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Binding of pro-migratory serum factors to electrospun PLLA nanofibers

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Abstract

Architecture of the poly(l-lactic acid) (PLLA) scaffolds is known to affect protein affinity and binding strength. Here, we demonstrate that nano-fibrous electrospun PLLA scaffolds reversibly absorb the pro-migratory serum factors that stimulate migration of vascular smooth muscle via an NFκB-dependent mechanism. Further, we demonstrate that mesenchymal stem cells seeded on the PLLA scaffolds do not enhance muscle migration but may maintain the ability of induced cells to migrate in an NFκB-independent manner. These findings further support the promising application of PLLA scaffolds for therapeutic angiogenesis and vascular graft engineering.

Keywords

Vascular smooth muscle cell; Mesenchymal stem cell; Poly L-lactic acid; Scaffold; Migration; Vascular bioengineering

1. Introduction

Angiogenesis, a complex process for new blood vessel formation, involves several important aspects: degradation of the basement membrane by proteases, proliferation and migration of endothelial cells (ECs), lumen formation, basement membrane assembly, recruitment of pericyte or vascular smooth muscle cells (VSMCs), vascular maturation, and finally blood flow. The recruitment of vascular smooth muscle cells and pericytes to the nascent blood vessel assures adequate morphology and controllable vascular permeability and therefore is necessary for success of therapeutic angiogenesis that is gaining appreciation as a clinically efficient and safe approach for ischemic vascular disease.

Polymeric scaffolds are commonly used to deliver progenitor cells to the sites of injury [1], including autologous bone marrow mononuclear cells, stem cells and endothelial progenitor cells that have been shown to promote neovascularization in ischemic heart disease and peripheral arterial disease [2]. Despite the rapid, within few days after implantation, loss of up to 90% of the transplanted originally seeded cells [2–4], cell seeding has been shown to improve repopulation of vascular grafts by host vascular cells [3;5–7]. Taking into considering that scaffold seeding with progenitor cells is performed in cell cultures using serum-supplemented media, we hypothesize that recruitment of the host cells to cell-seeded grafts and enhanced angiogenesis may be stimulated by the pro-migratory factors absorbed

on the polymeric scaffold during in vitro cell seeding, rather than the seeded cells themselves.

Indeed, poly(L-lactic acid) (PLLA) that is one of the most promising biodegradable polymers used in the fields of tissue engineering and in ischemic model of induced angiogenesis [4]; [8] has been shown to absorb proteins of serum that is a common additive to cell cultures. Further, the pore wall architecture of the PLLA scaffolds affects adsorption: pore walls with nano-fibrous architectures (diameter of fibers of 50–500 nm) created by thermally induced phase separation adsorbed four times more serum proteins than scaffolds with solid pore walls [9]. However, there is limited data on whether biological activities of the bound proteins persist in the polymeric scaffolds and whether such protein-loaded scaffolds can be efficiently used to deliver pro-migratory regulators of VSMC.

In the present study, we investigated the electrospun nano-fibrous PLLA scaffolds [10] for their ability to absorb and release the biologically active pro-migratory serum components using a short-term migration assay of VSMC towards the scaffolds, with a focus on the analysis of the molecular mechanisms that are required for this migration.

2. Materials and Methods

2.1 Cell culture

Human mesenchymal stem cells (hMSC) and human primary vascular smooth muscle cells (hVSMC) were purchased from Lonza, and rat A10 VSMC (rVSMC) were obtained from ATCC. hMSC were cultured in low-glucose Dulbecco's Modified Eagle Medium (DMEM) and VSMC were cultured in high-glucose DMEM. All cell cultures were supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin and grown at 37°C with 5% CO₂. Both hMSC and hVSMC were used at P4–6. Assay cultures were done for 6 hours in serum-free media (SFM).

