all sections. (**C**) Myeloperoxidase (MPO) assay of wounded corneal tissues. The MPO activity in *Lum*$^{-/-}$ mice is significantly lower than that of wild-type and *Lum*$^{-/-}$*,Kera-Lum*. Error bars indicate s.e.

Lumican is not necessary for myelopoiesis

It is known that lumican is also expressed in bone and cartilage (Ying et al., 1997); however, it remains unknown whether the lumican is involved in the maturation of PMNs during hematopoiesis. To examine this possibility, hematopoietic cells were isolated from the bone marrow of 5-week-old *Lum*$^{-/-}$ and *Lum*$^{+/-}$ mice that were injected intraperitoneally (IP) with casein and subjected to flow cytometry. Data shown in Fig. 2 and Table 1 demonstrated that there was no significant difference in the Ly6C/G^{+}, CD11b^{+} cell population between bone marrow cells isolated from *Lum*$^{-/-}$ (86.7%) and *Lum*$^{+/-}$ (87%) mice. This observation supports the notion that lumican is not necessary for PMN maturation during myelopoiesis in bone marrow.

Lumican modulates PMN extravasation into the peritoneal cavity after casein induction

To examine whether lumican modulates PMN extravasation, induction of PMN infiltration into the peritoneal cavity by casein injection was performed with *Lum*$^{+/-}$ and *Lum*$^{-/-}$ mice. Three hours after casein induction, cells were isolated from peritoneal lavages from experimental *Lum*$^{-/-}$ and *Lum*$^{+/-}$ mice (n=6) and analyzed by cytochemistry with Giemsa stain and flow cytometry. There was no significant difference in the total cell number between *Lum*$^{+/-}$ and *Lum*$^{-/-}$ mice; however, the percentage distribution of different leukocytes, for example, PMNs, monocytes and lymphocytes, varied greatly between the two genotypes as determined by Giemsa staining. A majority of the cell population in the lavage from *Lum*$^{-/-}$ mice was composed of lymphocytes (76.5 ±3.9%, n=8) in contrast to *Lum*$^{+/-}$ mice, where the majority of the cells in the lavage were PMNs (84.6±1.8%, n=8) (Fig. 3A). Several macrophages were found in each microscopic visual field from *Lum*$^{-/-}$ peritoneal cells; however, macrophages were scarce in *Lum*$^{+/-}$ animals. These observations suggest that the presence of lumican in blood vessels and tissues surrounding the abdominal cavity is necessary to facilitate PMN infiltration into the peritoneal cavity upon casein induction.

To corroborate our microscopy results, flow cytometry was performed to determine the leukocyte population present in the peritoneal cavity following casein induction. Results shown in Fig. 3B and Table 2 indicate that 41% and 97% of casein-induced cells in the peritoneal lavage were CD11b^{+}, Ly6C/G^{+} in *Lum*$^{-/-}$ and *Lum*$^{+/-}$ mice, respectively, consistent with impaired extravasation of PMNs in the absence of lumican.

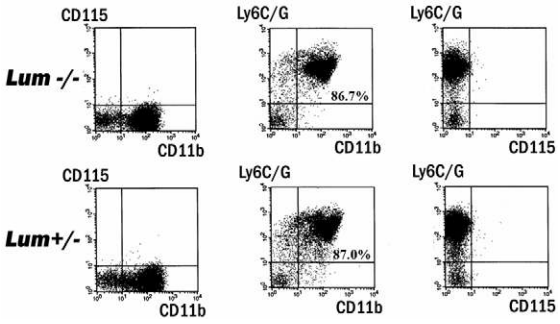

Fig. 2. Flow cytometry of non-erythroid bone marrow cells of *Lum*$^{+/-}$ and *Lum*$^{-/-}$ mice. Bone marrow cells were subjected to FACS analysis. There is no difference in the percentage of CD11b^{+} and Ly6C/G^{+} cells present in the bone marrow of *Lum*$^{-/-}$ and *Lum*$^{+/-}$ mice. CD115 recognizes macrophage-colony stimulating factor expressed on macrophages and monocytes, CD11b is a marker for leukocytes, Ly6C/G is a marker for neutrophils. CD8, CD4 and CD3 recognize cytotoxic T cells, T helper cells, and mature T lymphocytes, respectively (summary of FACS analysis is shown in Table 1).

Table 1. Summary of bone marrow cells from *Lum*$^{-/-}$ and *Lum*$^{+/-}$ mice[a]

Cell type	*Lum*$^{-/-}$	*Lum*$^{+/-}$
CD115	–	–
CD11b	+(>90%)	+
Ly6C/G	+(>90%)	+
CD115^{+}, CD11b^{+}	0%	0%
Ly6C/G^{+}, CD11b^{+}	86.7%	87.0%
Ly6C/G^{+}, CD115^{+}	0%	0%

[a]Statistics of mice shown in Fig. 2. There was no difference in the distribution of hematopoietic cell types in the bone marrow of *Lum*$^{+/-}$ and Lum$^{-/-}$ mice. CD115, macrophages; CD11b, leukocytes; Ly6C/G, neutrophils; CD8, cytotoxic T cells; CD4, T helper cells; CD3, mature lymphocytes. CD8, CS4 and CD3 were not determined.

Lumican promotes PMN extravasation from blood vessels

PMNs express integrins that bind extracellular matrix components (Merlin et al., 2001; Werr et al., 1998). Thus, it seems possible that the absence of lumican in blood vessels could directly alter PMN extravasation in *Lum*$^{-/-}$ mice. To examine this possibility, we determined the white blood cell population distribution in peripheral tail blood and circulating blood collected via heart puncture.