2.2 PLLA scaffold preparation

Biodegradable nanofibrous scaffolds were prepared by electrospinning of poly(L-lactic acid) (PLLA) (Sigma) in a 10–12% w/v hexafluoroisopropanol (HFIP) (Sigma) solution, as described in our previous study [10]. In brief, electrospinning was performed at room temperature, and at a rate of 3.0 ml/hr at 20 kV to form fibers with average diameter of 970 nm ($\pm$ 480 nm) [11]. The nanofibrous scaffolds were approximately 150 μ m thick with pore size $1.7 \pm 1.4 \mu$ m. Prior to use, PLLA scaffolds were cut into disks of 6 mm in diameter for 96-well plates and 11 mm in diameter for 24-well plates. Scaffolds were washed in 70% ethanol, then in PBS, and placed in the plate for each assay. Scaffolds that were to be seeded with hMSCs were coated with 0.03 μ g/cm² fibronectin. Cell seeding was performed at approximately 1.8×10^5 hMSCs per scaffold in 24-well plates, and 3.0×10^4 per scaffold in 96-well plates.

2.3 Migration assay

Modified chemotactic Boyden chamber system was used for the migration assays in 24-well plates. The permeable supports (Corning) had pore size of 8.0 μ m and were coated with collagen I (0.5 μ g/cm² in PBS) prior to use. VSMC were starved in DMEM supplemented with 0.25% FBS for about 16 hours prior to the migration assay, and were cultured in serum-free DMEM or indicated conditioned media for the 6-hour duration of the assay. VSMC density at plating was 2.2×10^4 cells per transwell chamber. After the assay cells in each transwell insert were fixed with 4% paraformaldehyde, un-migrated cells were scraped off from the top surface of the inserts with cotton tip applicator, and the migrated cells on

the lower side of the inserts were stained with DAPI and counted in four random fields per chamber under fluorescence microscope.

2.4 Luciferase assay

Stable reporter cell lines to analyze signaling network in VSMC migrating in response to serum factors have been established for intracellular pathways known to regulate cell migration in various types of cells. These included luciferase reporters for NFkB [12;13], MAPK/ERK [14], c-Myc [15], C/EBP [16], Notch [17], TGF- β [12;18], and cAMP/PKA [19]. Pathway-specific luciferase reporter lines of A10 rVSMCs and a control promoterless line were established by stable transfection with Cignal Reporter lentiviruses (Invitrogen) as described elsewhere [20]. Cell lysates were prepared using Promega reagents (cell lysis buffer) and freezing at -20°C . Luciferase activity was measured in lysates using Luciferase Assay System (Promega). Lactate dehydrogenase (LDH) activity in the lysates measured with BioVision Cytotoxicity Assay reagents was used as a reference to normalize the luciferase activity readings. Normalization of the luciferase activity data was performed by first dividing luciferase activity readouts by the average LDH read-out per sample, then subtracting the negative (promoterless) control cell line values from those of the experimental reporter cell lines, according to the manufacturer's instructions.

2.5 Inhibition of NFkB pathway in vitro

A cell-permeable NFkB inhibitor CAPE (Calbiochem) was used at a concentration of $15\mu\text{g/ml}$ during the 6-hour migration assays. The inhibitor was prepared in SFM and was added to both upper and lower chambers.

2.6 Protein preparation and gel electrophoresis

Media were collected after 6-hour incubation with pre-conditioned PLLA scaffolds and concentrated using Amicon ultra-0.5 centrifugal filter devices (Millipore) with 3kDa cutoff by a 40 min centrifugation at $10,000g$ at 4°C . The pre-conditioned scaffolds were stored at -20°C in Ripa buffer containing protease inhibitors (Pierce) until used for analysis. Both protein samples were fractionated on a 4–12% Bis-Tris gel (Invitrogen). Silver staining (Invitrogen) was used for detection of protein bands in each sample according to manufacturer's instruction.

2.7 Statistical analysis

The statistical analysis was performed with unpaired t-test. Values of variables are presented as the mean with standard deviation (SD). In all experiments, p values <0.05 were considered significant.

3. Results

3.1 Reversible protein absorption from serum to nanofibrous PLLA scaffold

We used a 10–12% w/v PLLA solution to prepare nanofibrous scaffolds with a random spinning pattern (Fig. 1A) [10]. The scaffolds were incubated in 10% FBS growth medium for three days (FBS-scaffold), and washed for 10 min with serum free-medium (SFM) to remove the remaining 10% FBS medium. To assure complete washing, the optical absorbance of the washing medium was monitored at 260 nm and the washing was considered complete when A_{260} of the wash solution was within 0.01 units of the fresh SFM. Ubiquitous coverage of the scaffold with serum proteins was detected by Eosin staining that revealed a significant increase in the bound dye only in scaffolds incubated in 10% FBS medium (Fig. 1B).