Peripheral blood obtained from the tail vein of *Lum*$^{-/-}$ and Lum$^{+/-}$ mice was used to prepare smears, which were subsequently examined by light microscopy (Fig. 4A). In *Lum*$^{-/-}$ mice, 27.2±4.2% (n=6) of white blood cells were PMNs (lymphocytes 65.5±4.5%, monocytes 3.3±1.3%); by contrast, only 11.7±0.91% (n=6) of white blood cells were PMNs, whereas the majority of the cells were lymphocytes or monocytes (86.5±1.1%) in *Lum*$^{+/-}$ mice. Three hours after casein injection, the percentage of PMNs increased to 64.7±3.1% and 38.6±3.8% in *Lum*$^{-/-}$ and *Lum*$^{+/-}$ mice, respectively (Fig. 4A,B). It is of interest to note that unlike the human, where most mature PMNs remain in circulation and extravasate into tissues only upon stimulation, for example by microbial infection or injury, in the mature mouse, PMNs extravasate from blood vessels and reside in tissues in the absence of stimulation (Nemzek et al., 2001; Surrat et al., 2001). These observations suggest that the presence of lumican in blood vessels facilitates the extravasation of PMNs under normal and inflammatory conditions.

After IP casein injection, the number of circulating white blood cells increased, as determined using a hemocytometer; however, there was no significant difference between *Lum*$^{-/-}$ and *Lum*$^{+/-}$ mice (Fig. 4C). To examine the distribution of circulating leukocytes, circulating white blood cells were examined by flow cytometry. As shown in Fig. 4D and Table 3, casein injection caused an increase in the PMN population found in the circulating blood as shown by an increase in CD11b^{+}, Ly6C/G^{+} cells from 25.9% to 58.2% in *Lum*$^{-/-}$ mice and from 15.5% to 42.9% in *Lum*$^{+/-}$ mice. The percentage of PMNs in circulation was higher in *Lum*$^{-/-}$ mice than in *Lum*$^{+/-}$ mice. This finding is consistent with the notion that lumican is required for PMN extravasation.

Anti-CD29 (integrin β1) antibody blocked lumican enhancement of PMN adhesion and migration in vitro

Our observation that *Lum*$^{-/-}$ mice had neutrophilia but fewer PMNs in the injured corneas suggests that lumican is necessary for the

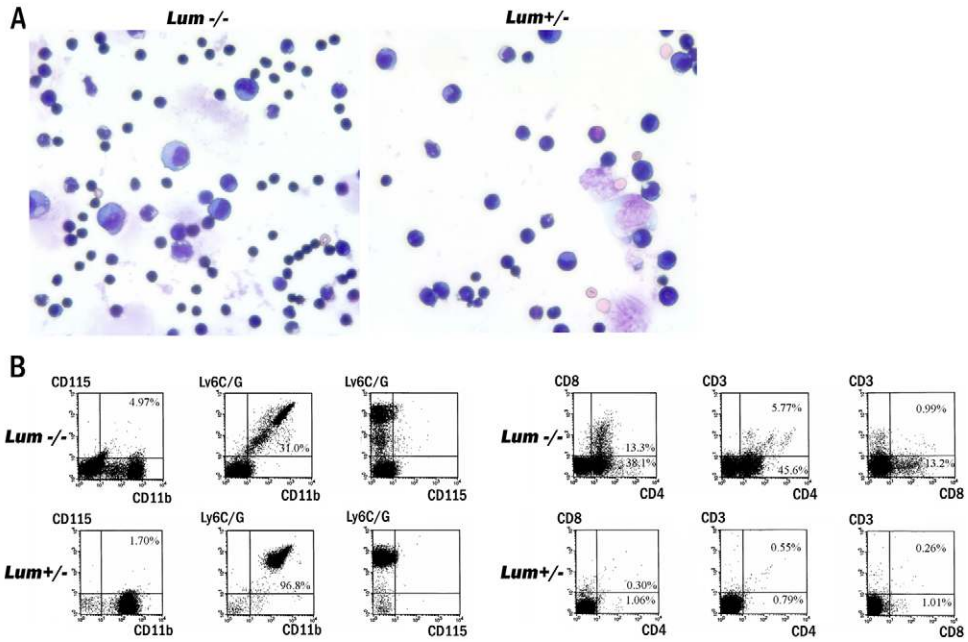

Fig. 3. White blood cell population in peritoneal lavages of *Lum*$^{+/-}$ and *Lum*$^{-/-}$ mice. Lavages prepared from experimental mice intraperitoneally injected with 2 ml of 5% casein in PBS. (**A**) Giemsa staining of cell smears shows that lymphocytes (76.5±3.9%, *n*=6) make up the majority of the cell population in the lavage from *Lum*$^{-/-}$ mice, whereas PMNs (84.6±1.8%, *n*=6) are the major cell type in *Lum*$^{+/-}$ lavages. (**B**) Flow cytometry shows 96% of the cells were CD11b^{+}, Ly6C/G^{+} in *Lum*$^{+/-}$ lavage, in contrast only 41.8% were CD11b^{+}, Ly6C/G^{+} and 45% of cells were CD4^{+} from the lavage of *Lum*$^{-/-}$ mice (summary of FACS analysis is shown in Table 2).