The reversibly bound serum-derived components were allowed to release into SFM for 6 hours at 37°C. Proteins that remained bound to the scaffold following the 6-hour incubation in SFM, were eluted with RIPA buffer containing protease inhibitors. Patterns of proteins reversibly attached and those bound to the PLLA scaffold were compared to the protein context of serum by SDS-denaturing gel electrophoresis and silver staining. As shown in Fig. 1C, the profile of proteins adsorbed by and released from the nano-fibrous PLLA scaffold was identical to that of the original FBS. High abundance proteins were putatively identified by their mobility according to previously published analysis [21]. Protein binding to the scaffold is reversible and only a minor proportion of few proteins remained bound to PLLA after 6 hours in SFM and could be eluted with RIPA buffer, although it should be also considered that these proteins can be reversibly released-bound to the scaffold due to their high abundance or high affinity to the scaffolds. This result demonstrates comprehensive protein binding by the electrospun nano-fibrous PLLA.

3.2 Serum factors released from nanofibrous PLLA scaffold promote migration of VSMC

To determine whether the released serum factors retain their biological pro-migratory activity after binding to electrospun PLLA scaffolds, we employed a Boyden chamber assay, in which the FBS-treated scaffolds were placed in the lower chamber filled with SFM and rat or human VSMC were seeded on the collagen type I-coated membrane in the upper chamber. Migration of the cells was visualized after 6 hours of incubation by DAPI nuclear staining. Both human and rat VSMC showed significantly higher levels of migration when co-cultured with FBS-PLLA scaffolds compared to control wells filled with SFM lacking the scaffold (Fig. 2 A). In contrast, the PLLA scaffolds incubated in SFM instead of 10%FBS for three days before the migration assay, failed to induced migration in either human or rat VSMCs (Fig. 2B), confirming that induction of VSMC migration was due to the serum-derived factors absorbed by PLLA. The tissue culture plastic that has minimal affinity to serum proteins had no effect on VSMC migration after a 3-day incubation in 10%FBS (data not shown).

Next, we examined the requirement for the nano-fibrous structure of PLLA on serum protein adsorption and release, by comparing the effects of electrospun scaffolds and solid beads. The PLLA beads were pre-treated with 10% FBS and tested for their ability to induce migration in rat VSMCs. As shown in figure 3A (grey bar), the FBS-treated PLLA beads did not induce migration in VSMC in contrast to FBS-treated electro-spun nanofibrous PLLA (black bar, $p=0.003$). We then analyzed serum protein absorbed by these different types of the PLLA scaffolds from the 10% FBS. The PLLA beads absorbed limited number of proteins (supposedly, BSA and Apo-A1 based on their mobility [21]) detected in the gel with Silver staining in contrast to the PLLA nano-fibrous scaffolds that absorbed almost all serum proteins (as shown in Fig. 1C). The inability of the PLLA beads either to bind many of the serum proteins or to induce cell migration further supports the importance of the scaffold structure for the purpose of delivering the biologically active proteins to cells.

3.3 Signaling pathway underlying migration in VSMC promoted by PLLA-retained serum factors

Migration of VSMC is a complex biological process regulated by various factors and signaling pathways (Table 1). To investigate which pathways contribute to migration induced by the factors released by electrospun PLLA pre-incubated with FBS, we analyzed seven major signaling cascades using stable rat luciferase reporter VSMC lines established in our previous studies [20]. We found that only NF- κ B signaling was activated in VSMC induced to migrate by the PLLA-retained serum factors (Table 1).

Quantitative analysis of luciferase activity in cell lysates revealed an approximately 50% induction ($p < 0.02$) in NF κ B signaling stimulated in VSMC by serum factors released from the electrospun PLLA scaffolds (Fig. 4A). To confirm specificity of the luciferase-based analysis, we employed a cell-permeable NF κ B inhibitor CAPE and observed that treatment of VSMC with this inhibitor prevented activation NF κ B signaling with the FBS-treated PLLA rendering luciferase reporter expression similar to control untreated VSMCs (Fig. 4A). Further, PLLA scaffolds pre-treated with SFM did not stimulate activity of the NF κ B signaling (Fig. 4B) indicating that the serum-derived factors rather than PLLA are required for the pathway activation.

3.4 Inhibition of NF κ B hinders migration of VSMC toward serum-treated PLLA scaffold

To examine whether activation of NF κ B was mediating increase in cell migration, the effect of CAPE on cell migration induced by serum factors absorbed on electrospun PLLA was analyzed. In both human and rat VSMC, CAPE treatment attenuated cell migration (Fig. 4C, D) indicating a critical role for NF κ B activation in this process.

3.5 MSCs modulate mechanism of VSMC migration induced by PLLA-borne serum factors

Seeding scaffolds with stem/progenitor cells has been suggested to have beneficial effects on neovascularization and repopulation of the vascular grafts with host cells [1]; [3]. Our results suggest that serum-derived factors absorbed by the PLLA scaffold may significantly contribute to regulation of VSMC migration. To determine the effect of mesenchymal stem cells on migration in VSMC induced by the PLLA-retained serum factors, we seeded the electrospun nanofibrous PLLA scaffolds with human MSC (180,000 cells per well in 24-well plates) and cultured in 10% FBS-containing growth medium for three days to allow cell attachment and distribution throughout the scaffold. 3D reconstruction of confocal analysis of the nuclear staining with DAPI demonstrated that hMSC formed monolayers on the surface and penetrated into the scaffold (Fig. 5A), indicating good attachment and survival on the PLLA nanofibers.

The MSC-seeded PLLA scaffolds were then used in migration assay on human VSMC. The presence of MSC in the scaffold did not significantly enhance migration as compared to the FBS-treated scaffolds (Fig. 5B) suggesting that serum factors absorbed on the nanofibers of PLLA are the major regulators of VSMC migration. Consistent with this notion, the hMSC seeded on the bottom of the Boyden chamber (in the absence of scaffold) failed to stimulate migration in human VSMCs (Fig. 5B). Nevertheless, we have also observed that while inhibition of the NF κ B signaling by CAPE blocked VSMC migration induced by the FBS-treated PLLA scaffolds, it could not completely attenuate the effect of the MSC-seeded scaffolds (Fig. 5C) suggesting that in the presence of MSCs serum-derived factors can regulate migration of VSMC in an NF κ B-independent manner.

Discussion

In the present study, we have focused on the ability of the electrospun nanofibrous PLLA scaffolds to absorb and release biologically active serum-derived factors that stimulate migration of VSMC. Controlled formation of the SMC-invested growing vessels is desired in various clinical situations and insufficient investment with SMC contributes to a high propensity for tumor cell shedding and possible metastasis as they are easily penetrated by tumor cells (reviewed by Owens [22]). At the same time, tight control over vessel growth is needed because abnormal angiogenesis underlies proliferative diabetic retinopathy, retinopathy of prematurity, sickle cell retinopathy, age-related macular degeneration, psoriasis, and tumor vascularization.

A novel finding of our study is the demonstrated biological activity of the PLLA-absorbed serum proteins to stimulate migration in VSMC via an NFkB-dependent mechanism. A critical role for activated NFkB has been described in TNF-alpha-induced VSMC migration associate with inflammation [13], and here we show that this signaling is also critical in mediating serum-induced VSMC migration. Several serum proteins have been shown to activate NFkB signaling in various cell types (Table 1). The PLLA beads incubated in 10% FBS fails to induced migration in VSMC and appear to bind less proteins that the nano-fibrils. Based on the mobility of serum proteins released from the FBS-treated PLLA nano-fibrils, a role for plasminogen, prothrombin, albumin, kininogen and IGF-1 in stimulating cell migration in VSMC can be postulated. While albumin is also bound by the PLLA beads, the relative levels of bound albumin is noticeably less than that bound by the non-fibrous scaffold of the same weight. Accounting for the dose-dependent regulation of NFkB signaling by albumin [23], a possibility exists that this protein may contribute to the reported induction on VSMC migration. Future studies addressing the precise roles of these serum proteins in VSMC migration may benefit generation of a sophisticated tissue engineered scaffold.

Our results describe the ability of the electrospun fibrous PLLA to absorb the full spectrum of serum proteins. In addition to biological factors and culture conditions, chemical and physical characteristics of the scaffold are also important [24]. In our experiments we did not observe variations in pH of the media conditioned for 6 hours by the FBS-treated scaffolds versus serum free cultures, indicating that differences in VSMC migration were not due to changes in acidity. However, we did observe a significant effect of the polymer structure on regulation of VSMC migration by electrospun nano-fibrous PLLA scaffolds versus PLLA beads. These results complement the previous study [9] demonstrating that specific protein-affinity and binding strength of the bioengineered scaffolds depend on their structures, and open novel approaches for targeted delivery of specific proteins by manipulating physical configuration of the scaffold.