extravasation of PMNs from blood vessels into tissues. To examine this hypothesis, we performed PMN adhesion and migration assays in vitro. We collected casein-induced PMNs from a peritoneal lavage from wild-type mice and seeded them onto dishes coated with arterial lumican (aLum, non-glycanated lumican core protein), bovine serum albumin (BSA), or keratan sulfate proteoglycan (glycanated) (KSPG, e.g. KS-Lum and KS-Kera) isolated from bovine corneas, as described previously (Funderburgh et al., 1991). The cell adhesion assay showed significantly more PMNs attached to the surface coated with aLum than those coated with BSA or KSPG (Fig. 5Aa), which is consistent with a previous report demonstrating that macrophages bind to non-glycanated lumican but not to KSPG (Funderburgh et al., 1997). The cell migration study using a Transwell cell migration assay demonstrated that more than twice the number of PMNs passed through the alum-coated membrane than through those coated with BSA and KSPG in response to chemoattractants MIP2 and f-MLF (Fig. 5Ab). These results indicate that lumican serves as a matrix to promote PMN extravasation. We next hypothesized that PMN adhesion and migration were the result of the interaction between PMN adhesion molecules such as integrins and lumican. To test this hypothesis, PMNs were treated with neutralizing antibodies against integrin β2 (anti-CD18) and integrin β1 (anti-CD29) or isotype control IgG. The treated PMNs were subjected to adhesion and migration assays using recombinant mouse GST-fusion lumican proteins (GST-mLum$_{17\text{-}338}$). Anti-CD29 neutralizing antibody significantly reduced PMN adhesion on GST-mLum$_{17\text{-}338}$-coated wells, whereas no significant difference between anti-CD18 and its isotype-treated PMNs were observed (Fig. 5B). We also observed that anti-CD29 neutralizing antibody significantly reduced PMN migration through a 3 μm pore coated with GST-mLum$_{17\text{-}338}$ (Fig. 5C,D).

Table 2. Summary of the distribution of white blood cells in casein-induced peritoneal lavages from *Lum*$^{-/-}$ and *Lum*$^{+/-}$ mice[a]

Cell type	*Lum*$^{-/-}$	*Lum*$^{+/-}$
CD115	–	–
CD11b	+	+
Ly6C/G	+	+
CD8	+	–
CD4	+ (45.6%)	–
CD3	–	–
CD115^{+}, CD11b^{+}	4.9%	1.7%
Ly6C^{+}, CD11b^{+}	41.8%	96.8%
CD8^{+}, CD4^{+}	13.3%	0.3%
CD3^{+}, CD4^{+}	0.79%	±
CD3^{+}, CD8^{+}	±	±

[a]Statistics of mice shown in Fig. 3. There were fewer PMNs (Ly6C/G^{+}, CD11b^{+}) in the lavage from *Lum*$^{-/-}$ mice than that of *Lum*$^{+/-}$ mice and significantly more CD4^{+} cells were present in *Lum*$^{-/-}$ than in *Lum*$^{+/-}$ mice.

PMNs fail to invade the injured cornea of *Lum*$^{-/-}$ mice in vivo

To examine further whether lumican has a direct role in PMN extravasation in vivo, DiO-labeled PMNs from a peritoneal lavage of wild-type mice were injected into *Lum*$^{-/-}$ and *Lum*$^{+/-}$ mice via the tail vein immediately followed by a 2 mm central corneal epithelial debridement wound. The appearance of green fluorescent cells in the cornea was monitored with a ZEISS epifluorescence stereomicroscope. In *Lum*$^{+/-}$ mice, about 50 DiO-labeled PMNs appeared at the limbal region of the injured cornea 10 minutes after injury, whereas few PMNs, if any, could be found in the injured cornea of *Lum*$^{-/-}$ mice (Fig. 6). One hour after injury, the number of PMNs at the limbal region remained the same as that seen at 10 minutes. Nevertheless, few PMNs could be found in the central cornea, suggesting that an immediate PMN extravasation occurs within 10 minutes of injury, but these PMNs do not invade into the injured cornea of wild-type mice even up to 60 minutes after epithelial debridement, suggesting that additional signal(s) are required for the invasion of PMNs into the stroma of injured corneas.

Discussion

We have previously demonstrated delayed wound healing in the corneal epithelium of *Lum*$^{-/-}$ mice as a result of impaired epithelium migration (Saika et al., 2000) and a decreased rate of epithelial cell

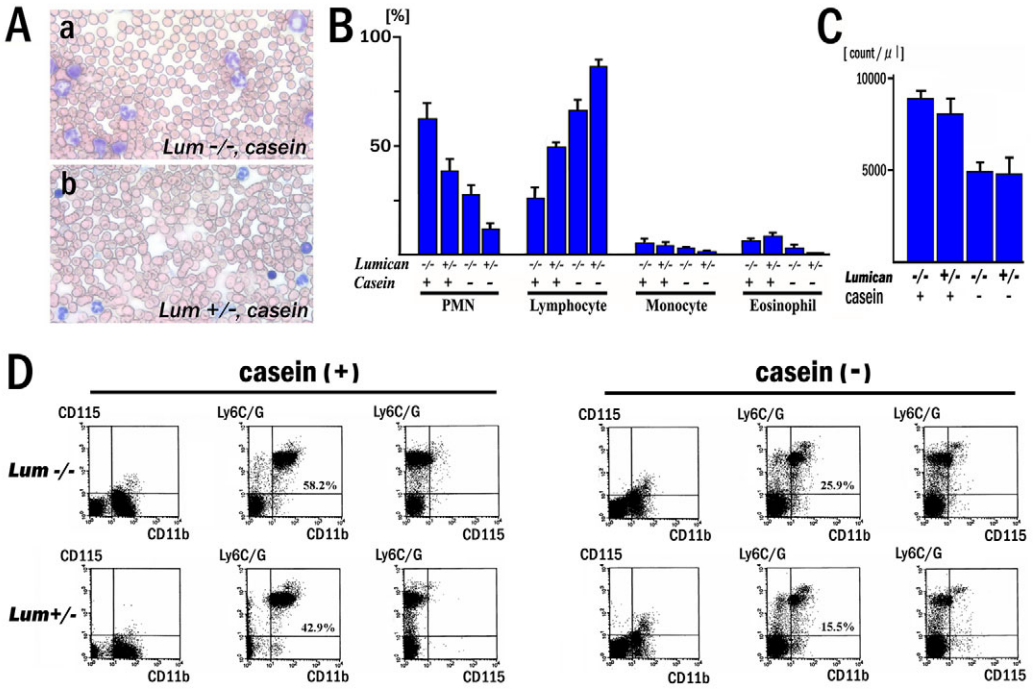

Fig. 4. Flow cytometry of peripheral and circulating blood before and after casein induction. Experimental mice were subjected to casein induction as described above. The cell type distribution in peripheral tail blood and circulating blood via heart puncture was analyzed by cytology of blood smears and flow cytometry. (**A**) After casein induction, cytology analysis with blood smear revealed that more PMNs are observed in *Lum*$^{-/-}$ compared with *Lum*$^{+/-}$. (**B**) Cell type distributions in peripheral tail blood determined by cytology with blood smear. The *y*-axis represents the percentage (%) of each cell type from the total leukocytes present within peripheral blood from *Lum*$^{+/-}$ and *Lum*$^{-/-}$ mice before and after casein induction. (**C**) Peripheral white blood cell counts increased by casein induction, but there was no significant difference between *Lum*$^{-/-}$ and *Lum*$^{+/-}$ mice. (**D**) Flow cytometry of peripheral white blood cells. In *Lum*$^{-/-}$ mice, the proportion of CD11b^{+} and Ly6C/G^{+} is larger than that of *Lum*$^{+/-}$ before and after casein induction (summary of FACS results is shown in Table 3).

proliferation following corneal epithelial debridement (Yeh et al., 2005). A hallmark sign of wound healing is inflammation, and we therefore hypothesized that ablation of *Lum* compromises the inflammatory response during the early events of wound healing following epithelial debridement. In the present study, we examined the possible roles of lumican on PMN extravasation into the injured cornea and found that the loss of lumican impaired PMN extravasation into the cornea following epithelial debridement. This is supported by several lines of evidence; the first being that the presence of lumican facilitates the invasion of PMNs into the corneal stroma as demonstrated by the lack of PMNs in injured corneas of *Lum*$^{-/-}$ mice. By using a mouse line in which lumican is expressed only in the cornea, we were able to rescue the PMN extravasation defect (Fig. 1). This is further supported by significantly lower levels of MPO enzyme activity in *Lum*$^{-/-}$ mice following epithelial debridement as well as a failure of PMNs to migrate into the injured cornea or extravasate into the peritoneal cavity upon casein induction (Fig. 1C, Figs 3, 4, 6, and Tables 2 and 3). Although the *Lum*$^{-/-}$*,Kera-Lum* mouse produces lumican only in the corneal stroma, the surrounding ocular blood vessels still lack lumican, highlighting two probable roles of lumican in the PMN extravasation process. First, the presence of lumican in the blood vessels helps to facilitate the exudation of PMNs out of the vessel and into sites of injury, but this is not entirely essential because PMNs were still present in the injured cornea of *Lum*$^{-/-}$*,Kera-Lum* mouse. This brings us to the second role of lumican, which highlights the importance of lumican in the cornea to facilitate the migration and invasion of PMNs.

The data presented here strongly support the ability of lumican to facilitate PMN exudation out of the blood vessels. To determine whether lumican serves to promote PMN extravasation during inflammation, we first showed that fewer CD11b and Ly-6C/G positive PMNs were present in the intraperitoneal lavage of *Lum*$^{-/-}$ mice when compared to that of *Lum*$^{+/-}$ mice following casein injection. Interestingly, a high proportion of the cell population in the peritoneal lavage of *Lum*$^{-/-}$ mice was CD4-positive T lymphocytes, whereas most (96%) of the casein-induced cells of *Lum*$^{+/-}$ mice were CD11b- and Ly-6C/G-positive PMNs (Fig. 3). A plausible explanation for this finding is that

Table 3. Summary of the distribution of circulating white blood cells following casein induction[a]

	+ Casein		– Casein	
Cell type	*Lum*$^{-/-}$	*Lum*$^{+/-}$	*Lum*$^{-/-}$	*Lum*$^{+/-}$
CD115	–	–	–	–
CD11b	+(>90%)	+	+	+
Ly6C/G	+(>90%)	+	+	+
CD115^{+}, CD11b^{+}	0%	0%	0%	0%
Ly6C^{+}, CD11b^{+}	58.2%	42.9%	25.9%	15.5%
Ly6C/G^{+}, CD115^{+}	0%	0%	0%	0%

[a]From mice shown in Fig. 4. Casein induction caused an increase of PMNs in the circulating blood in both *Lum*$^{+/-}$ and *Lum*$^{-/-}$ mice, but there were significantly more PMNs in the circulating blood of *Lum*$^{-/-}$ mice than that of *Lum*$^{+/-}$ mice. Numbers of cells expressing CD8, CD4 and CD3 were not determined.