Our study also tested the potential contribution of MSC in regulation of VSMC migration since progenitor cells are commonly used today to induce neovascularization upon injection or scaffold-mediated implantation. Although in our experiments seeding the scaffolds with MSC did not significantly enhance VSMC migration compared to scaffold-adsorbed serum factors, we have noticed that requirement for NFkB activity in VSMC migration is less stringent when MSC are present in the culture, as shown in figure 5B. This observation is interesting and suggests that in addition to the NFkB-dependent migration triggered by the serum factors, MSCs can promote migration of VSMC by an additional NFkB-independent mechanism. The effect of MSC on neighboring VSMC migration may be even more significant in clinical situation for controlled cellularization of vascular grafts *in vivo*, as suggested by previous studies showing a complete repopulation of the stem cell-seeded scaffolds by the host cells[3;5–7]. However, although initial cellularization of the graft is important, the continuous migration of VSMC may result in stenosis and needs to be restrained. Characterization of the molecular events underlying migration of VSMC into the scaffold, such as NFkB signaling identified in our study, is essential for advancement of vascular bioengineering through au fait manipulation with vascular cell behavior.

Current and developing strategies of vascular tissue-engineering aim to achieve endothelialization of vascular grafts without preprocedural cell seeding (reviewed in Melchiorri [25]). It seems intuitively correct that coordinate stimulation of cell migration in endothelial and vascular smooth muscle cells (VSMC) may benefit formation of the functional vessels. Our study demonstrates that PLLA fibrous scaffolds may be used to deliver serum factors able to stimulate cell migration in VSMC and likely, in endothelial cells to improve endothelialization and vascular graft outcomes. Further studies are needed

to address the potency of the PLLA-fiber-bound serum factors in regulation of endothelial cell migration.

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Figure 1. Nanofibrous PLLA scaffold reversibly binds serum proteins present in FBS
A 10–12% w/v poly L-lactic acid (PLLA) was used to fabricate random electrospun nanofibrous scaffold. **B** - Protein absorption on PLLA nano-fibrous scaffolds from 10% FBS was detected with Eosin Y staining, that shown ubiquitous bright staining only in FBS-treated scaffolds compared to untreated controls of scaffold incubated in serum-free medium (SFM). **C** - The scaffolds were incubated in 10% fetal bovine serum (FBS)-containing growth medium for 3 days, then washed in serum-free media (SFM) and incubated in fresh SFM for 6 hours. Conditioned media were collected after 6 hours. Proteins from concentrated conditioned media, PLLA scaffold elution, and fresh growth medium were fractionated on 4–12% polyacrylamide gel by denaturing electrophoresis and detected by silver staining.

Figure 2. Serum-derived factors absorbed on electrospun nanofibrous PLLA retain pro-migratory activity

A - Human or rat VSMC were induced to migrate in the Boyden chamber for 6 hours by FBS-treated PLLA scaffolds placed in serum-free media (SFM). Control wells were filled with SFM only. **B** - Migration of both human and rat VSMC was also measured in the presence of scaffold pre-treated with SFM and compared to SFM control wells, which did not show a significant difference. Three independent experiments were performed in triple replicates. *, $p < 0.05$; **, $p < 0.01$.

Figure 3. PLLA beads do not induce cell migration in rat VSMC

A – Migration of rat VSMC was induced by the PLLA nano-fibers or PLLA-beads pre-treated with 10% FBS. VSMC exposed to serum free medium (were used as a control). Three independent experiments were performed in triple replicates. *, $p < 0.05$; **, $p < 0.01$. **B** – PLLA beads were incubated in 10% fetal bovine serum (FBS)-containing growth medium for 3 days, then washed in serum-free media (SFM) and boiled in Laemmli denaturing buffer to release bound proteins that were fractionated on 4–12% polyacrylamide gel by denaturing electrophoresis and detected by silver staining.