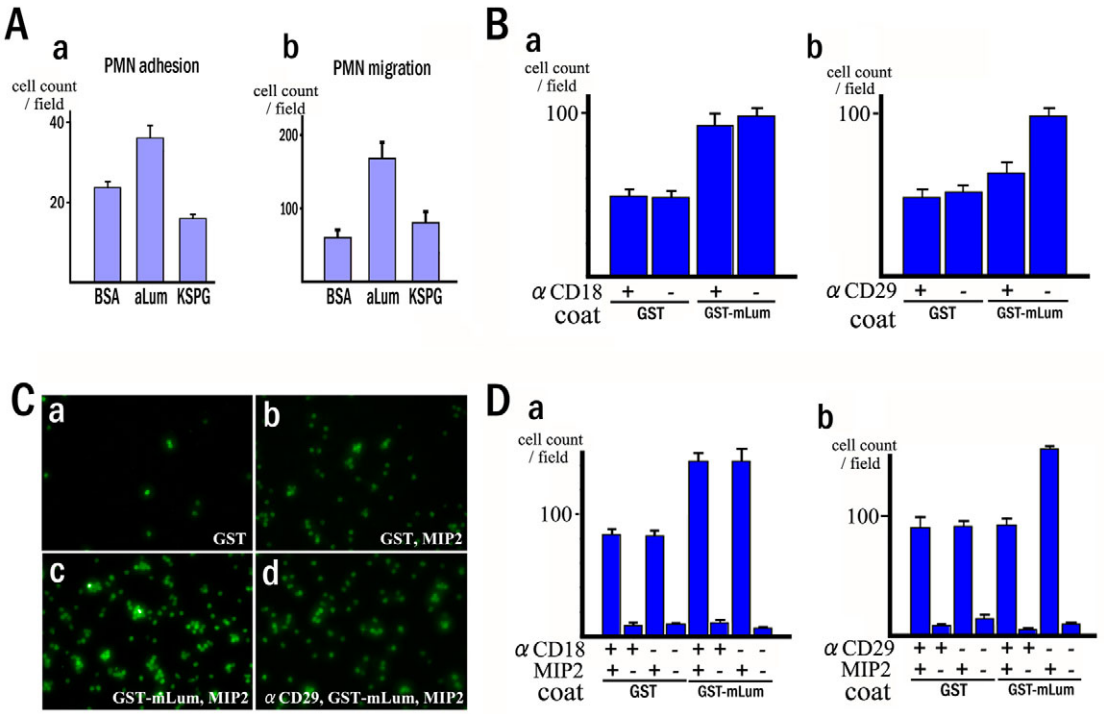

Fig. 5. PMN adhesion and migration assays in vitro. PMNs prepared from casein induced lavage of wild-type mice and used for adhesion and migration assays. (**A**) PMNs adhere better to non-glycanated lumican from aorta (aLum) coated culture chamber slides than to KSPG or BSA coated slides (a). More PMNs migrate through the Transwell coated with aLum than through those coated with BSA or KSPG (b). Error bars indicate 95% confidence intervals. (**B–D**) PMNs isolated from peritoneal lavage of wild-type mice treated with neutralizing anti-CD18 (integrin β2) and anti-CD29 (integrin-β) neutralizing antibodies and non-immune isotype IgG. The treated PMNs were then subjected to adhesion and migration analysis in culture wells coated with recombinant proteins, GST-Lum$_{17–338}$ and GST (control). (B) Anti-CD18 does not inhibit the binding of PMNs to the surface coated with GST-Lum$_{17–338}$ (a), whereas anti-CD29 inhibits the binding of PMNs to GST-Lum$_{17–338}$ (b). (C) Fluorescent microscope images of PMNs that migrated through the membrane coated with GST-GST-Lum$_{17–338}$. a, untreated PMNs seeded on a surface coated with GST; b, untreated PMNs on a GST-coated surface plus MIP2 in the lower chamber; c, untreated PMNs seeded on surface coated with GST-Lum$_{17–338}$ plus MIP2; d, PMNs treated with anti-CD29 seeded on surface coated with GST-Lum$_{17–338}$ plus MIP2. (D) Anti-CD18 does not inhibit migration of PMNs through the Transwell chamber (a), whereas anti-CD29 inhibited the migration of PMNs (b).

lumican is essential to facilitate PMN exudation from blood vessels under normal physiological conditions, as well as under inflammatory and wound-healing conditions, but its presence might not be necessary for lymphocyte exudation under these same conditions. Another possible explanation to explain the increased level of lymphocytes relative to PMNs within the lavage of *Lum*$^{-/-}$ mice is that there is an enhanced egress of lymphocytes from the bone marrow or an increase in lymphocyte extravasation in the absence of lumican. To further support this notion, there was a higher percentage of PMNs in peripheral

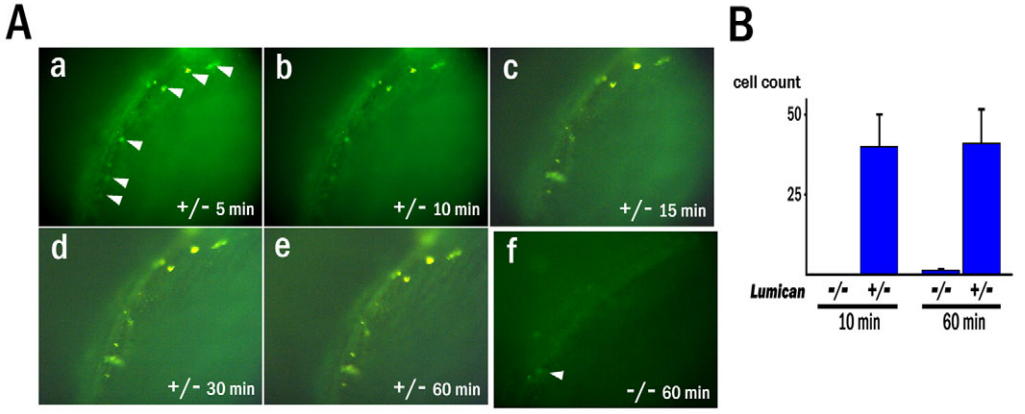

Fig. 6. PMN migration analysis in vivo. DiO-labeled casein-induced intraperitoneal PMNs from wild-type mice injected into *Lum*$^{+/-}$ and *Lum*$^{-/-}$ mice followed by corneal epithelial debridement. Corneas are observed using fluorescence microscopy. (**A**) Five minutes after wounding, DiO-labeled cells (arrowheads) appear at the edge of the limbus in *Lum*$^{+/-}$ mice. 60 minutes after wounding of *Lum*$^{+/-}$, the number of DiO-labeled cells remains about the same. In the wound area of *Lum*$^{-/-}$ mice, only one DiO-labeled cell (arrowhead in f) was observed. (**B**) DiO-labeled cell numbers counted 10 minutes and 60 minutes after wounding. No DiO-labeled cells were observed at 10 minutes (n=5) in *Lum*$^{-/-}$ mice. Error bars indicate 95% confidence intervals.

(Fig. 4A) and circulating blood obtained from *Lum*$^{-/-}$ mice than in *Lum*$^{+/-}$ mice (Fig. 4B,C and Table 3). After casein induction, *Lum*$^{-/-}$ mice had neutrophilia. This phenomenon might indicate that the intraperitoneal injection of casein stimulates PMNs to be released from the bone marrow into the circulating blood, but these PMNs cannot readily extravasate from the blood vessel into tissues under normal physiological conditions in mice or into the peritoneal cavity and tissues following injury and inflammation.

Why can lymphocytes, but not PMNs extravasate in the absence of lumican? The answer might lie, in part, in the types of receptors present on the two cell types or in varying responses to different chemokines. Previously it was observed that thioglycollate-induced macrophages adhere to non-glycanated lumican via specific cell surface receptors (Funderburgh et al., 1997). Monocytes, macrophages as well as neutrophils require integrin β2 for extravasaton (Bunting et al., 2002), suggesting a potential role for this integrin in the lumican interaction. Similarly, treatment with antibodies against β_2, α_M and α_L integrins blocks this migration (Lee et al., 2009). Interestingly, our data show that integrin β1, but not integrin β2 is necessary for adhesion and migration to sites of inflammation, because only neutralizing antibodies against integrin β1 had a significant effect on migration and adhesion (Fig. 5). Although these data appear contradictory to current thought, one must keep in mind that the PMNs used in the migration and adhesion assays were isolated from peritoneal lavage, whereby integrin β1 is probably already in an active conformation. It has been demonstrated that the activation of integrin β1 in PMNs is mediated via activation of integrin β2 binding to an extracellular ligand(s), whereby integrin β1 mediates binding to a RGD motif present in the ECM for PMN adhesion and locomotion. Murine lumican does not contain the integrin-β1-binding RGD motif, but contains the integrin-β2-binding motif LDV at the C-terminus. It is possible that lumican serves as a modulator of integrin β2 activation and subsequent β1 activation for PMN extravasation. We propose that binding of β2 integrin to lumican in the endothelium activates integrin β1 on PMNs, allowing them to interact with other extracellular molecules for extravascular tissue migration. This hypothesis is supported by Werr and co-workers (Werr et al., 1998), who showed that extravasation and interstitial tissue migration of PMNs in the rat is significantly reduced following treatment with a neutralizing antibody against integrin β1 (Werr et al., 1998).

Adhesion and migration assays were performed to support the role of lumican as a scaffold during the PMN extravasation process. Glycanation of lumican with keratan sulfate eliminated PMN adhesion and migration of casein induced PMNs from wild-type mice (Fig. 5). Furthermore, wild-type PMNs transfused via the tail vein of *Lum*$^{-/-}$ mice failed to invade into the injured cornea, whereas a significant number of PMNs appeared in the injured cornea of wild-type mice (Fig. 6). As mentioned earlier, lumican present strictly within the corneal stroma partially reduced the PMN invasion defect (Fig. 1). Taken together, these results strongly support the notion that lumican serves as a matrix to facilitate PMN extravasation during an inflammatory response. A likely possibility as to why lumican is required in the corneal stroma to aid in PMN invasion following epithelial debridement is its ability to facilitate a gradient for the CXCL1/KC chemokine. Our previous studies showed that CXCL1/KC is produced early after LPS injection and that lumican directly interacts with CXCL1/KC (Carlson et al., 2007; Lin et al., 2007). Our unpublished observations indicate that lumican is not necessary for the production of CXCL1/KC but rather might facilitate the establishment of a chemokine gradient that is required for neutrophil invasion into the corneal stroma upon KSPG degradation during the wound-healing process.

Future research will aim to identify the receptors for lumican on neutrophils (Wu et al., 2007) as well as further address the role of lumican in generating a chemokine gradient, which will shed light on the mechanisms by which lumican modulates various cellular processes. Taken together, our results support the notion that in addition to regulating the collagen fibril architecture within the corneal stroma, lumican also has a role in the ability of PMNs to exit blood vessels and invade damaged tissue.