Figure 4. Effect of serum-treated PLLA scaffold on NFκB activity in rat VSMC

Rat aortic VSMC line A10 was cultured in presence of PLLA scaffolds or in their absence (SFM only) for 6 hours following (A) a 3-day pre-incubation of the scaffolds or only cell culture wells with serum-containing media (t-Scaffold and t-well, respectively), or (B) a 3-day pre-incubation in SFM only. Panel (A) also shows cultures incubated for 6 hours in the presence of an NFκB-specific inhibitor, CAPE, added to the medium at 15μg/ml. Each migration assay was repeated 3–4 times with 3 wells per experimental condition. The values have been presented as fold change for simplified comparison. * indicates p-values < 0.05.

Figure 5. Migration of both rat and human VSMC toward PLLA scaffolds is significantly reduced following inhibition of NFkB activity

A - Mesenchymal stem cells (MSC) were seeded onto electrospun PLLA scaffolds three days prior to vascular smooth muscle cell (VSMC) migration assays to allow for proliferation and penetration of the cells in the scaffolds. Human aortic VSMC (**B**) and rat A10 aortic VSMC line (**C**) were cultured in the presence or absence of PLLA scaffold in serum-free media (SFM). When inhibition of NFkB was desired, a cell-permeable NFkB inhibitor, CAPE, was added to the culture media at a concentration of 15µg/ml. Human VSMC were also plated in the presence of MSC-seeded PLLA scaffolds with and without CAPE for analysis of NFkB inhibition in all previously tested conditions for this study. At

the assay endpoint, cells in each transwell insert were fixed with 4% paraformaldehyde, and the migrated cells on the lower side of the inserts were stained with DAPI and counted under fluorescence microscope. Each migration assay was repeated 3–4 times with 2–3 wells per experimental condition. * indicates p-values ≤ 0.05 and ** indicates p-values ≤ 0.01 .

Table 1

Changes in signaling pathways associated with migration in VSMC stimulated by serum factors release by electrospun PLLA

Signaling Pathway	Transcription Factor	Luciferase Activity in VSMC	Change with migration induced by electrospun FBS-PLLA	Role in Migration
NF-kB	NF-kB	Detectable	Increased	Shown [12;13]
MAPK/ERK	Elk-1/SRF	Detectable	no	Shown [14]
c-Myc	Myc/Max	Detectable	no	Shown [15]
C/EBP	C/EBP	Detectable	no	Shown [16]
Notch	RBP-Jk	Detectable	no	Shown [17]
TGF- β	SMADs 2/3/4	Not detectable *	no	Shown [12;18]
cAMP/PKA	CREB	Not detectable *	no	Shown [19]

Migration in stable cell lines of rat A10 aortic VSMC expressing luciferase reporters was induced by serum factors released into SFM by the FBS-treated PLLA scaffolds. Cells were harvested and lysed after 6 hours of incubation. Luciferase activity in cell lysates was normalized to total lactate dehydrogenase (LDH) activity representing relative cell number.

* indicates luciferase reporter functionality has been confirmed with known positive regulators such as Forskoline for PKA and TGF- β 3 for TGF- β . Each assay was repeated 3 times with 6 wells per experimental condition. p-values < 0.05 were considered significant difference.

Table 2

Serum proteins shown to activate NFkB signaling

Plasminogen (90 kDa) – Precursor of Plasmin (86 kDa)	Syrovets T, et al (2001) Plasmin-induced expression of cytokines and tissue factor in human monocytes involves AP-1 and IKKbeta-mediated NF-kappaB activation. <i>Blood</i> , 97(12):3941-50.
Prothrombin (72 kDa) – precursor of thrombin (36.7 kDa)	Delekta PC, et al. (2010) Thrombin-dependent NF- κ B activation and monocyte/endothelial adhesion are mediated by the CARMA3-Bcl10-MALT1 signalosome. <i>J Biol Chem</i> . 285(53):41432-42.
Albumin (66.5 kDa)	Pearson AL, et al. (2008) Albumin induces interleukin-6 release from primary human proximal tubule epithelial cells. <i>J Nephrol</i> . 21(6):887-93.
Kininogen (50 kDa)	Khan MM, et al. (2006) High-molecular-weight kininogen fragments stimulate the secretion of cytokines and chemokines through uPAR, Mac-1, and gC1qR in monocytes. <i>Arterioscler Thromb Vasc Biol</i> . 26(10):2260-6.
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