Materials and Methods

Animals

Animal care and use conformed to The Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Cincinnati. Lumican-knockout mice (Saika et al., 2000) (*Lum*$^{-/-}$) were crossed with C57BL/6J to get *Lum*$^{+/-}$. *Lum*$^{-/-}$ mice were obtained by breeding *Lum*$^{+/-}$ mice. *Lum*$^{+/-}$ mice did not exhibit any abnormalities in the corneal stroma or skin when compared with wild-type mice. Both *Lum*$^{+/-}$ and wild-type littermates were used as controls. Additionally, *Kera-Lum* transgenic mice in a C57BL/6J background (Meij et al., 2007), which express lumican by cornea stromal keratocytes under the control of the keratocan promoter, were crossed with *Lum*$^{-/-}$ to obtain bitransgenic *Lum*$^{-/-}$,*Kera-Lum* mice. These mice express lumican only in the corneal stroma. Male mice, aged from 5 to 6 weeks, were used in all experiments.

Wound healing experiment

Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). A drop of oxybuprocaine hydrochloride solution (Benoxyl 0.4% solution, Santen, Tokyo, Japan) was applied to the cornea. The center of the cornea was marked by a 2-mm-diameter skin biopsy punch. Corneal epithelium debridement was created with an Algerbrush II® (The Alger Company, Lago Vista, TX).

Histology and immunohistochemistry

Eyes enucleated from experimental mice were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, at 4°C overnight and embedded in paraffin. Sections (5 μm) were used for hematoxylin and eosin (HE) staining and for immunohistochemistry with rabbit anti-mouse PMN antibody (Cedarlane Laboratories, Ontario, Canada) followed by fluorescein-conjugated goat anti-rabbit IgG (H+L) (Vector Laboratories, Burlingame, CA).

Myeloperoxidase assay

A myeloperoxidase (MPO) assay (Williams et al., 1982) was modified and used to determine PMN numbers in the cornea of mice after epithelial debridement. Corneas excised 0, 12, 24 and 48 hours after epithelial debridement were homogenized with a Tissuemizer (Tekmar, Cincinnati, OH) in 0.5 ml of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide. Samples were freeze-thawed three times followed by centrifugation after which a 0.1 ml aliquot of the supernatant was added to 0.4 ml of 50 mM phosphate buffer containing o-dianisidine dihydrochloride (16.7 mg/100 ml) and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was monitored continuously for 5 minutes. The rate of change in absorbance at 460 nm was determined for each sample and with a standard curve generated using purified myeloperoxidase (#70021, Fluka) units of MPO/cornea were calculated. One unit of MPO activity is equivalent to approximately 2×10^5 PMNs/ml.

Recovery of white blood cells from the peritoneal cavity

Our previous studies showed that IP injection of casein yields a high proportion of neutrophils from the peritoneal cavity of wild-type mice (Sun et al., 2006). Experimental *Lum*$^{-/-}$, *Lum*$^{+/-}$ and wild-type mice were intraperitoneally injected with 2 ml of 5% (w/v) sterilized casein in phosphate buffered saline (PBS). Circulating blood (by heart puncture), peripheral tail blood, bone marrow and peritoneal lavage were collected 3 or 4 hours after casein injection. Blood, bone marrow and peritoneal lavage of experimental mice were diluted with PBS-2 mM ethylenediaminetetraacetic acid (EDTA). To isolate white blood cells, erythroid cells were removed from the specimens, with VitaLyse Erythrocyte Lysing Kit (BioE, St Paul, MN) according to the manufacturer's protocol. The white blood cells were subsequently subjected to flow cytometry as described below. Smears of peripheral blood from tail vein and peritoneal lavage were stained by Giemsa stain modified solution (Sigma) according to the manufacturer's protocol.

Flow cytometry

Peritoneal cells (1×10^6/ml) isolated as described above were suspended in 0.5 ml PBS and incubated with 0.5% normal rat serum (Santa Cruz Biotechnology, Santa Cruz, CA) in ice-cold PBS for 30 minutes to block nonspecific antibody binding. The cells were stained by adding 1 μg each of allophycocyanin (APC)-conjugated hamster anti-mouse CD3e (CD3 ε chain) monoclonal antibody (#553066, BD Biosciences, San Jose, CA), Alexa-Fluor-488-conjugated rat anti-mouse CD4 (#MCD0420, Caltag Laboratories, Burlingame, CA), and R-Phycoerythrin (R-PE)-conjugated rat anti-mouse CD8a (#MCD0804, Caltag Laboratories) for lymphocyte analysis and Alexa-Fluor-488-conjugated rat anti-mouse CD11b (#RM2820, Caltag Laboratories), APC-conjugated rat anti-mouse Ly-6C/G (Gr-1) (#RM3005, Caltag Laboratories) and R-PE-conjugated rat anti-mouse CD115 (#MCA1898PE, Serotec, Raleigh, NC) for white blood cell analysis and placed on ice for 30 minutes. After three washes with ice-cold PBS-BSA, the cells were examined by flow cytometry. Controls included cells stained with the monoclonal antibodies separately for color compensation as well as cells stained with combinations of isotype control (APC-conjugated hamster IgG1k #553974, BD Biosciences), Rat IgG2a Alexa Fluor 488 (#R2a20, Caltag Laboratories), Rat IgG2b R-PE (#R2b04, Caltag Laboratories) for lymphocyte analysis; Rat IgG2b Alexa Fluor 488 (#R2b20, Caltag Laboratories), Rat IgG2b APC (#R2b05, Caltag Laboratories), Rat IgG1 R-PE (#R104, Caltag Laboratories) for PMN analysis.

Recombinant lumican synthesis

Recombinant lumican protein was synthesized in a cell-free system. For the mouse lumican-coding DNA fragment, PCR was performed using mouse lumican cDNA as a template and the following primer set: mLum 49-76+Sac1 (CTCGAGCTCAGTGGCCAATACTACGATTATGACATCC) and mLum988-1017Spe1 (AAAACTAGTTAGTTAACGGTGATTTCATTTGCTACACG) for mouse Lumican without the signal peptide ($Lum_{17–338}$). PCR products were digested with *Sac*I and *Spe*I, and cloned into pEU-E01G (TEV)-N2 vector. Glutathione *S*-transferase (GST) fusion proteins were synthesized using a wheatgerm expression kit (WEPRO Series 1240G, Emerald BioSystems, Bainbridge Island, WA). Recombinant GST-$Lum_{17–338}$ protein was purified with Glutathione Sepharose 4B (GE Healthcare, Piscataway, NJ) followed by dialysis with PBS. GST was prepared as a control.

Purified lumican and GST-Lumican fusion protein coating

Tissue culture chamber slides (Labtec, Naperville, IL) and cell culture inserts (3 μm pore size, BD Biosciences) were pre-coated with lumican purified from bovine aorta (non-glycanated) (Funderburgh et al., 1991) or keratan sulfate proteoglycan (KSPG) (glycanated) from bovine corneas (Reigle et al., 2008) at 10 μg/ml for 60 minutes at 37°C in a humidified atmosphere containing 95% air, 5% CO_2. For neutralizing antibody studies, 24-well culture plates (Iwaki, Tokyo, Japan) and cell culture inserts (FluoroBlok™ Insert System, 3 μm pore size, BD Falcon™, San Jose, CA) coated with type 1 collagen, were coated with GST-$Lum_{17–338}$ or GST at 10 μg/ml in PBS for 60 minutes at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. Subsequently, protein solutions were removed and culture plates and cell culture inserts were dried at 4°C.

Preparation of antibody-treated PMNs

Enhanced green fluorescent protein (EGFP)-positive wild-type PMNs were incubated with purified anti-mouse CD18 (anti-integrin β2, Clone: M18/2, BioLegend, San Diego, CA), LEAF™ purified anti-mouse/rat CD29 (anti-integrin β1, Clone: HMβ1-1, BioLegend), purified rat IgG2a, k isotype control (RTK2758, BioLegend) or LEAF™ purified Armenian hamster IgG isotype control (HTK888, BioLegend) in PBS containing 0.5% BSA and 2 mM EDTA at 25°C for 30 minutes. PMNs were washed twice with ice-cold 0.5% BSA-PBS and diluted in PBS at the concentration of 10^5 cells/ml and 10^6 cells/ml for PMN adhesion and migration assays, respectively.

PMN adhesion analysis

Tissue culture chamber slides (Labtec, Naperville, IL) were pre-coated with lumican purified from bovine aorta (Funderburgh et al., 1991) (non-glycanated) or keratan sulfate proteoglycan (KSPG) (glycanated) from bovine corneas (Reigle et al., 2008) as described previously. After washing in PBS, the slides were blocked with 1% BSA for 60 minutes at 37°C in a humidified atmosphere containing 95% air, 5% CO_2. 1×10^4 PMNs in 100 μl RPMI-1640 containing 0.5% BSA were seeded in the tissue culture chamber slides and incubated for 60 minutes at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. The cell suspension was aspirated and the tissue culture wells were rinsed with PBS three times to remove non-attached cells. PMNs that adhered to the bottom surface of slides were fixed with 4% paraformaldehyde and stained by May-Light Giemsa staining solution. Randomly chosen fields were photographed and the cells in individual fields were counted. Average of cell numbers in five different fields from individual specimens was determined. Unpaired *t*-test was used for statistical analysis and $P<0.05$ was considered to indicate a significant difference.

PMN migration analysis in vitro

Cell culture inserts (BD Biosciences) were pre-coated with lumican and keratan sulfate proteoglycan (KSPG) from bovine aortas and corneas, respectively, as described above. After washing three times with PBS, inserts were blocked with 1% BSA for 60 minutes at 37°C in a humidified atmosphere containing 95% air, 5% CO_2. Inserts were ready for the experiment after washing three times with PBS. 1×10^5 PMNs in 100 μl RPMI-1640 containing 0.5% BSA were seeded and pre-incubated at 37°C in a humidified atmosphere containing 95% air, 5% CO_2 for 30 minutes to allow cells to settle on the surface before cell migration assays as described below. RPMI-BSA (750 μl) with murine MIP-2 (R&D systems, Minneapolis, MN) and f-MLP (Sigma) were added to the lower wells as a chemoattractant and the cells were then further incubated for another 90 minutes. PMNs that migrated toward the lower chamber and adhered to the bottom surface of wells were fixed with 4% paraformaldehyde and stained by May-Light Giemsa staining solution. Randomly chosen fields were photographed and the cells in the individual fields were counted as described above.

In vivo analysis of PMN extravasation

Casein-induced intraperitoneal cells from wild-type C57Bl/J6 mice were washed in ice-cold PBS twice and stained by Vybrant® DiO cell-labeling solution (Molecular Probes) according to the manufacturer's protocol. Cell populations were examined by Giemsa staining and the cell suspension, which was more than 95% PMNs, was used for tail vein injection. Ten minutes after injection of 10^6 cells in 0.2 ml PBS to wild-type and $Lum^{-/-}$ mice, a central corneal epithelial debridement wound was created as previously described (Saika et al., 2000). The appearance of green fluorescent PMNs in the cornea was determined using a ZEISS epi-fluorescence stereomicroscope (Stemi SV 11, Carl Zeiss, Germany).

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